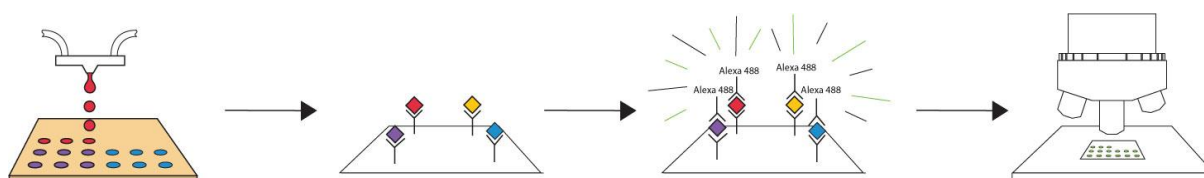# Miniaturized Multiplex Approaches for Prostate Cancer Diagnostics

- Detection of hK2 and Various Forms of PSA

Belinda Adler

Master's Thesis in Biotechnology, 30 hp

December 16th 2009

Department of Electrical Measurements

Lunds University, Sweden

Supervisor: Kerstin Järås

Examiner: Thomas Laurell

Abstract

Prostate cancer is the most common form of cancer amongst males in the western world. Today's diagnostic methods for prostate cancer that rely on PSA measurement are not satisfying. The level of prostate specific antigen (PSA) is determined in a blood test. Of the men with elevated PSA levels only 25 % have prostate cancer. This thesis aims to develop a proof of concept for a miniaturized multiplex microarray, which in a future perspective could be used as a diagnostic tool for prostate cancer. Different diagnostic methods are used today but none are separately sufficiently specific. The microarray based method in this thesis aims to a screening technique for simultaneous measurements of total, free and intact PSA as well as free human kallikrein 2 (hK2) in serum. The intermediate goal was a dual approach where PSA total and free are measured on two chips. The dual assay paved the way to the multiplex approach where different biomarkers simultaneously were measured on the same porous silicon chip. In both approaches a capture antibody was dispensed onto a porous surface. The chip was incubated with the biological sample and detected with a fluorescently labeled detector antibody in a confocal microscope. The intermediate dual assay was validated using 8 plasma samples obtained from the clinical routine analysis. Furthermore, three biomarkers (PSA total, free and intact) were successfully detected simultaneously on a porous silicon chip sized 3.5 x 3.5 mm.

Contents

Abbreviations

11B6	An antibody against hK2
2E9	An antibody against PSA
4D4	An antibody against PSA
5A10	An antibody against PSA
ab	Antibody
Alexa 488	Alexa Fluor 488
BPH	Benign Prostatic Hyperplasia
cDNA	Complementary Deoxyribonucleic Acid
CV	Coefficient of Variation
DELFIA	Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenedinitrilotetraacetic acid
$F(ab)_2$	Fragment of the antibody
Fc	Fragment of the antibody
FITC	Fluorescein Isothiocyanate
H117	An antibody against PSA
H117-biotin	Biotinylated H117
H50	An antibody against PSA
HF	Hydrofluoric Acid
hK2	Human kallikrein 2
HPR	Human Proteome Resource
HRP	Horseradish peroxidase
HUGO	Human Genome Organization
HUPO	Human Proteome Organisation
KTH	KTH Royal Institute of Technology
mAb	Monoclonal Antibody
mRNA	Messenger Ribonucleic Acid
MSI	Mean Spot Intensity
MQ	MilliQ
MWCO	Molecular Weight Cut Off
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCa	Prostate Cancer
PGP	Personal Genome Project
PMT	Photomultiplier
RIA	Radioimmunoassay
RNA	Ribonucleic acid
PSA	Prostate Specific Antigen
RT	Room Temperature
SA	Streptavidin
UMAS	Malmö University Hospital

1. Background and Methodology

1.1 Prostate Disease

Prostate cancer is the most common form of cancer and cause of death among men in the Western world. The disease is age related and three-quarters of the cases affects men aged 65 or above. [1] To diagnose prostate cancer today rectal palpation, transrectal ultrasound examination and a blood test to determine the prostate specific antigen (PSA) content are used. Each of those three separated methods has only 25-35 % chance of finding prostate cancer. [2] In all three methods combined the chance is 60 %. Furthermore only 25 % of those with slightly elevated PSA levels have prostate cancer. [3]

Instead of prostate cancer, a large part of the patients with elevated PSA have a benign prostatic hyperplasia (BPH). BPH is an enlargement of the prostate gland which is not considered a premalignant lesion. [4] The prostate grows with age and when the outer tissue of the prostate stops expanding, it can lead to compression of the urethral canal. The proliferation leads to an elevated PSA concentration in the serum. Infection can also cause temporarily elevated PSA level. The prostate have three different zones; central, transition and peripheral zone, see Figure 1. BPH often starts in the transition zone of the prostate and prostate cancer in the peripheral zone.
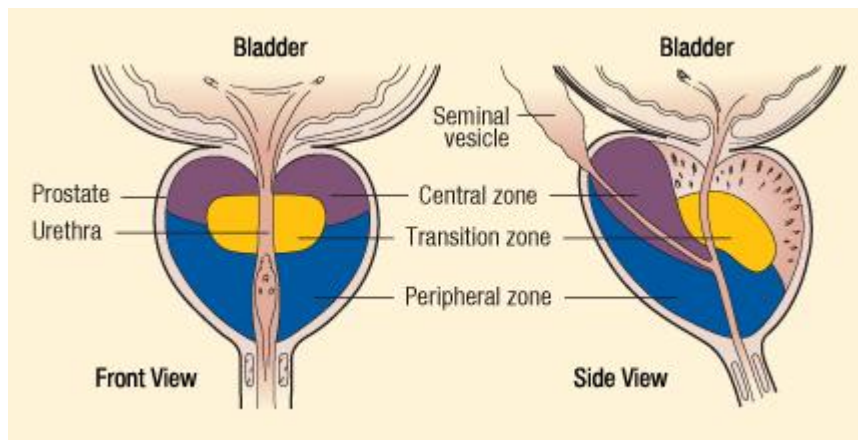

Figure 1 The prostate gland and its three zones, central transition and peripheral zone. [5]

1.2 Prostate Specific Antigen

Prostate specific antigen is a protein which can be detected in blood samples. PSA is secreted by the epithelial cells of the prostate gland. Its function is to break down the gelatin-like substance that holds the spermatozoa, such that the sperm can swim more easily through the cervix and fertilize the egg. The PSA is transported from the sectretory epithelial cells into the lumen, i.e. the sack-like structure of the prostate, in the form of proPSA. In lumen proPSA changes to active PSA, that later can be changed into inactive forms (Figure 2). It is likely that human kallikrein 2 (hK2) is the physiological activator of proPSA. [6] hK2 also belongs to the protein family of human kallikreins and corresponds to 10-50 % of the PSA expression. In addition PSA and hK2 are expressed extraprostatic in tissues and fluids.

The epithelium of the prostate gland is built up by a membrane, basal cells and secretory epithelium, see Figure 2. Both active and inactive PSA can leak though the epithelium into the blood circulation where it is found in the form of bound and free PSA. PSA in serum can be nicked and is then a part of the free PSA. Intact PSA is found in both the free and bound form in blood serum. The cells which form the blood-luminal barrier with its tight junctions are suggested to be the basal cells. [4] It is believed that prostate epithelial stem cells are found in the basal layer.
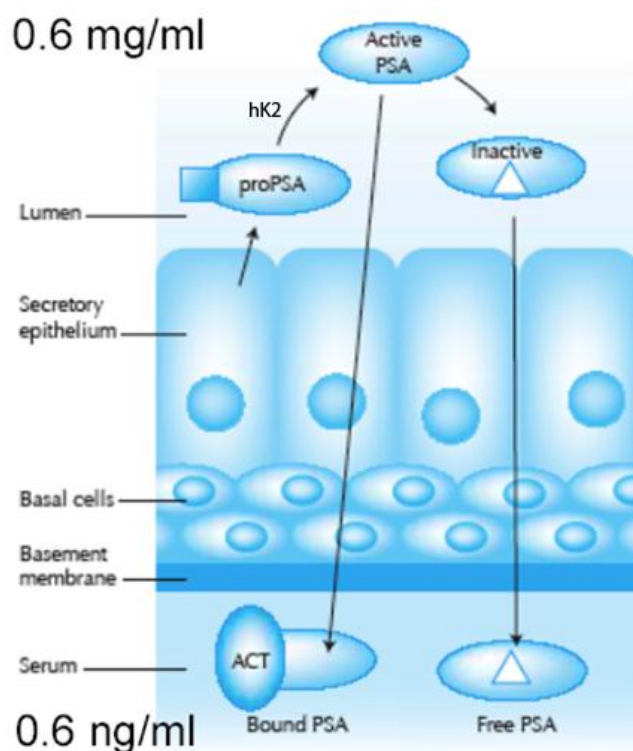

Figure 2 Structure of the prostate epithelium [7]

A small part of the PSA leaks into the blood vessels in healthy individuals. Malignant tissue is more unorganized and the walls between the prostate and the blood vessels can be broken. [2] Therefore increased PSA values in the blood are found in prostate cancer patients, even though the production of PSA is lower in the cancer cells. [2] The PSA concentration in the serum of a healthy person is approximately 0.6 ng/ml and in lumen 0.6 mg/ml, Figure 2. [7]

There is a grey zone in PSA concentration between men with BPH and prostate cancer, see Figure 3. The normal value for BPH is 0-10 ng/ml PSA in the serum whereas for the prostate cancer it is often set from 3 to over 100 ng/ml. [2] There is also a risk to have prostate cancer with a value below 3 ng/ml. Approximately 25 % of those with PSA values in the upper normal range have prostate carcinoma.
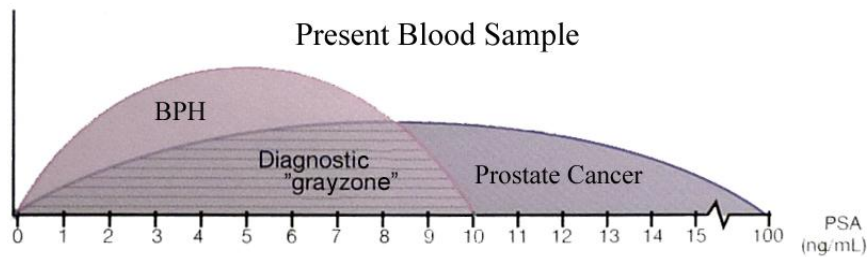
Figure 3 Present blood sample for PSA concentration in clinical diagnostics in Sweden [2]

In 1987 Stamely and co-workers observed that an elevated PSA level in serum correlated with prostate cancer (PCa) as well as disease stage. [8] PSA was then suggested to be a marker for prostate cancer. Amongst others Lilja reported about PSA as a biomarker for PCa and presented in 1987 a molecular cloning of PSA cDNA [9]. PSA correlates with both benign and malignant processes in the prostate. [4] There is evidence that the ratio between PSA free/total and hK2 are more closely related to prostate cancer. [10] Today both PSA total and the ratio PSA free/total are used in the clinic according to Liljas findings.

1.3 Proteomics

Proteome is a complement of proteins encoded from a genome which is expressed and modified by a cell, organism or tissue. The term proteomics derives from the two words *prote*in and gen*omics*. Marc R Wilkins coined the term in 1994 being a PhD student. [11] He is now a professor in Sydney, Australia. Proteomics is the study of proteins, particularly structure and functions, in a tissue or cell at a specific time under certain conditions. Within proteomics you can separate, identify and characterize proteins. [12]

Just prior to the proteomic era, the genes were studied. HUGO (Human Genome Organization) was one of the two organizations sequencing the human genome, published in Nature in February 2001. [13] The competitor Craig Venter and his company Celera finished at the same time and published in Science February 2001. [14] In the early years of 2000 George M. Church invented the Personal Genome Project (PGP). [15] A project that aims to develop a database of 100 000 people's gene sequences connected to their medical records. As of today ten people have already been screened and at this moment PGP are looking for additional 100 persons to incrementally expand the enrolment.

In the proteome field a still growing project called HUPO (Human Proteome Organisation) was started in 2001. [16] HUPO aids the development of international proteomic initiatives, so that the human diseases can be better understood. In Sweden Professor Mattias Uhlén at KTH started the Swedish Human Proteome Resource (HPR) program in 2003. [17] The HPR aims to explore the human proteome using antibody based proteomic methods. The project combines bioinformatics, proteomics and pathology.

Proteins are much more than just the genes. They can fold and modify after translation and act in different unexpected ways. For better understanding of the human body and to develop new diagnostic tools, we need to look into the proteins. When the proteomic

field began around 1994 the initial technique used was two-dimensional gel electrophoresis. During the last 10 years the research for novel disease biomarkers has revolutionized the development of mass spectrometry techniques as well as protein and antibody microarrays.

1.4 Protein Chip Technology

Protein chips and protein microarrays are used in methods to determine the presence and to some extent also the amount of protein in a sample. Protein chips can also be used to determine functionality such as phosphorylation. Antibodies are often used to bind the protein of interest, and it is then called antibody arrays.

The protein chip has originated from the immunoassay. In 1950 the Radioimmunoassay (RIA) was used by Yalow et al to present an immunoassay for plasma insulin in man. [18] This method is extremely sensitive and specific but involves radioactive labeling which causes radioactive waste and needs of radioactive protection. [19] From the RIA the nonradioisotopic labeling substance immunoassays were developed. Immunoassays are often carried out many at the same time. In 1991 the 96 well plate or microplate was patented by Pfizer and shortly after became commercialized and a standard definition. [20, 21]

Instead of working in a 96 well plate the antibody or biological sample can be arrayed onto a chip. The volumes can be reduced and numerous tests can be carried out simultaneously on the same chip in a high throughput manner. [22] This was foreseen almost 20 years ago by Ekins and co-workers [23, 24], suggesting a miniaturized format of the immunoassays to speed up the kinetics and improve the detection limit.

A technique that could be compared to the protein microarrays is the gene expression analysis. In this method oligonucleotides are arrayed onto chips. RNA is then isolated from a sample, reverse-transcribed into cDNA, labeled and hybridized onto the array. A reference sample is treated the same way but with a different label. [25] The result reveals the relative level of RNA in the sample compared to the reference. In this way expression analysis of malignant and normal PCa cells could be performed.

The breakthrough that gene expression analysis led to also implied the potential of the protein chip technology. It should be noted that the level of mRNA not necessarily correlates with the final protein level [26-28]. Today protein chip technology is a well established technique [29-31], but still more of a challenge than gene expression analysis, mainly since proteins, unlike oligonucleotides, have a vast diversity of surface characteristics and can be chemically modified by post-translational modifications.

The three common ways of detecting biomarkers on protein chips include:
1) direct labeling
2) reverse-phase antibody microarrays
3) sandwich immunoassays [32]

In the direct labeling technique illustrated in Figure 4 the capture antibody is dispensed and the biological sample, already labeled with the fluorophore, incubated on the chip. The readout is performed in a florescent microscope. This method has the disadvantage of being time consuming and challenging, because the biological sample needs to be

labeled. The two other techniques, reverse-phase and sandwich, omit the labeling procedure and are therefore better suited for medical diagnostics.
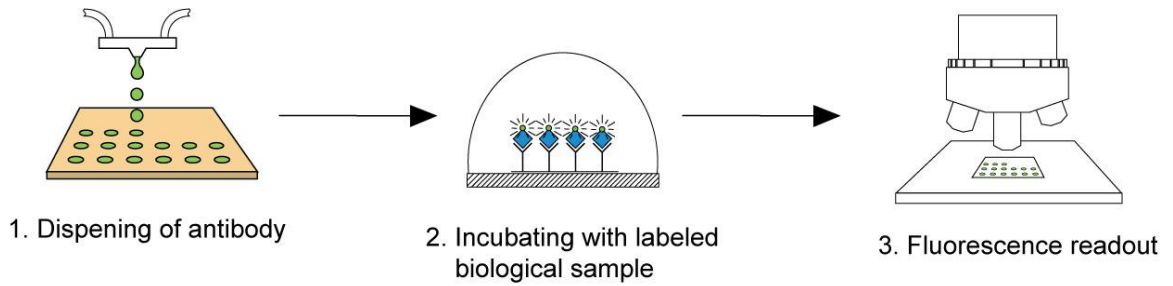

Figure 4 Direct labeling technique. Capture antibodies are dispensed onto the surface (1) and subsequent blocking and washing is followed by incubation with the fluorescently labeled biological sample (2). After another washing step the detection in a fluorescent microscope (3) is carried out.

In reverse-phase antibody microarrays, as shown in Figure 5, the biological sample is arrayed onto the surface. For detection fluorescently labeled detector antibodies are incubated with the chip that subsequently is evaluated in a microscope. [32]
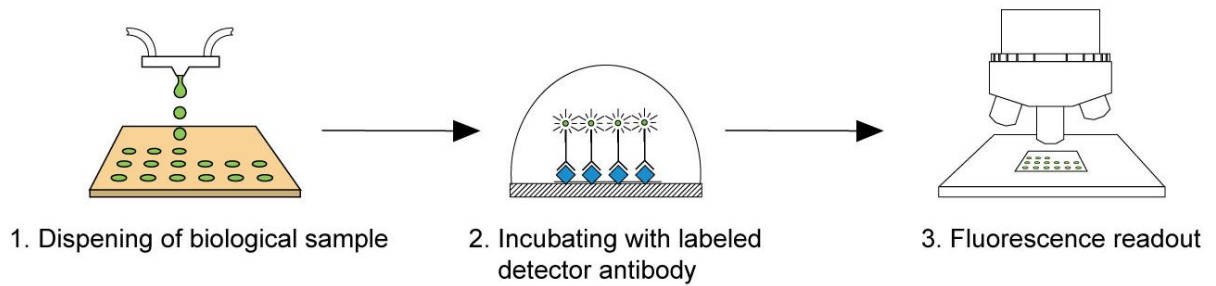

Figure 5 Reverse-phase antibody microarray [32]. Dispensing of the biological sample (1) is followed by washing and blocking steps before incubation with labeled detector antibody (2). More washing steps are performed and the detection is carried out in a fluorescent microscope (3).

The sandwich immunoassay, or sandwich antibody microarray, illustrated in Figure 6 has two different antibodies looking like a sandwich with the biological sample in the middle. The capture antibodies are dispensed onto the surface and incubated with the biological sample and then with the fluorescence labeled detector antibodies. The sample is detected in a fluorescent microscope. This method is more sensitive than the reverse-phase [32].
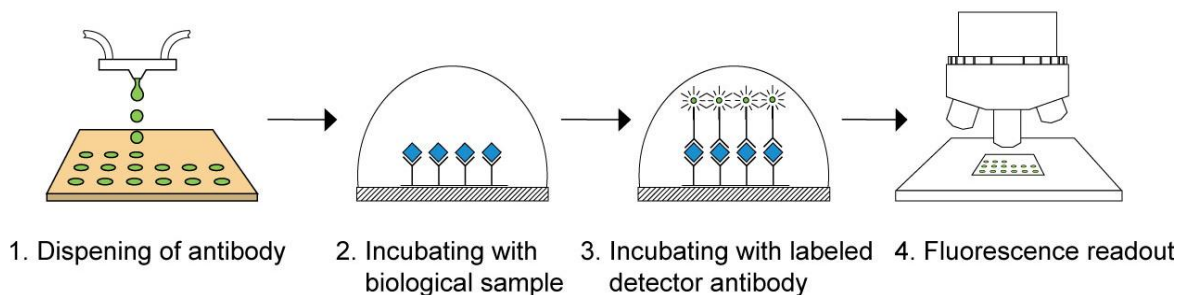

Figure 6 Sandwich immunoassay [32]. The capture antibodies are dispensed onto the surface (1), followed by blocking and washing steps prior to addition of the biological sample to the chip (2). After another wash the chip is incubated with labeled detector antibodies (3) in order to detect the proteins in a fluorescent microscope (4).

In the context of protein chip technology "Lab-on-a-chip" is a phrase worth noticing. It refers to a new technique where the laboratory work can be performed on an approximately square centimeter sized chip. The size of the chip means that smaller quantities of both analyte and biological sample can be used. This is an advantage in the medical sector, where often a limited amount of sample is accessible. There are also other positive effects like low fabrication and reagent costs and faster reaction times due to the low volumes.

1.5 Surfaces within Protein Chip Technology

The surfaces or substrates used within the field of protein microarrays require a big capacity to bind proteins without changing their functionality and construction. Three main groups of surfaces are: passively/physical adsorption, covalent coupling and affinity binding [33]. At the department of Electrical Measurements, we produce our own in-house developed porous silicon chips for protein chip technology. The porous surface enlarges the area offering a vastly increased analytical area.

1.6 Micro- and Nanoporous Silicon

Around 1957 Turner and Uhlir were the first to observe porous silicon [34, 35]. Since then porous silicon has gained interest in numerous applications such as medical therapy [36, 37], power technology [38, 39] and optical fibers [40]. As early as in 1996 the T. Laurell group produced porous silicon which was used to couple enzymes to the extended surface area. [41, 42] In 2002 the porous silicon surface was used as an immunoassay support for immobilization of antibodies. The antibodies bound HRP (horseradish peroxidase) labeled atrazine, which was detected by chemiluminescence. [43]

Anodization is a way to make the silicon chip porous. Silicon atoms can be dissolved from the surface in an electrochemical bath under electrostatic bias and/or illumination conditions. This is made in an in-house developed chamber as shown in Figure 7. The wafer is mounted with a liquid phase containing HF and a solvent on both sides and a lamp in front of the window. The charges will move across the bulk silicon and pores will form at the surface interface. Those pores will branch and develop nanostructures, see
Figure **8**. Figure 8 also describes a possible chemical reaction for dissolution of silicon.

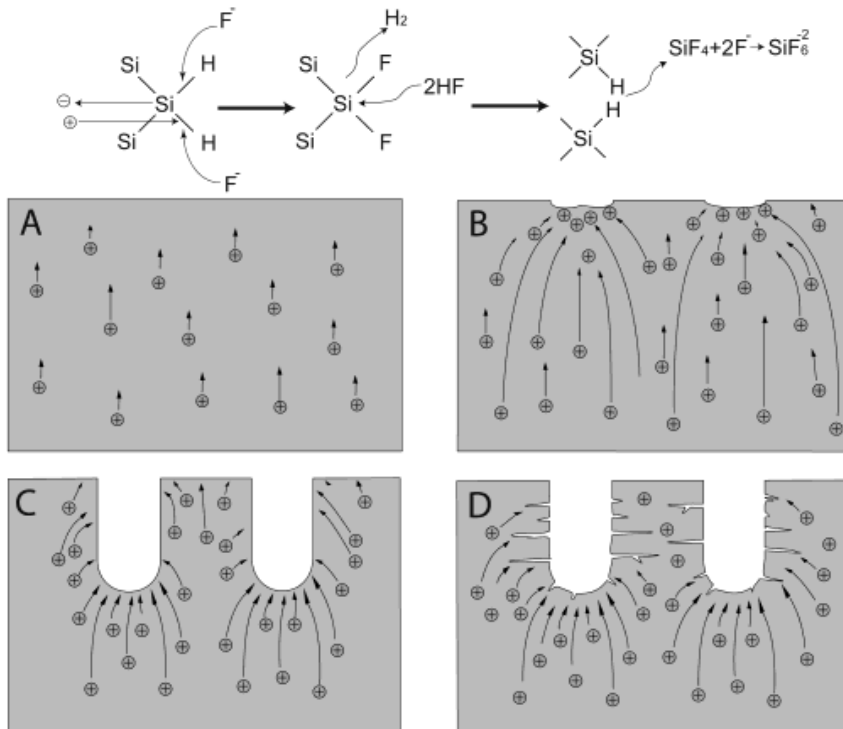**Figure 7** In-house developed chamber for anodization of silicon wafers.

Figure 8 Chemical pathway of silicon anodization and a schematic drawing of pore formation. Nano-/macro porous formation are created by migration of the charge in the silicon (A) followed by random nucleation (B) and then macropores form and propagate (C) and at last micropores forms branches. [44]

An interesting feature of porous silicon surfaces is that creation morphologies can display stringly hydrophobic behavior. Normally, when a droplet on a surface evaporates it leaves an outer ring of solid material. This phenomenon is called the coffee ring effect. [45] As the solvent evaporates from the drop, the contact angle is changed. Evaporation is more effective at the peripheral parts of the drop. The evaporative losses induce a convective transport inside the droplet outwards to the periphery of the droplet. The solid material in the drop is thus transported to the outer line where it crystallizes and a coffee ring effect can be seen in Figure 9. [12]
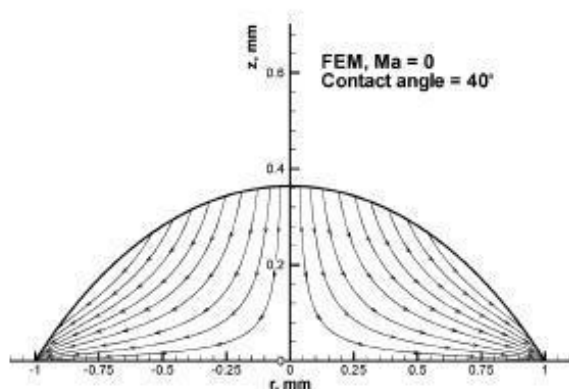

Figure 9 Transport flow of the solid particles in a drop [46]

The Lotus effect is a phenomenon where a droplet stays on a surface without wetting it. The surface behaves hydrophobically and the drop either stays on or rolls off the

surface. When the droplet lands on the surface it stays in drop form. The wetting of the surface can be more or less hydrophobic, thus the droplet touches the surface with a smaller or bigger contact area. This can be measured by the contact angle (θ), see Figure 10. If the surface is less hydrophobic, the droplet will spread out on the surface and the contact angle will be low, sometimes close to 0°. If the surface is more hydrophobic the angle can be up to 120° or more.
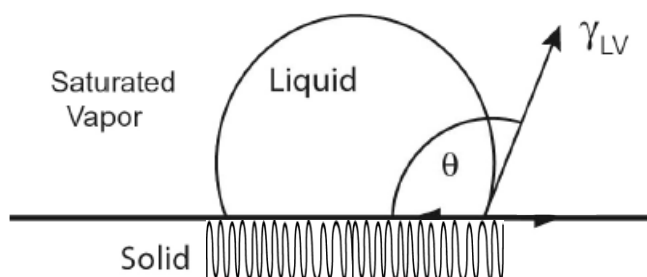

Figure 10 Contact angle (θ) of a drop on solid material with courtesy of K. Järås, Lunds University.

The surface chemistry of silicon is hydrophilic and proteins on the surface are prevented from denaturation as would have been the case on a material with hydrophobic chemistry. When the silicon is anodized the surface will however start to behave hydrophobically, corresponding to a high contact angle (θ) and a small contact area between the droplet and the chip. This is a clear surface profit within protein chip technology, since it increases the signal intensity and leads to improved limit of detection. In addition porous silicon chip reduces the coffee ring effect. [44] Comparisons have been made by Ressine et al between porous silicon surface and non porous glass slides.

1.7 Microdispenser

To deposit the capture antibody on the surface a spotter, also called arrayer, is required. At our department an in-house developed microdispensor is used [47]. The dispenser is made from a silicon plate with a nozzle to dispense the droplets. A piezo ceramic plate is pushing out the droplets. The dispenser can deposit droplets of approximately 100 pl. [47-49]

1.8 Streptavidin Alexa Fluor 488 and FITC-labeled Antibodies

To detect PSA on porous silicon chips, fluorescently labeled antibodies can be used. In this work two different approaches were addressed, one applying biotinylated anti-PSA antibodies and Streptavidin (SA) Alexa Fluor 488 conjugate, and the other utilizing FITC labeled anti-PSA antibody. Alexa Fluor 488 (Alexa 488) is exited at 495 nm while emitting at 519 nm and FITC excites at 494 nm and emits at 519 nm. A 488 nm argon laser was used to excite the fluorophores.

1.9 Epitope Map

On the Epitope map as shown in Figure 11 it can be seen which antibodies binds to which biomarkers (hK2 and various forms of PSA). To evaluate which monoclonal antibodies (mAb) that could be used for the assays in this work, the epitope map was studied, see Figure 11.
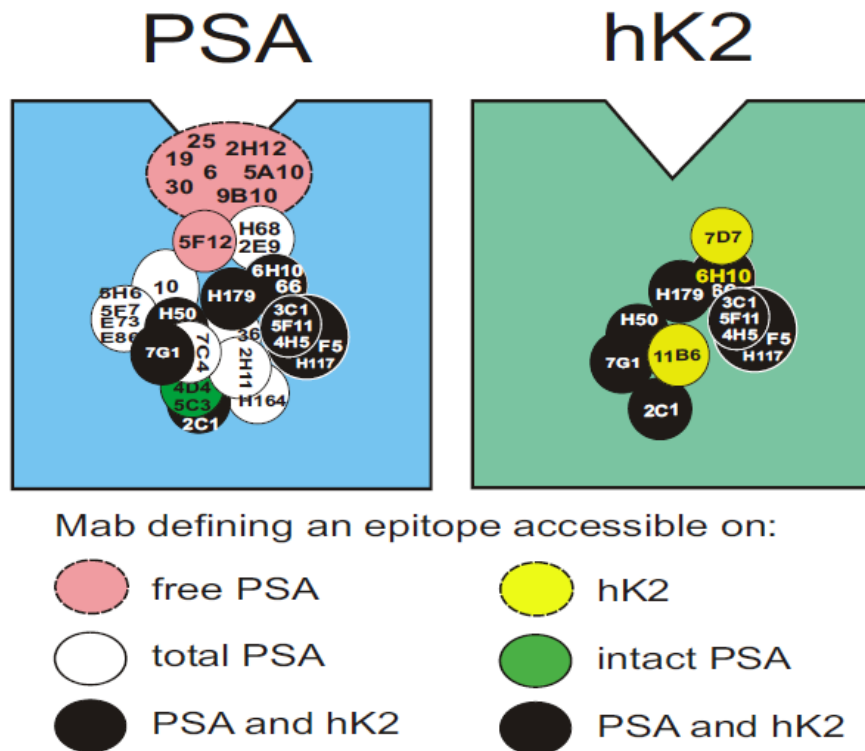

Figure 11 Epitope Map of PSA and hK2 showing which antibodies that bind the same epitope. With curtsey of Kim Pettersson's group, University of Turku, Finland [50]

1.10 Aims

Today in the clinical routine total and to some extent also free PSA are detected in the search for prostate cancer. The goal of this project was to try to improve the test in order to analyze different biomarkers simultaneously. First a dual approach was tried out, where one out of two chips was blocked. PSA free was blocked using unlabeled antibodies and the assay approach gave the relative concentrations of PSA total and free. In the second step, the multiplex approach, additional biomarkers were added to the same chip. The four markers tested were total PSA, free PSA, intact PSA and hK2 free.

In both steps different capture antibodies were dispensed onto a porous silicon chip in order to bind to different biomarkers in the biological sample. The assay was detected with a fluorescently labeled detector antibody using a confocal microscope.

2. Materials and Methods

2.1 Materials, Reagents and Proteins

The Addison Engineering double side polished silicon wafers used were p-type, boron-doped, with the crystal orientation 100 and resistivity 1-10 Ω cm. Hydrofluoric acid (HF), dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were obtained from Merck.

The 0.01M PBS buffer in powder form with pH 7.4 was bought from Sigma Aldrich. Milk powder and casein for blocking were obtained from Bio-Rad and albumin from Sigma Aldrich. PBST was made from 0.01M PBS and 0.05% tween 20. The mercoglas came from Merck and was provided by Malmö University Hospital (UMAS), Department of Pathology. Mouse IgG was obtained from Biodesign and ProStatus PSA Assay Buffer originally came from PerkinElmer but was kindly given by the staff at Clinical Chemistry, UMAS.

The antibodies H117, 2E9 and 5A10 were kindly given by Malmö University Hospital, Clinical Chemistry. The monoclonal antibodies were produced from mice, as earlier described. [10] The antibodies 4D4 and 11B6 produced from mice were kindly provided by Kim Pettersson's group at the Department of Biotechnology, University of Tukru, Finland. [50, 51] 11B6 F(ab)$_2$ was produced by enzymatic bromelain digestion of 11B6 mAb. [52] The F(ab)$_2$ fragments had a size of 95-110 kDa in comparison to 150 kDa for an intact antibody. 2E9-FITC was labeled in-house [32]. Streptavidin-Alexa 488 (SA-Alexa 488) was obtained from Invitrogen.

2.2 Samples from the Clinical Routine

Eight EDTA-plasma samples containing PSA and hK2 were taken from the clinical routine at Malmö University Hospital. The female plasma was obtained from a healthy volunteer. A titration series of 700, 70, 7 and 0.7 ng PSA tot/ml was made from one of the patient samples (original total PSA concentration of 3640 ng/ml) and the female plasma.

2.3 Fabrication of Nanoporous Silicon Chip

The fabrication was performed by anodic dissolution of a p-type, boron-doped silicon wafer. The silicon wafer was mounted in a two compartment cell, developed in-house, Figure 7. The electrolyte consisted of HF/DMF 1:10. A 100 W halogen lamp was staged 10 cm from the window of the back chamber. A constant current of 90 mA was applied to the wafer. The electrochemical etching took place for approximately 45 min. The wafer was diced into pieces sized 0.1380 x 0.1380 inches, equivalent to 3.5 x 3.5 mm, by a General Signal Micro Automation 602M saw.

2.4 Dialysis

The dialysis unit Slide-A-Lyser from Pierce with a molecular weight cut off (MWCO) 3.5 kDa, was soaked in water before use to remove glycerol from the membrane. To change buffer from Tris to PBS and to remove sodium azide (a bacteriostatic

preservative) of the antibody suspension the proteins were dialyzed in 500 ml 0.01M PBS at 4°C. The buffer solution was changed six times, and thereby the Tris buffer was theoretically diluted 75 000 times.

2.5 Biotinylation

N-Hydroxysuccinimidobiotin from Sigma Aldrich was dissolved in DMSO to the concentration of 3.14 mg/ml. The biotin solution was incubated with the antibody H117 which had the concentration 1.16 mg/ml. The molar ratio between biotin and H117 was 18.7. The room temperature incubation lasted 4 h and 20 min on end over end rotation. The solution was dialyzed (2.4 Dialysis) and the H-Hydroxysuccinimidobiotin was theoretically diluted 30100 times.

2.6 Dual Assay

The dual assay was performed to detect the total as well as the free PSA concentration. Half of all the chips were used to detect total PSA. The capture antibody H117 (0.5 mg/ml) was immobilized by physical adsorption on the silicon chip by the in-house developed piezoelectric microdispenser [47]. The volume of a dispensed droplet was approximately 100 pl, and the drops were arrayed onto the chip with a distance of 150 µm. To remove loose bindings the chips were washed three times in 10 ml, 5 ml and 5 ml PBST respectively, each 5 min on a shaker at room temperature (RT). The chips were put in wells of a 96 well plate to separate the reactions and make them easier to handle. Blocking was performed by incubating the chips in 5 % milk solution (nonfat dry milk diluted in PBST) to reduce unspecific bindings. The microarrays were subsequently washed with 150 µl PBST three times, 5 min at RT, 500 rpm. 30 µl of the biological sample was incubated on each chip for 60 min at RT, 500 rpm. Washing was performed with 150 µl PBST three times, 5 min at RT, 500 rpm. Each chip was incubated with 30 µl of the labeled detector antibody (33 µg/ml 2E9-FITC), for 120 min in the dark at RT, 500 rpm. The chips were washed with 150 µl PBST three times, 5 min at RT, 500 rpm, quickly dipped in MQ water and dried with pressurized air. The detection was performed in a confocal microscope (Olympus BX51WI) with the software Fluoview 300 and the following settings: PMT: 595, gain: 3.1, offset: 0%, emission: 510 nm and above, no filter, 20 x objective and 100% laser.

To detect free PSA, a second chip was run in parallel for each plasma sample, using a blocking antibody. When the first half of the chips were incubated with 2E9-FITC for 120 min as described above, the rest of the chips were blocked for the first 60 min with 0.5 mg/ml antibody 5A10 (free PSA specific antibody) and the last 60 min with 2E9-FITC. The antibody 5A10 was bound to the free PSA in the biological sample. By subtracting the intensity from the chip without 5A10 blocking with the chip blocked with 5A10, the signal corresponding to free PSA in the sample could be calculated.

2.7 Multiplex Assay

The multiplex approach aimed to detect total PSA, free PSA, intact PSA and free hK2 on the same chip. The capture antibodies 2E9 (0.7 mg/ml), 5A10 (0.7mg/ml), 4D4 (0.6 mg/ml) and 11B6 (0.6 mg/ml) were immobilized by physical adsorption on the silicon chip by the in-house developed chip-based piezoelectric microdispenser [47]. The volume of a dispensed droplet was approximately 100 pl, and the drops were arrayed

onto the chip with a distance of 150 µm. To remove loose bindings the chips were washed three times in 10 ml, 5 ml and 5 ml PBST respectively, each 5 min on shaker at room temperature (RT). Each chip was put in a well in a 96 well plate to separate the reactions and make it easier to handle. Blocking was performed by incubating the chips with 1 % casein or milk in PBS to reduce unspecific bindings. The chips were subsequently washed with 150 µl PBST three times, for 5 min at RT, 500 rpm. 30 µl of the biological sample was incubated on each chip for 60 min at RT, 500 rpm. Each chip was washed with 150 µl PBST three times, 5 min at RT, 500 rpm. Each chip was incubated with 30 µl of the detector antibody H117-biotin (5.65 µg/ml) in PBST with 1% casein, for 60 min at RT, 500 rpm. The microarrays were subsequently washed with 150 µl PBST three times, 5 min at RT, 500 rpm and then incubated with 30 µl SA-Alexa 488 (11.3 µg/ml in 0.01M PBS) for 30 min in the dark at RT, 500 rpm. Washing was performed with 150 µl PBST three times in the dark, 5 min at RT, 500 rpm. The chips were quickly dipped in MQ water and dried with pressurized air. Detection was performed in a confocal microscope (Olympus BX51WI) with the same settings in the software Fluorview 300 as described in section 2.6.

2.8 Mean Spot Intensity (MSI) and Limit of Detection (LOD)

Approximately 500 droplets were dispensed per chip. With the 20 x magnification roughly 5 x 5 spots could be viewed in the same microscope image. The whole chip was visually inspected and two to five images saved. The picture with the highest spot intensity was used to quantify the spots. Another criterion that the chosen image had to fulfill was a 3 times 3 spot area without air bubbles or scratches. In addition, to each of the 3 x 3 spots quantified by the circle method, the background, was measured next to the spot. The background area analyzed was the same as the area of the spot. Mean spot intensity was based on the nine spot intensities subtracted by the background respectively. Standard deviation and coefficient of variation (CV) were calculated for each chip.

Limit of detection was defined as the lowest analyte concentration generating a mean spot intensity equal to or above MSI of the negative control plus two standard deviations based on the measurement of the negative control.

2.9 DELFIA

ProStatus™ PSA Free/Total DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) from PerkinElmer, Turku, Finland was used as a reference assay. [53] To detect total and free PSA three antibodies were used in the sandwich assay. H117 captured both free and bound PSA and two antibodies differently fluorescence-labeled were used as detector antibodies. 5A10-Europium recognized free PSA and H50-Samarium detected total PSA by binding to both free and bound PSA. This method or a slightly modified version of it is today utilized in the clinical routine analysis at Malmö University Hospital.

To detect PSA intact 5A10 was used as a capture antibody and 4D4 which was europium labeled was used as a detector antibody. For free hK2 11B6 was used as a capture antibody and 6H10 as the detector antibody.

3. Results and Discussion

In this thesis four potential prostate cancer biomarkers from the kallikrein family have been addressed. Main focus has been assay development, and to show proof-of-concept for a multiplex approach on our porous silicon surfaces. Assay development has been performed to optimize different factors of the assays. The findings related to assay improvements are presented in section 3.1. To bring the thesis closer to a multiplex approach the first step was a dual assay performed on two chips, where PSA total and free were measured. In paragraph 3.2 the dual assay results from eight EDTA plasma samples obtained from patients undergoing clinical PSA testing are presented. The second step was to detect various biomarkers on one porous silicon chip, the so called multiplex approach. Separate titration series of the four biomarkers as well as a final proof-of-principle detection of several biomarkers on the same chip are shown in section 3.3.

3.1 Assay Development

In order to get the laboratory work optimized a number of factors needed to be evaluated, such as dispenser and etching, blocking, background and spot focus.

3.1.1 Dispenser and Etching

When the antibodies were dispensed onto the porous silicon chips it was important to achieve homogeneous droplet formation without satellite effects. Homogeneous droplet formation is a prerequisite for adequate spot detection and comparison of signal intensity between the chips. The first chip batch produced gave rise to very high hydrophobicity of the surfaces. During dispensing the droplets bounced away from the porous silicon layer of approximately every second chip. The superhydrophobic chips had to be replaced by less hydrophobic ones from the same batch. In the second chip batch produced, the etching time was reduced to 40 minutes and the surfaces were slightly less hydrophobic and therefore gave no bouncing effect. The hydrophobicity did not only depend on the etching time but also on the resistivity of the chip batch in question.

The satellite effect shown in Figure 12 could be avoided by changing the voltage over the dispenser. The optimal settings were approximately 4 V on the Frequency Generator and 15 V on the Voltage Regulator.
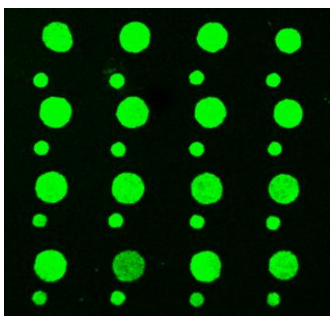

Figure 12 Satellite effect. Small unwanted droplets between the dispensed droplets.

3.1.2 Blocking

To avoid unspecific binding of the antibodies, blocking was performed after dispensing the capture antibody. Three different blocking agents were tested: 5% milk, 1% casein and 5% albumin. The assay was performed as described in section 2.7. The total PSA concentration was analyzed for one of the EDTA-plasma samples (31.1 ng PSA total/ml). The data for each blocking agent was based on two chips treated equally. On each chip two images were selected and on each image the background were measured three times. The background area was based on a square with the following dimensions: x= 400μm, y=50μm.

In Table 1 the mean of the background intensities are shown for the blocking agents milk, casein and albumin. Casein had the lowest mean and in addition the lowest CV and had thereby blocked the most. Milk was the second best alternative but was delivered in powder form and sometimes harder to prepare because it was not solubilised as the casein. Casein was used as an easy and good blocking agent in the rest of the assays described in this thesis.

Table 1: Background intensity results for blocking optimization.

Chip	Background Intensity	CV n=12
Milk	$7.6 \cdot 10^6$	0.20
Casein	$7.2 \cdot 10^6$	0.05
Albumin	$9.9 \cdot 10^6$	0.16

3.1.3 Background

In order to avoid background intensity caused by salt formation of the buffer the chips were dipped in MQ water for five seconds and dried with pressurized air. Even though this was a procedure used previously, an optimization was made and two other methods were tried out. The first was to not dip the chip into MQ water but dry with pressurized air. The second technique was to neither dip the chip into MQ water nor dry with pressurized air, instead directly measure in the microscope. The background optimization was mainly developed for the intact PSA assay with the antibody 4D4, an antibody sensitive to washing. By evaluating the background using 4D4 it is made sure that the quick dip in water does not reduce the spot intensity. The EDTA-plasma from the clinical routine with concentration 31.1 ng PSA total/ml was used in the analysis.

The different methods gave high spot intensity on all three chips. The results for the MSI and the background measurements can be seen in Table 2. The background was measured the same way as in 3.1.2 except that only one chip per method was performed. As anticipated the chip dipped in MQ water and dried had the lowest background value, see Table 2. The chip which was only dried had a high homogenous background and the chip directly measured had a more heterogeneous and stained background. The method with the lowest background had unfortunately slightly lower MSI but the low background value was to prioritize to not jeopardize the LOD.

Table 2 Results for background minimization using the three different dipping and drying procedures.

Method	MSI	CV n=9	Background intensity	CV n=6
MQ + dry	$18.0 \cdot 10^6$	0.07	$4.3 \cdot 10^6$	0.09
dry	$18.5 \cdot 10^6$	0.08	$8.9 \cdot 10^6$	0.12
nothing	$18.8 \cdot 10^6$	0.14	$9.5 \cdot 10^6$	0.41

3.1.4 Spot Focus

Microscopic measurements were performed with oil immersion. A drop of MQ water was placed on the chip under the cover glass prior to detection. The cover glass must lie flat to bring focus on nine spots simultaneously. Difficulties arose when lifting the cover glass to place it parallel to the small chip beneath. In addition the intensity is reduced when lifting the glass extensively, therefore two lifts were considered maximum. Instead of MQ water Mercoglas was tried under the cover glass.

Mercoglas was a poor option since it is a polymer reflecting light from the laser and made the whole chip glow. Instead of Mercoglas MQ water was used in an in-house produced Plexiglas slide with an indentation for a chip. This arrangement prevented the cover glass from tilting as much and the focus could be brought to nine spots or more simultaneously. The only reason to lift the cover glass with this method is to get rid of air bubbles which maximally takes two lifts.

3.2 Dual Assay

On the way towards a multiplex approach the dual assay was developed. This assay, in which PSA total and free are detected, is based on two chips (Figure 13). The same capture and detector antibodies, H117 and 2E9-FITC respectively, are used in both microarrays. On one of the chips the signal of PSA free is blocked by addition of the antibody 5A10. The mean spot intensity for PSA total is measured on the first chip. The MSI of PSA free is calculated as the MSI difference between the first and the second chip.
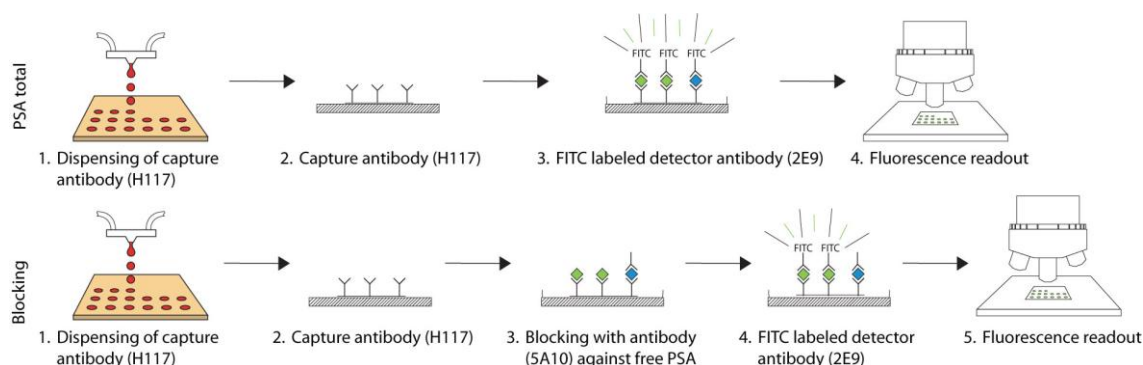

Figure 13 Dual assay procedure. Two chips (upper and lower row respectively) are used simultaneously. On the lower one the PSA free is blocked before detection. The chips are first dispensed with capture antibody H117 (1) followed by washing and blocking steps. Then the lower chip is blocked against PSA free with antibody 5A10 (3 lower) and subsequently incubated with the FITC labeled detector antibody 2E9 (3 upper and 4 lower). The last step, after yet another washing, is the fluorescent readout. The upper chip generates the total PSA signals, and signal intensities of upper chip minus lower is proportional to the PSA free concentration.

The dual assay approach was evaluated with eight EDTA-plasma samples from the clinical routine. All samples were diluted to get a final concentration of 10-20 ng/ml in the dual assay. Table 3 shows the results from the DELFIA and microarray measurements. CV was calculated based on 9 adjacent array spots. The results are also visualized in the graph of Figure 14, in which mean spot intensity is plotted against PSA concentration as measured by the DELFIA. Note that both MSI of PSA tot and PSA free are plotted against total PSA concentration by the DELFIA. In this way the results of free and total PSA are shown beneath each other, indicating that they belong to the same analysis. In all of the eight plasma samples except for sample number two, the mean spot intensity was higher for PSA total compared to the blocked chips, thereby establishing a successful shielding by 5A10 antibodies.

Table 3 Dual assay. DELFIA and MSI results with CV for total and free PSA

Sample	PSA total DELFIA (ng/ml)	MSI*	CV n=9	PSA free DELFIA (ng/ml)	MSI*	CV n=9
1	3640	$7500 \cdot 10^6$	0.08	329	$3100 \cdot 10^6$	0.20
2	2260	$770 \cdot 10^6$	0.11	326	$-16 \cdot 10^6$	-6.40
3	907	$400 \cdot 10^6$	0.18	152	$200 \cdot 10^6$	0.34
4	88.5	$38 \cdot 10^6$	0.26	31.3	$9.7 \cdot 10^6$	0.87
5	85.7	$82 \cdot 10^6$	0.16	12.4	$52 \cdot 10^6$	0.25
6	80.7	$82 \cdot 10^6$	0.11	10.9	$32 \cdot 10^6$	0.31
7	31.1	$5.6 \cdot 10^6$	0.09	8.52	$2.8 \cdot 10^6$	0.22
8	20.3	$3.8 \cdot 10^6$	0.09	4.02	$0.94 \cdot 10^6$	0.46

* The measured MSI was multiplied with the dilution factor generating the MSI presented in the table.

In sample number two the cover glass on the PSA total chip was lifted more than twice to adjust it horizontally and by that the PSA free value got negative. This experiment was performed before the glass slide with the indentation was used. In Figure 14 the free PSA value is missing because it is not possible to logarithm a negative value.

All samples, except for number two and four, decreased successively in MSI with decreasing total PSA concentration as measured by DELFIA. The MSI for PSA total and free on sample number four had lower intensities and the corresponding CVs were raised. This could have been caused by a number of things, such as moving cover glass extensively, laboratory errors or possibly dilution errors. The same titration pattern appeared with PSA free but here also a divergent value on sample number two was present.
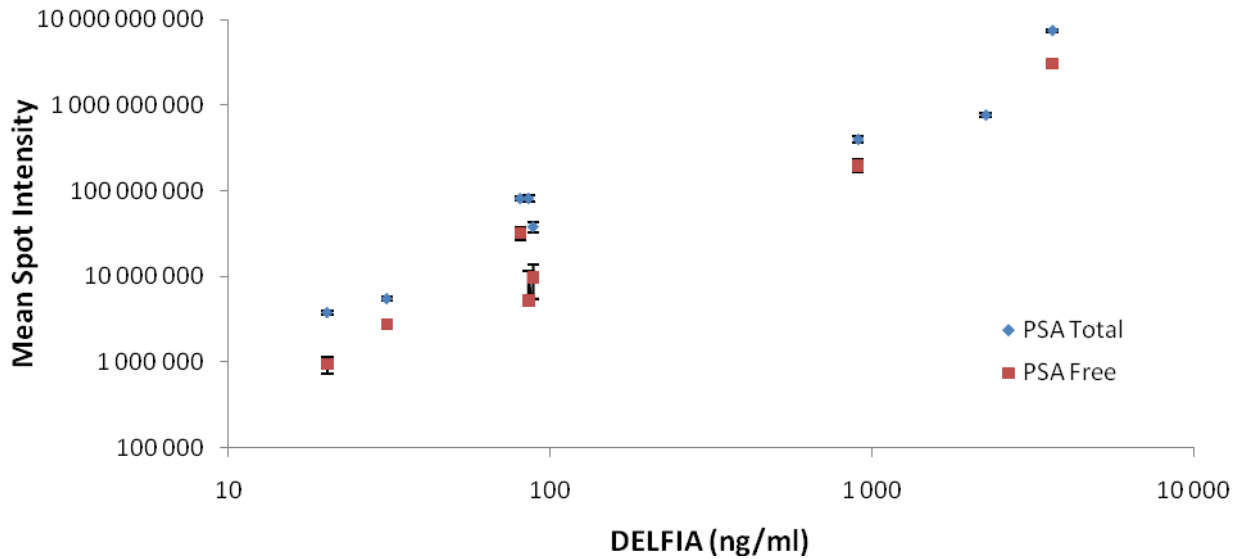
Figure 14 MSI results for total and free PSA with standard deviations are plotted against the DELFIA results for total PSA. Note that both axes are logarithmic.

In the future the aim is to make a true quantitative approach to measure total and free PSA in a dual assay. This requires a standard curve which is run together with the plasma samples to be analyzed. Since the MSI of the total and free PSA measurements are clearly separated from each other the possibility of developing a true quantitative dual assay seems reasonably high.

3.3 Multiplex Assay

The aim of the multiplex assay was to detect four different biomarkers on one porous silicon chip simultaneously as shown in Figure 15. To achieve the goal the antibody couples for total PSA, free PSA, intact PSA and free hK2, see Table 4, were evaluated one by one before a possible combination on the same chip. With use of the Epitope Map, see Figure 11, the capture antibodies in Table 4 were selected. To detect all biomarkers simultaneously on one chip the detector antibody should preferably be the same. The detector antibody was biotinylated and coupled to Streptavidin-Alexa 488 to be detected in the confocal microscope.
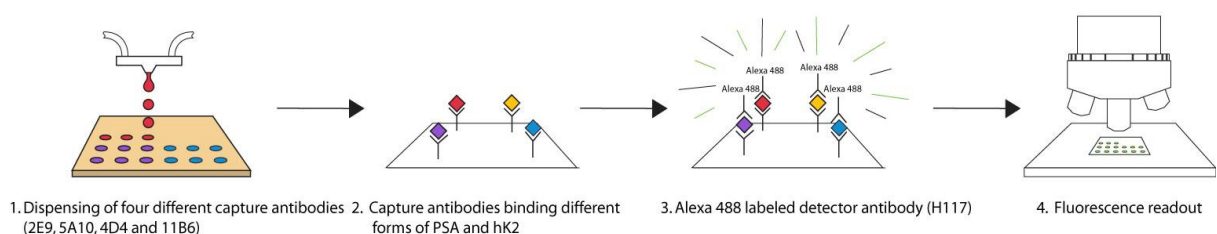

1. Dispensing of four different capture antibodies (2E9, 5A10, 4D4 and 11B6)
2. Capture antibodies binding different forms of PSA and hK2
3. Alexa 488 labeled detector antibody (H117)
4. Fluorescence readout

Figure 15 Fourplex assay. The four different capture antibodies (2E9, 5A10, 4D4 and 11B6) are arrayed onto the chip (1). After a blocking step and washing the biological sample is added (2), different forms of PSA and hK2 bind to their specific antibodies. The detector antibody H117-biotin and streptavidin-Alexa-488 are added (3) and then detected in the fluorescent microscope (4).

Table 4 Capture and detector antibody couples used in the assays

Biomarker	Capture ab	Detector ab
PSA total	2E9	H117
PSA free	5A10	H117
PSA intact	4D4	H117
hK2 free	11B6 F(ab)$_2$/mAb	H117

3.3.1 Total PSA

Total PSA was analyzed by capture antibody 2E9 and detector antibody H117 as illustrated in Figure 16. H117 was biotinylated and coupled to SA-Alexa 488. In Figure 17 the MSI for total PSA in the dilutions series ranging from 0.7 to 700 ng/ml PSA total is plotted against the results of the reference assay. The mean spot intensity increased with increasing PSA concentration as measured by the DELFIA. The MSI of the highest PSA concentration was not linear with the other three MSI values plotted. The reason is the exceptionally high analyte concentration above the upper detection limit of the confocal microscope. As a control female plasma was analyzed and no signals could be detected (data not shown). LOD of the assay was 0.7 ng/ml.
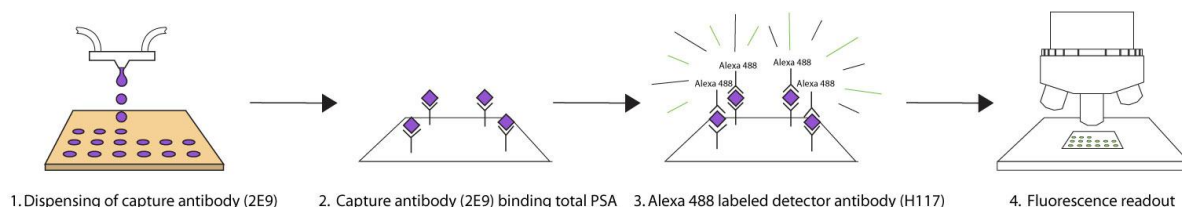

1. Dispensing of capture antibody (2E9)   2. Capture antibody (2E9) binding total PSA   3. Alexa 488 labeled detector antibody (H117)   4. Fluorescence readout

Figure 16 Total PSA detection. Dispensing of capture antibody 2E9 (1) and incubation of the biological sample (2) before detection by the detector antibody H117-biotin coupled to streptavidin-Alexa-488 (3) in the fluorescent microscope (4).
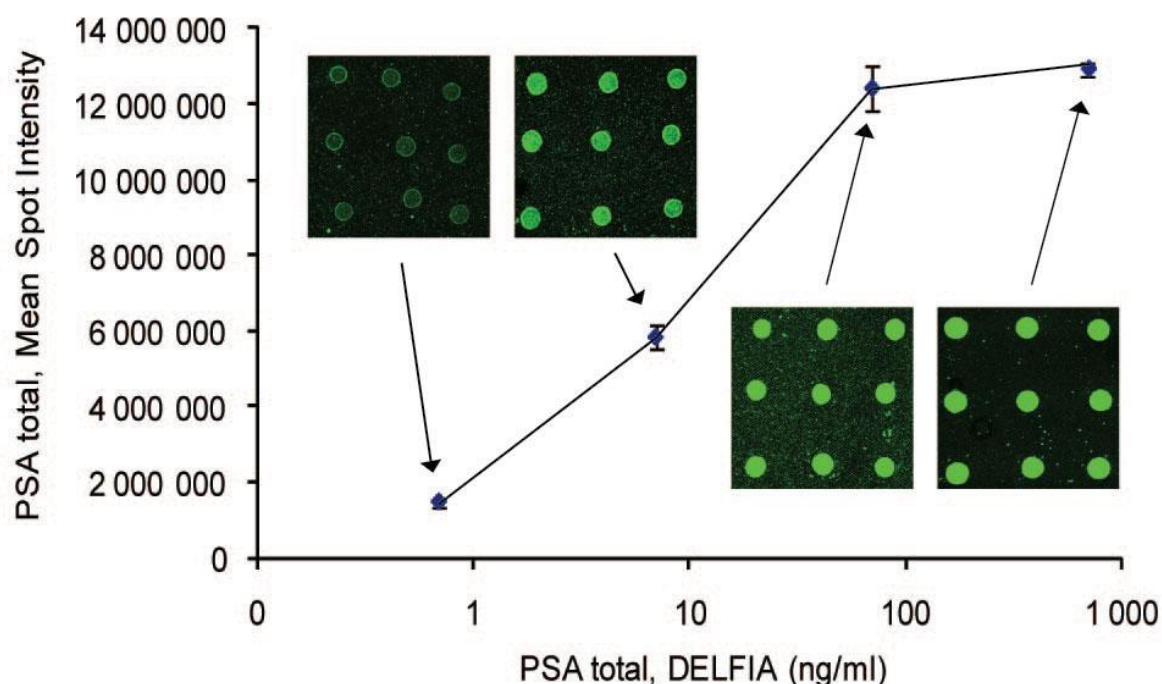

Figure 17 MSI results of total PSA assay plotted against DELFIA values from UMAS. The error bars represent the standard deviations calculated from nine inset images.

3.3.2 Free PSA

Free PSA was analyzed by capture antibody 5A10 and detector antibody H117-biotin, which was coupled to SA-Alexa 488 to be detected (Figure 18). In Figure 19 the results from the dilutions series 0.7-700 ng/ml total PSA is shown. The x-axis displays the DELFIA results and the y-axis the MSI with standard deviations included. The MSI increased with the increasing DELFIA values as shown in Figure 19. The DELFIA result for the lowest concentration was 0.05 ng/ml. This concentration seemed to be below detection limit of our microarray and could unfortunately not be detected. As a control sample female plasma was analyzed and no signals could be detected (data not shown). Limit of detection for the assay was 4.7 ng/ml. Spots were visible on the concentration for 0.5 ng/ml see Figure 19, but the high background generated an unfavorable limit of detection.
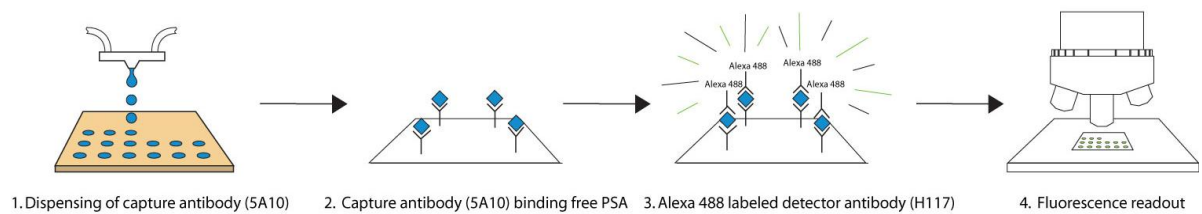

1. Dispensing of capture antibody (5A10)   2. Capture antibody (5A10) binding free PSA   3. Alexa 488 labeled detector antibody (H117)   4. Fluorescence readout

Figure 18 Free PSA detection. The detector antibody 5A10 was spotted onto the chip (1) followed by incubation of the biological sample (2) and at last detection by fluorescently labeled (streptavidin-Alexa-488) detector antibody H117-biotin (3) in the microscope (4).
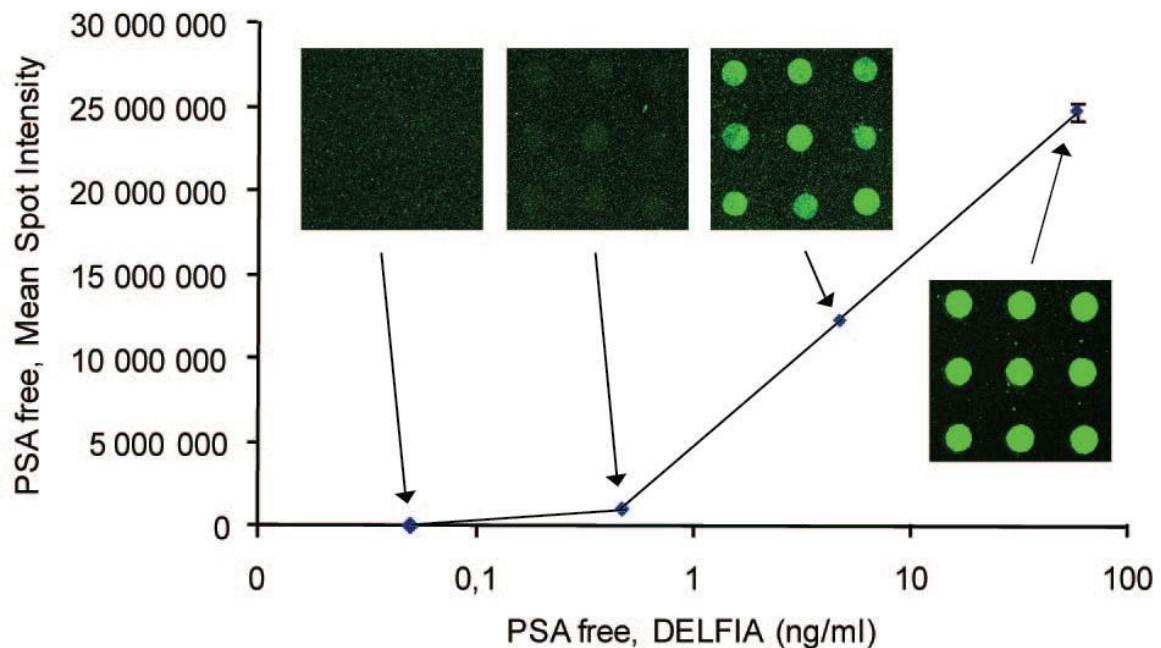

Figure 19 MSI results plotted against DELFIA values for PSA free. Standard deviations were calculated from the nine inset images.

3.3.3 Intact PSA

Intact PSA was captured with the antibody 4D4 and detected with the antibody H117-biotin and SA-Alexa 488 as illustrated in Figure 20. As shown in Figure 20, intact PSA was difficult to detect straightforward with the antibodies selected. The titration series with PSA total concentration of 0.7-700 ng/ml gave rise to low signals and no differences in MSI for the four plasma samples, see Figure 21. The MSI was only based on 1-3 spots and thereby no standard deviations are shown. On one of the chips only 2 out of approximately 500 spots were visible. The two lowest PSA intact concentrations in the titration series were not detectable by the DELFIA either.

It turned out that the antibody 4D4 had a high k_{off} and thereby probably the antigen dissociated from the antibody in the many washing steps. By incubating without washing after addition of the biological sample the bindings were maintained. Only one washing step before detection and the quick dip in MQ water were kept. This improvement gave rise to a higher signal (data not shown). To make sure that the quick dip in MQ water did not affect the binding of PSA the background optimization as described in 3.1.3 was performed with 4D4 analyzing intact PSA.
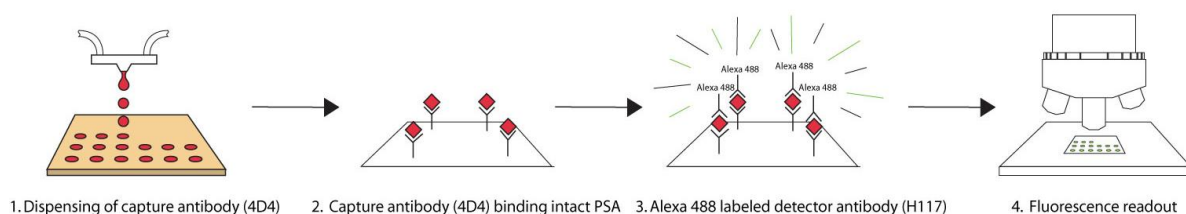

1. Dispensing of capture antibody (4D4)   2. Capture antibody (4D4) binding intact PSA   3. Alexa 488 labeled detector antibody (H117)   4. Fluorescence readout

Figure 20 PSA intact detection. Spotting of capture antibody (1) subsequent incubation with biological sample (2) detection with SA-Alexa 488 and detector antibody H117-biotin (3) in the confocal microscope (4) are performed.
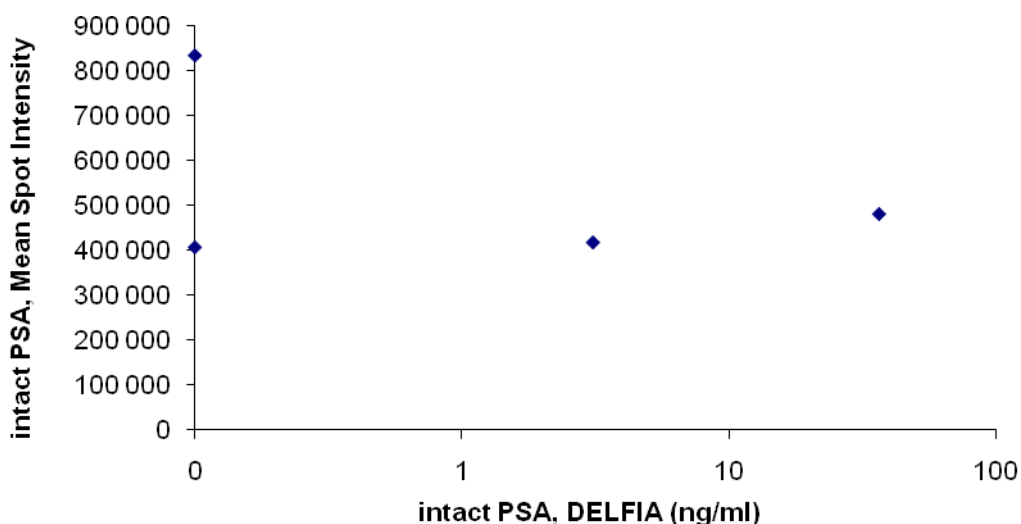

Figure 21 PSA intact results. MSI measurements plotted against DELFIA assay concentrations.

3.3.4 hK2 free

To detect hK2 free both 11B6 F(ab)$_2$ and 11B6 mAb were tested as capture antibodies and H117-biotin-SA-Alexa 488 was used for detection (Figure 22). The titration series with PSA total concentrations of 0.7-700 ng/ml was used together with a negative control consisting of female plasma.

The DELFIA could not measure any hK2 in the two biological samples with lowest hK2 concentrations, since the concentration was below the LOD of the assay. All of the microarrays had extremely high mean spot intensity with F(ab)$_2$ as capture antibody, see Figure 23. Even the negative control sample was extremely high (data not shown). The MSI was based on only 1-3 spots therefore no standard deviations could be calculated. Using monoclonal antibodies as capture molecule gave similar result. The antibodies had been frozen prior to use but have proved to perform well in other situations. The assay was later run with both PSA Assay Buffer and mouse IgG to remove heterophilic antibodies, i.e. antibodies binding to the Fc region, and human-α-mouse-antibody respectively. Neither of those methods got rid of the false signal. It should be noted that only one female plasma donor was tried out, and that both the negative control as well as the titration series (0.7-700 ng/ml tot PSA) contained this female plasma.
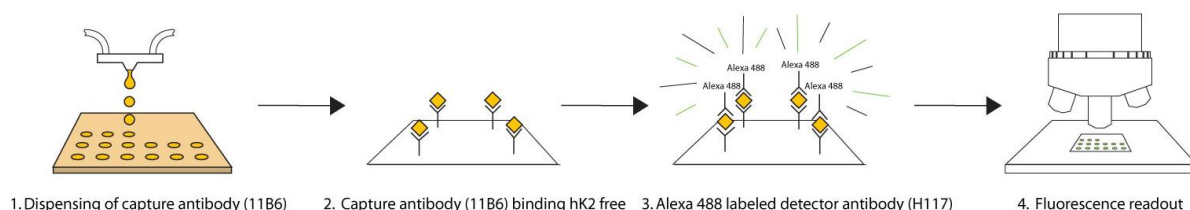

1. Dispensing of capture antibody (11B6) 2. Capture antibody (11B6) binding hK2 free 3. Alexa 488 labeled detector antibody (H117) 4. Fluorescence readout

Figure 22 Free hK2 detection. The capture antibody 11B6 was dispensed onto the porous silicon chip (1) and then incubated with the biological sample containing hK2 (2). Detector antibody H117-biotin and streptavidin-Alexa-488 (3) were used to measure the intensity in the fluorescence readout system (4).
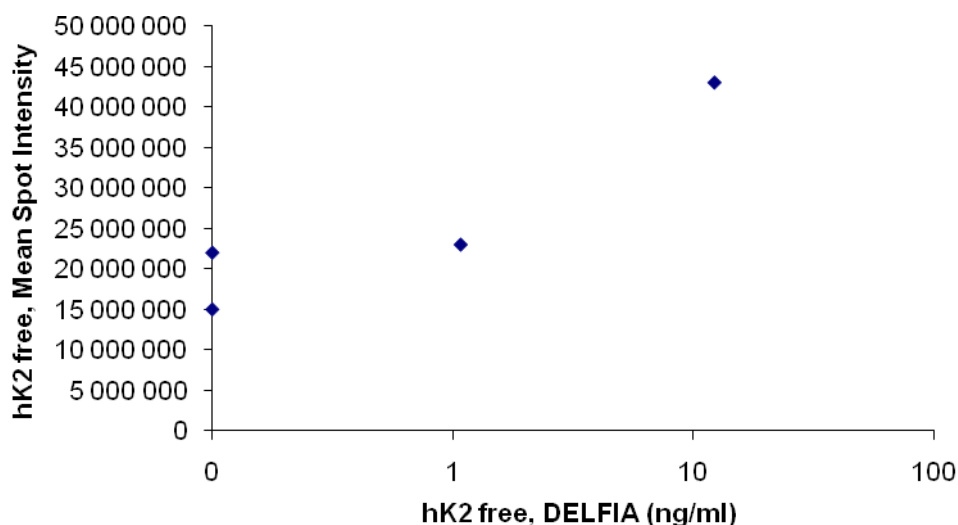

Figure 23 hK2 free results for the MSI measurements plotted against the DELFIA reference assay.

3.3.5 Triplex

Three of the four potential antibody couples generated promising results according to the data presented above. hK2 needs to be evaluated more before usage in a multiplex approach. PSA total, free and intact were now put together to a multiplex approach. The capture antibodies 2E9, 5A10 and 4D4 for PSA total, free and intact respectively were dispensed on the same chip as shown in Figure 24. The titration series with the concentration of 0.7-700 ng/ml PSA total and a negative control were added to five different chips. Only one washing step and the quick dip in MQ water was performed after the biological sample was added, due to PSA intact, as described in 3.3.3.
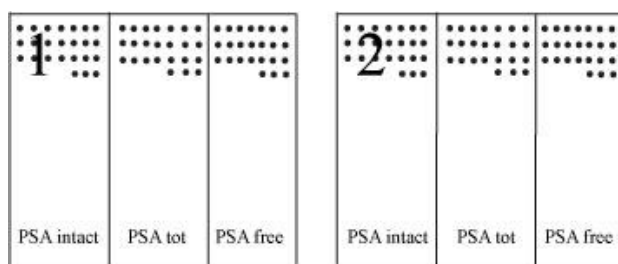

Figure 24 Triplex spotting design on the chips sized 3.5 x 3.5 mm.

All three biomarkers were successfully detected in the fluorescence microscope for all concentrations in the dilution series except the lowest concentration for PSA free, as shown in Table 5 and Figure 25. The triplex assay gave three increasing titration curves with increasing DELFIA values, see Figure 26. The different PSA forms are all plotted against the DELFIA value for the PSA total concentration. In this way the results of total, free and intact PSA are shown beneath each other, indicating that they belong to the same analysis. The intensity corresponding to a PSA total concentration of 700 ng/ml was so high that it superseded the detector in the confocal microscope. The two highest concentrations 70 and 700 ng/ml got similar values even though a clear difference could be seen by visual inspections of the screen. Since the background intensity, rising with analyte concentration, did not reach the top altitude it subtracts the intensity value of the 700 ng/ml substantially generating a lower MSI than the concentration of 70 ng/ml. Therefore artifacts with decreased intensities at the highest analyte concentrations are present in Figure 26.

Table 5 Triplex assay. DELFIA and MSI results with CV for total, free and intact PSA

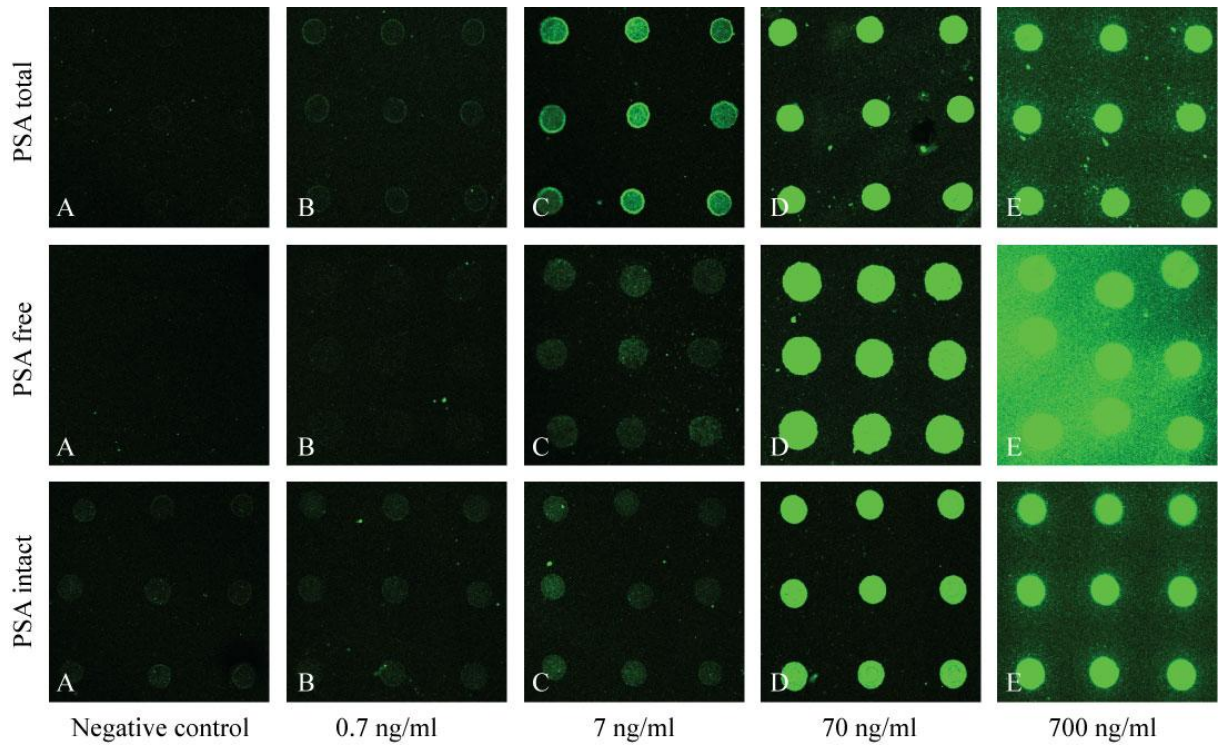|	PSA total			PSA free			PSA intact		
	DELFIA (ng/ml)	MSI	CV	DELFIA	MSI	CV	DELFIA (ng/ml)	MSI	CV
0.7	630	$0.46 \cdot 10^6$	0.21	57	$0.45 \cdot 10^6$	0.30	36	$0.62 \cdot 10^6$	0.12
7	66	$5.1 \cdot 10^6$	0.25	4.7	$1.8 \cdot 10^6$	0.31	3.1	$1.4 \cdot 10^6$	0.29
70	6.3	$15 \cdot 10^6$	0.04	0.47	$37 \cdot 10^6$	0.04	-	$14 \cdot 10^6$	0.10
700	0.76	$12 \cdot 10^6$	0.18	0.05	$21 \cdot 10^6$	0.19	-	$17 \cdot 10^6$	0.07

Figure 25 Images from the fluorescence microscope of PSA total, free and intact at the different PSA concentrations. A-images represent the negative control and B-E are the dilution series 0.7-700 ng PSA total/ml. At the images E the concentration are over the upper LOD of the samples.
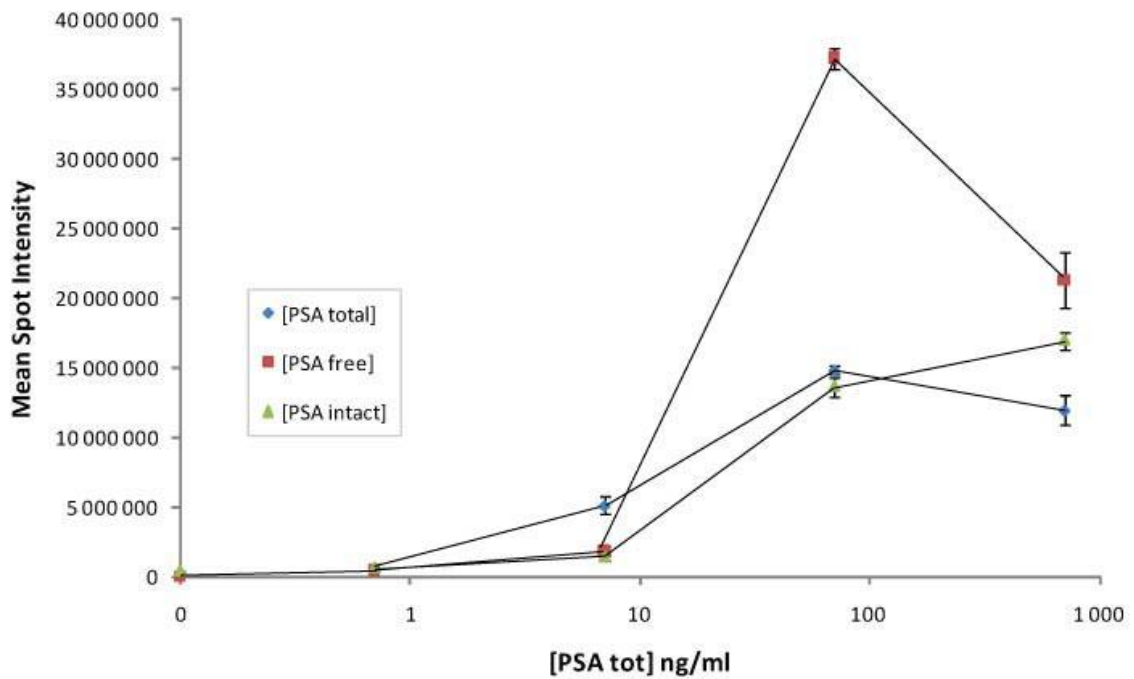

Figure 26 Triplex results. MSI plotted against DELFIA results. The values in origo represent the negative female plasma.

Unfortunately a low signal was detected from the negative control (female plasma) on PSA intact and PSA total. This probably has to do with the fact that many of the washing steps were removed due to the high k_{Off} of 4D4. If 4D4 can bind to PSA intact even though a slightly more stringent washing is performed the faint signals of the negative controls could probably be washed away.

The lowest analyte concentrations in the dilution series for PSA total and intact and the second lowest for PSA free could successfully be detected (Table 6). LODs for the different PSA forms in the triplex assay can be seen in Table 6. It should be noted that LOD for PSA intact is as low as 0.03ng/ml. In addition the analyte concentrations could possibly be titrated a bit further to improve the LODs even more. It is likely that the LODs could be improved in this thesis compared to earlier work at the department, since there is a possible signal amplification effect, due to the biotinylation of H117. The more biotin coupled to the detector antibody, the more streptavidin-Alexa 488 could be bound and the signal will be amplified.

Table 6 LOD for PSA total, free and intact in the triplex assay

|	LOD (ng/ml)
PSA total	0.8
PSA free	0.5
PSA intact	0.03

4. Conclusions and Further Research

The detection of PSA as well as other PCa biomarkers can be scaled down on microarrays and used in the clinical diagnostics and prognostics. The miniaturized technique entails lower consumption of biological samples as well as reagents. Järås et al made a study in 2007 [32] on different miniaturized microarray techniques on 3D porous silicon chip and concluded that sandwich assays are the best choice. Because PSA not only codes for prostate cancer but also for example BPH, various markers need to be addressed to achieve a better screening for prostate cancer. In this thesis the first steps towards a multiplex sandwich approach has successfully been demonstrated. The first goal, to make a dual assay to measure PSA total and free was accomplished. In the future this assay aims to make a true quantitative approach to measure PSA total and PSA free. The second aim of the thesis, to show proof-of-principle of a multiplex approach, was also fulfilled. Three different forms of PSA were measured simultaneously on one porous silicon chip. Further optimization needs to be done regarding the washing steps. The assay for hK2 needs to be investigated in detail in order to try to extend the microarray from a triplex to a fourplex assay. In the future other suitable biomarkers connected to prostate cancer can be tried out and added to the multiplex assay.

5. Populärvetenskaplig sammanfattning på svenska

Prostatacancer är den vanligaste formen av cancer för män i väst-världen. Sjukdomen är åldersrelaterad och tre fjärdedelar av de drabbade är 65 år eller äldre. För att diagnostisera prostatacancer kan en markör i blodet mätas. Denna markör, kallad prostataspecifikt antigen (PSA), uttrycks i större mängd i blodet vid prostataförändringar. För att kunna hitta prostatacancer och skilja sjukdomen från bland annat godartad prostataförstoring skulle fler markörer kunna detekteras samtidigt.

Inom vården idag mäts nivåerna av den totala mängden PSA och ibland även fritt PSA. Fritt PSA är en del av den totala mängden PSA och består av PSA som inte är bundet till något annat protein. Mätningarna idag skiljer inte tillfredställande på godartat prostataförstoring och prostatacancer vilket behöver förbättras. I detta examensarbete har fyra markörer, även kallade analyter, undersökts i ett miniatyriserat format. De fyra markörerna som undersöktes var förutom totalt och fritt PSA, intakt PSA, vilket hittas i både det fria och bundna formerna av PSA, samt humant kallikrein 2 (hK2) som till hör samma familj som PSA.

För att mäta koncentrationen av en sorts analyt i blodet användes två olika antikroppar. Markörerna i blodprovet fångades upp med hjälp av antikroppar som var fästa på ett poröst kiselchip med storleken 3,5 gånger 3,5 mm likt bilden nedan. Kiselchipen utgör en bra fästyta och är egenutvecklade. För att detektera hur många analyter som fastnat på antikropparna användes en annan antikropp som var fluorescensmärkt, och således kunde mängden markörer mätas i ett fluorescensmikroskop.

1. Första antikroppen fästes   2. Markörerna fångas upp   3. Andra antikroppen fäster in   4. Mätning i mikroskop
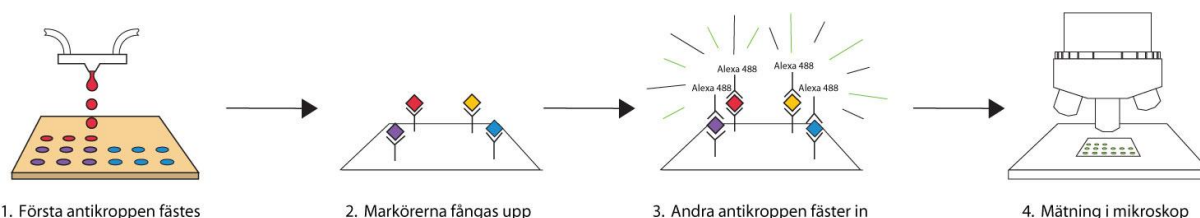
För att kunna fånga upp de olika markörerna på ett och samma chip behövs olika antikroppar, var och en specifik mot enbart en av analyterna. För att detektera de bundna biomarkörerna behövs en annan antikropp som har bindningsförmåga till alla markörerna och som är märkt med en, i ett mikroskop lysande, molekyl. Var och en utav dessa antikroppspar med tillhörande biomarkör testades för sig innan de sammanförandes på ett enda chip.

Det visade sig att antikroppsparet för hK2 inte fungerade tillfredställande och måste utvärderas mer innan det går att använda. De tre andra antikroppsparen för markörerna totalt PSA, fritt PSA och intakt PSA fungerade efter olika optimeringar bra och kunde således samtidigt mätas på samma chip i en såkallad triplex modell.

6. Acknowledgements

7. References

1. Parkin, D.M., et al., *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.
2. Abrahamsson, P.-A., ed. *Prostatacancer - Information för patienter och anhöriga.* 2003.
3. van Gils, M.P.M.Q., et al., *Innovations in serum and urine markers in prostate cancer current European research in the P-Mark project.* European Urology, 2005. **48**(6): p. 1031-1041.
4. Sävblom, C., *The Kallikrein-Related Peptidases hK2 and PSA with Emphasis on Genetic Variation, Secretion, and Sperm Motility*, in *Faculty of Medicine* 2008, Lunds University: Lund.
5. *Prostate UK.* [cited 25.11.09]; Available from: http://www.prostateuk.org/prostate/aboutprostate.htm.
6. Kumar, A., et al., *Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2.* Cancer Research, 1997. **57**(15): p. 3111-3114.
7. Scardino, P. and K. Slawin, eds. *Atalas of the Prostate.* 2006, Springer-Verlag New York, LLC.
8. Stamey, T.A., et al., *Prostate-Specific Antigen as a Serum Marker for Adenocarcinoma of the Prostate.* New England Journal of Medicine, 1987. **317**(15): p. 909-916.
9. Lundwall, A. and H. Lilja, *Molecular-Cloning of Human-Prostate Specific Antigen Cdna.* Febs Letters, 1987. **214**(2): p. 317-322.
10. Lilja, H., et al., *Prostate-specific antigen in serum occurs predominantly in complex with alpha 1-antichymotrypsin.* Clinical Chemistry, 1991. **37**(9): p. 1618-25.
11. Wilkins, M.R., *From Proteins to Proteomes: Large Scale Protein Identification by Two-Dimensional Electrophoresis and Amino Acid Analysis.* Biotechnology, 1996. **14**.
12. Ressine, A., *Development of protein microarray chip technology - An outlook towards proteomics and diagnostics -*, in *Dept. Electrical Measurements.* 2005, Lund Institute of Technology: Lund.
13. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
14. Venter, J.C., et al., *The sequence of the human genome.* Science, 2001. **291**(5507): p. 1304-51.
15. *Personal Genome Project* [cited 17.08.09]; Available from: http://www.personalgenomes.org.
16. *Human Proteome Organisation.* [cited 18.08.09]; Available from: http://www.hupo.org.
17. *Human Proteome Resource.* [cited 09.10.29]; Available from: http://researchprojects.kth.se/index.php/kb_1/io_8632/io.html.
18. Yalow, R.S. and S.A. Berson, *Immunoassay of Endogenous Plasma Insulin in Man.* Journal of Clinical Investigation, 1960. **39**(7): p. 1157-1175.
19. Gosling, J.P., *A Decade of Development in Immunoassay Methodology.* Clinical Chemistry, 1990. **36**(8): p. 1408-1427.

20. Venn, R.F., et al., *96-Well solid-phase extraction: a brief history of its development.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2005. **817**(1): p. 77-80.

21. Merson, D.J.R.C.C., 3 Havelock Place, Ash Nr. Canterbury Kent, CT3 2AB, GB), Bojanic, Dr. Dejan (3 Moss End Mews, Vine Close, Ramsgate Road, Kent, CT, 11 7BW, GB), *An assay tray assembly.* 1995, Pfizer Limited (Ramsgate Road, Sandwich, Kent, CT13 9NJ, GB),PFIZER INC. (235 East 42nd Street, New York, N.Y., 10017, US).

22. Dupuy, A.M., S. Lehmann, and J.P. Cristol, *Protein biochip systems for the clinical laboratory.* Clin Chem Lab Med, 2005. **43**(12): p. 1291-302.

23. Ekins, R., F. Chu, and E. Biggart, *Multispot, multianalyte, immunoassay.* Ann Biol Clin (Paris), 1990. **48**(9): p. 655-66.

24. Ekins, R.P., *Multi-analyte immunoassay.* J Pharm Biomed Anal, 1989. **7**(2): p. 155-68.

25. Hofman, P., *DNA microarrays.* Nephron Physiol, 2005. **99**(3): p. p85-9.

26. Anderson, N.L. and N.G. Anderson, *Proteome and proteomics: new technologies, new concepts, and new words.* Electrophoresis, 1998. **19**(11): p. 1853-61.

27. Gygi, S.P., et al., *Correlation between protein and mRNA abundance in yeast.* Mol Cell Biol, 1999. **19**(3): p. 1720-30.

28. Linck, B., et al., *Messenger RNA expression and immunological quantification of phospholamban and SR-Ca(2+)-ATPase in failing and nonfailing human hearts.* Cardiovasc Res, 1996. **31**(4): p. 625-32.

29. MacBeath, G. and S.L. Schreiber, *Printing proteins as microarrays for high-throughput function determination.* Science, 2000. **289**(5485): p. 1760-1763.

30. Templin, M.F., et al., *Protein microarray technology.* Trends in Biotechnology, 2002. **20**(4): p. 160-166.

31. Zhu, H., et al., *Global analysis of protein activities using proteome chips.* Science, 2001. **293**(5537): p. 2101-2105.

32. Jaras, K., et al., *Reverse-phase versus sandwich antibody microarray, technical comparison from a clinical perspective.* Analytical Chemistry, 2007. **79**(15): p. 5817-25.

33. Hall, D.A., J. Ptacek, and M. Snyder, *Protein microarray technology.* Mechanisms of Ageing and Development, 2007. **128**(1): p. 161-167.

34. Uhlir, A., *Electrolytic Shaping of Germanium and Silicon.* Bell System Technical Journal, 1956. **35**(2): p. 333-347.

35. Turner, D.R., *Electropolishing Silicon in Hydrofluoric Acid Solutions.* Journal of the Electrochemical Society, 1958. **105**(3): p. C55-C56.

36. Pramatarova, L., et al., *Porous silicon as a substrate for hydroxyapatite growth.* Vacuum, 2004. **76**(2-3): p. 135-138.

37. Rajaraman, S. and H.T. Henderson, *A unique fabrication approach for microneedles using coherent porous silicon technology.* Sensors and Actuators B-Chemical, 2005. **105**(2): p. 443-448.

38. Aravamudhan, S., A.R.A. Rahman, and S. Bhansali, *Porous silicon based orientation independent, self-priming micro direct ethanol fuel cell.* Sensors and Actuators a-Physical, 2005. **123-24**: p. 497-504.

39. Presting, H., et al., *Porous silicon for micro-sized fuel cell reformer units.* Materials Science and Engineering B-Solid State Materials for Advanced Technology, 2004. **108**(1-2): p. 162-165.

40.   Vincent, G., *Optical-Properties of Porous Silicon Superlattices.* Applied Physics Letters, 1994. **64**(18): p. 2367-2369.

41.   Drott, J., et al., *Porous silicon as the carrier matrix in microstructured enzyme reactors yielding high enzyme activities.* Journal of Micromechanics and Microengineering, 1997. **7**(1): p. 14-23.

42.   Laurell, T., et al., *Enhanced enzyme activity in silicon integrated enzyme reactors utilizing porous silicon as the coupling matrix.* Sensors and Actuators B-Chemical, 1996. **31**(3): p. 161-166.

43.   Yakovleva, J., et al., *Microfluidic enzyme immunoassay using silicon microchip with immobilized antibodies and chemiluminescence detection.* Analytical Chemistry, 2002. **74**(13): p. 2994-3004.

44.   Ressine, A., et al., *Macro-/nanoporous silicon as a support for high-performance protein microarrays.* Analytical Chemistry, 2003. **75**(24): p. 6968-74.

45.   Deegan, R.D., et al., *Capillary flow as the cause of ring stains from dried liquid drops.* Nature, 1997. **389**(6653): p. 827-829.

46.   Hu, H. and R.G. Larson, *Analysis of the microfluid flow in an evaporating sessile droplet.* Langmuir, 2005. **21**(9): p. 3963-71.

47.   Laurell, T., L. Wallman, and J. Nilsson, *Design and development of a silicon microfabricated flow-through dispenser for on-line picolitre sample handling.* Journal of Micromechanics and Microengineering, 1999. **9**(4): p. 369-376.

48.   Onnerfjord, P., et al., *Picoliter sample preparation in MALDI-TOF MS using a micromachined silicon flow-through dispenser.* Analytical Chemistry, 1998. **70**(22): p. 4755-60.

49.   Miliotis, T., et al., *Capillary liquid chromatography interfaced to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an on-line coupled piezoelectric flow-through microdispenser.* J Mass Spectrom, 2000. **35**(3): p. 369-77.

50.   Vaisanen, V., et al., *Development of sensitive immunoassays for free and total human glandular kallikrein 2.* Clinical Chemistry, 2004. **50**(9): p. 1607-1617.

51.   Nurmikko, P., et al., *Production and characterization of novel anti-prostate-specific antigen (PSA) monoclonal antibodies that do not detect internally cleaved Lys145-Lys146 inactive PSA.* Clinical Chemistry, 2000. **46**(10): p. 1610-1618.

52.   Vaisanen, V., et al., *Intact free prostate-specific antigen and free and total human glandular kallikrein 2. Elimination of assay interference by enzymatic digestion of antibodies to F(ab ')(2) fragments.* Analytical Chemistry, 2006. **78**(22): p. 7809-7815.

53.   Mitrunen, K., et al., *Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum.* Clinical Chemistry, 1995. **41**(8 Pt 1): p. 1115-20.