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Assay development for quantification of monoclonal IgG in Gyrolab Bioaffy™

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Assay development for quantification of monoclonal IgG in Gyrolab Bioaffy™

Malin Rehnholm

Sammanfattning

När kroppen ska försvara sig mot främmande ämnen såsom bakterier och virus bildar kroppen antikroppar som är en stor del av kroppens egna försvarssystem, immunförvaret. Utan immunförvaret skulle vi inte klara av att stå emot minsta infektion och det är därför nödvändigt för överlevnad. Antikroppar binder till det främmande ämnet, antigenet och presenterar det för celler som har till uppgift att bryta ner antigen för att undvika dess överlevnad.
På senare år har det visats att förloppet för somliga sjukdomar såsom vissa typer av cancer, infektiösa sjukdomar samt autoimmuna sjukdomar kan mildras och kanske även botas genom att behandla patienten med antikroppar riktade mot den cancerogena eller infektiösa substansen. För denna behandling krävs tekniker för syntetisk framställning av antikroppar i stor skala och med god produktivitet. För att kunna utveckla odlingssystem med hög produktivitet krävs tillgång till enkla, snabba och robusta metoder för att kunna kvantifiera IgG.

Det finns olika typer av antikroppar och i detta projekt, genomfört på Gyros AB, Uppsala, har den mest förekommande antikroppen immunoglobulin G (IgG) använts för att utveckla en metod för att kunna kvantifiera koncentrationer mellan 20 µg/ml upp till 4000 µg/ml. Målet var att skapa en robust metod med effektiv tidsanvändning som kan användas för kvantifiering av olika subklasser av IgG. Protein G och fragment Z är namnen på två rekombinanta immunoglobulinbindande proteiner från bakterier som har använts för att fånga upp samt detektera antikroppar i provet. De detekterande proteinerna är märkta med ett fluorescerande reagens och signalen som genereras är proportionell mot antalet IgG molekyler i provet så länge systemet ej är mättat av instrumentella eller biologiska skäl.

Slutsatsen är att man beroende på önskat mätområde kan anpassa metoden genom att använda de fångande och detekterande reagenserna i olika kombinationer. Högst IgG koncentration kan kvantifieras med Fragment Z som både fångande och detekterande reagens. Det tycks dock som om koncentrationer inom det låga mätområdet går förlorade.
Nästa steg blir att jämföra denna metods prestanda gentemot andra metoder utvecklade på Gyros AB, Uppsala. Försök på relevanta serumprover med känd koncentration borde analyseras för att utvärdera metodens pålitlighet.

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Abbreviations

IgG	Immunoglobulin G
CDC	Complement-dependent cytotoxicity
ADCC	Antibody dependent cellmediated cytotoxicity
CHOP	Cyclophosphamide, doxorubicin, vincristine and prednisolone
CD20	Cluster of differentiation number 20
hTNFα	Human tumor necrosis factor alpha
Mabs	Monoclonal antibodies
CD	Compact disc
Fc	Fragment crystallizable
Fab	Fragment antigen binding
SpA	Staphylococcal protein A
SpG	Streptococcal protein G
Fz	Fragment Z
PG	Protein G
BSA	Bovine Serum Albumin
PBS	1 x Phosphate buffered saline: 150mM PB, 0.15 M NaCl, 0.2% NaN_3. pH 7.4
PBS-T	PBS with 0.01% Tween 20
ELISA	Enzyme linked immunosorbent assay
RIA	Radioimmunoassay
PMT	Photo Multiplier Tube
LIF	Laser Induced Fluorescence

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1 Introduction

1.1 *The great need for correctly measuring antibodies in a sample.*

Hundreds of companies worldwide are today working with the development of monoclonal antibodies with a purpose to treat patients with certain disorders and extensive studies have resulted in several therapeutic products being available on the market. The first monoclonal antibody registered for pharmaceutical use was approved in 1986 [1]. Approximately 20 monoclonal antibodies have so far been registered, approximately hundred product candidates are in clinical evaluation and more than 500 monoclonal antibodies in pre-clinical phases, respectively. This market involves more than $15 billion per year [2]. Treatments with monoclonal antibodies alone or in combination with other conventional therapies have resulted in a higher recovery frequency than previously. Hitherto, monoclonal antibodies have been applied on a regular basis in the fields of cancer, autoimmunity, inflammation and in infectious diseases. Antibodies are major constituents of the adaptive immune system and are involved in identifying and binding foreign substances, antigens, transporting and presenting them to T cells, macrophages and other cell types of the immune system that are responsible for eliminating antigens. By treating the patient with antibodies directed against the antigen, one can with the aid from the patients´ own immune system eliminate foreign substances and the disease can be mitigated.

Rituximab was the first monoclonal antibody to be registered in Sweden for treatment of malignant disorders. Rituximab (Mabthera) is a chimeric human/mouse anti-CD20 antibody that is used in the treatment of B cell lymphomas. In order to function properly, effector functions recruited from the patients own immune system such as complement-dependent cytotoxicity (CDC) and antibody dependent cellmediated cytotoxicity (ADCC) is required to obtain full effect. This antibody is directed against CD20, a cell membrane bound ion channel protein present on both normal as well as malignant pre-B-lymphocytes and mature B-cells [3].

A clinical study was performed on 130 patients suffering from follicular lymphoma and they were all treated with Rituximab. 57% of the patients responded well and progress was seen after approximately nine months [3]. Another study done in 2002 by Coiffier et.al.[4] reported that overall survival was increased in patients with diffuse large-B-cell lymphoma when treated with the standard treatment CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) plus Rituximab compared to treating with CHOP alone [4].

Remicade is a recombinant chimeric monoclonal antibody directed against hTNFα that can be used for treating patients suffering from certain severe forms of Crohns disease but also other autoimmune disorders. 108 patients with mild to severe forms of Crohns disease took part in a study and a one-time-dosage of Remicade resulted in 81% of patients demonstrating response after 4 weeks that was maintained in 42% of patients after 12 weeks [5]. The dosage of antibody based drugs varies in different disorders depending on degree of severity and desired effect but given the prevalence of disease, the dosage required and the frequency and duration of treatment it can be stated that huge quantities of monoclonal antibodies must be supplied to meet these needs. A typical treatment schedule can be to initially administer 5 mg Mab/kg bodyweight into the patient followed by two identical infusions two and six weeks later. If

response is not seen additional treatments can be made [5]. A 60 kg patient with Crohns disease would therefore require 300 mg per treatment resulting in more than 1g of monoclonal antibodies per patient and year. These are enormous quantities and the pressure on developing more efficient processes to produce Mabs in order to meet these needs is therefore constantly rising. The most important response parameter, although not the only one, during development of such processes is quantifications of IgG. Hence methods that conveniently and accurately quantify monoclonal IgG in the appropriate concentration range will be requested.

Researchers worldwide are motivated to develop more efficient, more precise and less expensive manufacturing techniques. To meet these needs larger cell culture tanks for increased productivity will be required as more companies addressing this market.
Autoimmune disorders, cancer and various infections are diseases where antibodies are expected to contribute to recovery, although additional therapies might still be necessary. Antibodies directed against tumour cells in cancer patients are sought for as well as antibodies functioning as scavengers in infectious or autoimmune diseases meaning that the antibodies function as receptors which bind antigens and present them to the immune system where they can be eliminated.

Monoclonal antibodies are today widely used. Initially they were produced by a B cell hybridoma, a cell line created when fusing a normal B cell with an immortal B cell tumour line. The first hybridoma secreting monoclonal antibody was described in 1975 and during the late 1970s a lot of progress was done with this technique [1]. All antibodies produced from one clone are essentially identical and two clones can never produce identical antibodies [6]. The specificity for different antigens and epitopes do therefore vary as well as the affinity. There are five classes of human immunoglobulins, IgG, IgA, IgM, IgD and IgE. IgG and IgA can be further divided into various subclasses [6]. Each class has different effector functions and these properties are used when one wishes to amplify different subclasses. Most monoclonal antibodies belong to the IgG class of immunoglobulins, and in particular subclass IgG1.
Today there is a great need to develop quantitative immunoassays for monoclonal IgG since this type of molecule is used for treating various diseases. The desired properties would be to employ an assay where a certain subtype of IgG can be distinguished and quantified from either pure or complex samples. Another important parameter to consider is to cover a wide range of concentrations to avoid dilutions of the analyte as well as the time consumed to quantify a sample. Measuring many samples in parallel without affecting the outcome is required.

1.2 Gyrolab Bioaffy

Gyrolab Bioaffy is a tool developed by Gyros AB where sensitive sandwich immunoassays based on the antibody-antigen interaction automatically are performed.
The technique uses a compact disk (CD) with 14 separate segments. In each segment there are 8 identical microstructures that each one can analyse 1 sample. This results in 112 identical microstructures per CD, each capable of carrying out a single individual test.
The CD is put into a Gyrolab Workstation where reagents and other liquids are transferred into the CD in an automated way. This reduces the risk of pippetting errors and makes the system more efficient and timesaving. In one batch 5 Gyrolab Bioaffy CDs can be run, i.e 560 data points can be generated in 4-5 hours.

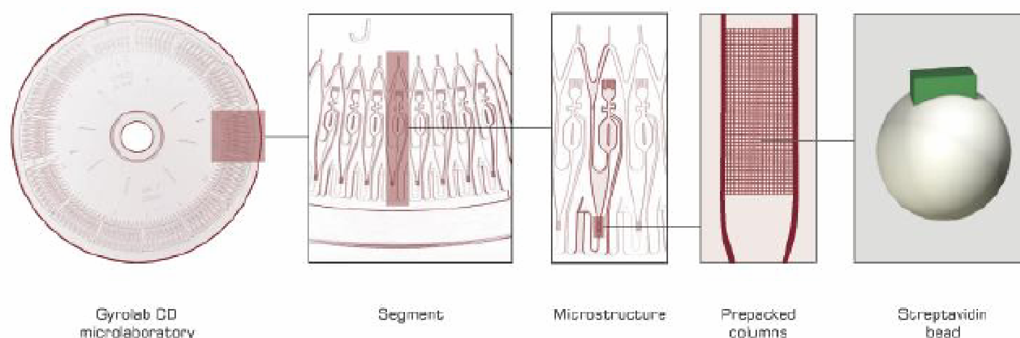


Figure 1. The Gyrolab Bioaffy CD to the left contains 14 separate segments, one segment being marked. Each segment consists of 8 microstructures (middle picture). The column in every microstructure is usually packed with Streptavidin-coated beads (to the right).
Picture used with permission from Gyros.

In each microstructure there is a microcolumn prepacked with streptavidin coated particles and any biotintinylated biomolecule can become attached to the solid surface. The capacity of the column can be adjusted by using particles with various porosity and the amount of immobilized streptavidin. Compared to solid particles, porous particles allow higher concentration of capturing reagent being attached to the column.
Each segment has a common inlet and a common channel that distributes wash solution and reagents to all microstructures within that segment (figure 2.). In each microstructure there is an individual inlet where the sample is added. To prevent overflow and to control the volume that passes through the column, the structure is equipped with a channel for excess liquid. Hydrophobic barriers are placed strategically to prevent unwanted liquid movement within the microstructures. Precise liquid movement in the structures is of great importance and hence the surface chemistry is optimised to achieve the appropriate conditions. Capillary action and a hydrophilic coating in the microstructure enable the liquid to efficiently be drawn into the microstructures to the hydrophobic barrier [7, 8].
The reagents and samples are kept in 96-well microplates. Wash solutions, capturing reagents, samples and detecting reagents are transferred from the microplate to the CD by needles in an automatic robotic arm that rapidly dispenses correct volumes in a pre-programmed order.
When spinning the CD the hydrophobic breaks are overcome by the centrifugal force acting on the liquid and the defined liquid volume passes rapidly through the column. Thus, interactions between analyte and reagents take place under flow conditions. Flow rate is affected by changing the rotational speed but also characteristics of the liquid and design of the structure are of importance. The detection is done by a laser induced fluorescence (LIF) detector that is integrated in the instrument.
The small volume required enables more samples to be analysed in shorter times. For one segment the required volume of capture and detecting reagent is 2.5 µl and 420 nl of analyte is consumed per structure which is a lot less than other available techniques as for example ELISA (Enzyme linked immunosorbent assay). Up until recently CDs with a volume definition chamber of 200 nl has been used. The dynamic range for a given analyte usually covers 3-4 orders of magnitude.

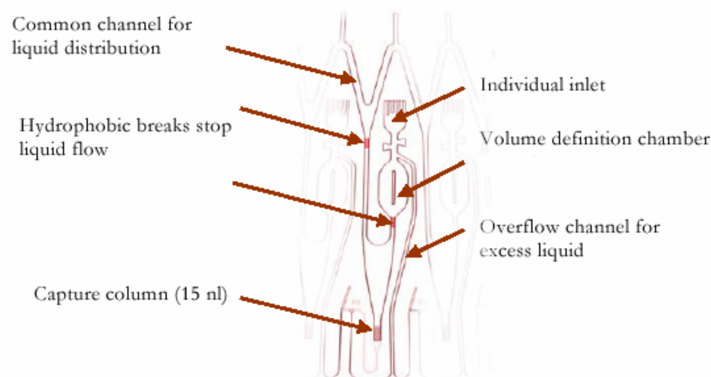


Figure 2. Design of an individual microstructure. The common channel distributes wash solution, capture and detecting reagent to the whole segment and the sample of interest is added through the individual inlet. The structure of the volume definition chamber varies between various CDs. All reagents are passed through the capture column when spinning the CD and part of the reagents get immobilized upon binding with appropriate reagents. Picture used with permission from Gyros.

1.3 Immunoassays

1.3.1 Sandwich Assay

A number of immunoassays have been developed to measure different ranges of concentrations with different specificity making them suitable at different situations depending on which parameters one is interested in. The most common assay is the sandwich assay where usually two different antibodies with specificity and appropriate affinity for the same antigen are used to bind the antigen or vice versa when quantifying antibodies. When quantifying antigen the antibodies are not allowed to bind to overlapping parts of the epitope on the antigen or to epitopes that are in common for several antigens since this may cause misleading results [6]. An assay where the concentration of analyte is known is set up in order to create a standard curve where concentration is plotted versus the response. This curve is used as a reference when calculating the concentration of the unknown sample. Most widely used immunoassays are probably ELISA and RIA (Radioimmunoassay). These methods are sensitive and cheep but are also time consuming and are neither automatically handled unless costly investments are made, nor efficient in sample usage.

1.3.2 Gyrolab Bioaffy: Sandwich Immunoassay

The Gyrolab Bioaffy system is an open system designed to have a well defined chemical interface to which customer defined reagents can be immobilized. Each reaction structure contains a small pre-packed particle based column coupled with streptavidin. Each subunit of streptavidin can bind one molecule of biotin. The affinity is so high that the interaction can be regarded as a covalent binding since only denaturating chemicals can separate these molecules upon binding [7]. The small size of biotin (244 Da) compared to streptavidin (60000 Da) is that small that it does not affect the sterical characteristics of the tetramer.
The reagent used for capturing the antibody is labelled with biotin and when added to the solid surface the capturing reagent is attached. The analyte at unknown concentration is bound when added to the capturing reagent. Unbound analyte will be washed away. It is important to avoid saturation, which occurs when there is a lack of streptavidin-coated particles or too low

concentrations of capturing solution in relation to the amount of sample. This results in analyte not being able to bind and flows through giving misleading results since the amount of captured antigen should be in excess to the total amount of analyte in the sample. The detecting reagent is labelled with the fluorescent Alexa 647 dye which absorbs light at 650 nm and has a fluorescence maximum at 668 nm [33]. This labelled detecting reagent is then automatically added in controlled volumes but in excess to the reaction and the signal is detected and transformed into values that can be analysed. The choice of capturing and detecting reagents used in an assay depends on what target protein one wishes to measure.
When targeting antibodies it is not necessarily the epitope of the antibody that is involved in the reaction. In this project the interaction between the Fc region of the antibody and the antigen is utilized (figure 3).

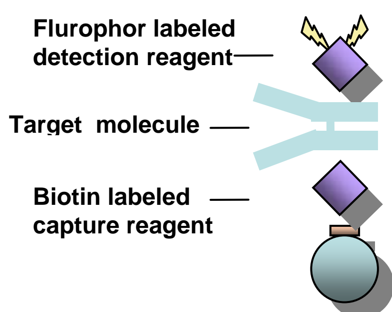


Figure 3. Sandwich immunoassay. Streptavidin coated particles are immobilized on a solid surface. The biotinylated capturing reagent binds to the streptavidin coated particles and captures the target molecule. The detecting reagent attaches to the target protein and emits a signal that can be detected. When working at a nanoliter scale only very small volumes of the sample is required.

1.4 Optimizing the working range of an immunoassay

In order to achieve a wide dynamic range but also addressing an appropriate concentration range it is important to consider parameters that affect the working range in the assay. Obviously, if possible it is more convenient to be able to measure samples without pre-treatment such as dilutions. In Gyrolab Bioaffy there are at least 4 major factors that have to be considered. A smaller sample volume in the volume definition chamber will allow less molecules to pass through the column. Capture molecules with low affinity for an interaction will bind less analyte compared to molecules showing high affinity. Higher concentrations can therefore be added before reaching saturation in the column. A faster flow will result in fewer molecules being captured and quantified. Changing the density of capturing ligand in the column is the fourth factor that can influence the working range of the assay and increase the capacity of the column.
These four parameters; volume, affinity, sample flow and column capacity, have been studied in this project to achieve a wide dynamic range and an appropriate working range in relation to concentrations of analyte found in real samples. Time for analysis is another important factor to optimise. Rapid analyses will not only deliver results early on but also increase the potential capacity of the analysis system thereby improving the utilization of the instrument investment.

1.5 200 nl in CDBA2 versus 20 nl in CDE13

Working at a nanoliter scale requires only very small volumes of sample. This is a huge benefit when handling samples with limited volume. Gyros AB has developed a CD named Gyrolab Bioaffy 1C which has been the product CD for some time. The volume definition chamber is designed to have a volume of 200 nl. Depending on the reagent set up used, the IgG concentration range has been in the interval of ~0.1-100 µg/ml. Lately, experiments have been performed on a CD having microstructures with a volume definition chamber of only 20 nl, i.e. the volume is reduced by a factor 10. If the sample volume is reduced and thereby the absolute amount of IgG in the sample, higher concentrations of IgG are needed to saturate the column capacity. Therefore higher concentrations of IgG can be quantified by reducing the volume. Studies on the 20 nl sample volume CD have revealed that the concentration range therefore is moved upwards compared to results from using Gyrolab Bioaffy 1C. Gyrolab Bioaffy 1C might be useful when handling low concentrations of IgG while a CD with reduced sample volume is beneficial when working with high concentrations. The 20 nl sample volume CD is a prototype with only 24 structures of 20 nl in each CD.

1.6 Background

1.6.1 Protein A

Protein A is a protein that can be found on the cell surface of most strains of *Staphylococcus aureus* gram-positive bacteria. These bacteria synthesize the protein and also present it on the surface by covalently linking it to the peptidoglycan part of the cell wall. Additionally, in certain strains, around 8-30% of protein A is secreted during the exponential growth phase [10]. Protein A has for decades been a very useful tool in immunobiology due to its affinity for the Fc region of IgG from many species. Radioimmunoassays, study of cell surface antigens, immunohistochemistry, purification and quantitative determination of immunoglobulins and its fragments are some of the applications employing staphylococcal protein A (SpA).
SpA was first noticed in 1940 but it took until 1964 before it got its name “Protein A” [11, 12]. Protein A has a molecular weight of 42000 Da and is a single polypeptide chain. It is composed of five repetitive immunoglobulin binding regions; E, D, A, B and C which are homologous to approximately 80% and consist of 58 to 62 amino acid residues respectively. These regions can be found on the surface of the bacteria whereas the sixth region X is believed to be attached to the cell wall [13, 14, 15]. Protein A is very stable to heat and other denaturing agents and is therefore easy to handle and to use [10].
Immunoglobulins demonstrate two different types of reactivity for protein A[16, 17].The classical interaction involves the interface of the CH2-CH3 domains of the Fc portion of human IgG1, IgG2 and IgG4 [16, 17, 18, 19]. Similarly, a number of species of IgGs demonstrate Fcγ binding although the affinities involved may vary substantially. In addition, immunoglobulins may also interact with protein A through the variable domain of the heavy chain of many different immunoglobulin classes and across species, which is named the alternative protein A reactivity. More specifically it involves the V_HIII class of the framework region of the variable domain of immuoglobulins. However, this interaction involves a significant proportion of polyclonal IgG, IgA, IgM and IgE [10, 15, 18, 20, 21].

a) b)

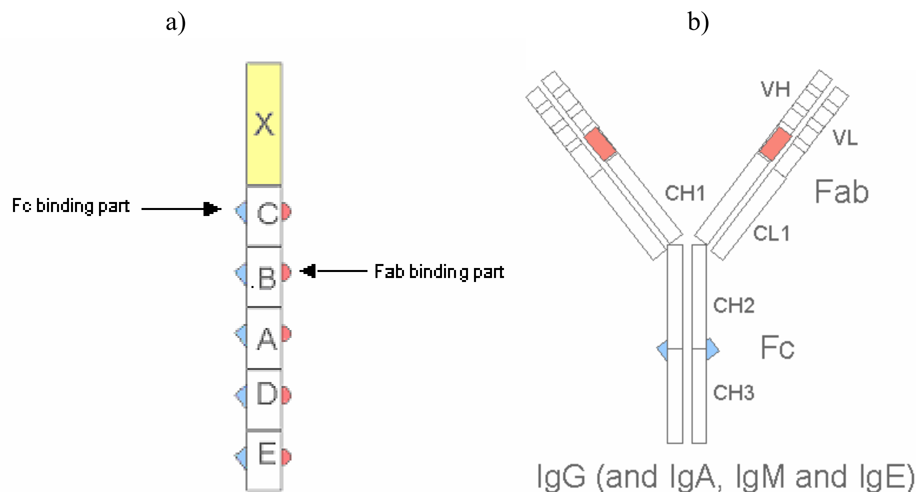


Figure 4. a) A simplified model of Protein A. The five homologous regions are named A, B, C, D and E and they all have a binding site for both the Fc region of an IgG (blue parts) and the $F(ab')_2$ region (red parts).
b) An immunoglobulin molecule. The sites where protein A binds to the immunoglobulin are marked with color. Picture used with permission from Gyros AB

It has been shown in crystallographic studies that the Fc portion of an immunoglobulin has two binding sites for the B fragment of SpA that can bind simultaneously [16, 23]. However, in liquid phase it has been difficult to prove beyond doubt that simultaneous binding of fragment B to both heavy chains may occur [16, 23].
Fragment B, with a size of 6.6 kDa consists of two anti-parallel alpha-helices that are held together by a beta-like structure. The two SpA molecules bind on opposite sides of the Fc region at the boundary between the C_H2 and C_H3 domains [11, 17].

1.6.2 Fragment Z

In several biochemical assays and biotechnological applications one uses antibodies to capture, detect and measure antigen. There are some disadvantages when using antibodies which can be circumvented by replacing it with protein A. Higher yields, reduced incubation times and less unspecific binding are some of the benefits [10]. Therefore scientists today are searching for novel binding proteins that can be used instead of antibodies to detect IgG.
One of these proteins is the Z domain, a 58 aa residue that is a mutated form of the B domain of the *Staphylococcus aureus* protein A [24]. The amino acid alanin at position 29 has been replaced with a glycin in the second helix of the Z domain and is believed to cause changes responsible for loss of the $F(ab')_2$ binding site [20]. The $F(ab')_2$ regions of immunoglobulins have structural differences and SpA does therefore not bind equally well to all immunoglobulins. The synthetic fragment Z can therefore be used to achieve the same reactivity for all immunoglobulins since they all have the two Fc regions in common which fragment Z bind to. Due to its affinity and specificity for the Fc region fragment Z may hypothetically be used as both a capturing and detecting reagent in sandwich immunoassays.
In this project fragment Z has been used in different assays as both capturing and detecting agents in different combinations with protein G. The reason why fragment Z has been used is to reduce the affinity for immunoglobulins, creating an analytical situation where larger

concentrations of IgG can be used to form a stable complex enabling an assay for higher concentrations of immunoglobulins to be measured. The samples have been different subclasses of immunoglobulins from mostly human but also bovine and mouse. Other immunoglobulins have also been analysed to get an estimation about the reliability of this technique.

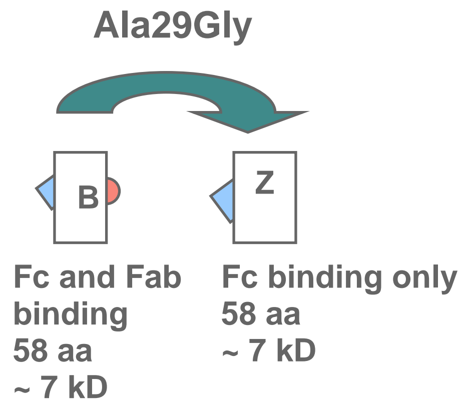


Figure 5. Modification of fragment B of SpA results in the formation of fragment Z.
Picture used with permission from Gyros AB

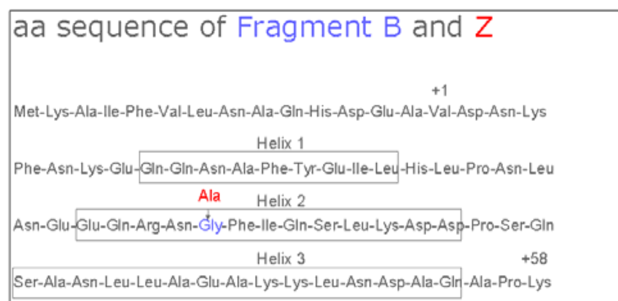


Figure 6. The amino acid sequence of fragment B vs. fragment Z. Fragment Z is a modified form of fragment B and the amino acid glycin at position 29 has been replaced by an alanin.
Picture used with permission from Gyros AB

1.6.3 Protein G

Streptococcal protein G (SpG) is a bacterial surface protein which is expressed on the cell wall of Streptococci. The native molecule binds with high affinity to immunoglobulin G (IgG) but also to serum albumin. The recombinant protein G has two homologous domains with high affinity for the Fc region but also two other homologous sites that bind to the $F(ab')_2$ region on IgG [26, 27, 28, 29,30]. Compared to fragment Z, the ability of protein G to bind both to the Fc region and to the F(ab´)2 region (figure 7.) results in a higher probability that an immunoglobulin will become captured when using protein G as the capturing reagent. The ability of protein G to bind in the interface between CH2 and CH3 of the Fc region but also to the CH1 domain of $F(ab')_2$ [28] makes it an attractive reagent for quantification of IgG. There is a greater chance that the two heavy chains in the Fc region of the captured immunoglobulin will be exposed to the detecting reagent when using protein G as capturing reagent as when compared to using fragment Z, which only has the Fc-binding region that can bind to the immunoglobulin. When one of the heavy chains in the Fc regions is bound to the capturing

reagent as in the case of capturing with fragment Z it might become more difficult for the second heavy chain of the Fc region to get exposed to the detecting reagent.
According to Åkerström *et al*.[31] protein G binds somewhat better to most subclasses of IgG, although the affinity varies considerably between species [28]. It has been shown that protein G also binds monoclonal antibodies from mouse IgG1, IgG2a, IgG2b and IgG3 to some degree. This statement is although very vague since Eliasson *et al*. (1987) [21] state that protein A bind human IgG1κ, IgG1λ, IgG2κ, IgG2λ, IgG4κ and IgG4λ slightly better than what protein G does while protein G binds IgG3 much better. This is explained by the lack of Fc reactivity shown by the rather rare IgG3. This results in fragment Z not being able to detect this subclass [32].

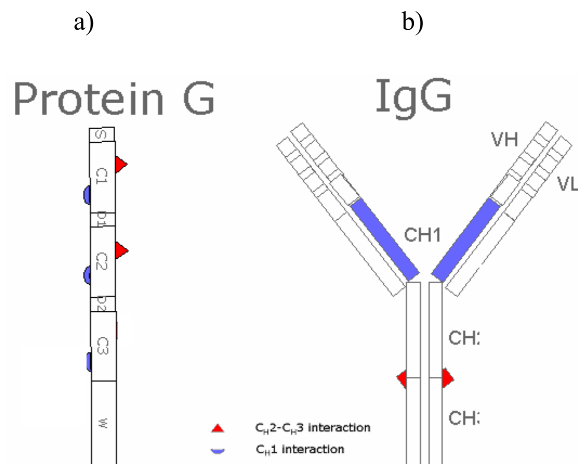


Figure 7. a) Streptococcal protein G consists of albumin and IgG-binding binding functions. There are two homologous domains for both types of interactions [25].
b) An immunoglobulin G molecule with its protein G binding sites displayed. The red triangles symbolise the region that is responsible for binding to the constant chain. The blue half-circles show the sites with affinity for the $F(ab')_2$ region. Two types of interactions are therefore involved in the IgG-binding.
Picture used with permission from Gyros.

2 Aim of the project

There were several goals with this project. The main goal was to develop an assay quantifying monoclonal human IgG in a broad concentration range covering high IgG concentrations using fragment Z and protein G. These two proteins should be investigated as both capturing and detecting reagents.
It was of great interest to study the differences achieved when using a 20 nl versus a 200 nl CD. Important parameters to evaluate with respect to sample volume included the working and dynamic range i.e. the span of various concentration being correctly quantified. Optimization of the time used for analysis without loosing assay performance was another parameter of interest since it has implications on the total analysis capacity of Gyrolab Workstation as well as the outcome when using various assays. Different combinations achieved by changing the four parameters; volume, affinity, sample flow and column capacity will hopefully result in an assay with the desired properties. Another question to answer was whether the assays would be capable of measuring concentrations up to ~5 g/L thereby avoiding dilution of samples in diluents.

Main goals with the project

- Determine the working concentration range of IgG for different assay configurations
- Study the effects of changing sample volume and flow rate through the column
- Avidity and affinity; do these parameters affect the outcome?
- Quantify several samples using the assays Fz/Fz and PG/Fz; is there a difference in quantified concentration depending on assay configuration?
- Optimize the method
- Study time-usage, where can time be saved?

3 Materials and Methods

3.1 Biotinylated Fragment Z and Protein G

Biotinylated fragment Z (Affibody, Bromma, Sweden) and protein G (PIERCE, Rockford, IL) were used as capturing reagents in the experiments. Both these reagents bind to the Fc part of several immunoglobulins from various species [33]. Protein G does in addition show binding properties to the $F(ab')_2$ region of many immunoglobulin classes [28]. Both biotinylated reagents were purchased at a concentration of 1 g/L. The purchased fragment Z has a molecular weight of 28.2 kDa [34] indicating that it is provided as a tetramer with disulfide bonds since one fragment Z molecule has got a size of 7 kDa.

3.2 Immunoglobulin preparations

Optimization of the assays was done using various analytes to study the effect of their characteristics. Several subclasses of human, mouse and bovine immunoglobulins were analyzed and standard curves were generated for all analytes. The analytes were serially diluted into concentrations spanning the dynamic range of the assay i.e. covering the interval from approximately 5000 µg/ml to 0.8 µg/ml which fulfilled the goal.

Human polyclonal IgG (Fitzgerald, Concord, U.S) was used when optimizing the concentrations of capturing and detecting reagent since the other reagents available could not be found at sufficient concentrations. After establishing the basic adjustments human IgG1κ, IgG2κ, IgG3κ and IgG4κ (SIGMA, Stockholm, Sweden) were analysed as well as human IgM (SIGMA, Stockholm, Sweden), Fcγ fragments (Bethyl, Montgomery, Texas, U.S) and $F(ab')_2$ (Calbiochem, Darmstadt, Germany) to establish the specificity of the assay. Mouse IgG1 (LabAs Ltd, Tartu, Estonia), IgG2aκ (R&D Systems, Abingdon, England), IgG2b (R&D Systems, Abingdon, England) and bovine polyclonal IgG (LABORA, Upplands Väsby, Sweden) were also analysed to determine the degree of reactivity in the assay. These analytes are listed in Table 1. Plotting standard curves in a graph provides information that can be used when studying the characteristics and properties of the analytes. For every standard curve created a blank was also run. These experiments were all performed on CD´s of 200 nl due to the limited number of structures in a CD of 20 nl and since the only interest is the relative response within the assay.

	Analyte	Sample number	Concentration (mg/ml)
Human	Polyclonal IgG	R-1892	24.0
	IgG1κ	R-1886	1.2
	IgG2κ	R-1887	1.0
	IgG3κ	R-1888	1.1
	IgG4κ	R-1890	1.0
	IgM	R-1889	0.8
	$F(ab')_2$ fragment	R-1897	4.2
	Fc fragment	R-1898	1.0
Mouse	IgG1	R-1003	7.7
	IgG1	R-1004	7.3
	IgG1	R-1055	5.0
	IgG1	R-1056	13.9
	IgG2Aκ	R-1328	1.0
	IgG2Bκ	R-1396	1.0
Bovine	Polyclonal IgG	R-1418	10.0

Table 1. Analytes used to optimize the assays. For mouse there were several IgG1 reagents available and experiments were done on all of them to visualize possible differences. Human polyclonal IgG was the most useful reagent due to the high concentration.

3.3 Labeling Fragment Z with Alexa Fluor® 647

Fragment Z was labeled with Alexa Fluor® 647 Monoclonal Antibody Kit (Molecular Probes), a kit that not only provides an easy way to label low concentrations of monoclonal and polyclonal antibodies with the Alexa Fluor 647 dye but also larger proteins such as fragment Z. The properties of this dye are those that stable dye-protein conjugates are formed and these absorbs light at 650 nm and have and fluorescence maxima around 668 nm [35]. The labeling was essentially done according to the protocol. Only half the amount (50 µg) of fragment Z required was used for one tube with reactive dye. The pH was adjusted to 7.5 – 8.5 by adding one-tenth volume (4-5 µl) of 1 M sodium bicarbonate buffer. The mixture of fragment Z and the reactive dye was then let to incubate at rotation for about 3 hours. Separation of Alexa-labelled fragment Z from unlabeled protein was done with the Slide-A-Lyzer® Mini Dialysis Units Plus Float (PIERCE, Rutherford, IL). This is a membrane with a cut off of 3.500 kDMW. The membrane was placed in a floating plate and put in a cup filled with 1x PBS-0.01% Tween making the membrane stay in contact with the solution. A magnetic stiring in the cup kept the solution in circulation. After approximately 15 minutes the mixture of reactive dye and fragment Z was pipetted onto the membrane. A lid was put on the memebrane and the cup was covered with foile to avoid light and it was left like that over night. The membrane allowed unincorporated ALEXA 647 dye to pass while the labeled fragment Z was kept in the membrane. After purification the volume in the membrane was measured. Since the protein is very small, the concentration could only be estimated assuming that all fragment Z had been labeled with ALEXA 647 and that it still was kept in the membrane.

By diluting the detection reagent 1:10, 1:30 and 1:100 and comparing the standard curves created it was possible to decide which dilution that gave the highest response and the lowest background signal.

3.4 Assay development

The most suitable concentrations for the assay of biotinylated fragment Z was determined by serial dilution of the reagents to generate a standard curve where the response was studied. A broad dynamic range with a low background signal for a standard curve is sought after as well as the possibility to measure higher concentrations of the antibody before the slope of the curve flattens out. In this application particular attention was addressed to the high end of the standard curve to find conditions for accurate quantification of monoclonal IgG corresponding to concentrations of optimized cell culture supernatants that may exceed 1 mg/ml (1 g/L). The concentration used for biotinylated fragment Z was set to 50 µg/ml. This had previously been done for protein G and the amount had been set to 100 µg/ml. The concentration differences are not believed to affect the outcome since previous experiments performed in the laboratory showed that there was no difference in response when quantifying with either 100 µg/ml or with 50 µg/ml of biotinylated fragment Z. 50 µg/ml was then chosen since lower amounts would be required. However this project was mostly regarding developing an assay for quantifying human monoclonal IgG and therefore the majority of time was spent on creating optimal standard curves.

3.5 Calculating the concentration of a unknown sample.

In order to accurately quantify IgG in unknown samples, a relevant standard at known concentration must be used. For consistency it is important that the reference and the sample are analyzed in the same way with the same reagents. The reference sample was diluted in 1xPBS-0.1% BSA in factors of 5 or less over the whole range of concentrations and the response from all dilutions were plotted against the concentrations to create a standard curve. The responses of the samples of unknown concentrations were compared to the standard curve and the amount of protein in the sample could be calculated.

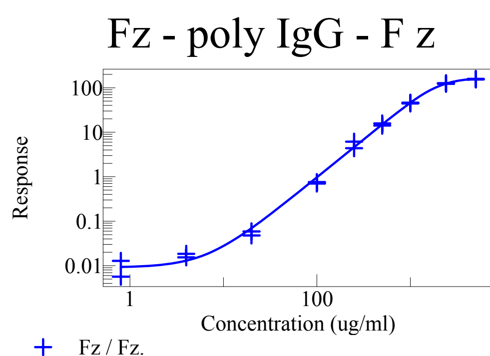


Figure 8. A standard curve prepared with biotinylated fragment Z as capturing reagent, polyclonal IgG as analyte and Alexa labeled fragment Z as the detection reagent. The response was plotted on the Y-axis versus concentration on the X-axis.

3.6 Dilution studies

Gyros AB has developed diluents that are appropriate for several assays. In order to determine if the considered assay reagents should be diluted in these diluents or in other buffers several experiments were performed in which capture, analyte and detector were diluted in either 1x PBS-0.01% Tween (15 mM PB, 150 mM NaCl, 0.02% NaN_3, 0.01% Tween 20) or 1x PBS-

0.1% BSA (15 mM PB, 150 mM NaCl, 0.02% NaN_3, 0.1% BSA). Dilution studies where the detecting reagent was diluted in Detection Reagent Diluent (Gyros) and the other reagents were diluted in either 1x PBS-0.01% Tween or 1x PBS-1% BSA were also done.

3.7 Negative Controls

For verification of the specificity of the assay control reactions should be run in parallel with the ordinary reactions. It is important to investigate weather there is any unspecific binding to the column that has to be regarded or not. An unsaturated Streptavidin column that exposes free biotin binding sites may also create non-specific interaction versus the ALEXA dye in the detecting reagent. Therefore studies of non-specific capture must include saturation of the streptavidin binding column with a neutral protein not interacting with IgG. For this purpose biotinylated BSA (PIERCE, VWR International, Stockholm), biotinylated HSA (Human-Serum-Albumin) –binding reagent (PIERCE, Sigma, Stockholm) and biotinylated anti-HSA-affibody (PIERCE, Affibody, Stockholm) were all tested as control capturing reagents. The labeling with biotin (PIERCE, Stockholm) was done at the lab according to the protocol supplied with the NHS-LC-Biotin compound. The NHS-LC-Biotin is a molecule with a molecular weight of 341.41 Da which is equipped with a spacer arm that enhances binding with streptavidin. The capture and analyte were diluted in 1x PBS-0.01% Tween and the detector in Detection Reagent Diluent (Gyros) respectively. 200 nl CDs were used and the detection was done using a filter reducing the signal by a factor 300. PMT was set to 1%, 5% and 25%. For each standard curve a blank consisting of 1x PBS-1% BSA was run to control the background signal and was designated an additional negative control.

3.8 Optimizing the assays by modifying the method.

Some steps in the method were studied in order to determine if they could be either modified or even removed to get a more efficient method for quantifying IgG with the two assays investigated. Parameters such as capture wash, detection wash, analyte spin and detection reagent spin were studied. Different combinations of these changes were analysed and modifications were done until the standard curve lost robustness (Appendix 1). The analyte spin should in a CD of 200 nl sample volume generate a liquid force rate of 1 nl/sec. By increasing the spin speed and thus the flow rate to approximately 2.0 nl/sec it was studied whether higher concentrations could be quantified due to the faster flow. The same changes were investigated for the detection reagent spin.

3.9 Assay preparation

The assays studied in this project followed the workflow below which is described in the Gyrolab™ Workstation User Guide Version 7.1 [7].

- Prepare lists e.g. sample lists
- Create batch
- Prepare solutions and microplates
- Start-up and prime Gyrolab Workstation
- Prepare Gyrolab Control software to run batch
- Load Gyrolab Workstation
- Start run
- Finish run and unload Gyrolab Workstation
- Data analysis

3.9.1 Preparation of lists

Before being able to run a CD there are two types of lists that must be prepared, a reagent and a transfer list, both created as Excel files. Reagent type, position in the microtiter plate and concentration of standards can be found in the reagent list. The transfer list complements the reagent list by telling the instrumnet into which structure or segment the reagents should be transferred. This information is imported into the software when creating a batch. A batch is created for each run and is a collection of the information given in the lists but it also contains the method that should be used and all other information required for a run. Samples and standards are diluted according to the regent list and are transferred to the microtiter plates. Before loading the CDs and microtiter plates into the Gyrolab Workstation and starting a run the instrument must be primed with pump and wash liquid.

3.9.2 Detection and data analysis

The quantitative measurement of protein is done by using the laser induced fluorescence (LIF) detector integrated in the Gyrolab Workstation LIF. The detector uses HeNe laser @ 632.8 nm as its light source. During the detection step the laser moves from the periphery towards the centre as the CD is rotating and data is given for all structures in one CD simultaneously. The software Gyrolab Evaluator is used to create standard curves for analysed individual datapoints and to calculate concentration of the unknown samples. The data from each column is integrated in the software Gyrolab Viewer and can be displayed as a graphical representation in two or three dimensions showing possible outliers and other factors that have to be considered (figure 9). A column profile should ideally have a high signal in the top of the column which then rapidly decreases along the path, but will depend on the assay and the affinity between the analyte and the capturing reagents.

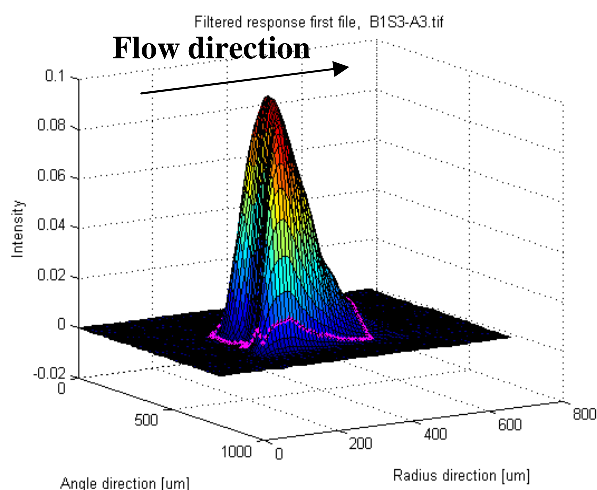


Figure 9. A good column profile should show response within the integration area (pink line) with a intensity peak at the beginning of the column. The intensity should then rapidly decrease in radius direction.

3.10 Bioaffy 1C v1 CDE13 and Bioaffy 1C v3

This project included studies on two types of CDs and therefore different methods were used. Bioaffy 1C v1 CDE13 was the main method used on the CD having 20 nl sample volume but modifications in this method was done when trying to optimize time usage. Bioaffy 1C v3 (Appendix 1) was used when doing experiments on the CD having 200 nl sample volume but modifications were also sometimes made for the same purpose. Both methods consist of the same operations although the time required differs in the methods.
After the needles initially had been cleaned with Bioaffy wash station solution 1 (15 mM PB, 150 mM NaCl, 0.02% NaN_3, 0.01% Tween 20) the structures including the columns were washed twice with 1x PBS-0.01% Tween to soak the streptavidin coated particles and the wash liquid was removed by spinning the disc. Upon addition of biotinylated capture reagent a binding reaction occurred between the biotin and the streptavidin resulting in immobilization of the capture reagent. Sample was added after washing away unbound capture reagent. Two further washes took place followed by background detection (section 3.9.2). Finally the detection reagent was added followed by four washes and the second detection was done.

3.11 Real Samples

To verify the functionality of the Fz/Fz and PG/Fz assays, 10 samples from sample set I (Collaborator I) with concentrations spanning the interval from 5 µg/ml to 1630 µg/ml were quantified. All samples were analyzed in undiluted form and the enclosed standards from the collaborator were used to calculate the unknown concentrations of the samples. The samples from Collaborator I had earlier been analysed by Biacore, HPLC and by another assay at Gyros. The results were all compared to estimate the reliability in the assays of interest. A reference control with a known concentration was also available.

3.12 Detecting the signal with different PMT-levels

When the generated signal reaches the detector it is amplified by a Photo Multiplier Tube (PMT). Depending on the concentration in the sample and the amount of labeled detecting reagents, different levels of amplification can be made to reach a desired response signal. The amplification can be controlled and modified by changing the PMT level and the higher the PMT, the more response signal is amplified i.e. for high concentrations and high degrees of labeling it is appropriate to detect with a low PMT. The level of PMT detection can easily be controlled to achieve a satisfying response.
Previously, at Gyros, high concentrations of samples, i.e high response levels from the LIF, have been solved by introducing a filter that reduces the signal intensity with a factor of 300. Thereby similar PMT settings have been used as when quantifying lower concentrations of other proteins. In this project however it was investigated whether it was possible to reduce the level of PMT with a factor of 300 instead of using the filter. For various PMT levels column profiles using Gyrolab Viewer were observed and conclusions were drawn based on the shape of these profiles. A well chosen PMT setting should return a column profile with a low background signal. When quantifying IgG using a filter the PMT settings are usually set to 1%, 5% or 25%, although in this project where similar measurements were performed without the filter the PMT was set to number as low as 0.002% up to 0.05%. The method can be modified to detect several PMT levels in one run [9].

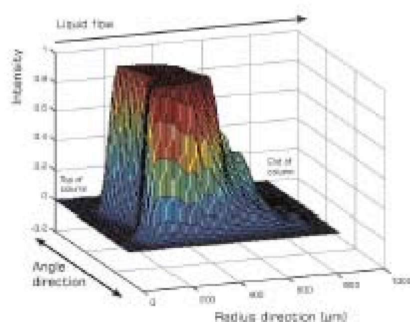


Figure 10. PMT saturation. The signal is too high to be detected. To avoid this phenomena the PMT should be reduced.

3.13 Time optimization

Time equals money and therefore it is of great interest to improve time usage when doing analysis without affecting the system performance negatively. In these experiments various modifications were done in the methods and runs were performed where the only interest was to save analysis time without loosing assay performance. Capture wash, detection wash, analyte spin and detection reagent spin were parameters that were modified. In addition it was examined how much time that could be saved if pre-immobilized fragment Z was in place in the column when the CD is put into the instrument. This modification is not practically feasible in product terms at this stage but the experiment was done to simulate how much time that possible could be saved in future experiments. In order to get some numbers on how long it in theory would take to run the still non-existing CD with 14 segments of 20 nl, CDBA2´s with 14 segments were tested in dummy runs with methods that were designed for CDE13 containing only 3 complete segments of 20 nl structures. The time usage was compared to runs where no modifications had been done. (Appendix 1). Calculations on time saved were done and the goal was to run one CD´s of 20 nl below 40 minutes. For each run a Gyrolab

Report was available which is a report that contains detailed information about what occurred in the run, including a time log for all events that occurred during a batch run.

4 Results

4.1 Are Fz/Fz and PG/Fz two appropriate assays?

Initial results with quantification human polyclonal IgG with the Fz/Fz and PG/Fz assays indicated that they both have potential for measuring concentrations spanning a broad concentration range (figure 11). It was decided that these two assays should be further studied in this project in order to develop an assay that eventually will be suitable for quantifying monoclonal antibody in a variety of concentrations.

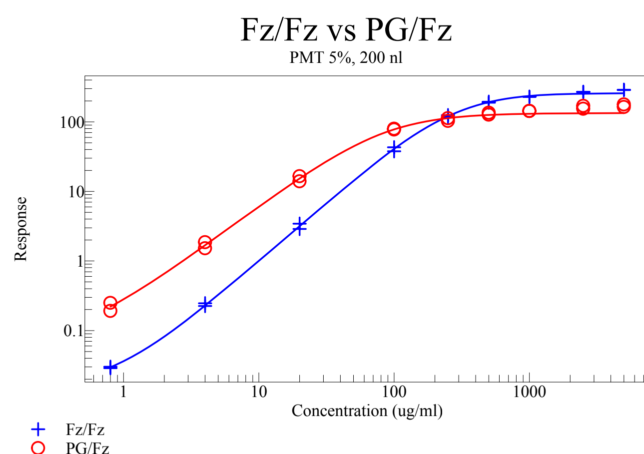


Figure 11. Initial experiments performed with Fz/Fz and PG/Fz quantifying human polyclonal IgG on a 200 nl CD. The standard curves indicate that lower concentrations can be quantified with the PG/Fz assay while the Fz/Fz assay is more suitable for higher concentrations.

4.2 Can the method be optimized regarding usage of time?

Modifications of the method Bioaffy 1Cv1 CDE13 was done in order to study the robustness of the assays considered in this project. The original method contains two particle washes, two capture reagent washes, two analyte washes and four detection reagent washes. The capture spin has been set to 8000 rpm for 64 seconds and analyte spin has been set to 2500 rpm for 72.5 seconds while detection reagent spin was set to 6000 rpm for 263 seconds (Appendix 1.). These parameters and their importance in a methodological perspective had not previously been fully investigated and therefore modifications of the method were done and the results were compared to those results given using the original method. Capture wash, detection wash, analyte spin and detection reagent spin were those parameters studied. The goal with these modifications is to reduce time usage in order to increase analysis capacity in Gyrolab Workstation. Time can be saved if the sample would pass through the column faster without affecting the performance and this could theoretically occur by spinning the CD at a higher rpm.

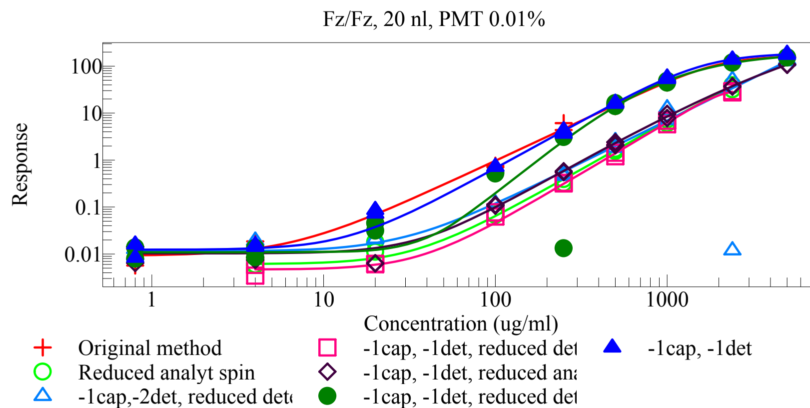


Figure 12. Robustness of Fz/Fz assay when quantifying human polyclonal IgG. Method modifications are done in the original method and the different responses are plotted to get a picture of the robustness. Modifications tend to result in standard curves being shifted more or less to the right. Most changes occur when the analyte spin is modified. Removing 1 capture reagent wash and 1 or 2 detection washes does not seem to effect the curve as much as when reducing spin time. Reduced spin time is compensated with a higher speed to allow all proteins to pass.

The results shown in figure 12 indicated that the standard curve of the original method has a concentration range of almost 3 orders of magnitude spanning from 5 µg/ml to 2000 µg/ml. Removing one capture and detection wash yielded a standard curve with a higher background signal than the original method although the curve flattened out at similar levels. Removing a second detection wash gave a curve parallel to the original one except that it could not quantify as low concentrations. On the other hand higher levels could be measured before saturation. Concentrations from 20 µg/ml to 3000 µg/ml could be quantified. Reducing time of analyte spin in addition tended to result in a curve shifted to the right although this has not yet been fully investigated. The robustness seemed to be more affected by reducing detection reagent spin. Compared to the original method the concentration range at low concentrations was lost by a factor 10.
The optimized standard method was set to include only one capture reagent wash and three detection reagent washes. The analyte and detection reagent spin was set to 32.5 seconds and 69 seconds respectively.
These modifications were finally tested on the two assays studied in the project and the results can be seen in figure 13. Human polyclonal IgG was used as the analyte. Comparing the modified method to the original for Fz/Fz (figure 13a) showed that by modifying the method the concentration range got narrower. The same comparisons for PG/Fz (figure 13b) showed a more robust method and the concentration range was not affected. When comparing the two assays using the original method it was obvious that the concentration range was shifted to the right for the assay with Fz/Fz and the same was seen for the modified method although fragment Z as the capturing reagent gave a narrower concentration range (figure 13 c and d). Finally standard curves using different CDs were studied and for both assays it could be stated that higher levels of protein can be dealt with using the 20 nl CD (figure 13 e and f).

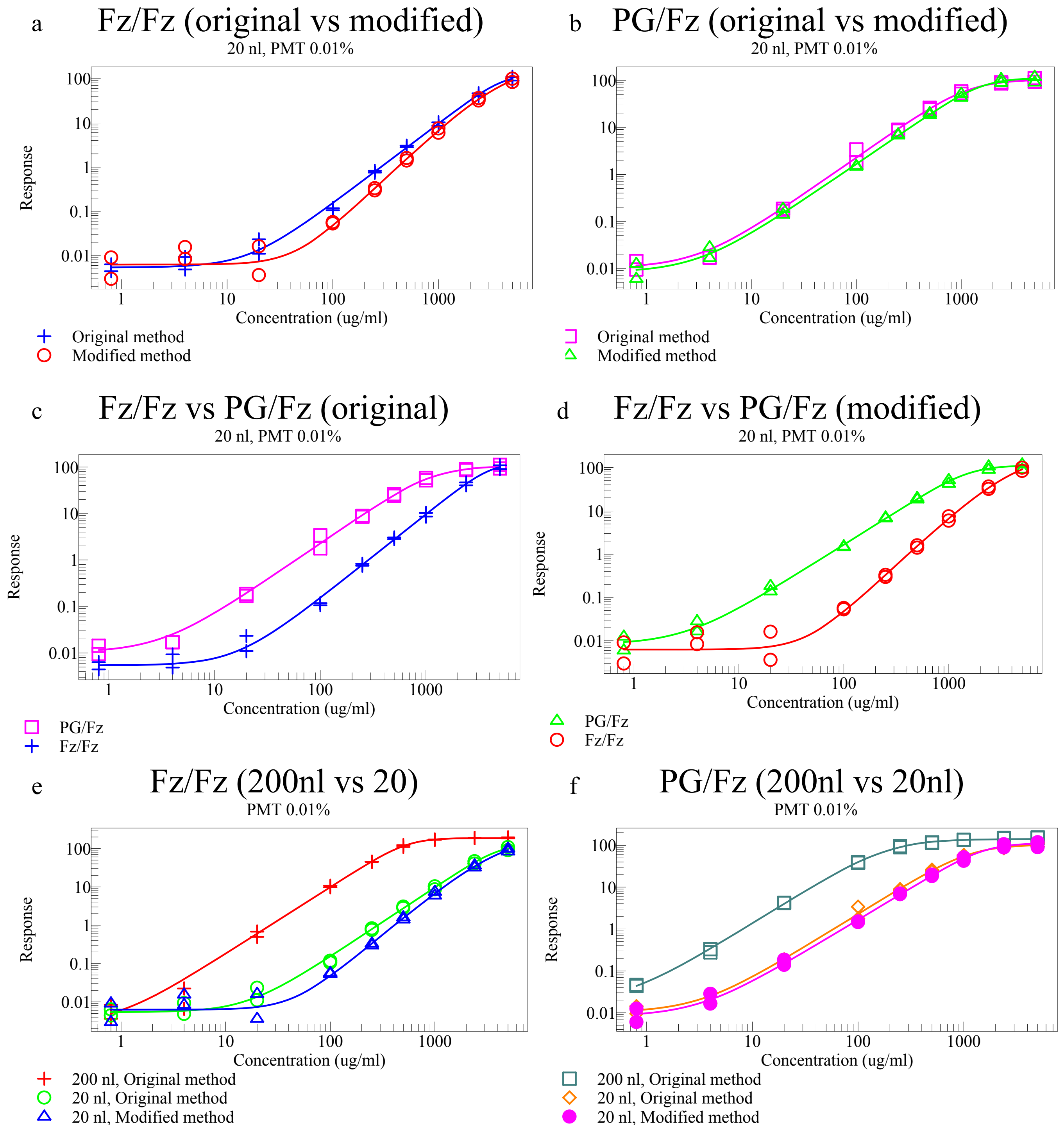


Figure 13. Comparisons of standard curves from Fz/Fz and PG/Fz assays detecting human polyclonal IgG using 200 and 20 nl sampe volume. Results after using the original method is compared to those given using a modified method where one capture reagent wash and one detection reagent wash has been removed. The time of both analyte and detection reagent spin has been reduced although the spin is faster.

a) Fz/Fz. Original method vs modified.
b) PG/Fz. Original method vs.modified.
c) Fz/Fz vs PG/Fz (modified method)
d) Fz/Fz vs PG/Fz (original method)
e) Fz/Fz. 200nl vs 20nl
f) PG/Fz. 200nl vs 20nl

When changing the parameters reported in chapter 4.2 the time usage was affected and these time changes were noticed and analysed. The results can be seen in Appendix 1. It is important to keep the assay as unaffected as possible and it is therefore necessary to consider how much method parameters that can be changed while maintaining the desired performance. The method deleting one capture wash and one detection reagent wash in combination with reduced analyte spin and detection reagent spin would in theory save more than 12 minutes compared to running the original method on a full CD of 20 nl (Appendix 1, blue marks). The most extreme modification would be to use Fz covalently coupled to capture particles which would eliminate method steps related to attaching biotinylated Fz to Streptavidin beads.

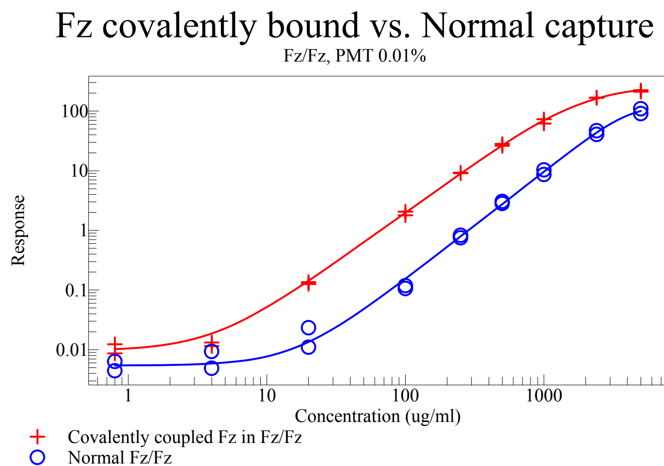


Figure14. Normal capture attachment vs. Fz covalently coupled to capture particles when quantifying human polyclonal IgG with Fz/Fz. Normal capturing generates a curve covering a higher concentration range than covalently coupled Fz. The reason is not clear and further experiments should be performed.

This modification would reduce the overall time usage with almost 8 minutes. The standard curve with Fz covalently coupled to capture particles is compared to the results generated with the original method and these can be seen in figure 14. Fz covalently coupled gives a curve shifted to the left quantifying lower concentrations than the original method.

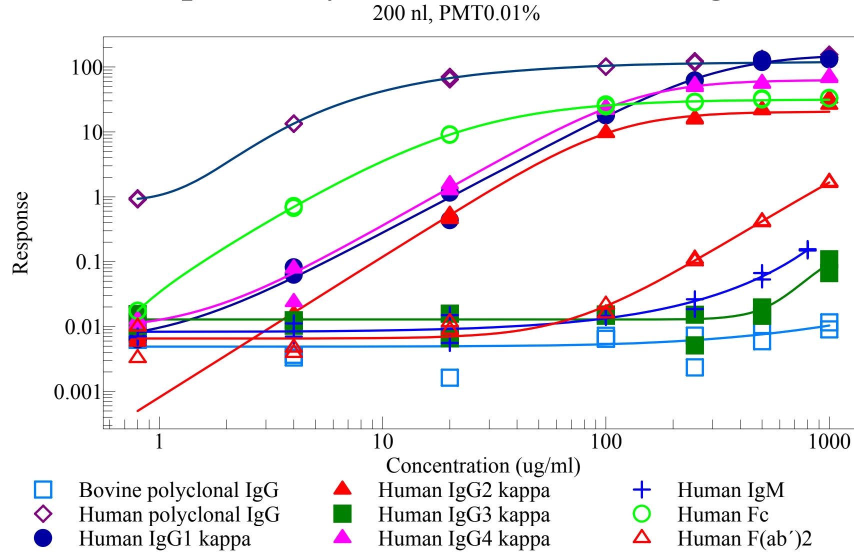
4.3 Analysis of different immunoglobulin subclasses

4.3.1 Human subclasses

Stability of the Fz/Fz assay was established by testing various subclasses of immunoglobulins of human and bovine origin. The results demonstrated in figure 15 present the standard curves detected with PMT 0.01%. Of the analytes tested, human polyclonal IgG had the highest response signal to fragment Z at low concentrations. The curve generated using human Fc fragment had a lower background signal than human polyclonal IgG although higher concentrations could be quantified before the curve flattened out. Human IgG1κ, IgG2κ and IgG4κ showed as expected responses very similar to each other while there hardly was any response for IgG3κ. Lack of binding was also seen for human IgM, human $F(ab')_2$ and bovine polyclonal IgG. It has been discussed whether the assay would detect bovine immunoglobulins since these could exist in various samples including cell culture serum. These results were consistent with what was expected although the high response seen for polyclonal IgG was somewhat confusing.

a)

PG/Fz, Specificity for various immunoglobulines



b)

Fz/Fz, Specificity for various immunoglobulins

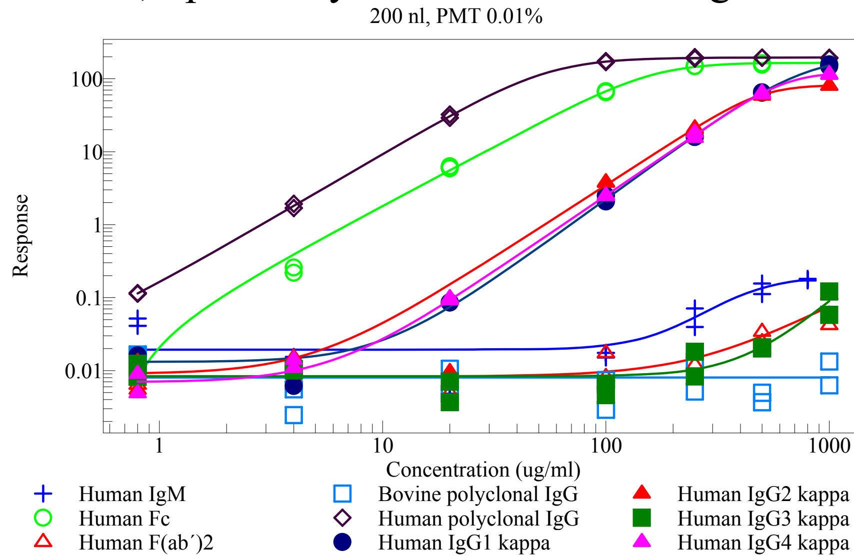


Figure 15. Human and bovine subclasses of immunolobulins. PMT 0.01%. 200 nl CD.
a) PG/Fz assay specificity is tested for various immunoglobulins. The high response of polyclonal IgG can not be accepted without further experiments. Human Fc fragments come next followed by IgG4κ, IgG1κ and IgG2κ. No or very low response can be seen for $F(ab')_2$, IgM, IgG3κ and bovine polyclonal IgG.
b) Fz/Fz assay specificity is very similar to the PG/Fz assay. Polyclonal IgG gives the highest response followed by human Fc fragments. The response curves of IgG1κ, IgG2κ and IgG4κ come next with curves almost identical. No response is generated for IgG3κ, $F(ab`)_2$, IgM and bovine polyclonal IgG.

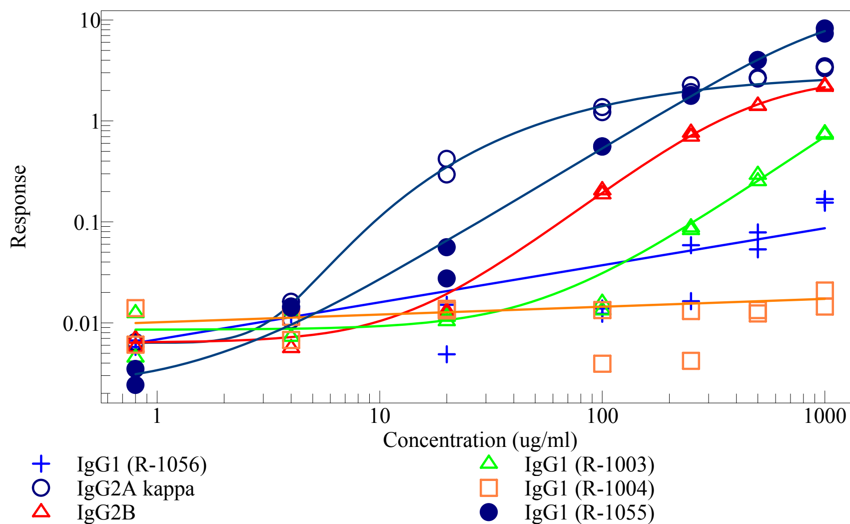
4.3.2 Mouse subclasses

The results presented in figure 16 a and b indicated that Fz/Fz binds stronger to IgG2aκ than what PG/Fz does even though it was difficult to draw conclusions on the concentration range. PG/Fz could quantify concentrations of IgG2b at lower concentrations than PG/Fz although the curve flattened at a lower response signal. One sample of IgG1 bound stronger than IgG2b in both assays but the other samples showed hardly any response in the Fz/Fz assay. Protein G as capture did however show more of a response for these samples of IgG1 but it was not possible to draw any conclusions based on these values. The various samples of IgG1 showed different responses and no pattern could be distinguished.

a)

PG/Fz, Specificity for mouse immunoglobulins

200 nl, PMT 0.01%



b)

Fz/Fz, Specificity for mouse immunoglobulins

200 nl, PMT 0.01%

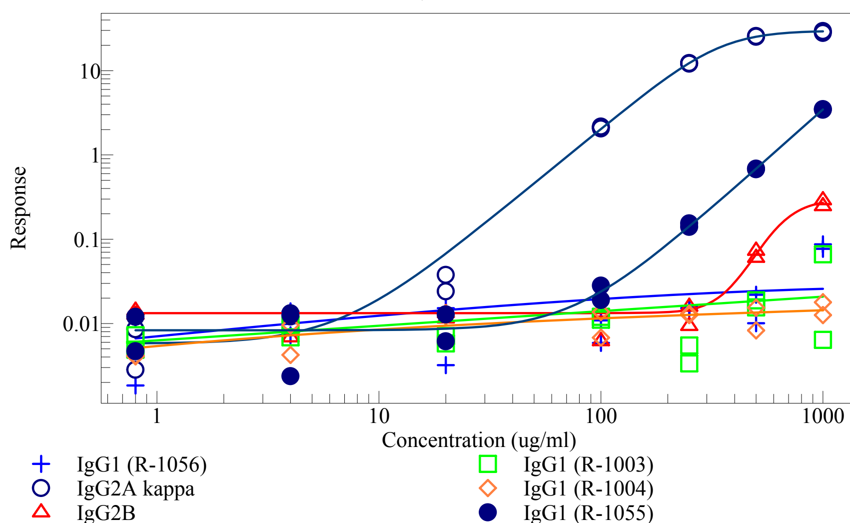


Figure16.

a) PG/Fz specificity tested on various mouse immunoglobulines. IgG2a (filled circles) show a somewhat odd standard curve which is not fully reliable. Compared to the other assay the concentration range is a lot narrower. IgG1(open triangles) has a low background signal and the slope covers a broad range of concentrations. IgG2b (filled triangles) can be quantified at concentrations approximately 10 times lower than when using Fz/Fz but the curve starts to even out around 500 µg/ml. The other samples containing IgG1 (crosses, open squares) show higher signals than the other assay does but one sample of IgG1 (open circles) does not show any response at all.
b) Various mouse immunoglobulins are tested for specificity of the Fz/Fz assay. Highest response is seen for IgG2aκ (filled circles) and thereafter comes a sample containing IgG1 (open triangles) and IgG2b (filled triangles). No response is seen for the other samples containing IgG1 (open squares, crosses and open circles).

4.4 Control studies

IgG1κ was used as the analyte and the concentration range tested spanned from 0.16 µg/ml to 500 µg/ml. PG/Fz was used as a reference. The maximum response was low for all reagents before flattening including the PG/Fz assay. Higher PMT of 5% and 15% levels did give a higher response (not shown). The background signal of BSA and HSA-binding capture were approximately 2-3 times lower than that of anti-HSA affibody which also showed the highest response before flattening. None of the three candidates were ideal as a negative capture control but since biotinylated BSA had the lowest response at high concentrations and a low background signal it was chosen as the negative control. Compared to the assay with protein G and fragment Z the maximum response differed approximately with a factor 10.

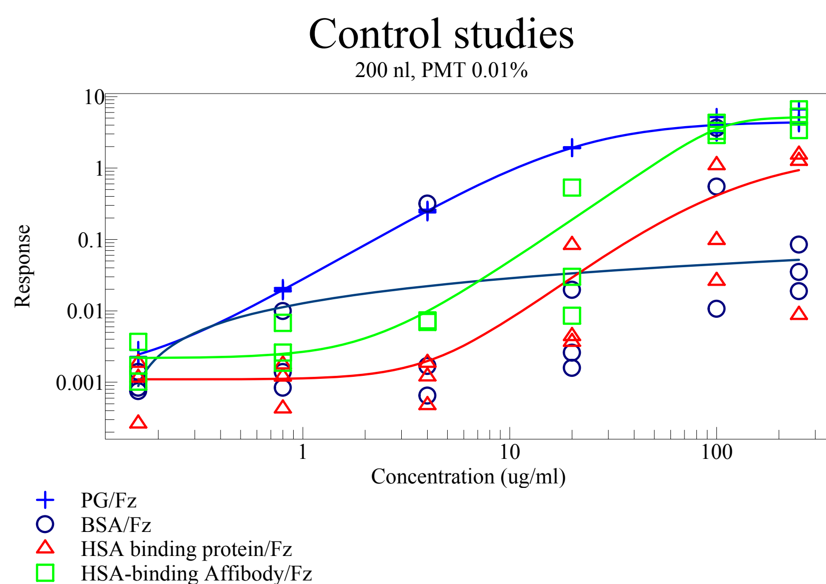


Figure 17. Response given for various control capturing reagents when quantifying human IgG1κ. Biotinylated BSA (blue open circles) was chosen as the most appropriate control since the response flattens before reaching 1µg/ml and stays at this level unaffected by increased concentrations of analyte.

4.5 Are diluents required for accurate determination of IgG concentrations?

The results from testing various diluents are presented in figure 18. The experiment when diluting all reagents i.e. capture, analyte and detector in 1x PBS-0.01% Tween (filled diamonds) gives similar results as observed for 1x PBS-1% BSA (filled squares). Diluting

the capturing reagent in diluent (Gyros, Uppsala, Sweden) does not seem to affect the result (open triangles and crosses).

It was established that the capture should be diluted in 1x PBS-0.01% Tween while the analyte and detecting reagent preferably should be diluted in 1x PBS-1%BSA.

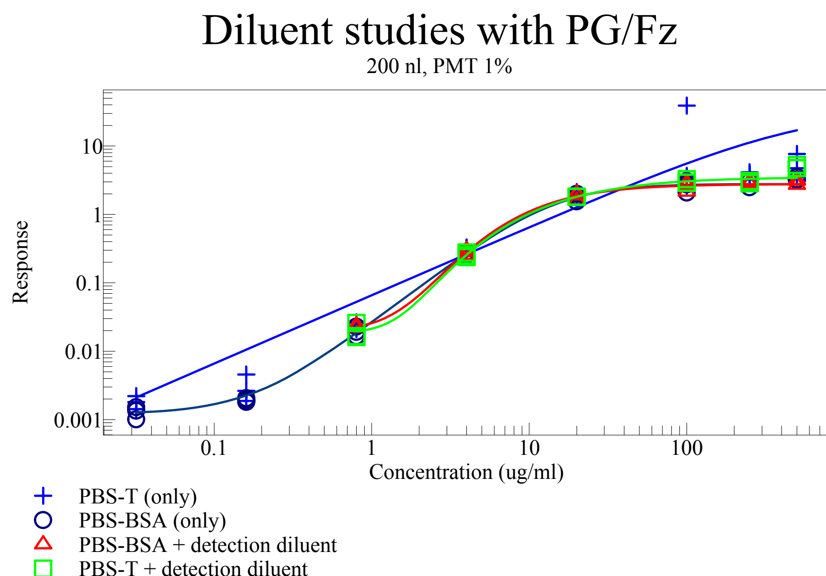


Figure 18. Several combinations of diluents were examined in order to establish if certain diluents are required for accurate concentrations of IgG. Diluting all in PBS-T gives the most linear curve although even better results were observed when diluting capture in PBS-T and analytes and detector in PBS-BSA respectively (not shown).

4.6 PMT

Column profiles are generated using the Gyrolab Evaluator program. Different PMT levels returns column profiles with various intensity. Figure 19 is an example of how column profiles with different PMT measuring the same concentration of 2400 µg/ml can look like. PMT 0.002% gives a good shaped curve although the intensity is not as high as required. The maximum intensity is 1 but it is not even reached when detecting with the highest PMT of 0.05%. This profile has a shape indicating PMT saturation since the peak is not as significant as for the other. PMT saturation means that the current is lacking enough capacity to detect the whole signal and therefore this can cause misleading results. The curve does not decline as rapidly as desired after reaching its maxima and the intensity stays high even further down the column. PMT 0.01% gives a good column profile although for this PMT level the intensity is also very low. By detecting with the same PMT in different instruments it was shown that the intensity signal varies indicating that the signal detected is dependent on the instrument and the amount of light it allows through. This is an important fact to consider when doing experiments on more than one instrument.

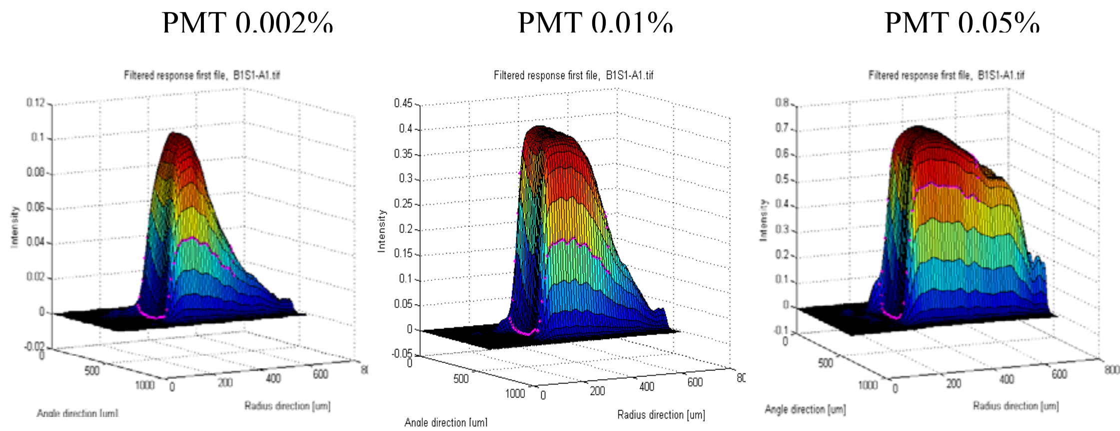


Figure 19. Column profiles generated using the Gyrolab Evaluator program. Different PMT levels result in changed intensity and shape of the profile. The concentration above is 2400 µg/ml.

An experiment was performed where standard curves at different PMT levels were compared. Fragment Z was used to both capture and detect human polyclonal IgG.
The results were also compared to a reference run, an indentical run performed on an intrument where detection was done with a filter and the PMT was set to 5%. The results can be seen in figure 20. All standard curves lie within the same concentration range even if the background signal and slope varies. Detecting with PMT 0.01% gave the curve with the broadest concentration range and therefore it was decided that this PMT should be used in future experiments.

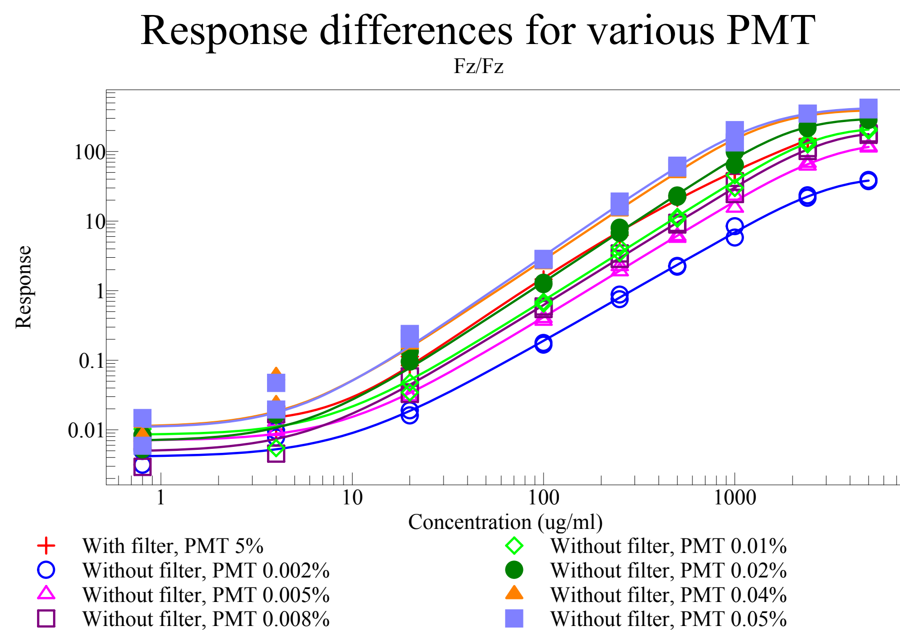


Figure 20. Various PMT give different responses when quantifying human polyclonal IgG. A slope that covers a broad concentration range is desirable. In the above experiment, detecting with PMT 0,01% gives the curve with the steepest slope. The lower the PMT, the lower is the background signal. Considerations have to be made wheather a low background signal is more important than measuring higher concentrations. Various runs can have different PMT adjustments.

4.7 Real samples

The results generated when quantifying real samples were analysed in Gyrolab Evaluator and comparisons were made between Fz/Fz, PG/Fz and a third assay previously developed by Gyros. Measurements had also been performed using Biacore and HPLC (Table 2.). It is not possible to decide which of the two assays studied in this project that gives the most precise value since there is no obvious pattern distinguished. The results are more easily interpreted in figure 21 where the samples are plotted against all techniques.

Results given from different techniques when quantifying the samples from Collaborator I

	FZ-FZ	PG-FZ	Gyrolab	Biacore	HPLC
#1	65	75	50	50	56
#2	28	38	24	24	30
#3	21	10	6	5	11
#4	38	33	22	21	28
#5	71	92	56	55	68
#6	205	295	227	231	240
#7	524	594	508	468	529
#8	1285	1184	1218	1016	1293
#9	1505	1460	1825	1457	1597
#10	1605	1655	1811	1626	1682
Control 1260 mg/L	1575	2088	1345	1278	

Table 2. The values listed above are quantified concentrations (µg/ml) generated by the two assays studied in this project and as a comparison the concentrations generated by three other techniques are shown. Ten samples from collaborator I were analysed and a control sample of known concentration was run as a reference.

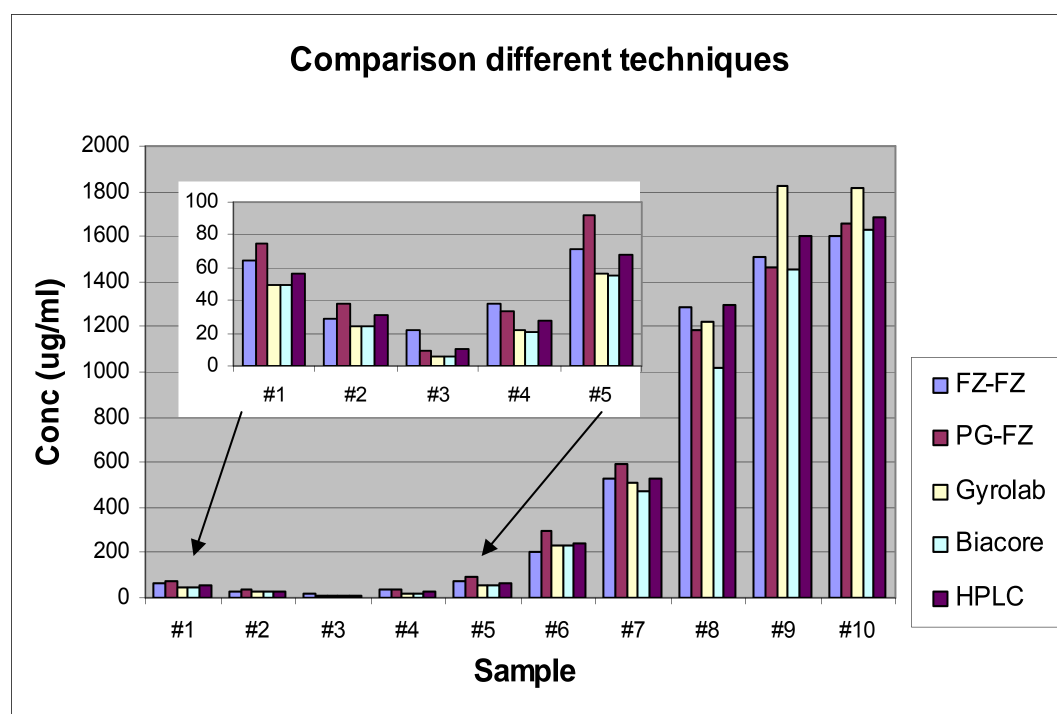


Figure 21. The results shown in Table 1 are plotted for each sample. The graph indicates that the two assays Fz/Fz and PG/Fz manage to quantify the concentrations as well as the other techniques.

5 Discussion

The aim of this project was to develop an assay for quantifying high concentrations of human monoclonal IgG using Gyrolab Bioaffy. In order to generate a very generic immunochemistry recombinant versions of bacterial polypeptides (Fz and PG) were selected for assay development.
CD´s having a sample volume of 20 nl will within a short time become a product at Gyros. Therefore it was important to investigate the performance of these assays when quantifying immunoglobulins using a 20 nl CD. Until now CD´s of 20 nl has only existed as a prototype with 3 segments per CD. In order to simplify experiments and increase data generating capacity, most studies were performed on 200 nl CD´s when only relative results were of importance, although the aim was to develop an assay for measurements on 20 nl.

Several experiments confirmed that reduced sample volume enables quantification of higher IgG concentrations, independent of assay, which is illustrated as response curves covering a higher concentration range. It does unfortunately seem like the lower limit of quantification is increased when using less sample volume. Increasing sample flow over the column does also seem to produce response curves that are somewhat shifted towards higher concentrations of IgG.

Different combinations with either Fz or PG as the capturing reagent and Fz as detecting reagent has been employed to study assay performance for IgG quantification. The combination of using Fz as both capturing and detecting reagent had previously not been evaluated and the results indicate that this assay allows higher concentrations to be quantified than PG/Fz although the working range is narrower. It is not excluded that other combinations where protein G also is used as detecting reagent could generate good results. Before deciding which assay that is the most appropriate assay, combinations with PG/PG and Fz/PG should be evaluated.

Protein G has 6 binding sites for IgG reactive with the Fc and CH1 regions of IgG, respectively while fragment Z has 2 sites reactive with the Fc region only. This fact could perhaps explain why lower concentrations of IgG can be quantified with the PG/Fz assay. Hypothetically, using the Fz/Fz assay at low IgG concentrations there is a risk that the only two Fz binding sites positioned on the Fc region might get saturated to the immobilized capturing Fz. This would then prevent binding of the detecting Fz yielding low signal at low IgG concentrations. Since PG in addition has binding sites for the Fab region there is a greater chance that the Fc region on IgG will remain exposed for further reaction with the detection reagent. At higher IgG concentrations this phenomenon is reduced due to a competeing situation between a lot of IgG and there is a lower risk that two Fz will bind the same IgG.
Also, when Fz is used as capturing reagent and binds to one of the two binding sites on the CH2-CH3 region of an IgG there might be a sterical hindrance making it more difficult to expose the second unbound Fz binding site of the antibody. Protein G, which is a larger protein, might statistically bind the Fab region in 50% of the cases and thereby still exposing the Fc region for the detecting Fz. This could be an explanation to why a broader concentration range is observed for PG/Fz assay.

It has previously been discussed whether the binding sites for SpA and SpG on Fc might overlap and thus prevent both proteins to bind simultaneously. Three dimensional studies on

mouse IgG2a performed by Kato *et* al. [36] suggest that the binding of both protein A and protein G to the Fc region is competitive meaning that their binding sites overlap. These results are based on both solution and crystal structure studies. The opposite was later reported by Aybay [37] who claimed the prescence of two independent SpG and SpA binding sites in the Fc region of mouse IgG. The results presented in this report agree with the studies performed by Aybay. If there would be an overlap the assay PG/Fz would probably not have generated such a high signal. The studies done by Kato and Aybay were performed on mouse immunoglobulins and perhaps this is not fully applicable to human and other species.

More studies on the robustness of PG/Fz should be done since most attention was paid towards the other assay. The studies performed where method parameters were modified suggest that PG/Fz is a very robust assay.
The results observed for the two assays indicate that PG/Fz is an appropriate assay when quantifying polyclonal IgG concentrations in the lower concentration range from 2 μg/ml up to 1000 μg/ml while there are signs that Fz/Fz can be used when measuring high IgG concentrations from 20 μg/ml up to more than 4000 μg/ml. Which one of these two assays that is to recommend depends on the concentration of IgG that are to be measured.

Some variation of results could be seen when using different instruments. Performing the experiments on the same instrument is therefore necessary and the conditions should always be the same. Variations in temperature, humidity or using various particle batches are factors that might influence the result. Until there is a supplier of ALEXA 647 labelled fragment Z it is necessary to do the labelling procedure at the lab which has not been optimised.

The studies made on real samples from Collaborator I showed very similar results compared to HPLC, Biacore and Gyrolab and both assays used in this project have capacity to quantify samples with concentrations in the higher range although lower concentrations are more uncertain to quantify. It is difficult to see a clear response pattern for the two assays and conclusions about which of the two assay that gives the most precise result is difficult to draw. It would be desirable to quantify samples from other collaborators before making clear statements although the results seem promising especially at high IgG concentrations.

A simple way of evaluating the specificity of the assay is to exchange critical binding reagents for appropriate controls. In this project biotinylated BSA was chosen as a capturing control. This was used to see if there was any unspecific binding directly to the column of IgG without specific interaction with the capture ligand. The responses seen for this control indicated some unspecific interaction to the BSA column at high IgG concentrations. Several explanations can be found for this behaviour. For instance, one explanation is that polyclonal IgG do contain antibodies directed against BSA and that the response seen in the control studies to some extent reflect the presence of such antibodies. The latter is supported by the observation that when monoclonal IgG (IgG1κ) was tested in control reactions no response was obtained (data not shown). The worst scenario indicated that ~1% of the the signal generated for polyclonal IgG could be due to non-specific trapping of IgG in the column (figure 17). More control studies was not done in this project due to time limitation.

Several mouse immunoglobulins were tested for the purpose to define the specificity of the Fz/Fz and PG/Fz assay. In general mouse immunoglobulins show a less reactivity to interact with protein G and protein A than human and other species do [38]. According to the litterature, [10, 21] mouse IgG2, IgG2b and IgG3 bind to Protein A while only some types of IgG1 bind. The

same is believed for protein G with the differences that it is believed to bind IgG2b stronger and IgG2a weaker than what Protein A does. Both Protein A and protein G show affinity for IgG3. However, it has been suggested that increasing the salt concentration and adjusting the pH might improve the affinity of some mouse IgG [38]. The results presented in this project show that the specificity for mouse subclasses varies between the assays although they show a similar pattern. In general it can be said that the PG/Fz assay have higher specificity for most subclasses even though the curve of IgG2A flattens a lot earlier. More studies on different mouse immunoglobulins should however be made before making any statements.

Human and bovine immunoglobulins were also tested for specificity in the assays. It was obvious that human polyclonal IgG gives the highest response followed by human Fc fragments. IgG1κ, IgG2κ and IgG4κ show similar responses while human IgM, $F(ab')_2$, human IgG3κ and bovine polyclonal IgG show hardly any response. These results were expected [10, 21]. The fact that bovine polyclonal IgG does not generate a response is very valuable since bovine polyclonal IgG might be found in real cell culture samples. If the assay would detect bovine polyclonal IgG misinterpreted assumptions could easily be made.
These results are however not completely reliable. The high response of human polyclonal IgG compared to the other subclasses makes one doubt if the concentrations really are what they are supposed to be. In addition, we have no knowledge of how large proportions in the tested immunoglobulin preparations that might appear in aggregated form resulting in miscalculated concentrations.

Polyclonal IgG was used as analyte in most experiments. This is because we lacked monoclonal antibodies with high enough concentrations in the laboratory. In addition, polyclonal IgG are antibodies that are not produced from one single clone but from many different cells although all antibodies produced are directed towards the same antigen but to different epitopes on the antigen. Monoclonal immunoglobulins are identical and therefore it is easier to produce a response using polyclonal IgG since the probability of having some IgG with specificity for the assay is increased.

The time usage is an important parameter to study in order to develop a technique that is as efficient as possible in terms of instrument capacity. It was studied if it was possible to run a CD below 40 minutes. 5 CD´s could then be run in approximately 3 hours and 20 minutes enabling 3 runs of 5 CD´s per normal working day of 8 hours. This would result in a capacity to quantify 1680 data points per day.
Changes were therefore done in the method to examine if some parameters could be modified to reduce time without affecting the performance of the assay. Removing 1 capture reagent wash, 2 detection reagent washes in combination with reducing analyte and detection reagent spin resulted in 1 CD being run in just 39 minutes. If using covalently bound Fz, 1 CD will only take 36.5 minutes using the original method or even less if also modifying the method. Making these changes may, however, affect the performance of the assay. The modifications do influence the result and it is therefore necessary to determine how many changes that are allowed without affecting the performance of the assay too much. Instrument capacity is another factor that perhaps could be improved. Washing needles and spinning the CD are examples of events that should be run in parallel in order to increase effectiveness. Also, several minutes are required at the end of each run to generate the results and perhaps this time usage could be reduced. Modifications in the software could therefore probably increase time usage more than changing steps in the method. These are all factors that should be looked over in order to create a product as efficient as possible.

Recently it was noticed that the fragment Z we have been working with is purchased as a tetramer instead of a dimer as we assumed. Reducing the tetramer will break the disulfide bonds between the two sets of dimers. This was not done in this project but with this knowledge we will probably perform the labeling of ALEXA 647 according to the protocol in future experiments since the molecule is big enough for separating ALEXA dye from labelled fragment Z with a spin column instead of the dialysis membrane that was used. However, the results given here indicate that it is not necessary to reduce the tetramer since good values were generated anyhow. Reducing will cause loss of material and the detection reagent will probably also become less stabile.

If more time was given additional studies on the different assays using both PG and Fz as capturing reagents would be done and the main focus would be to determine which one of these that has the broadest application range.
The assays studied in this project are not fully optimised. A more appropriate capturing control reagent should be used and more replicates should be done but the project has exposed the opportunities of developing assays for quantifications of higher concentrations using the 20 nl CD. It seams possible that different assays could be useful for different purposes. If further studies would show that covalently bound Fz would generate good result, this change could mean that no other modifications would be necessary to optimise time usage. Therefore I believe that more efforts should be put on this potential change.
Within short a new CD having 20 nl sample volume and 112 structures will be released. It will be interesting to study the assays presented in this work in the new CD and see if it gives the results expected.

6 Acknowledgments

I would like to thank my supervisor, Dr Mats Inganäs at Gyros AB for excellent support and for giving me the opportunity to do this master degree project at Gyros. I would also like to thank all the employees at Gyros for being very friendly and giving answers to all my questions and letting me become part of the group.

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Appendix 1

Optimization of time being consumed for one CD.
Runs are performed on CDBA2 but the method is adjusted as if it was a 14 segment CD with 20nl.

The aim is to run a CD within or below 40 minutes.

1. Original method, (Bioaffy 1c V3) 3 PMT detect, 48 samples + standard curve (duplicate)

2. Original method, 3 PMT detect, 96 samples (oneplicate) and one standard curve (duplicate)

3. One removed capture reagent wash, one removed detection reagent wash, 1 PMT detect. 48 samples and standard curve (duplicate)

4. One removed capture reagent wash, two removed detection reagent washes, 1 PMT detect. 48 samples and standard curve (duplicate)

5. One removed capture reagent wash, one removed detection reagent washes, reduced analyte and detection reagent spin. 1 PMT detect. 48 samples and standard curve (duplicate)

6. One removed capture reagent wash, one removed detection reagent washes, reduced analyte spin. 1 PMT detect. 48 samples and standard curve (duplicate)

7. One removed capture reagent wash, one removed detection reagent washes, reduced detection reagent spin. 1 PMT detect. 48 samples and standard curve (duplicate)

8. Covalently bound Fz. Original method. 1 PMT detect, 48 samples and one standard curve (duplicate).

9. Covalently bound Fz. One removed detection reagent wash, reduced analyt and detection reagent spin. 1 PMT detect, 48 samples and one standard curve (duplicate)

Results

	1	2	3	4	5	6	7	8	9
Initial needle wash	95	95	95	95	95	95	95	95	95
Particle wash 1	119	119	119	119	119	119	119	119	119
Particle wash spin 1	40	41	40	40	40	41	40	40	40
Particle wash 2 structure	63	62	63	63	63	62	63	63	63
Particle wash 2 common	0	0	0	0	0	0	0	0	0
Particle wash spin 2	39	37	39	39	39	37	39	39	39
Capture reagent addition	138	139	138	138	138	139	139	0	0
Capture reagent spin	69	69	69	69	69	69	69	0	0
Capture reagent wash 1	76	76	76	76	76	76	76	0	0
Capture reagent wash spin 1	60	59	60	60	60	59	59	0	0
Capture reagent wash 2	63	63	0	0	0	0	0	0	0
Capture reagent wash spin 2	57	56	0	0	0	0	0	0	0
Analyte addition	688	1086	688	688	688	688	688	688	688
Analyte spin	74	73	74	74	32	32	73	73	32
Analyte wash 1	66	66	66	66	66	66	66	66	66
Analyte wash spin 1	40	39	40	40	40	39	40	40	40
Analyte wash 2	62	61	62	62	62	61	62	62	62
Analyte wash spin 2	28	29	28	28	28	29	28	28	28
CD alignment	8	7	8	8	8	7	8	8	8
Detect background PMT 1	84	83	84	84	84	83	84	84	84
Detect background PMT 2	85	85	0	0	0	0	0	0	0
Detect background PMT 3	84	83	0	0	0	0	0	0	0
Spin out	72	72	72	72	72	13	72	72	72
Detection reagent	139	138	139	139	139	138	139	139	139
Detection reagent spin	264	263	264	264	69	264	69	264	69
Detection reagent wash 1	64	66	76	76	76	66	76	76	76
Detection reagent wash spin 1	60	59	60	60	60	59	60	60	60
Detection reagent wash 2	73	73	73	73	73	73	73	73	73
Detection reagent wash spin 2	47	39	47	47	47	39	47	47	47
Detection reagent wash 3	61	63	61	0	63	63	61	61	61
Detection reagent wash spin 3	40	39	40	0	26	26	39	39	39
Detection reagent wash 4	63	63	0	0	0	0	0	39	0
Detection reagent wash spin 4	28	26	0	0	0	0	0	29	0
CD alignment 2	6	8	6	6	6	8	6	6	6
Detect PMT 1	82	83	82	82	82	83	82	82	82
Detect PMT 2	86	86	0	0	0	0	0	0	0
Detect PMT 3	86	86	0	0	0	0	0	0	0
Total tid (per skiva)	**3209**	**3592**	**2669**	**2568**	**2420**	**2615**	**2461**	**2392**	**2088**
	53min, 29sek	59min 52sek	44min 29sek	42min 48sek	40min 20sek	43min 35sek	41min 1sek	39min 52sek	34min 44sek