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Developing and characterizing an assay for prostate specific antigen

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Abstract

Detection of biomarkers in complex sample environment (e.g. plasma) is problematic, due to large amounts of non-specific binding. The non-specific binding affects the analytelevels negatively, which causes a loss of sensitivity. The possibility to minimizing the effects of non-specific binding in plasma measurements in Biacore was studied. By using Biacore S51, some of problems related to the non-specific binding could be minimized satisfactory.

Keywords

Biacore, Surface Plasmon Resonance, SPR, non-specific binding, plasma, Prostate Specific Antigen, PSA.

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Developing and characterizing an assay for prostate specific antigen

Fredrik Noborn

Sammanfattning

Biomarkörer har idag stor betydelse inom bland annat diagnostik. Ett problem vid detektion av biomarkörer är att mätningen sker i komplexa provmiljöer (t ex plasma), vilket orsakar stora mängder ospecifik inbindning. Den ospecifika inbindningen påverkar analysnivåerna negativt, vilket försämrar mätningens känslighet.

Syftet med arbetet var att utveckla en proteinassay med hög känslighet. Denna assay användes sedan som modellsystem för att studera effekter av olika åtgärder för att hantera problem vid mätning i plasma. Som analyt valdes prostataspecifikt antigen (PSA), ett protein som används vid diagnosticering av prostata cancer. PSA är ett bra modellsystem eftersom det endast återfinns i försumbara nivåer hos kvinnor, vilket betyder att kvinnlig plasma kan användas som nollprov.

Den utvecklade assayen fungerade mycket tillfredsställande i buffert där detektionsnivån var under 1 ng/ml. Vid mätning i plasma orsakade den ospecifika inbindningen som väntat problem. Det var inte möjligt under arbetets gång att utveckla en fullständig metod för att hantera alla individuella skillnader i ospecifik inbindning mellan olika plasma prov. Vissa problem relaterat till ospecifik inbindning kunde däremot minimeras genom att utnyttja Biacore S51. Detta instrument gav möjlighet att kompensera för de individuella skillnaderna mellan olika plasma prov på ett tillfredsställande sätt.

Examensarbete 20 p i Molekylär bioteknik programmet

Uppsala universitet mars 2005

Table of contents

1. Introduction

Biomarkers play an important part in today's research and diagnostics. Detection of biomarker is always problematic since the detection is made in a complex sample environment (e g. plasma), which causes large amount of binding from components other than the analyte of interest (non-specific binding). This type of binding affects the sensitivity of the assay detection and is therefore not desired.

The problem of this matter is partially related to the SPR technique that is used in Biacore. Other screening methods such as ELISA, which uses labels to detect biomolecular interactions, is not affected by this problem to the same extent [1].

The objective with this work was to find ways to limit the non-specific binding and thereby facilitate plasma measurements in Biacore. An assay was first developed in buffer. This assay was then used as a model system in plasma to study the effects of different measures taken in order to minimize the effects of non-specific binding.

The model analyte chosen for this work was prostate specific antigen (PSA), which is used as a marker for diagnosing prostate cancer. This cancer form is the most common among men and constitutes about 30 % of all diagnosed cancer cases [2].

PSA provides a good model system since it is measurable to a reasonable extent in males only, which means that female plasma can be used as zero samples [3]. Another advantage is that it is a well-characterized protein with high clinical interest, which means that it is easy to find suitable antibodies for PSA detection on the commercial market.

1.1 The Biacore instrument

1.1.1 SPR

Surface Plasmon Resonance (SPR) is a phenomenon that occurs in thin metal films at an interface between media of different refractive index. When light strikes the interface, it undergoes total reflection if the incident angle is large enough and if the light comes from the media with higher refractive index. Total reflection creates an electromagnetic wave, evanescent wave, which travels a short distance into the media with lower refractive index [4]. The total reflection causes a distinct reduction of intensity of the incoming light at specific angles. This phenomenon is called SPR, and is used in Biacore instruments to study molecular interactions.

The angle at which SPR occurs depends on several factors. One factor is the refractive index of the non-reflecting side of the interface. A mass change on this side induces a change in the refractive index and hence also a change in SPR angle [4]. This correlation makes it possible to measure the mass changes on the non-reflecting side in real-time.

In the Biacore instrument, light from different angles strike the interface to see at which angle SPR occurs. When molecular interactions take place on the non-reflecting side it leads to an increase in mass, which will cause a change in SPR angle. A change in the SPR-angle implies that the mass bound to the surface has changed, and a curve in which the amount of molecule bound to the surface is plotted versus time can be made. This curve is referred to as “sensorgram” in Biacore terminology.

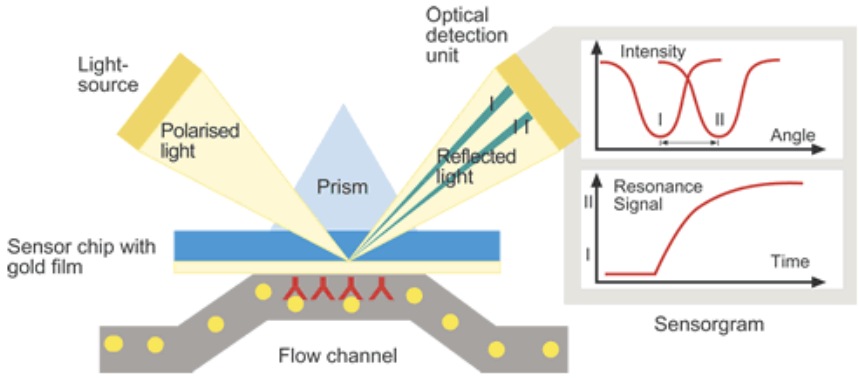


Figure 1: The principle of SPR-detection. A mass change at the non-reflecting side (here illustrated with antibody-antigen binding), produces a shift in the SPR-angle. The shift causes a change in intensity, which is registered by the optical detection unit. Figure from Biacore AB.

1.1.2 The microfluidic system

In Biacore instrument, the samples are supplied to the sensor surface with a microfluidic system. The microfluidic system contains a series of channels and valves in a plastic block, the Integrated Microfluidic Cartridge (IFC).

The IFC is designed differently depending on the Biacore model. In Biacore's S51 instrument the IFC has three detection spots (figure 2). Two of the spots (detection spot 1 and 2) are normally used for ligand binding whereas the third is used as a reference. The structure of IFC in S51 makes it possible to immobilize different ligands on the two detection spot and have a simultaneously flow of different reagents over the two spots.

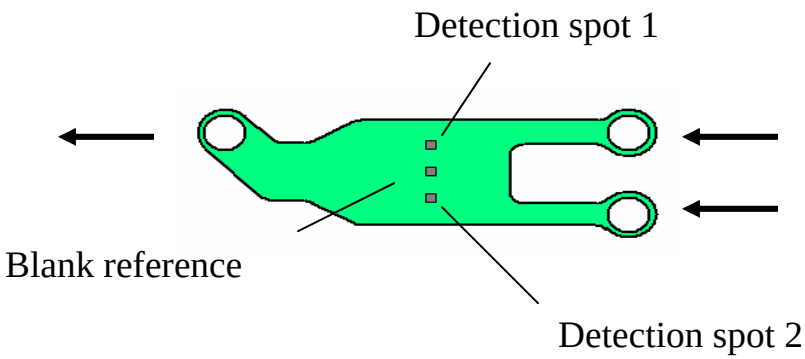


Figure 2: The integrated microfluidic cartridge (IFC) in Biacore S51. The IFC has a Y-shaped structure and has two separate inflows for buffer/sample and one waste outflow. By controlling the flow rate during the coupling step, protein can be immobilized on one side or the other on the flow cell (spot 1 or 2). The middle spot cannot be used for ligand binding as is normally used as a reference spot. Figure from Biacore AB.

In Biacore 3000 there are four parallel detection spots where each spot can be used for ligand binding. Contrary to S51, BC 3000 only allows a single reagent flow over the detection spots. The reagent can be injected in either a serial or parallel manner.

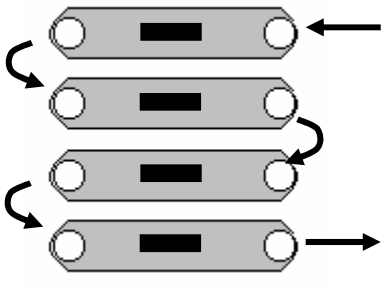


Figure 3: The integrated microfluidic cartridge (IFC) in Biacore 3000. The IFC has four parallel detection spot over which a flow can be directed in either a serial or parallel manner. Here, a serial flow over the detection spots is shown. Figure from Biacore AB.

1.1.3 The sensor chip

The sensor surface in the Biacore instrument is an exchangeable part, referred to as a chip. The Biacore chip is composed of a thin layer of gold on a supporting glass layer. Silver actually generates a stronger signal, but gold is more durable and chemically inert, qualities that are preferred, and is therefore the choice of metal in the Biacore chip. The gold film is in turn covered with a covalently bound matrix (attached via an alkane linker layer) on which biomolecules can be immobilized [5].

The most generally applicable chip is Sensor Chip CM 5, with a surface matrix of carboxymethyl dextran, which provides a hydrophilic environment for the surface interaction. Molecules immobilized to these dextran molecules will be able to move quite freely in all dimensions.

1.1.4 The Sensorgram

The interaction between two interaction partners in a Biacore system is studied in a sensorgram. The interaction consists of two phases: the association phase and the dissociation phase (figure 4). In the association phase, the injected analyte binds to the immobilized ligand, which causes a mass change on the surface. This mass change induces a change of response in the sensorgram. When the injection phase of the analyte is over, the analyte will start to dissociate from the ligand, resulting in a decrease of mass on the surface and consequently a loss of response. A sensorgram can give information about a various characteristics of the studied system such as: kinetics, specificity, concentration and affinity.

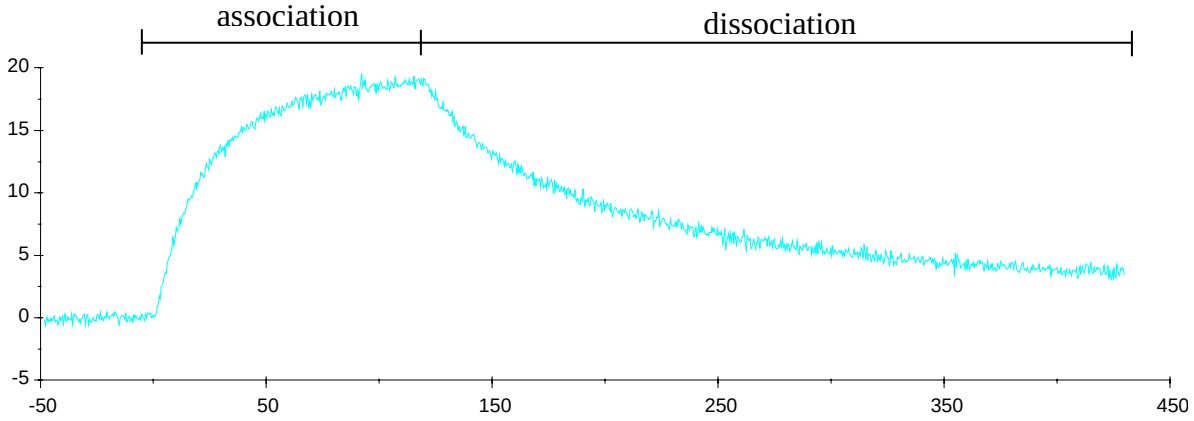


Figure 4: Sensorgram showing the two phases of a reagent injection: association- and dissociation phase. Figure from Biacore AB

1.1.5 Immobilization

Immobilization is the procedure when the one of the interaction partners, the ligand in Biacore terminology, is attached to the matrix on the gold layer. There are two major immobilization strategies, covalent coupling and immobilization via capturing antibody.

Covalent coupling is when the ligand is attached to the matrix by a covalent link. There are several types of covalent coupling methods, where amine coupling is the most frequently used.

Immobilization via capturing antibody relies on the high affinity between a capturing antibody and a desirable ligand. Different capturing molecules can be immobilized depending on what kind of ligand that is being captured. An often used antibody for capturing procedures is rabbit anti mouse (RAM), which is used to capture monoclonal mouse antibodies.

1.1.6 Regeneration

Regeneration is the process of removing bound analyte from the sensor chip surface after analysis of a sample, in preparation for the next analysis cycle. The regeneration procedure breaks the link between the captured analyte and the ligand using a regeneration solution.

An incomplete regeneration or loss of the binding activity from the surface will impair the performance of the assay. Each assay has its optimal regeneration condition and it is therefore crucial to find an adequate regeneration in order to have a successful assay [6].

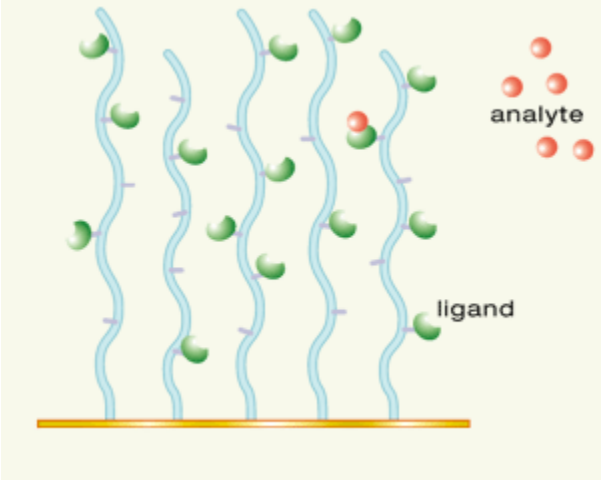


Figure 5: Showing when an analyte comes loose from the ligand during regeneration. Figure from Biacore AB.

1.2 The model analyte - Prostate Specific Antigen (PSA)

Prostate specific antigen (PSA) is a single chain glycoprotein and has a weight of 33 kDa. It constitutes of 237 amino acids and belongs to the kallikrein family of serine proteases PSA is produced by epithelial cells in the prostate gland and is secreted into the urethra during ejaculation. Its physiological function is to liquefy seminal plasma by proteolyses gelforming proteins [2].

PSA is present in both a single- and complexed state in the body. The most abundant complex of PSA is the one it forms with antichymotrypsin (ACT), a protease inhibitor. PSA can also form complexes with other proteins, such as human kallikrein (hk2) and alpha-2-macroglobulin (A2M). Normally the fraction of complexed protein constitutes of about 90% of the total amount of PSA in the body [8].

PSA is used as a marker for diagnosing prostate cancer, which makes it important for clinical research. Prostate cancer is the most common cancer form among men and represents 30 % of all cancer cases. In the diagnostic procedure the blood level of PSA is measured: a normal value is between 2-5 ng/ml, depending on individual and age [2]. An increased level is used as an indication for prostate cancer. Voices have been raised for a screening test of the male population, in similarity to the mammography test on women. However, the PSA test has certain constraints. An increased PSA level can only be used as an indication for prostate complications in general and not prostate cancer in particular. This is because an increased PSA level overlaps with other prostate complications such as: benign prostate hyperplasia and prostatitis. This fact, together with individual variations in normal PSA level, generates many false positives for the PSA test. In order to make a correct diagnose of prostate cancer the PSA test should always be complemented with clinical examination.

Although the PSA test has its limitations it is still very useful. The test can be used as an indicator of how the patient responds to the cancer treatment. A decrease in PSA level is an indication of retrogression of cancer and vice versa.

A lot of research is made in order to improve the PSA tests. It has been found that if the amount of PSA in a free state is measured together with PSA in complex state, the accuracy of cancer prediction is improved. For example, in a benign tumour the fraction of complexed PSA decreases to a level of about 70-80%, whereas the in malign tumours and normal cases the level is 90% [8]. In other words, by measure the fraction of complexed PSA it possible to distinguish malign and benign tumour forms.

The high clinical interest for PSA together with the fact that it is only detectable in men makes it suitable as a model system for assay development.

2. Materials and methods

2.1 Materials

2.1.1 Instruments and software
Biacore 3000
Biacore S51

Biacore 3000 Control Software version 3.2
Biacore 3000 Evaluation Software version 3.2

Biacore S51 Control Software version 2.0 Beta 3
Biacore S51 Evaluation Software version 1.2

2.1.2 Reagents
Sensor chip CM5, Research grade, Biacore AB.
Sensor chip CM5, Series-S, Biacore AB.

Rabbit anti mouse-immunoglobulins (RAM), Biacore AB.
Anti-myoglobin, clone 2F9.1, Biacore AB.

Amine coupling kit, Biacore AB, containing:
N-hydroxysuccinimide (NHS)
N-ethyl-N'-(dimethylaminopropbyl)corbodiimide (EDC)
1 M ethanolamine hydrocholoride pH 8.5 (etanolamine-HCl)

10 mM acetate buffer, pH 3.5, 4.0, 4.5, 5.0, 5.5, Biacore AB.
10 mM glycin-HCl, pH 2.0, 2.5, 3.0, Biacore AB.
50 mM NaOH, Merck
1 M HCl, Merck
Triflouracetic acid (TFA), Merck-Schuchart
HBS-EP, Biacore AB.
CM-Dextran, sodium salt, Fluka.
1 M Formic acid, Riedel-de-Haën.

Prostate specific antigen, A32874H, Biosite.

Antichymotrypsin, 50104H, Biosite.

Monoclonal mouse anti-human PSA:
M86343M, clone PS1, No. 1883, Biosite.
M86506M, clone 5A6, No. 1884, Biosite.
M86433M, clone PS2, No. 1887, Biosite.

gc5, provided by Prof. U-H Stenman, Helsinki University, Finland

Plasma samples, Akademiska Sjukhuset, Uppsala

S0004 03	922832,	# 1
S0004 03	213846,	# 2
S0004 03	922826,	# 3
S0004 03	922851,	# 4
S0004 03	213521,	# 5
S0004 03	214892,	# 6
S0004 03	214395,	# 7
S0004 03	922837,	# 8
S0004 03	213809,	# 9
S0004 03	922836,	# 10

2.2 Methods

2.2.1 Immobilization

Amine coupling (covalent immobilization)
Amine coupling is a type of covalent immobilization, which uses cabodiimine/hydroxysuccinimide chemistry to create a covalent link between the matrix and the free amino groups on the ligand. The covalent link is achieved when carboxymethyl groups are modified with a mixture of NHS (N-hydroxysuccinimide) and EDC (N-ethyl-N'- (dimethylaminopropbyl)corbodiimide)) introducing N-hydroxysuccinimide esters into the surface matrix (figure 6). The esters react with amines on the ligand forming an amide bond [8].

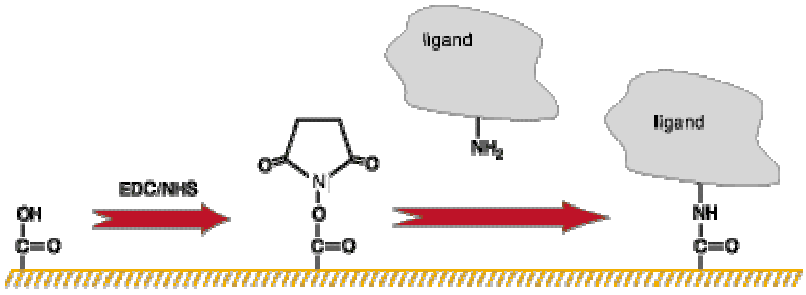


Figure 6: Scheme of amine coupling. Reactive esters are introduced to the surface by a mixture of EDC/NHS. The esters react with amines on the ligand and create a covalent link between the matrix and the ligand. Figure from Biacore AB.

After injection of the ligand, the remaining esters are deactivated by an injection of ethanolamine-HCl. A typical sensorgram where the ligand has been immobilized by amine coupling is shown in figure 7.

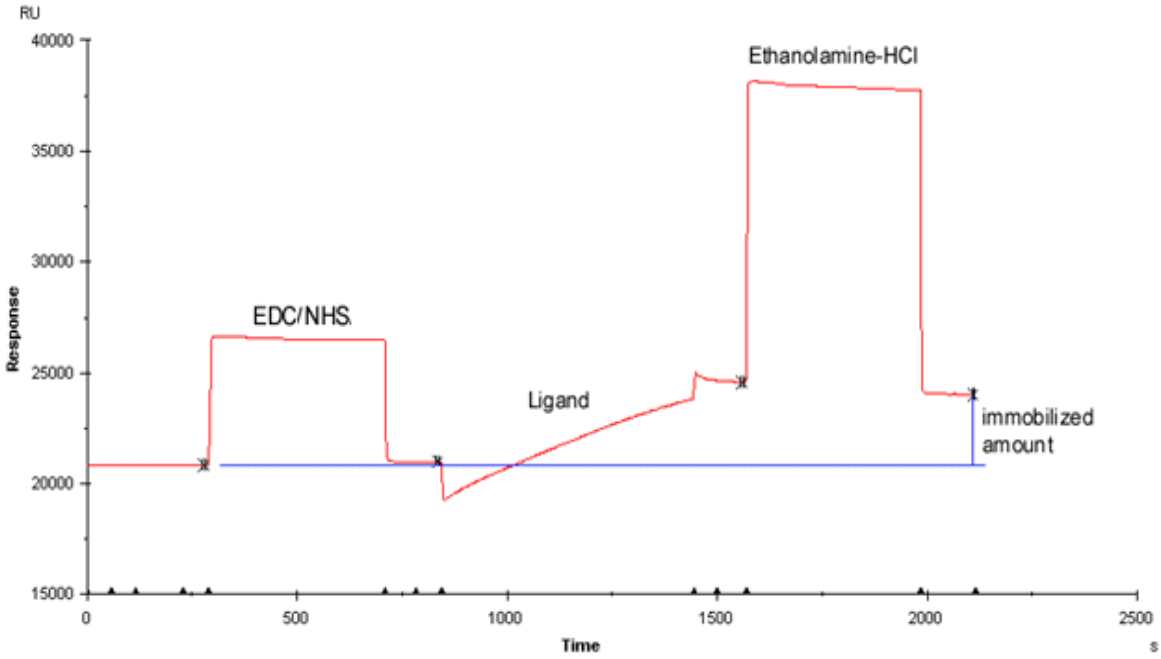


Figure 7: A typical sensorgram where the ligand has been immobilized by amine coupling. The level of immobilized ligand corresponds to the difference in baseline level before and after injection of ligand. Figure from Biacore AB.

Immobilization of macromolecular ligands is usually performed from dilute ligand solutions (10-50 µg/ml or less), but the immobilization levels often correspond to a protein concentration in the dextran matrix in the range of 10-20 mg/ml or more [4]. This is achieved through electrostatic pre-concentrations of ligands in the dextran matrix.

At pH values above 3.5, the carboxymethylated dextran at the sensor chip surface is negatively charged and the electrostatic attraction provides an efficient way to concentrate positively charged ligands at the surface.

A primary requirement for the electrostatic pre-concentration at the surface is that the pH of the ligand solution is between 3.5 and the isoelectric point of the ligand, so that the surface and the ligand carry opposite net charges. In addition, the electrostatic interactions involved in pre-concentration are favored by a low ionic strength of the coupling buffer. In general, covalent immobilization of proteins is best performed with 10mM buffer solutions with a pH above 4. The pre-concentration effect on an unactivated sensor chip under different buffer conditions can be measured in order to determine the optimum coupling conditions [4, 5].

When amine coupling was performed, EDC and NHS were prepared according to the instructions included in the Amine coupling kit. Different injection times for EDC/NHS, ligands and ethanolamine-HCl were tested throughout the work in order to obtain preferred ligand levels.

Immobilization via capturing antibody
Immobilization via capture uses an antibody that is covalently immobilized at the surface to capture a second type of antibody (ligand). The method relies on high affinity binding between the capturing antibody and the ligand [8]. The ligand is then ready to detect the analyte of interest. After the experiment, a regeneration solution removes both the ligand and the analyte, making it possible for new ligands to be captured. This immobilization technique is very fast and is often used in experiments were several different ligands needs to be tested or when regeneration conditions cannot be established.

The capturing antibodies were immobilized using amine coupling. An EDC/NHS mixture was injected for 10 min followed by a 7 min injection of the ligand (RAM, 30µg/ml in acetate buffer pH 5.0). Ethanolamine-HCl was injected for 7 min to deactivate the surface and a pulse of Glycine pH 1.8 was injected to remove non-covalently bound material.

2.2.2 Assay

2.2.2.1 Sandwich assay
An often-used method for signal enhancement in Biacore is the sandwich assay. The method is used in cases were the analyte generates very low response, and/or to increase the specificity of the assay (as in this case) [6].

The sandwich method is an extension of the single step direct approach: after analyte has bound to the surface-attached ligand, a secondary antibody (the enhancer) is injected to bind to the analyte in order to enhance the analyte response. An important criterion is that the two antibodies are specific to different epitopes on the antigen, i.e. be able to bind the analyte independently of each other.

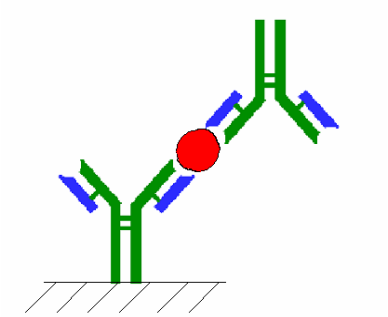


Figure 8: Schematic picture of a sandwich assay. Showing the ligand (bottom), analyte (middle) and enhancer (top).

In complex sample environments (e. g. plasma) responses arising from sources other than binding of analyte will complicate the interpretation of the result. Such responses may arise from binding of non-analyte molecules in the sample to the ligand or the dextran

matrix [4]. However, by introducing a second antibody (enhancer), which binds to the analyte, response arising from the specific binding is enhanced whereas the response from non-specific binding is not. However, the situation during measurements in plasma is often complicated by the fact that a substantial downward drift in the response caused by dissociation by the non-specifically bound molecules. This is further complicated due to great individual variations in non-specific binding among plasma samples.

2.2.2.2 Reference subtraction

In Biacore S51 it is possible to perform a special type of reference subtraction not possible in Biacore 3000. This feature makes it possible to compensate for both the drift and individual variations in non-specific binding.

In plasma, the response generated by PSA is only a fraction of the response caused by non-specific binding. After the end of the sample injection, the dissociation of non-specifically bound material is going on. Then, during the injection of the enhancement reagent, this dissociation still occurs contributing to the obtained response. In many cases this contribution is far larger than the specific response. By performing the assay in two parallel spots and excluding the enhancement injection in one of the spots, allowing running buffer to flow over one spot (reference spot) the contribution from analyte binding can be resolved.

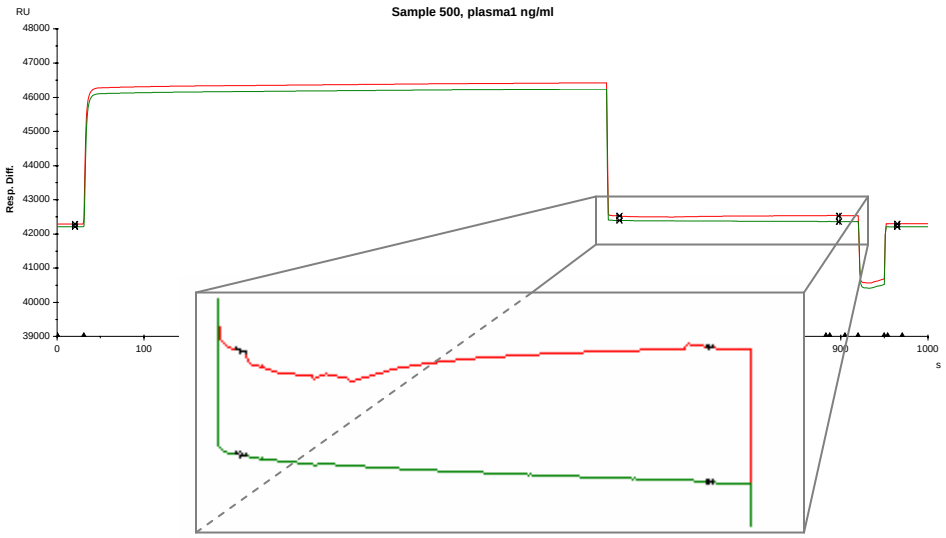


Figure 9: A typical sensogram for measurement of PSA in plasma. The three phases of the assay can be seen: primary response, enhancement step and regeneration. The enhancement step has been enlarged in order to show the difference between the reference spot (green) and the spot over which enhancer is injected (red). Dissociation can be seen on the reference spot, while the enhancement injection can be seen on the other spot.

2.2.2.3 Regeneration condition studies

The choice of regeneration solution is of great importance in order to have a high performance assay. The solution should be able to regenerate the surface completely, so that all ligand is made free. At the same time conditions must be gentle enough to the ligand antibody, and not damage it in any way. In an assay with an ideal regeneration solution the amount of analyte that binds to the surface in each repetitive analysis cycle is constant [6].

In a regeneration test the analyte is injected a repeated number of times over the surface. After each injection the surface is regenerated with the solution of interest. From the result an analyte vs. cycle plot is made. The procedure is repeated with different solutions.

A decrease in analyte response during repetitive injections of the same regeneration solution candidate (typically five cycles) can be explained by loss of capacity, either caused by damage to the ligand or insufficient regeneration. Insufficient regeneration is also manifested in baseline level increase. Damage to the ligand is often accompanied by a slight decrease in baseline level. The main objective of a good regeneration procedure is to maintain constant analyte response during repetitive injections of the same sample concentration.

2.2.2.4 Experimental procedures

Dilution series were made in order to create calibration curves. Samples were prepared stepwise, by a 1+1 dilution procedure (e.g. 1000 ng/ml, 500ng/ml, 250ng/ml, 125ng/ml, …, 1.95ng/ml). Each sample was run in duplicate and 11 blank samples (buffer only) were run before the dilution series in order to determine the limit of detection (LOD). Five start-up cycles were first run for the conditioning of the flow system. A start-up cycle consists of a complete analysis cycle with the highest analyte concentration sample injected. After the five start-up cycles, the blank samples were run, followed by the calibration solutions. The calibrants were run from the lowest to the highest concentration. Finally, the samples were run.
For each sample, a corrected relative response for the enhancement injection was taken.

2.2.2.5 Evaluation

The dilution series generated a set of corrected relative responses from which a calibration curve was constructed. The curves were provided by BIAevaluation software, using a 4 parameters fitting equation to calculate the curve.

The limit of detection (LOD) is the lowest analyte concentration that can be detected but not necessarily quantified as an exact value. LOD is primarily a function of the signal-to-noise ratio in the measurements itself, and is set in relation to statistical variations for blank samples [6]. A commonly used value is 3*SD, where SD is the standard deviation of replicate measurements on blank samples.

LOD does not include any factor relating to experimental variations in source, compositions or preparations of samples.

The precision of an assay describes the agreement between results obtained from multiple measurements on the same homogeneous sample.

The precision of an assay is expressed in “coefficient of the variation” (CV) in this work. For a set of replicate measurements, the standard deviation is given by,

$$SD = \sqrt{(1/(n-1))\sum (y_i - \overline{y})^2}$$

where n =number of measurements
and y = response for a given measurement.

The coefficient of variation is given by

CV(%) = SD/mean * 100

The two commonly used parameters for CV values are: CV_{dose} and $CV_{response}$. The $CV_{response}$ value reflects the consistency of response values for a given concentration, while CV_{dose} reflects the consistency with which a given response value can be related to analyte concentration.

2.2.3 Preparation of plasma samples

The plasma samples were centrifuged for 10 min at 4000 rpm and thereafter filtered through a 0.22 µm filter.

Another problem with plasma is the high degree of unspecific binding. A way to reduce this problem is to dilute the plasma with CM-dextran. By adding CM-dextran, the unspecific binding that is caused by attractions to the dextran matrix can now bind to the dextran in the solution instead. The plasma samples were diluted 1+1 in HBS-EP containing CM-dextran (10mg/ml).

3. Results

The assay development procedure was divided into two parts. First, a preliminary assay was developed and characterized for buffer conditions. Then, the assay was modified in order to be applied to measurements in human plasma.

3.1 Assay development

The assay development procedure includes everything from choosing the instrument type to the selection of reagents. In order to perform this, an assay was developed using buffer conditions. It is important to have a functional preliminary assay that generates satisfying results in buffer before adopting the assay for plasma measurements.

3.1.1 Choice of reagents

3.1.1.1 Ligand characteristics

An important part of the assay development is the selection of a suitable ligand. The assay performance depends to a great extent on the ligand and in order to make the optimal choice there are several aspects to consider.

The most important ligand characteristic is specificity. The ligand should first of all have specificity for the analyte of interest and variants thereof. In this work there were two analyte variants of interest: PSA in a free state and PSA in a complexed state. It has been found that by measuring the level of these two states (instead of measuring PSA in free state alone), the reliability of prostate cancer prediction is improved. It is therefore of particular clinical interest for assays win which these two states can be measured.

The objective was to develop an assay for free PSA and depending on the progress of the work also develop an assay for detection of total PSA. Two types of antibodies were available: antibodies with specificity to free PSA and antibodies with specificity to total PSA, where “total” refers to specificity for both free- and complexed PSA. The available antibodies and their specificities, given by the company from which they were purchased, are shown in table 1.

Table 1: Specificity of the available antibodies.

Ligand	Specificity
1883	free PSA
1884	total PSA
1887	total PSA
gc5	no information

The first step in characterizing the antibodies was to test their binding to the analyte. A suitable ligand must have high affinity to the antigen of interest in order to obtain a sensitive assay. A further requirement for sandwich assays is that the analyte should have a low dissociation rate from the ligand, otherwise a substantial part of the analyte maybe lost before and during the injection of the second antibody.

An experiment was made to get basic knowledge about the binding properties of the antibodies. 1883, 1884, 1887 and gc5 were immobilized one at the time by using high affinity capture on a rabbit anti mouse immunoglobulin surface. After the immobilization, 1 µg/ml of PSA was injected over each ligand and the response was studied. Since the binding capacity of the surface is proportional to the amount of immobilized ligand, the response has to be normalized to achieve comparable result. The immobilized amount of 1883 was used as reference and the fraction of other immobilized ligands in respect of 1883 were calculated. The obtained factor was multiplied with the response and a normalized response was obtained. The result is shown in table 2. The sensorgrams were normalized according to the same principle and are shown in over-lay plot in figure 10. The sensorgrams have been aligned at a point before the PSA injection.

Table 2: The table shows the analyte responses (RU) after injection over the ligands. Ligand concentrations were 30 µg/ml and the analyte concentration 1 µg/ml.

Ligand	Analyte response (RU)	Immobilized level (RU)	Normalized analyte response (RU)
1883	425	1271	425
1884	155	2022	97
1887	69	1844	47
gc5	128	1344	120

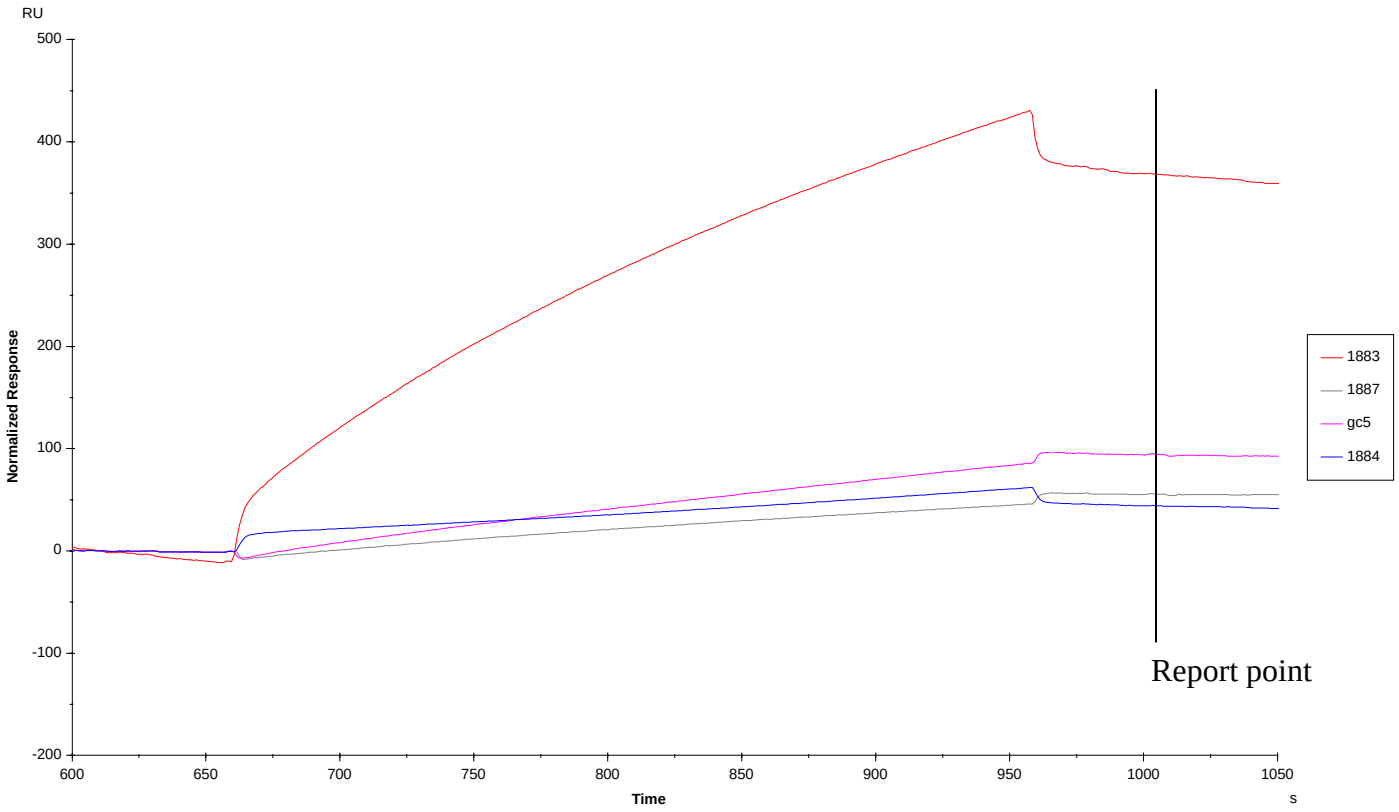


Figure 10: The over-lay plot shows the differences in binding levels between the ligands. Ligand concentrations were 30 µg/ml and the analyte concentration 1 µg/ml.

The experiments showed that 1883 has the best binding capacity of PSA of the tested antibodies.

3.1.1.2 Selecting a suitable antibody pair

A suitable sandwich pair must recognize and bind to different epitopes on the analyte. This is necessary since the sandwich assay is based on the principle of selectivity in two steps, where the ligand and enhancer binds independently on different analyte sites. An epitope mapping was made in order to select an antibody pair.

The antibodies were tested in different combinations in order to find pairs that bind the analyte simultaneously. The antibodies (1883, 1884) were immobilized one at a time on the surface by using high affinity capture on a RAM surface. Anti-myoglobin was used as a blocking antibody to block the remaining free binding sites on RAM. 1 µg/ml of PSA was injected followed by 30 µg/ml of the second antibody. Formic acid (1 M) with 30 seconds of contact time was injected to regenerate the surface. The procedure was repeated until all combinations had been tested (Table 3).

Table 3: The table shows the second antibody response (RU) for every combination of antibodies. 1883-1887 is a suitable sandwich pair.

	2nd Antibody				
1st Antibody		1883	1884	1887	gc5
	1883	ND	162	1080	109
	1884	922	ND	537	66

ND: Not determined

1883 and 1887 appears to work well in pair since they generate a high response. The high response shows that the antibodies recognize two different epitopes on PSA, which is required for PSA detection. The overall specificity of the sandwich pair is determined by the specificity of the ligand, since it is the first selection step of the assay. 1883-1887 will therefore have an overall specificity to free PSA because of 1883's specificity to free PSA.

Applying the same principle of selectivity, a sandwich pair for detecting total-PSA should have a ligand specific to total PSA. This means that 1884 is a suitable ligand for detecting total PSA. The table shows three pairs with the required specificity, but only two of them give a satisfying response: 1884-1883 and 1884-1887.

The sandwich pairs for future assay development were: 1883-1887 for free-PSA and 1884-1887 for total-PSA.

3.1.1.3 Assay conditions

An assay procedure is in many cases a compromise between the analytical and practical aspects. For concentration assays the objective is always to obtain the best possible specificity and sensitivity. The general guideline to achieve this is to use high reagent concentrations and long injection times. However, there are always practical constraints when choosing these parameters because high reagent concentrations and long injection times leads to large reagent consumption and long cycle time. In order to find the optimal assay parameters both the analytical and practical aspects must to be considered.

An experiment was made to find a set of parameters that generated an adequate analyte response. The investigated parameters were; ligand concentration, enhancer concentration and contact time, analyte concentration and contact time for the analyte injection. The starting point for assay development was 100 ng/ml of PSA and 35µg/ml of ligand antibody (1883, 1884). The analyte injection was set to 10 minutes with a 5µl/ml flow. The concentrations and injection times were chosen on the basis of prior knowledge at Biacore. These settings gave satisfying results were therefore kept throughout the whole work.

Another experiment was made in order to find a suitable concentration and injection time for the enhancement step. The objective was to find a condition that saturates all binding sites on PSA in a short period of time (to save time in the assay), without using too much reagent. A common procedure is to use a short injection time using high antibody concentration. In order to ensure robust conditions in the enhancement step the second antibody concentration was optimized.

A range of enhancement concentrations (0 – 50 µg/ml) were injected with 3 minutes contact time (flow 5 µl/min). The other assay parameters were set according to the conditions that were determined in previous experiments. The analyte concentration was 100 ng/ml. A response – concentration graph was made from the results (figure 11).

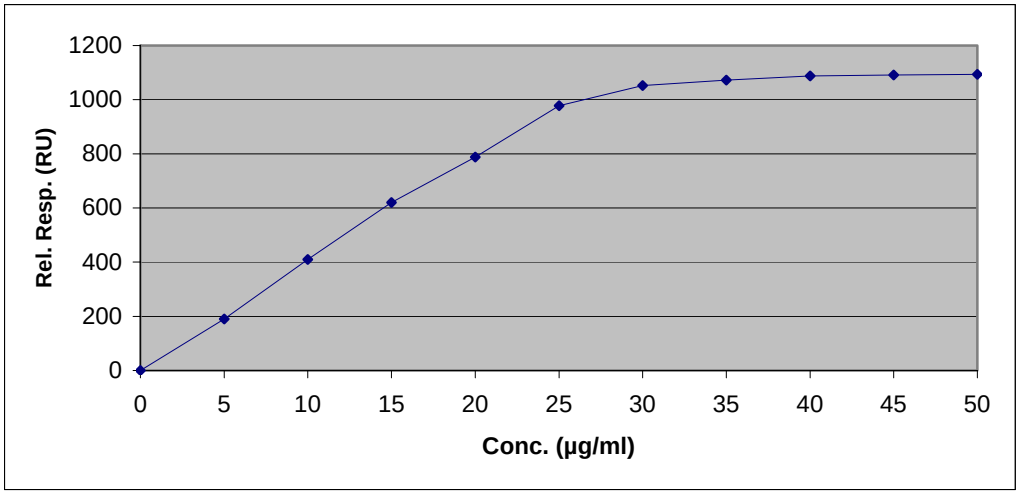


Figure 11: Response as a function of enhancer concentration. The injection time was 3 minutes and the flow was 5 µl/min.

The graph shows that for low enhancement antibody concentrations there is a proportional relationship between response and concentration. When the concentration exceeds 25 – 30 µg/ml the curve levels off, which is due to saturation of binding sites. An increase of enhancement concentration above 30 µg/ml will therefore not lead to an increase of response. However, the enhancer concentration was set to 35 µg/ml in order to have a margin to the saturation concentration, since a small concentration deviation at this level will not affect the response to a big extent.

Finally, all assay parameters were tested in an assay. The parameters were set according to what had been determined in previous experiments. The sensorgram of the cycle are shown in figure 12.

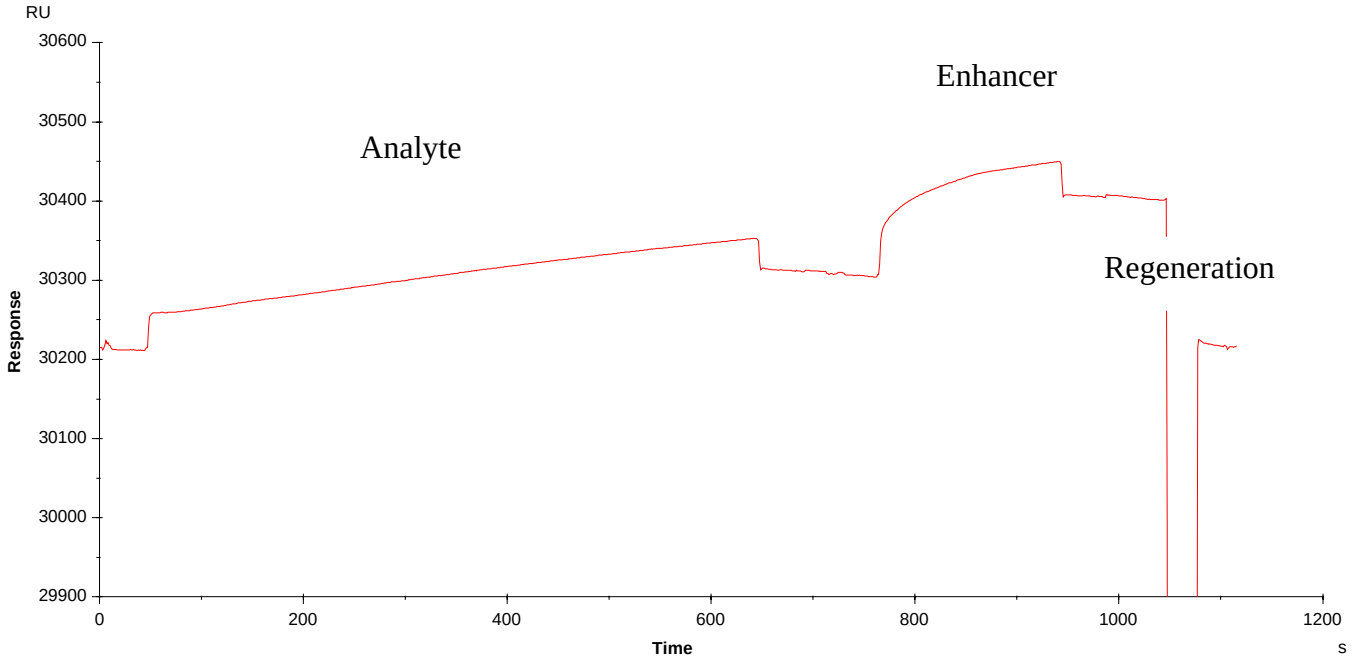


Figure 12: Sensorgram showing an analysis cycle. The enhancer concentration is 35 µg/ml and the analyte concentration 100 ng/ml. The injection time was 10 minutes for the analyte injection and 3 minutes for the enhancer.

The sensorgram shows the complete analysis cycle; binding of analyte, enhancer and finally regeneration. The sensorgram shows that the amount of bound analyte is increasing throughout the injection phase, whereas the enhancer has almost reached a maximum level. This means that the sensitivity of the assay can be improved by extending the analyte injection time (but not the enhancer). However, an increased injection time would give a longer analysis cycle, which may not be practical for the final assay. The injection times were therefore kept at these present values, but with the knowledge that an extension of analyte injection time would increase the sensitivity.

3.1.1.4 Subclass analysis

The antibodies that were used in this assay are mouse monoclonals of IgG-type. There are four subclasses of IgG: IgG1, IgG2a, IgG2b and IgG3, each affecting the measurements in different ways in terms of non-specific binding. The subclasses 2a and 2b are not desired in plasma measurements, since they cause high amounts of non-specific binding. This phenomenon is explained by their binding to the human complement factor (Clq); an abundant protein in blood which is a part of the body's complement system [9]. The most suitable subclass for plasma measurements is IgG1, due to its relatively low levels of non-specific binding.

In order to determine the subclass of the monoclonal antibodies a subclass analysis was made. The company from which the antibodies were purchased also makes an analysis. However, those analyses are not always to be trusted and therefore a further test may be necessary.

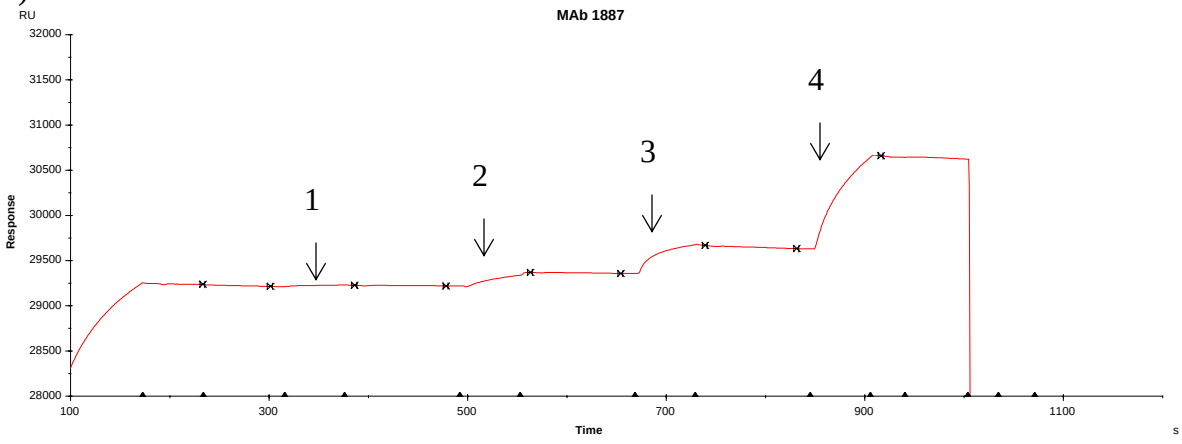
Although a preparation of monoclonal antibodies is expected to consist of a unique protein, there might be some contamination of other antibodies, due to the production procedure. The contamination consists of native polyclonal antibodies of all classes and subclasses without reactivity towards PSA. In plasma measurements, where the desired subclass is IgG1, other subclasses have implications on the assay since they may cause non-specific binding. The contamination of other antibodies of undesired subclasses is therefore a problem in assays.

In addition to determine the subclass of the monoclonal antibody, a subclass analysis can also be used to measure the level of contaminating antibodies.

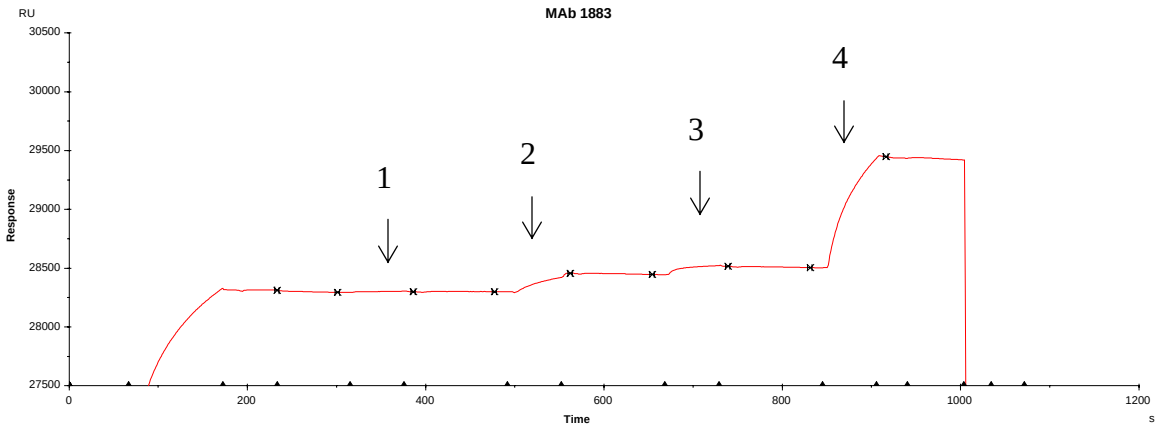
The antibodies 1883, 1884 and 1887 were immobilized by using high affinity capture on a RAM surface. The concentration of the antibodies were 35µg/ml.

After the capturing step, the subclass reagents anti-IgGx (3, 2b, 2a, 1) were injected in successive order over the ligand. Formic acid (1M) was used as regeneration solution. Sensorgrams are shown in figure 13.

a) 1887



b) 1883



c) 1884

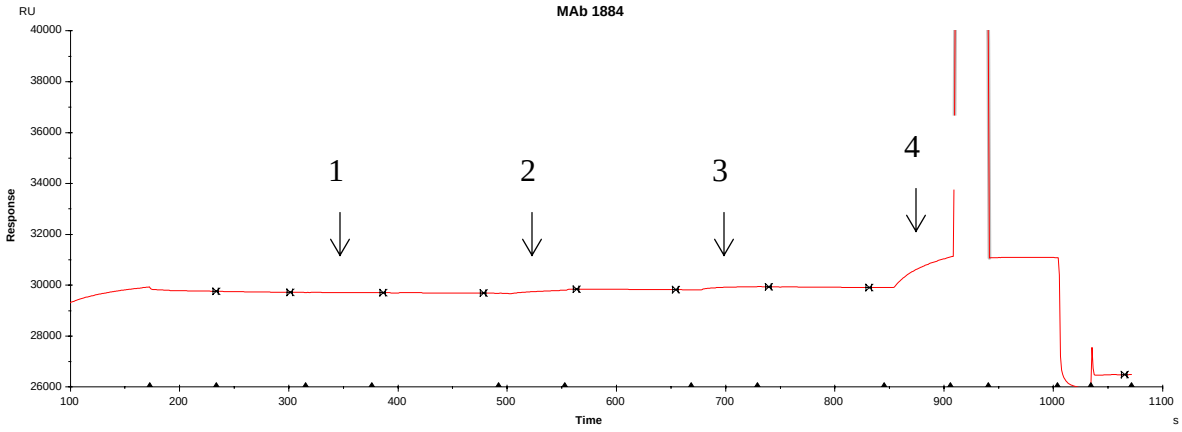


Figure 13: Sensorgrams from the subclass analysis made on the three different PSA-antibodies. 1: injection of anti-IgG1, 2: injection of anti-IgG2b, 3: injection of anti-IgG2a and 4: injection of anti-IgG1.

The results show that the major part of all preparations consist of IgG1, and only small fractions of the others subclasses. Although the small fraction of contamination, it is still believed to affect future assay performance in a negative way. It is therefore considered necessary to minimize the contamination, which can be done by optimizing the immobilization procedure.

3.1.1.5 Choice of immobilization conditions

In assays aimed for concentration determination, a general strategy to obtain good assay performance is to use high ligand levels, which enable measurement of low analyte levels. However, too high immobilization levels may create steric obstacles that will limit the enhancer to bind the ligand-analyte complex.

A pH scouting experiment was made to find a suitable immobilization condition for 1883. 1883 was diluted in acetate buffers with different pH: 5.5, 5.0, 4.5, and 4.0. The ligand concentration was 35 µg/ml and the contact time of the ligand injection was 10 minutes.

Table 4: Immobilized amount of 1883 at different pH.

pH	Immobilized amount (RU)
4.0	6971
4.5	8052
5.0	9168
5.5	4018

The table shows that pH 5.0 gives the highest immobilization level. Although the immobilized amount is important in assay performance, it is not the only factor of interest. The subclass analysis experiment showed that the ligand preparation consists of several subclasses (IgG1, IgG2a, IgG2b and IgG3), where 2a and 2b are disadvantageous in assays measurements since they induce large amount of non-specific binding.

The pH affect proteins differently during immobilization. The monoclonal antibody is a protein with a defined isoelectric point whereas the polyclonal antibodies display a wide range of isoelectric points. Therefore, it may be possible to find a condition that favors immobilization of the desired monoclonal antibody [6].

Anti-IgGx (1, 2a, 2b and 3) were injected over the immobilized surfaces. The response after each injection was recorded and the percent of IgG2a, IgG2b and IgG3 with respect to total immobilized amount was calculated. The result from the subclass analysis is shown in table 5.

Table 5: The table shows the amount of IgG2a, IgG2b and IgG3 in percent of total amount of immobilized ligand.

pH	Undesired subclasses (%)
4.0	3.1
4.5	5.7
5.0	5.1
5.5	25

The subclass analysis shows that pH 4.0 generates the lowest fraction of IgG3, IgG2a and IgG2b, in respect of total immobilized ligand. According to this table, pH 4.0 is the best option for immobilization.

To compensate for the decreased immobilized level that is obtained at pH 4.0 (table 4), the ligand injection time was extended to 20 minutes. At this injection time the immobilized level became about 14 000 RU, which is a satisfying result. When the instrument type later was changed from BC 3000 to S51 a loss of immobilization level was observed. To compensate for this loss, the ligand concentration was raised from 35 to 45 µg/ml.

3.1.2. Choice of regeneration conditions

A study was made to find a suitable regeneration condition and contact time for regeneration of analyte in buffer. A wide range of glycin buffers with different pH was tested, and for each pH different contact times (120, 60, 30 and 15 seconds) were tested. The flow during the regeneration injection was 20 µl/min.

1µg/ml of PSA was injected followed by the regeneration condition. The procedure was repeated three times for each condition. Normally, the mildest regeneration condition is injected first and the harshest in the end of the study. Analyte response - cycle plots were made to analyze how the regeneration affects the ligand's binding ability. In addition to this, baseline - cycle plots were made to see if material accumulates on the surface after each run. The obtained result showed that the range of pH buffers could at a closer interval of 2.5-2.2.

A regeneration experiment was made, this time with a pH range of 2.5 - 2.2 and contact time of 30 seconds (figure 14).

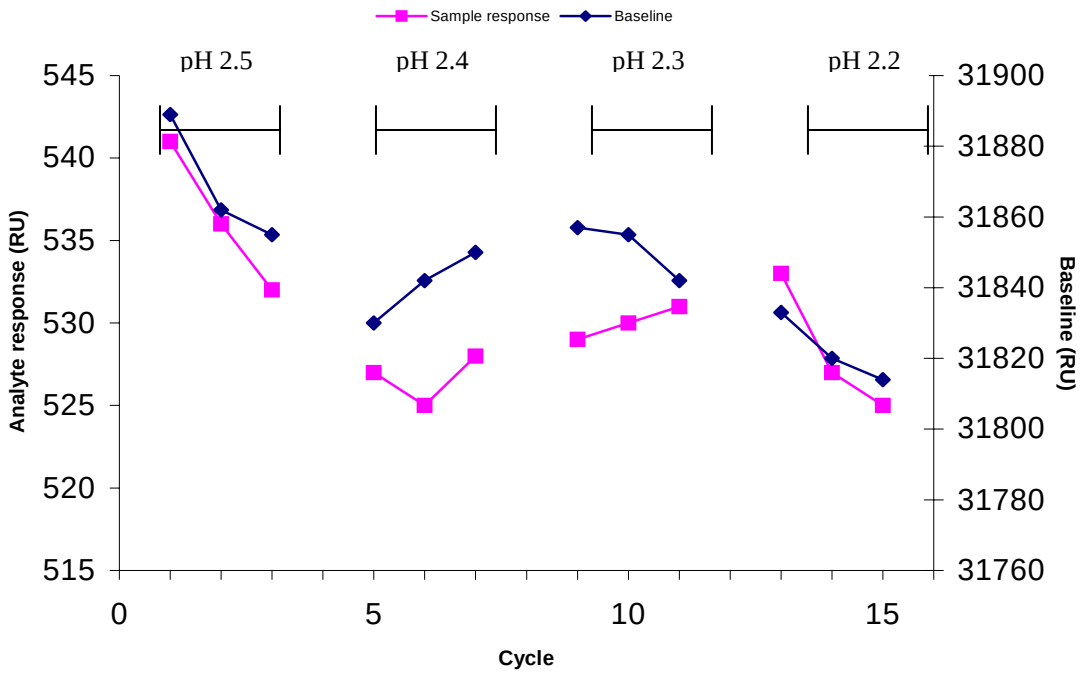


Figure 14: Result plots from regeneration studies on 1883 with four regeneration buffers with varying pH. The red plots correspond to analyte responses (left axis) and the blue plots are the values of the baseline response before the analyte injection in each run (right axis).

Ideally, the analyte response and the baseline should be constant. A drifting trend for the analyte response indicates that either the ligand is losing activity or that material is accumulating on the surface. Concerning the baseline, an increasing level is an indication that material accumulates on the surface, which is due to ineffective regeneration. According to this study, pH 2.3 seems to be a good regeneration condition for 1883 since both the sample response and baseline levels are almost constant.

Before implementation of a regeneration condition it is important to verify the long-term effect of the condition. An experiment was made were the same sample was injected a repeated number of times (25). The obtained result showed constant analyte response levels for all cycles. Hence, glycin pH 2.3 with 30 seconds contact time (flow 20 µl/min) was chosen as regeneration condition.

Glycin pH 2.3 was then tested as regeneration solution in plasma to see whether it had ability to remove the non-specific binding caused by plasma. An experiment was made with ten different plasma samples, where repetitive injections were made and followed by a regeneration injection with glycin pH 2.3.The experiments showed that the baseline increased after each cycle. This means that another regeneration solution is needed, which can regenerate non-specifically bound components.

An experiment was made in order to find a condition that has the ability to remove unspecific binding. At this stage, there were no concerns taken whether the solution would damage the antibody. The solutions that were used are listed below:

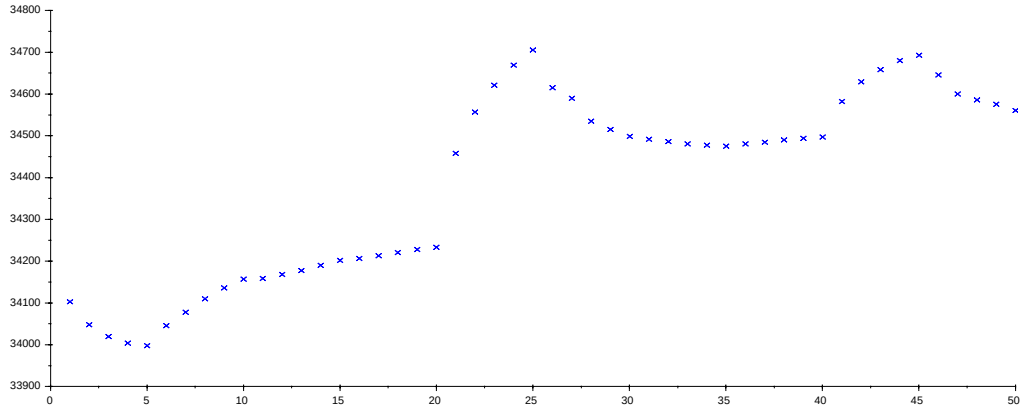
- NaOH (mM): 5, 10, 20, and 50
- HCl (mM): 1, 3, 5 and 10
- Etanolamine-HCl (mM): 8.5, 9 and 10
- NaCl (M): 5
- TFA (%): 0.1

None of the solutions had the ability to completely remove the non-specific binding. The result obtained in this study did not differ significantly compared to the study were glycin pH 2.3 was tested, and it was therefore meaningless to add a second regeneration injection of the solutions listed above to the injection of glycin pH 2.3.

3.1.3 Handling non-specific binding

An experiment was made in order to study the plasma samples with respect to non-specific binding.

The plasma samples were injected in the order 1 to 10 over a surface at which 1883 had been immobilized. Each sample was injected five times and glycin pH 2.3 was used to regenerate the surface (figure 15).



1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10

Figure 15: Baseline response as a function of sample number (1 to 10).

A decreasing baseline indicates that the regeneration solution has the ability to remove non-specific binding. In contrast, if the baseline increases it indicates that non-specific material accumulates on the surface after each cycle.

Plasma 1 and 10 can be considered as non-problematic with respect to non-specific binding, since they both have a decreasing baseline. Almost all other plasma samples have an increasing baseline, which makes them more problematic. Plasma samples 6 and 7 are hard to judge, although they both show a decreasing baseline response. Since 5 has

an extremely high increase in baseline level, the decrease for the following plasma samples is probably due to its background dissociation of accumulated material rather than the binding of 6 and 7.

3.1.3.1 Dilution of plasma

The results from previous experiments showed the difficulties with finding a satisfying regeneration solution for plasma samples. The present solution manages some of the plasmas but not all. The individual differences in terms of non-specific binding make it hard to find a solution that works in each single case.

Another way to handle the problems related to non-specific binding is by diluting the samples. The plasma samples were therefore diluted 50 % in buffer. The buffer consisted of HBS-EP, diluted 9+1 with 10 mg/ml CM-dextran in milli-Q water. Adding dextran is a recognized method to decrease the non-specific binding in plasma samples. The idea is that the material in the sample, which has affinity to the dextran surface at the sensor chip, will bind to the dextran in the solution instead.

3.1.3.2 Reference subtraction

The individual differences between the plasma samples cause problem for assay performance. The dissociation behavior will have an impact on the recorded enhancement response. The response in this step will be the sum of the responses from both non-specific binding and from the binding of the second antibody. Further, due to the variations among individual plasma samples, this must be handled differently for each sample.

In order to minimize this problem the instrument was changed form BC 3000 to S51. The S51 instrument provides a flow cell with two parallel detection spots. The unique property of S51 is that a flow with different solutions can be run over the two spots simultaneously, which gives a way to compensate for the individual dissociation rate in the plasma sample. By using one detection spot to measure the complete sandwich cycle (sample- and enhancement injection), whereas the other spot is leaved to dissociate freely during the enhancement, it is possible to measure the dissociation separately. By subtracting the measured dissociation from the total response, a corrected response is obtained. This method is called “reference subtraction”.

The difference between the flow cell of S51 and BC 3000 (considering this experiment) is schematically shown in figure 16.

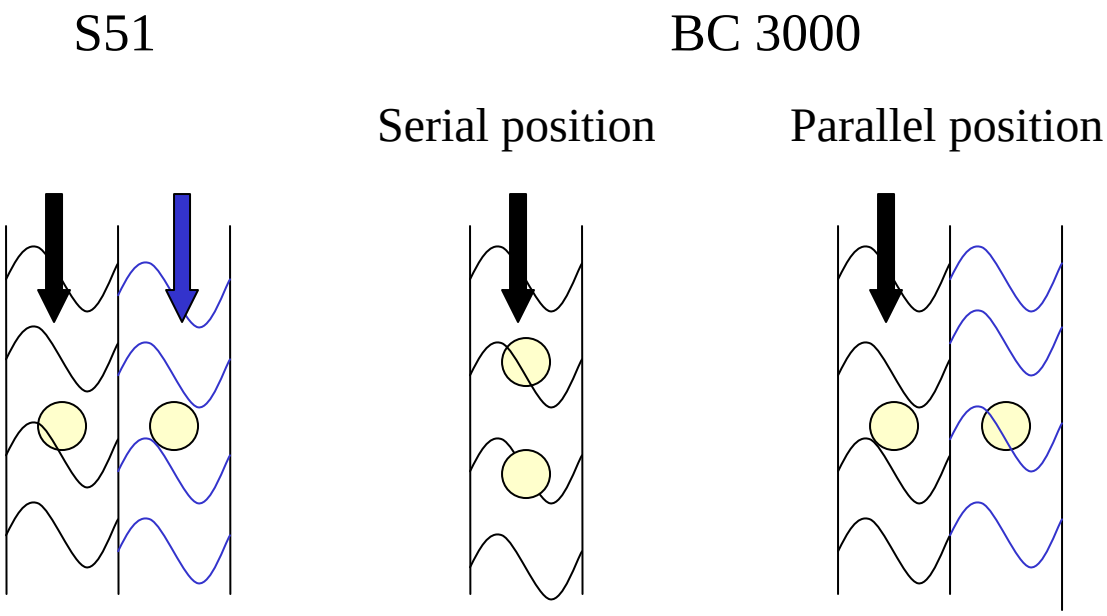
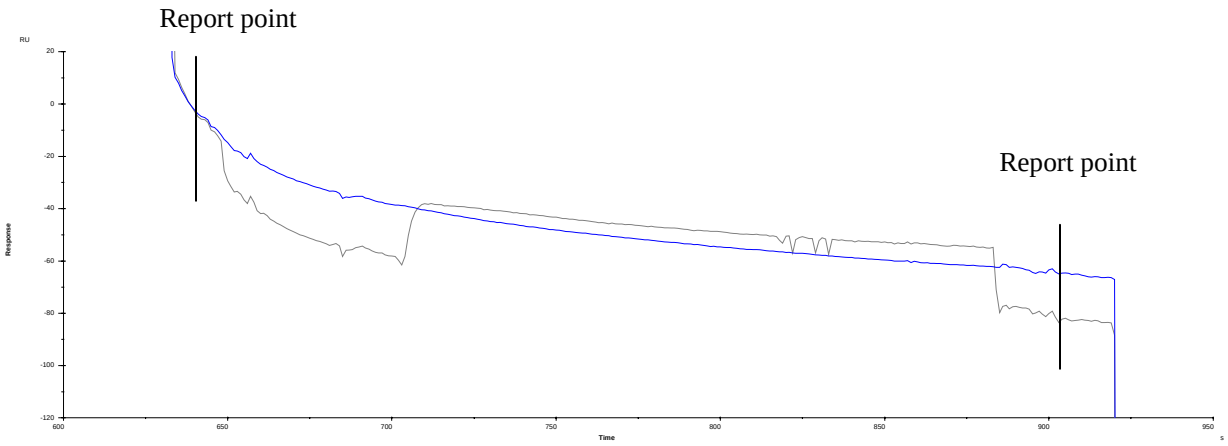


Figure 16: A schematic picture of the difference between the flow cells in S51 and BC 3000. In BC 3000 there are two ways for the detection, serial and parallel. An arrow symbolizes a flow of reagent; the black color symbolizes enhancement reagent and the blue running buffer.

An example of S 51's ability to handle the individual differences in dissociation rate is shown in figure 16. Two blank samples from plasma 1 and 4 were used (figure 17a and 17b). The figures show the sensorgrams for the measurement spot (1) and the spot that is used as reference (spot 2), during the enhancement step. The enhancer is injected over spot 1 (gray lines), whereas buffer flows over spot 2 (blue lines). By subtracting the measured dissociation in spot 2 from spot 1 (spot 1-spot2), a corrected response can be obtained.

a,



b,

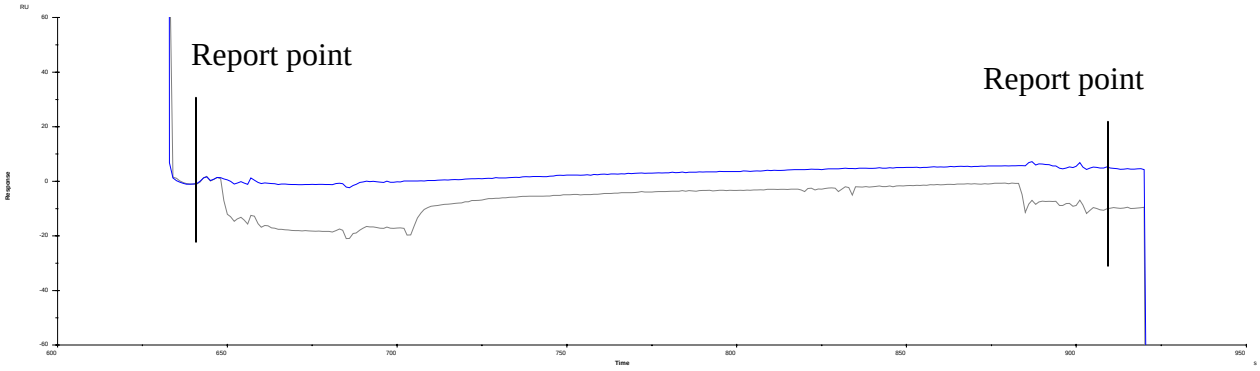


Figure 17: The sensorgrams illustrate the use of the reference spot, where the dissociation of the non-specific material can be determined separately. The differences in dissociation rate between plasma 1 and 4 can be studied in 8a and 8b (blue line).

As can be seen in table 6, the corrected response for the relative responses are within a much smaller range between the different plasma than for spot one alone. This shows the importance of "reference subtraction" for this assay.

Table 6: Relative response values for blank samples (plasma 1 and 4) run in S51. By using two spots a corrected response (Corr. Resp.) can be calculated. The difference in response between the two plasmas is less for the corrected response then for spot 1 alone.

sample	**Spot 1 (RU)**	**Spot 2 (RU)**	**Corr. Resp. (RU)**
Plasma 1	-70.16	-55.99	-14.17
Plasma 4	-9.59	3.84	-13.43

The results show that S51 instrument provides a unique way to compensate for individual variations. However, the corrected responses for the blank samples were negative, although they were supposed to be close to zero. This finding was odd since the two spots are almost treated in the same way. The only thing that differs between the spots is during the enhancement step (1887 is injected over spot 1 and running buffer flows over spot 2). This indicates that some component in the enhancement solution affects the surface in some way.

In order to study this hypothesis, the enhancer's buffer was changed from its original buffer to regular HBS-EP buffer by size exclusion chromatography. This procedure improved the measurement, although not to full extent.

In order to optimise the effect of reference subtraction, the report points were set to immediately before and after the enhancement injection. This adjustment gives the best possible enhancement response and thereby an improvement of assays performance. A single test run was made to confirm the positions of the report points. Arbitrary plasma

was spiked to a concentration of 500 ng/ml. The sensorgram is shown in figure 18, where the enhancement phase has been enlarged to show the positions of the report points.

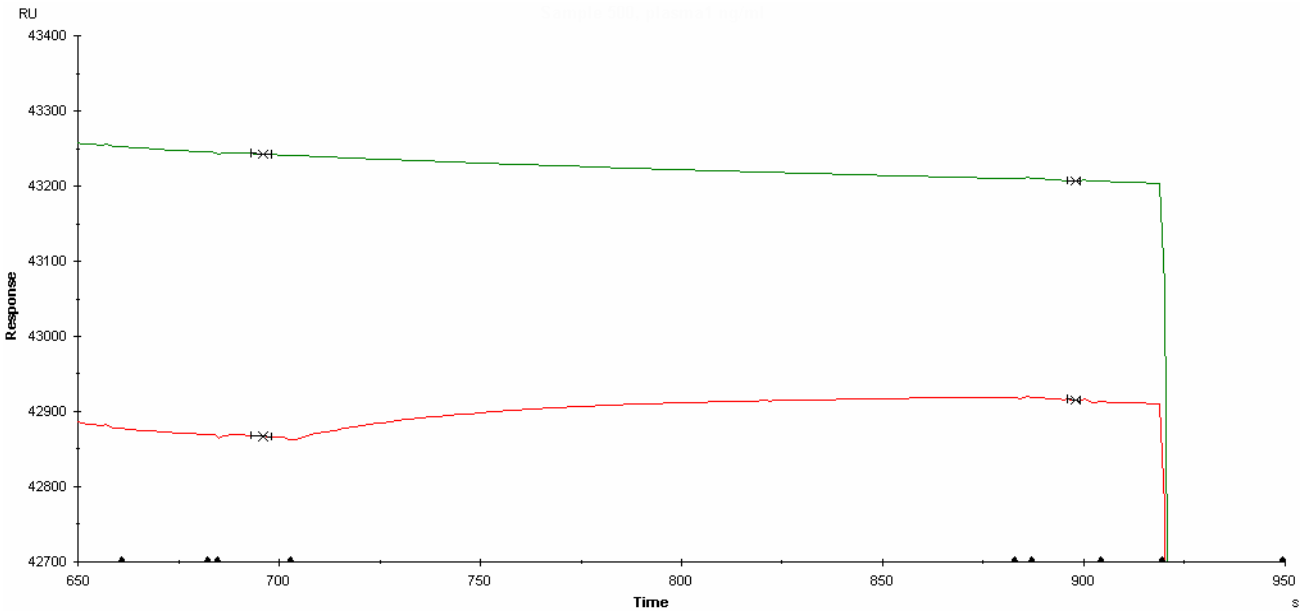


Figure 18: Sensorgram showing the position of the report points: immediately before and after enhancement injection.

3.2 Assay in buffer

The next step was to test the assay sensitivity and precision in buffer. A calibration curve was tested together with 10 blank samples in order to determine the limit of detection (LOD). The experiment was made on S51 with the sandwich pair.

The calibration curve was prepared in the range 200-0.195 ng/ml PSA by serial 1+1 dilutions. Each sample was run in duplicate and the enhancer concentration was 35µg/ml. The result is shown in figure 19 and table 7.

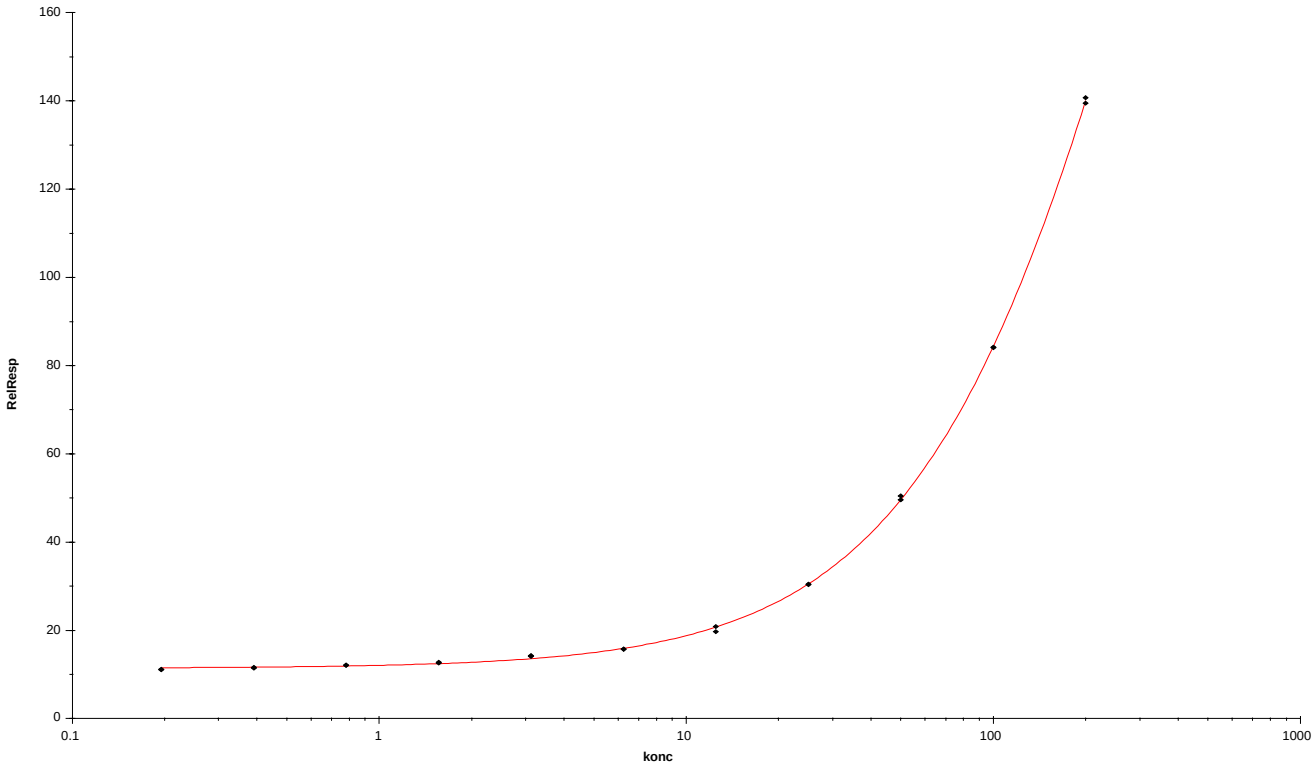


Figure 19: Calibration curve for PSA in buffer. The range of PSA is between 200 - 0.195 ng/ml. The curve was obtained by using corrected response values (spot 1- spot 2).

Table 7: Responses obtained for the dilution series of PSA (200 - 0.195 ng/ml).

Conc. (ng/ml)	**0.195**	**0.391**	**0.781**	**1.56**	**3.12**	**6.25**	**25**	**50**	**100**	**200**
Duplic.1 (RU)	11.08	11.46	12.08	12.58	14.07	15.68	19.70	30.35	84.12	139.95
Duplic.2 (RU)	11.12	11.61	12.14	12.79	14.24	15.83	20.89	30.50	84.20	140.36

The result showed that the precision of the assay is very good. The 10 blank samples gave an average value of 11.6 RU (SD=0.29), which corresponds to a limit of detection of 0.27 ng/ml.

3.3 Assay in plasma

In the experiments the plasma samples were diluted in HBS-EP with 10% CM-dextran (10 mg/ml) to a concentration of 50%.

A response difference will be observed in plasma compared to buffer, simply because the viscosity difference between the two solutions. The viscosity affects the masstransfer of analyte from the sample solution to the surface. A higher viscosity leads to a slower analyte transfer to the surface, and consequently a loss of response. It is therefore expected that the responses will be lower in plasma compared to buffer.

Using a buffer diluent containing 40% human serum albumin will increase the viscosity of the sample and thereby mimic the plasma situation in terms of masstransfer of analyte from the sample solution to the surface. It is therefore expected that the responses will be lower in plasma compared to buffer. By the introduction of the diluent, this effect is compensated for. The calibration curve was prepared in a range of 30 - 250 ng/ml PSA, by serial 1+1 dilution. A calibration curve was also made ordinary buffer in order to study the difference in response between the two conditions (figure 20).

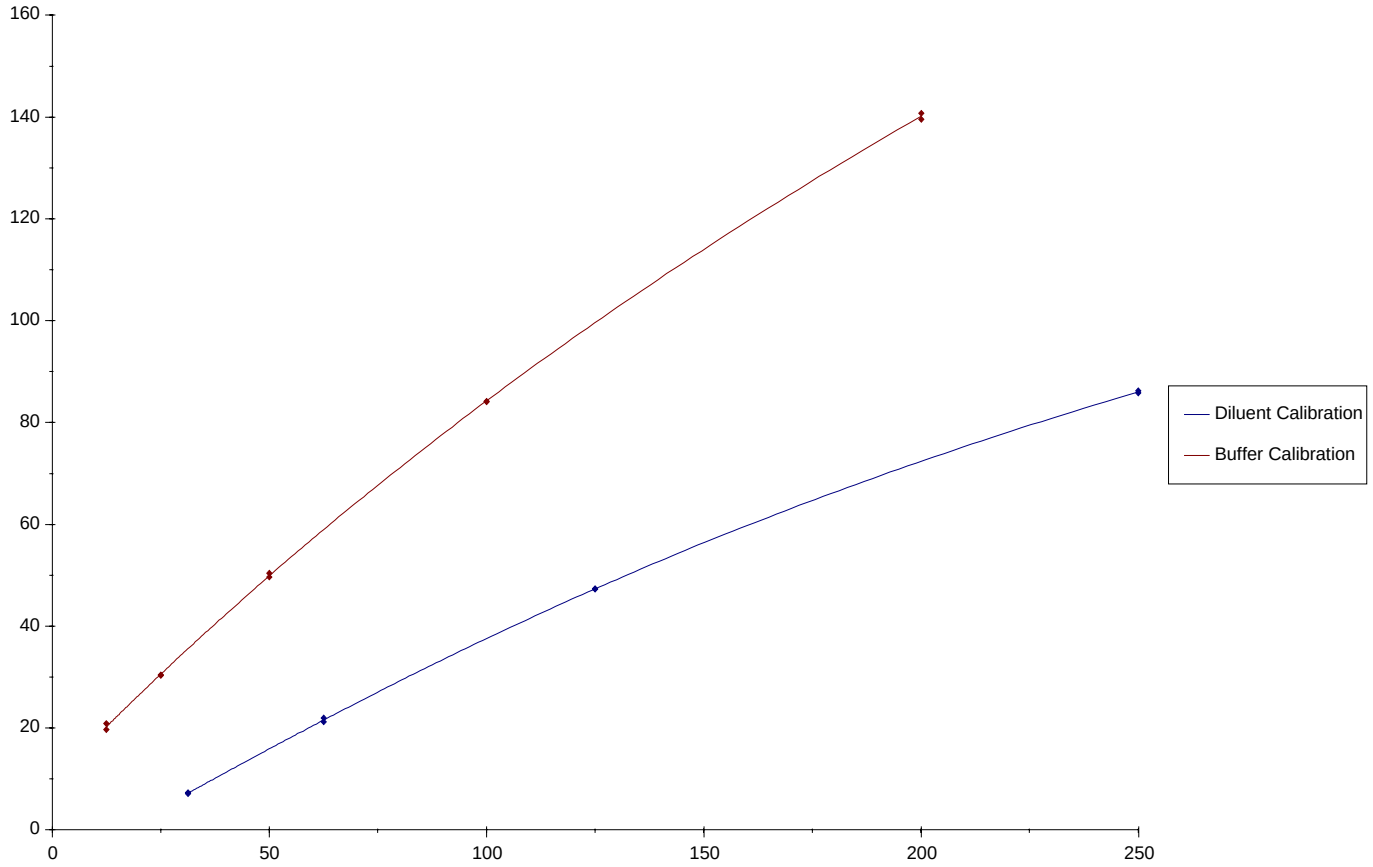


Figure 20: Calibration curves for PSA (250 - 30 ng/ml) in buffer (red) and diluent (blue).

A significant difference is observed between the two conditions.

A calibration curve was made by spiking analyte into plasma 1, due to its relatively low level of non-specific binding. The series was prepared by 1+1 serial dilution in the range 3.9 - 1000 ng/ml. The range in plasma was within a higher interval compared to the range in buffer, since a loss of sensitivity was expected.

An additional calibration curve was made in diluent to study the viscosity effects in diluent compared to plasma. In an ideal case the diluent curve and plasma curve should generate identical curves, since the diluent and plasma has the same viscosity.

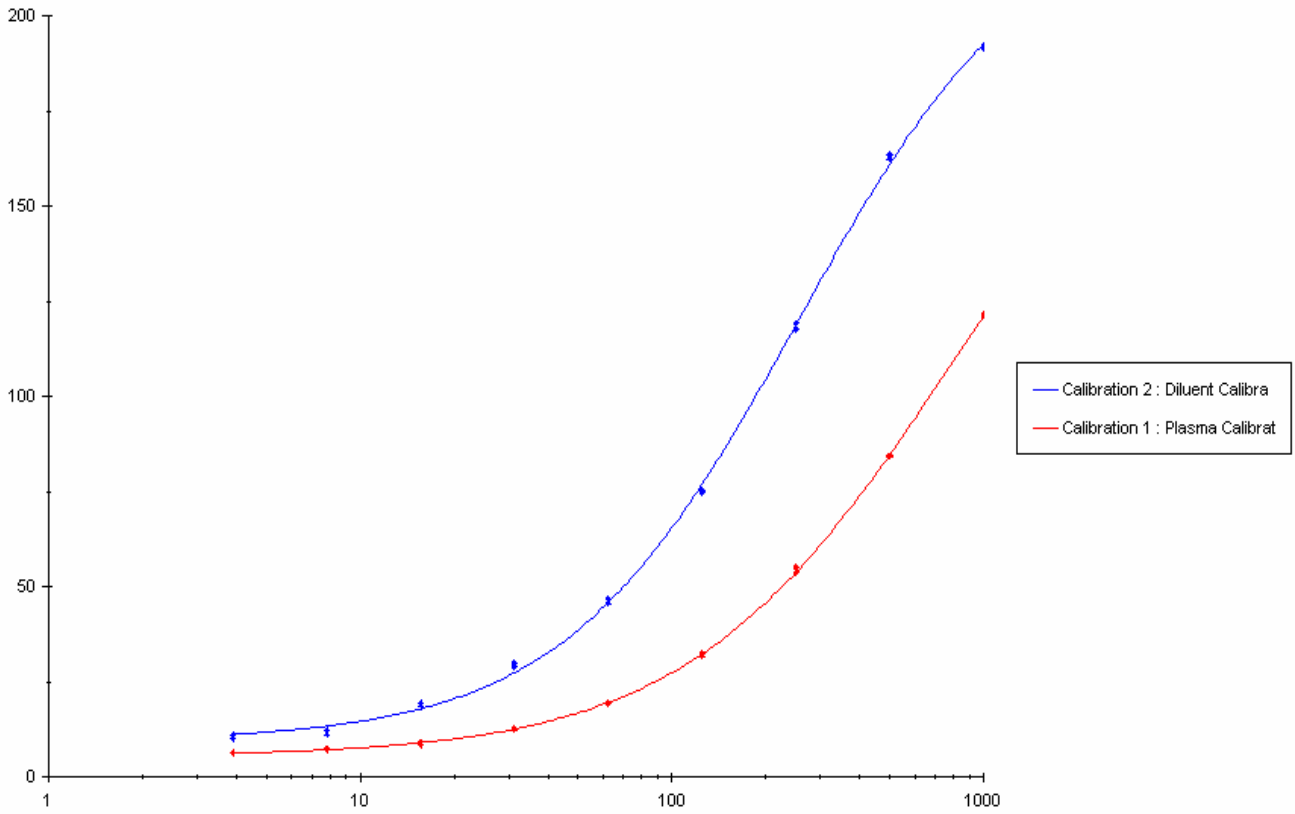


Figure 21: Calibration curve for PSA in plasma 1 (red) and HSA-solution (blue). The response (RU) is plotted as a function of concentration (ng/ml). The concentration range was 3.9 - 1000 ng/ml. The curve is obtained by using corrected response values (spot 1-spot 2).

A big difference in sensitivity between the two assay solutions can be observed. The blank samples from the HSA-solution gave an average value of 8.42 RU (SD= 0.87), which gives a LOD-value of 3.7 ng/ml. The corresponding measurement of the blank samples in plasma gave the average of 7.24 RU (SD=46.9 ng/ml) and a LOD-value of 46.9 ng/ml. Hence, the conclusion is that the PSA analyte recovery is low in plasma. One hypothesis may be that this is an effect of complexing of free PSA with some of its natural binding partners in the plasma.

3.3.1 Recovery of PSA-spiked samples

In order to test the assay's ability to handle individual differences between the plasma samples, a recovery experiment was made. The recovery refers to the correlation between the measured and expected concentration in a sample, where the expected concentration constitutes of a spiked sample. The measured concentration is based on a calibration curve obtained in one individual plasma. In this experiment, measured concentrations were based on a calibration curve obtained in plasma 1 and calibration curve obtained in diluent.

Plasma 1-9 were spiked in the following concentrations: 500ng/ml, 200 ng/ml, 50ng/ml and 0 ng/ml. The measured concentrations for plasma 1-9 are shown in table 8-9.

Table 8: Table shows the measured concentrations of plasma 1-9, based on calibration curve in plasma 1.

Spiked concentration (ng/ml)					
	Plasma	**0**	**50**	**200**	**500**
Measured PSA concentration (ng/ml)	**1**	LOW	37.0	137.9	396.7
	1	LOW	37.8	135.9	387.4
	2	LOW	15.0	191.8	433.3
	2	LOW	10.7	185.6	425.1
	3	LOW	33.8	140.0	343.1
	3	LOW	28.4	137.0	350.4
	4	LOW	32.4	161.4	379.6
	4	LOW	32.8	147.6	383.3
	5	LOW	34.9	127.5	285.3
	5	LOW	28.9	124.5	287.1
	6	95.7	165.3	329.6	628.2
	6	79.8	154.3	318.4	624.3
	7	19.6	110.7	280.8	575.9
	7	-	115.9	284.7	572.9
	8	160.0	437.1	593.8	964.7
	8	163.7	400.1	599.6	879.2
	9	238.5	206.2	342.7	687.8
	9	237.8	209.1	379.3	655.4

LOW: The measured response is below the range of the calibration curve.

Table 10: Table shows the measured concentrations of plasma 1-9, based on calibration curve in diluent.

Spiked concentration (ng/ml)					
	Plasma	**0**	**50**	**200**	**500**
Measured PSA concentration (ng/ml)	**1**	LOW	9.6	43.2	114.1
	1	LOW	7.6	42.6	116.6
	2	LOW	LOW	57.7	124.0
	2	LOW	0.325	59.3	126.2
	3	LOW	8.3	43.2	104.2
	3	LOW	6.2	42.6	102.3
	4	LOW	7.8	46.2	113.0
	4	LOW	7.9	50.4	112.0
	5	LOW	8.7	39.0	86.8
	5	LOW	6.4	40.0	86.4
	6	9.0	30.1	73.6	149.1
	6	13.7	33.1	76.5	150.0

	7 **7**	LOW -	19.4 18.0	64.9 63.9	136.9 137.6
	8 **8**	62.0 82.0	103.7 94.6	141.9 143.2	226.3 207.5
	9 **9**	31.7 32.6	44.9 44.2	89.2 79.9	156.5 164.2

LOW: The measured response is below the range of the calibration curve.

The experiment shows that there is great variation in the measured concentrations between the plasma samples. The variation is observed both for the measured concentrations based on the plasma 1 calibration curve and for the measured concentrations based on diluent calibration curve. The experiment shows that it is not possible, under present assay conditions, to use calibration curves obtained in plasma 1 or diluent, as reference for assay measurements.

A recovery curve for plasma 1 is shown in figure 22. The spiked-concentrations were plotted against the measured-concentrations, based on the calibration curve in plasma 1. The slope coefficient of the line gives the level of recovery.

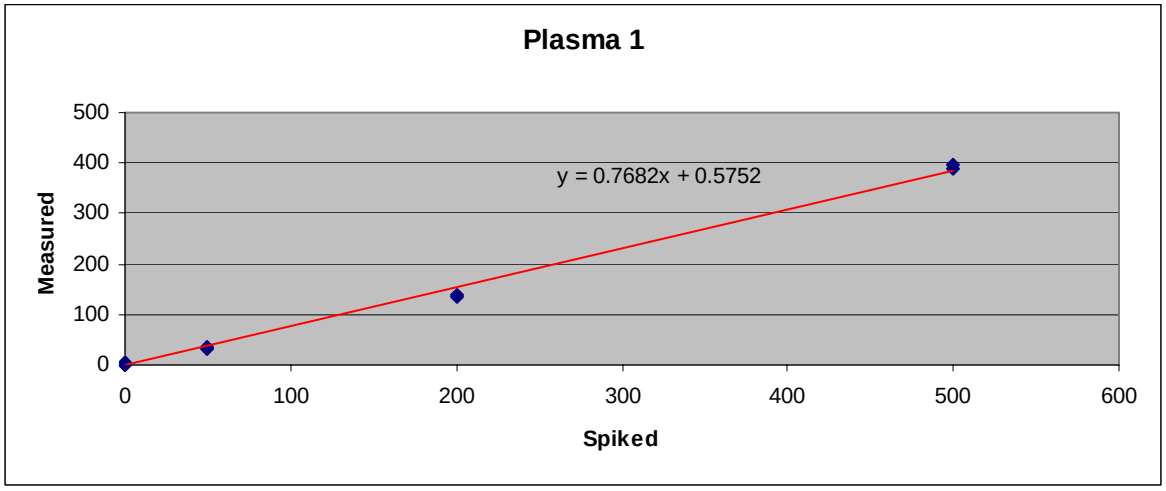


Figure 22: The spiked-concentrations plotted against the measured-concentrations for plasma 1, based on calibration curve in plasma 1. The level of recovery is 77 %.

3.3.2 Assay analysis - sensorgrams

In order to study the effect of "reference subtraction" on assay performance, over-lay plots were made from the obtained sensorgram in the recovery experiment.

Three different over-lay plots are shown, each plot focus on different aspects of the reference subtraction. The separate sensograms have been aligned at a common point before the enhancer injection and the enhancement step have been enlarged.

The first overlay-plot (figure 23) shows the 9 different plasmas during the enhancement step for the blank samples. The sensograms have been obtained by subtracting reference spot from the active spot (spot 1 – spot 2) and they are aligned at a point before the start of enhancement injection.

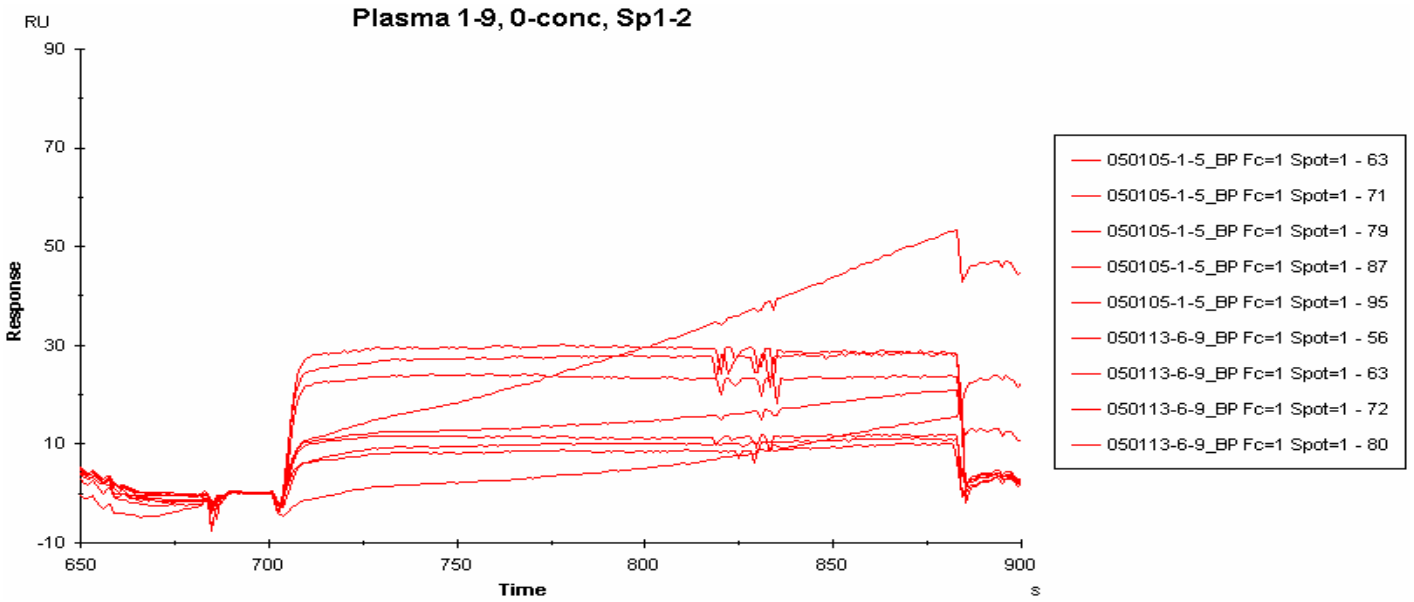


Figure 23: An overlay-plot for blank samples (plasma 1-9) during the enhancement step. The sensograms have been obtained by subtracting the reference spot (spot 2) from the active spot (spot 1).

The over-lay plot shows three sensorgram with substantial background binding of the enhancement Ab (plasma 6, 8 and 9). Remaining plasma samples show <5 RU of non-specific binding.

Next overlay-plots (figure 24 and 25) show the sensorgram from the concentration series of PSA (3.9 - 1000 ng/ml) in plasma 1. The sensorgram in figure 24 shows the active spot (spot 1) and figure 25 shows the reference spot (spot 2).

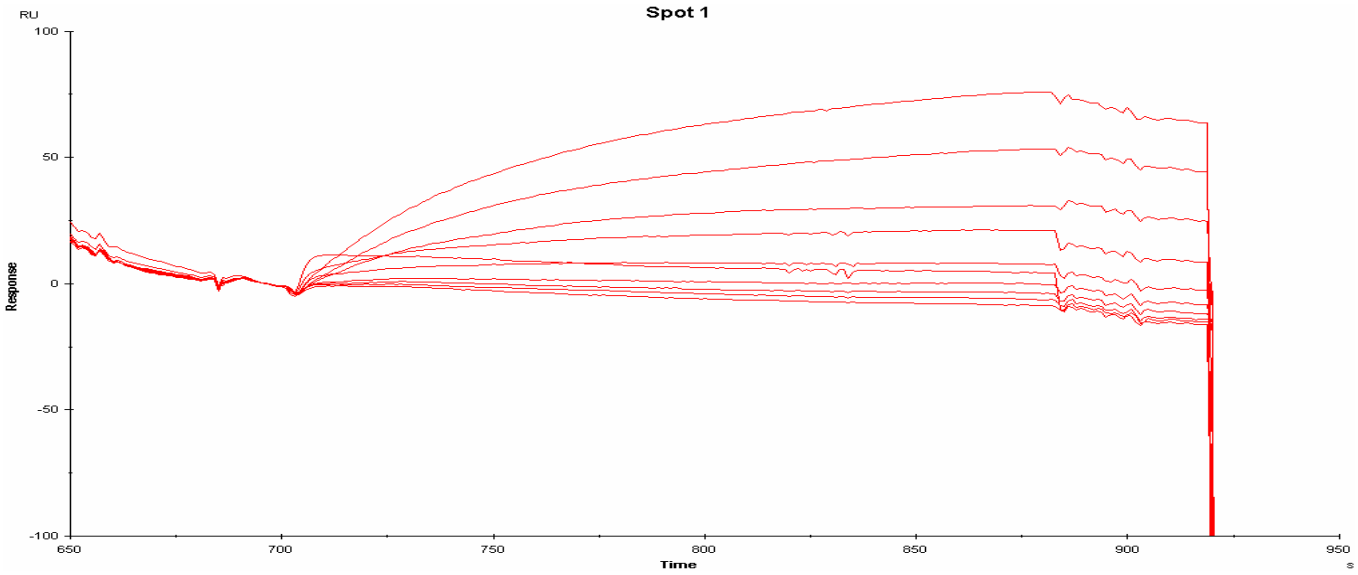


Figure 24: An overlay-plot for plasma 1 on the active spot (spot 1) in a concentration range of 0-1000 ng/ml.

The over-lay plot shows the different binding rates of enhancer reagent on spot 1. The differences are due to the different concentrations of PSA that was bound to the surface during the analyte injection step. A large amount of bounded PSA gives more binding sites for the enhancer, and consequently higher binding rate.

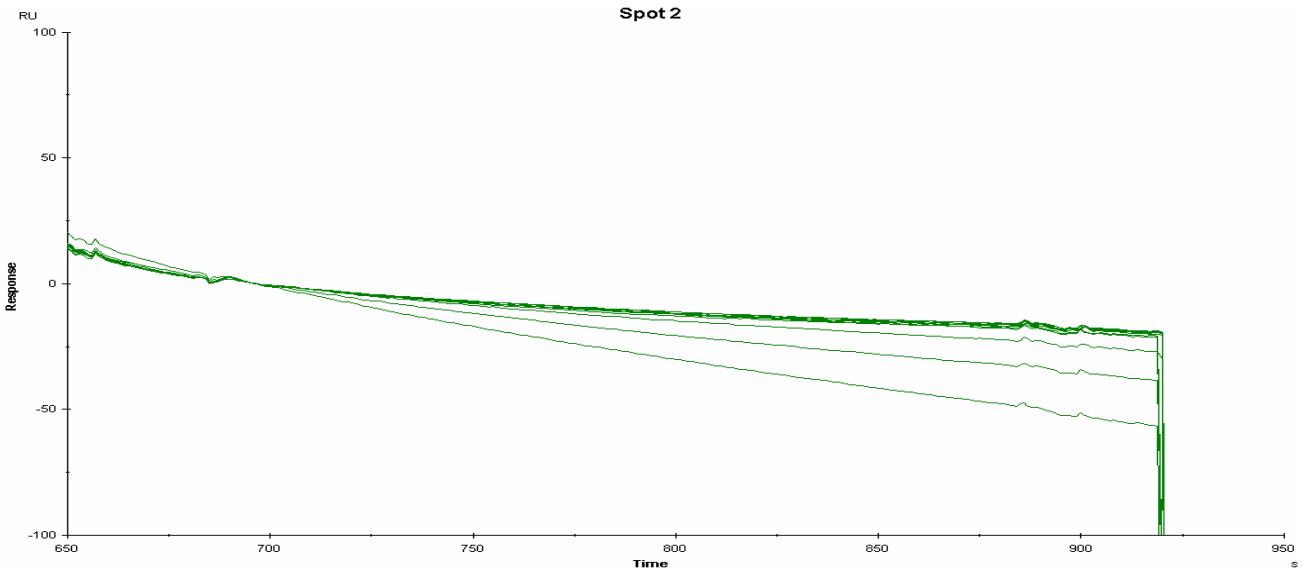


Figure 25: An overlay-plot for plasma 1 on the reference spot (spot 2) in a concentration range of 0-1000 ng/ml.

The overlay-plot shows the different dissociation rates of enhancer antibodies on spot 2. The difference in dissociation rate is due to the difference in PSA-concentration that was bound to the surface during the analyte injection phase.

3.4 Attempts to find PSA in a complexed form

The correlation between raised levels of PSA and prostate cancer is not as clear as it first seemed. It is now known that other types of prostate complications also increase the level of PSA. This makes cancer prediction uncertain and an additional marker is therefore needed to improve the diagnostic prediction.

It has been found that if the amount of PSA in a free state is measured together with PSA in complex state, the accuracy of cancer prediction is improved. The most abundant PSA complex is the one it forms with anti-chymotrypsin (ACT), a protease inhibitor. It is therefore a big clinical interest for methods were both types of PSA states could be measured.

In this work attempts were made to detect PSA in complex with ACT (c-PSA). For this task 1883-1887 was used as antibody pair, which is specific for free-PSA (f-PSA). The total amount of PSA (t-PSA) can be defined as: t-PSA= f-PSA + c-PSA.

The idea was to measure a known concentration of f-PSA, in a solution where no ACT has been added. In this solution the f-PSA correspond to t-PSA. In order to measure a presumed decrease of f-PSA, ACT was added to a solution with the same f-PSA concentration as the first solution. If ACT forms complex with PSA the amount of f-PSA will decrease, and will thereby be detected as a decrease in response.

Two solutions were prepared in buffer. One solution with PSA in a concentration of 1 µg/ml and the second were PSA and ACT was mixed in a concentration of 1 µg/ml and 5 µg/ml respectively.

Five repetitive cycles with f-PSA were made, followed by five cycles of PSA/ACT – solution. Two reference cycles were also made with a 5 µg/ml ACT-solution. Two sets of experiments were made; one incubated for 5 minutes in room temperature whereas the second solution was incubated for 5 days in 35 ºC. The responses from the experiment where the PSA/ACT solution was incubated for 5 days in 35 ºC is shown in table 9.

Table 9: Determination of f-PSA in i) a PSA solution of 1 µg/ml, ii) a solution containing PSA and ACT (1 and 5 µg/ml, respectively) and iii) in a ACT solution (5 µg/ml). The PSA/ACT solution had been incubated for 5 days in 35 ºC. No decrease in response can be seen for the PSA/ACT solution.

Cycle	PSA (RU)	PSA/ACT (RU)	ACT (RU)
1	138	147	10
2	139	147	10
3	140	147	
4	140	148	
5	139	149	

No decrease in response could be seen for the PSA/ACT solution. There was no difference in result between the two incubation conditions. No further attempts were made to find PSA in complex form.

4. Discussion

The main objective of this work was to develop an assay for PSA. The first part of the work was focused on finding appropriate reagents for this purpose. To assess the quality of the reagent, a preliminary assay in buffer was developed. This assay was then used as a model system in plasma to study the effects of different measures taken in order to minimize the effects of non-specific binding.

The developed assay proved to work extremely well in buffer, where the precision and sensitivity were most satisfying. When the assay was run in plasma the assay performance was affected in a negative way. This difference in assay performance between buffer and plasma was expected. The main part of the work has been to make modifications of the assay in order to get the best possible assay performance in plasma. Although certain improvements were made, a complete assay for plasma measurements could not be accomplished during the time of the work.

A main issue during the work was to deal with problems related to the individual differences between plasma samples. The individual differences cause trouble for assay performance, which is due to the unique dissociation behavior during the enhancement step for each plasma sample. The dissociation behavior will have an impact on the recorded enhancement response. The response in the enhancement step will be the sum of the responses from both non-specific binding and from the binding of the second antibody. However, by changing instrument form BC 3000 to S51, it was possible to compensate for this effect by using the S51's unique designed IFC.

The IFC in S51 is normally used to compensate for systematic disturbances when handling low weight molecules and had never been tested on assay performance in plasma at Biacore before. The function proved to be very useful to handle problems related to non-specific binding. Comparative studies were made on BC 3000 and S51, and the results show great improvements when it comes to handle the individual differences in plasmas. The "off set" differences between plasmas become much lower, making detection possible in cases were in would be virtually impossible without it.

Although the introduction of S51 improved the assay in a great extent, it is still a long way from being a functional assay for PSA detection in plasma. The assay does not have a regeneration condition that is capable of handling the individual differences in plasma samples. The assay also has a sensitivity problem, where the detection limit is too high in order to be of clinical importance. In the following text some improvements of the assay are discussed.

A way to make the assay applicable to a clinical situation would be to improve the regeneration phase. If a regeneration solution that prevents an accumulation of non-specific binding after each cycle could be found, the assay performance would probably be improved to a great extent. Although a big variety of solutions were tested, no one has yet proved to work on all plasmas. Further screening tests might result in a solution that works better than the present one.

Another way to reduce the non-specific binding would be to introduce some sort of purification step. Maybe some sort of chromatography or spin columns could be used in order to purify the plasma. However, this can be quite complicated since the method must be able to distinguish between specific (PSA) and non-specific substances.

To further improve the sensitivity, it might be interesting to look closer on the low recovery of PSA. In a diluent buffer, 500 ng/ml of PSA gave a response about 140 RU. In spiked plasma condition the same concentration only yields about 40 RU (depending of plasma sample). This means that there are about 100 RU differences between the two conditions. A certain loss was expected, but not this great.

Perhaps is some sort of complex formed in plasma between PSA and ACT (or other proteins). This complex might prevent the capturing antibodies from recognizing the epitopes on PSA, leading to low responses. However, this hypothesis was not confirmed by the experiments that were made to see if PSA forms complex with ACT in buffer, which all showed to be negative. Perhaps are PSA and ACT forming a complex after all, induced by something in plasma that is not present in buffer. Or maybe was the purchased ACT some sort of purified form that could not be used for these types of experiment. An interesting thing would be to develop an assay for total-PSA and compare a blank sample with a situation were free-PSA have been added to a sample. If the level of total-PSA increases it would indicate the added PSA forms complex in plasma.

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