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Microscale measurement of kinetic binding properties of monoclonal antibodies in solution using Gyrolab

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Abstract

The numbers of monoclonal antibodies approved for therapeutic use has increased rapidly over the last decade. As a consequence, precise and robust kinetic characterization techniques are crucial in order to select the best suitable candidates. A kinetic characterization method was developed in Gyrolab with automated sample transfers. The characterization was performed in solution in a mixing CD, containing an integrated nanoliter mixing chamber with affinity binding columns. Association rate constants were determined for four anti-TSH antibodies with values ranging from $3x10^5$ $M^{-1}s^{-1}$ to $10x10^5$ $M^{-1}s^{-1}$. The antibodies were ranked according to k_{ass}. Reproducibility tests indicated that the method was robust and reproducible with CV-values around 10%.

Keywords

Affinity, association rate constant, Bioaffy, characterization, Gyrolab, immunoassay, kinetics, microfluidics, monoclonal antibody, screening

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Populärvetenskaplig sammanfattning

Antikroppar är proteiner som kroppens immunförsvar använder för att identifiera olika främmande ämnen. Det kan röra sig om bakterier, virus eller andra främmande molekyler. En antikropp binder till en specifik del av det främmande ämnet. Tack vare detta används de allt mer inom läkemedelsbranschen, där man tar fram antikroppar mot olika sjukdomar. Vid framställning av antikroppar så eftersöks den antikropp som har de, för ändamålet, bästa egenskaperna. Därför är det viktigt att veta hur bra de binder till sin målmolekyl, hur fort det sker och hur länge de sitter kvar.

Gyrolab™ är ett automatiserat bioanalytiskt laboratorium som tillåter snabba analyser under en timme i en CD-skiva. Ytterst små mängder prov kan analyseras, vilket är en stor fördel eftersom mängden antikroppar som främställs ofta är högst begränsad. Detta projekt har utvecklat en metod som gör det möjligt att mäta hur snabbt antikroppar binder till sin målmolekyl. Till skillnad från andra tekniker så sker reaktionen mellan antikropp och målmolekyl i lösning, vilket är fördelaktigt eftersom man vill efterlikna det biologiska förloppet i så hög grad som möjligt. Metoden gör det möjligt att jämföra olika antikroppar med varandra. Detta kan komma att underlätta valet av potentiell antikroppskandidat vid läkemedelsframställning som en är lång och arbetssam process.

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This study has been conducted as a Master's thesis by Fredrik Johansson from Uppsala University with the supervision from Gyros AB. The report follows the basic structure designated by the University for students in the Molecular Biotechnology Engineering Programme. The thesis can be found through the Uppsala University thesis database. Fredrik Johansson has had the supervision of Johan Engström from Gyros AB and the scientific supervision from Mats Inganäs, Gyros AB.

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1. COMMON ABBREVIATIONS

Ab	Antibody
Ag	Antigen
Alexa-mAb	mAb labeled with an Alexa Flour®
Alexa-TSH	TSH labeled with an Alexa Flour®
BIA	Bridging immunoassay
BSA	Bovine serum albumin
BSA-b	Biotinylated BSA
CD	Compact disc
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and drug administration
IAA	Indirect antibody assay
Ig	Immunoglobulin
K_A	Association constant (M^{-1})
k_{ass}	Association rate constant ($M^{-1}s^{-1}$)
K_D	Dissociation constant (M)
k_{diss}	Dissociation rate constant (s^{-1})
LIF	Laser-induced fluorescence
M	Molar (Mol L^{-1})
mAb	Monoclonal antibody
μTAS	Microscale total analysis system
MP	Microtiter plate
QCM	Quartz crystal microbalance
RIA	Radioimmunoassay
S/N	Signal to noise ratio
SPR	Surface plasmon resonance
STD	Standard deviation
TSH	Thyroid stimulating hormone
TSH-b	Biotinylated TSH
[X]	Concentration of compound X

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2. INTRODUCTION

2.1 ANTIBODIES

In the beginning of the 20th century Paul Ehrlich described the concept of a *magic bullet* that could be designed to target a specific disease [1]. Years later, in 1975, Kohler and Milstein developed a procedure called hybridoma technology producing proteins similar to Ehrlich's hypothesized magic bullets [2]. These proteins were antibodies with high specificity towards their target molecule, or antigen (Ag), and appeared to be the ideal drug molecules. Antibodies (Abs), or Immunoglobulins (Igs), are proteins used by the immune system for the neutralization of foreign objects [3].

Antibodies are Y-shaped and consist of two light chains and two heavy chains, illustrated in figure 2.1. The variable regions of the light and heavy chains confer the specificity of an antibody [4]. Abs are divided into five classes according to their heavy chain structure: IgA, IgD, IgE, IgG and IgM. Different classes have different properties and act on different functional locations [4, 5].

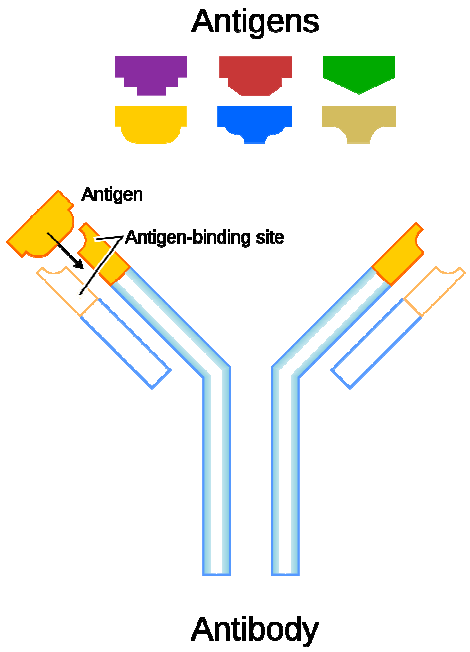

Figure 2.1 – Schematic representation of an antibody. The antibody binds to a specific part (epitope) of the antigen through its binding site (paratope).

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All existing therapeutic antibodies are IgG's. They make up 75% of the plasma antibodies produced in the body and can act as activators of the complement [4, 5]. The hybridoma technology, along with other modern techniques, generates monoclonal antibodies (mAbs) [6]. In contrast to polyclonal antibodies, mAbs originate from identical parent cells and share the same amino acid sequence and epitope specificity [7].

The long half-lives, high potency, good stability and high specificity make antibodies suitable as pharmaceuticals [3]. With increasing immunological knowledge and technological advances, the production of therapeutic antibodies has increased substantially over the last decade [7]. Over 20 mAbs have been approved by the Food and Drug Administration (FDA) and more than 100 are estimated to be under development [8].

2.2 ANTIBODY AFFINITY AND KINETICS

Affinity is the quantitative association strength between an antibody binding site and a monovalent antigen [9]. The binding includes all noncovalent interactions such as hydrogen bonds, hydrophobic effects, ion forces and van der Waals forces [10]. The affinity determines the exact number of antigen molecules that will be bound by the antibodies at any given concentration. Consequently affinity determination is critical in the development and production of therapeutic antibodies.

Since most therapeutic antibodies are bivalent and some multivalent, affinity is sometimes influenced by other factors [6]. *Avidity* is one such effect in which multivalent binding to an antigen results in a cooperative antigen-antibody interaction [9]. Sometimes *sterical hindrance* occurs when binding of antigen to one antibody binding site inhibits the binding of a second antigen to the other antibody binding site.

There are numerous methods available for affinity measurements. The most commonly used are based on label-free biosensors, including SPR (Biacore) [6], interferometry (ForteBio) [11] and QCM (Attana and Q-sense) [12, 13]. These methods use sensor surfaces where one of the interactants is immobilized and connected to a transducer. When the other interactants are flown across the surface the interactions can be monitored in real time. Other methods include ELISA [14] and Kinexa [15], which are label-based technologies where the reaction takes place in solution, thus allowing free interactions between the interactants [9, 16].

The interaction between a bivalent antibody and an antigen can be described by equation E1. First an antigen (Ag) binds to one antibody binding-site (Ab) at a certain association rate. Later a second

antigen is bound to the other binding-site at another association rate. If there are no effects due to avidity or sterical hindrance, both association rate constants ($k_{ass,1}$ and $k_{ass,2}$) and dissociation rate constants ($k_{diss,1}$ and $k_{diss,2}$) are equivalent and the system can be described through equation E2 [6]. This simplification is frequently being made by most methods and will be applied throughout this thesis.

(E1) $$Ab + Ag \underset{k_{diss,1}}{\overset{k_{ass,1}}{\rightleftarrows}} Ab \cdot Ag \underset{k_{diss,2}}{\overset{k_{ass,2}}{\rightleftarrows}} Ag_2 \cdot Ab$$

(E2) $$Ab + Ag \underset{k_{diss}}{\overset{k_{ass}}{\rightleftarrows}} Ab \cdot Ag$$

2.2.1 AFFINITY CONSTANTS

Most biosensor methods calculate the association constant (K_A) by measuring the association rate constant and dissociation rate constant and use equation E3 [6]. Another approach to derive K_A, employed by Gyros, is to use quotient of the antibody-antigen concentrations (equation E4) [6].

(E3) $$K_D = \frac{1}{K_A} = \frac{k_{diss}}{k_{ass}}$$

(E4) $$K_D = \frac{1}{K_A} = \frac{[Ab] \cdot [Ag]}{[Ab \cdot Ag]}$$

The dissociation constant (K_D) is determined in Gyrolab by mixing a constant concentration of antibody (Ab_{tot}) with a concentration series of antigen. The free antibody concentration at equilibrium is measured for different antigen concentrations and the response values (Resp) can be plotted against the antigen concentration (Ag_{tot}).

In order to relate the response values to free antibody concentration a standard curve is included in the analysis and used for calculations. If there is a linear relation between response and antibody concentration for the concentration used, K_D can be calculated through equation E5 [17].

(E5) $$Resp = \left([Ab_{tot}] - [Ag_{tot}] - K_D + \sqrt{([Ab_{tot}] - [Ag_{tot}] - K_D)^2 + 4K_D[Ab_{tot}]} \right) \frac{Resp_{max} - Resp_{min}}{2[Ab_{tot}]} + Resp_{min}$$

2.2.2 KINETIC CONSTANTS

Assuming that antigen is in excess, the free antibody concentration can be described as a function of time according to equation E6 [17]. Using relatively short reaction times makes the contributions of

the dissociation rate constant insignificant. This expression can be used to measure the association rate constant (k_{ass}).

This is accomplished by having a constant concentration of both antibody and antigen and varying the mixing time. With increasing mixing times, increased amounts of antigen-antibody complexes will be formed. This results in a decreasing exponential curve when measuring the remaining free antibody in the mixture. The response values are fitted to the exponential decay model with baseline (equation E7). The parameter A is the initial response and C is the response background of the reaction. By combining the theoretical expression in equation E6 with the exponential model in equation E7, k_{ass} can be calculated using parameter B and the antigen concentration (see equation E8).

(E6) $$[Ab](t) = [Ab_{tot}] \cdot e^{(-k_{ass}[Ag]t)}$$

(E7) $$y(t) = A \cdot e^{(Bt)} + C$$

(E8) $$-k_{ass}[Ag] = B \Rightarrow k_{ass} = -\frac{B}{[Ag]}$$

2.3 GYROLAB

2.3.1 GYROLAB TECHNOLOGY

Gyrolab is a microscale total analysis system (μTAS) [18], which utilizes microfluidics together with immunoassay techniques in a compact disk (CD) using small sample and reagent volumes. With automatic liquid transfers and an experimental time in the hour range Gyrolab is potentially a high throughput system [19]. Until now Gyrolab perform quantification of biomolecules for applications in the areas of pharmacokinetics, pharmacodynamics and bioprocesses. Additionally Gyrolab have shown to be suitable for interpretation and ranking of affinity interactions.

2.3.2 GYROLAB CD

All Gyrolab analysis steps takes place on a CD's containing microstructures. Capillarity is utilized for reagent influx and the centrifugal force both for volume definition as well as for flowing the reagents over the column. To date there are three different Bioaffy CD's. They differ in the volume definition chamber with either 20 nL, 200 nL or 1000 nL. There is also a newly developed mixing CD,

containing a chamber, which allows two or more analytes to be mixed before they are flown over the column. Figure 2.2 shows a conceptual large-to-small scale illustration of the Bioaffy CD's [19].

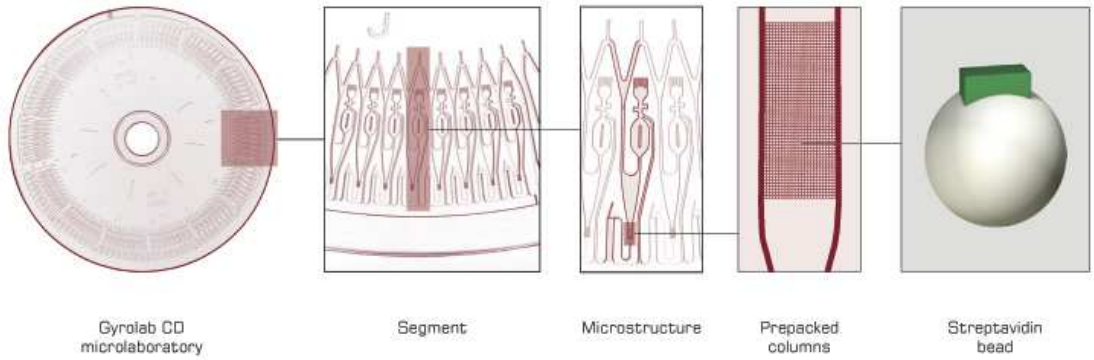

Figure 2.2 – A large-to-small scale overview of the Bioaffy CD. Bioaffy 20 nL and 200 nL contains 112 individual structures and Bioaffy 1000 nL contains 96. The mixing CD employs the same large-to-small scale concept as the others but with another structural design and has 48 individual structures.

Figure 2.3 illustrates the design of an individual microstructure for the regular Bioaffy CD's (left) and the mixing CD (right). Both contain two inlets, volume definition areas and hydrophobic barriers. All in all the design of the structures ensures an invariable sample volume and increases the reliability of the response data [19].

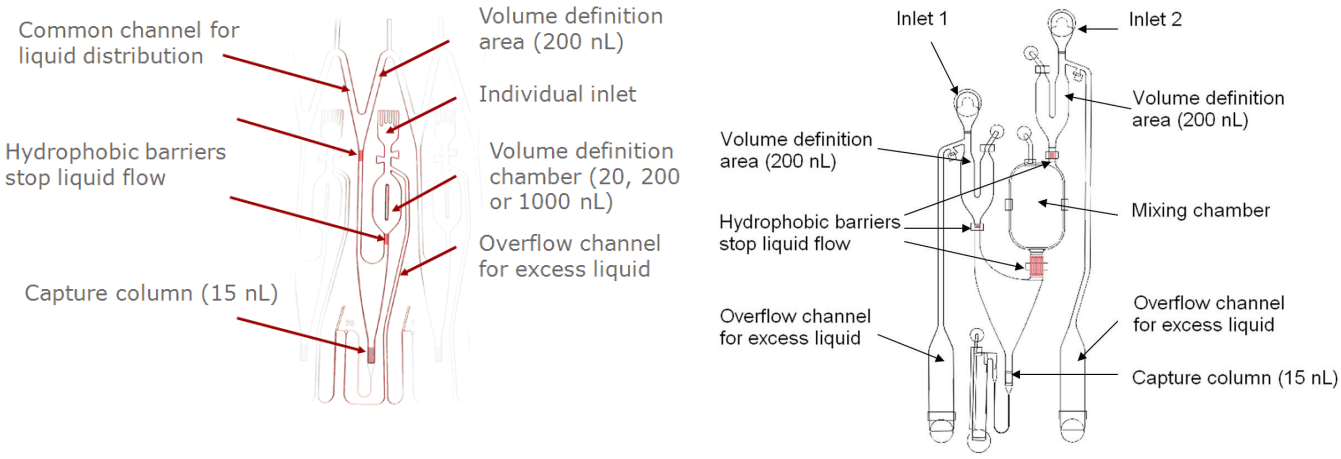

Figure 2.3 – A schematic overview of the structures in the regular Bioaffy CD's (left) and the mixing CD (right). In the regular Bioaffy CD the common inlet is used for capture and detection reagents and is interconnected with seven other structures. The hydrophobic barriers together with the volume definition area ensure that a consistent volume is distributed for each structure. The individual inlet is usually used for sample addition. The inlets of the mixing CD are used for different reagents for different assays. The mixing chamber allows for up to six liquid aliquotes to be mixed and has a stronger hydrophobic barrier.

Kinetic studies in solution have previously been made outside the CD in a mictrotiter plate (MP) with limitations in mixing times and difficulties regarding automatization [17]. The new Gyrolab mixing CD has the potential of solving these issues, making it a good candidate for kinetic studies.

2.3.3 IMMUNOASSAYS

A wide range of immunoassays are applicable in Gyrolab. These offer sensitive analyses in the search for new therapeutic antibodies against target molecules. Immunoassays are also used either to determine the presence or concentration of a biomarker as an indicator for a biological state [20].

Gyrolab utilizes the high affinity interaction between streptavidin and biotin in order to couple reagents to the microscale column. Streptavidin contains four subunits with a total size of 60 kDa. Each streptavidin subunit is able to bind one biotin molecule. The interaction occurs noncovalently with an association constant of 10^{15} M^{-1}; higher than most antigen-antibody interactions [21]. Biotin can be conjugated to a protein via the amine group. This allows for essentially any biotinylated protein to be coupled to the streptavidin-coated beads on the column and to be used as a capturing reagent [6].

The two assays used in this Master's thesis for mAb quantification are an indirect antibody assay (IAA) and a bridging immunoassay (BIA) shown in figure 2.4. As a capturing reagent both assays use a biotinylated antigen, which binds to the streptavidin-coated beads. The antigen is subsequently bound by the antibody with specificity towards that antigen. The reaction is finalized with the binding of a detecting reagent. For the IAA the detection reagent is a fluorescent antibody. The BIA on the other hand uses a fluorescent antigen as detection reagent, which utilizes the bivalent properties of the intact antibody. The fluorescent dye used for labeling is *Alexa Fluor 647*. This dye absorbs light at 650 nm and emits light at 688 nm, which is suitable for the laser-induced fluorescent (LIF) detection system in Gyrolab Workstation.

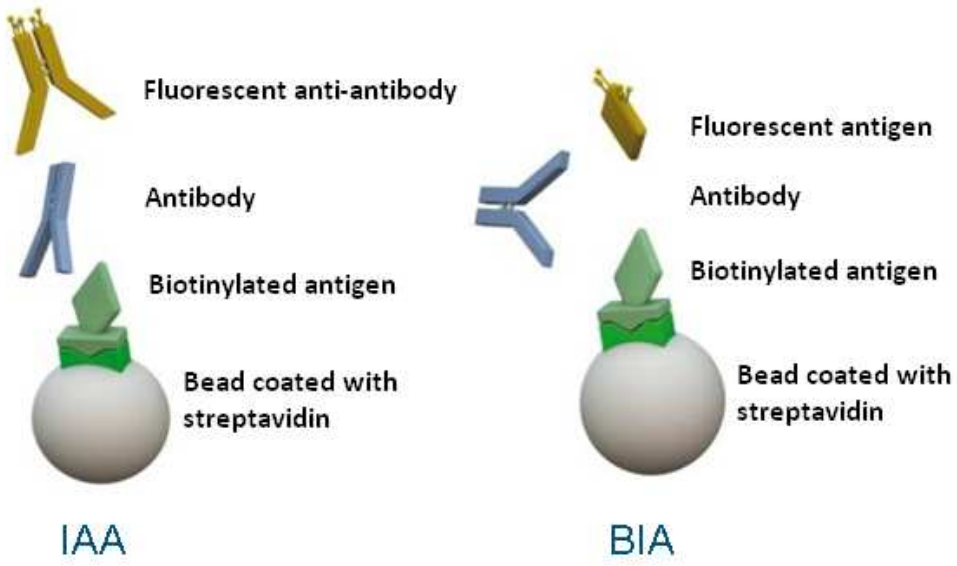

Figure 2.4 – The two assay formats used. The left picture illustrates an indirect antibody assay (IAA) and the right picture illustrates a bridging immunoassay (BIA). A biotinylated antigen is immobilized to the column via biotin-streptavidin interaction followed by addition of an antibody with specificity towards that antigen. In the IAA the detecting reagent is a fluorescent anti-antibody and in the BIA a fluorescent antigen.

2.3.4 REAGENT SYSTEM

The reagent system chosen was human thyroid stimulating hormone (TSH) together with four anti-TSH antibodies. Table 2.1 show the antibodies and their kinetic constants determined with the Biacore method. In addition, Medix have provided K_D values determined with another technique, radioimmunoassay (RIA). These values differ substantially for some antibodies (see appendix 2). However, the values presented in table 2.1 were determined with the same assay format under similar conditions and will be used for comparison throughout this thesis.

Table 2.1 - Kinetic constants**.** Medix Biochemica provided kinetic constants for each of the four anti-TSH antibodies determined with the Biacore technique.

mAb no	K_D (M)	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	Method of detection
5401	1.3×10^{-8}	5.2×10^{4}	6.9×10^{-4}	Biacore
5404	3.3×10^{-9}	1.4×10^{5}	4.3×10^{-4}	Biacore
5407	1.7×10^{-9}	2.3×10^{5}	3.9×10^{-4}	Biacore
5409	5.6×10^{-10}	3.2×10^{5}	1.8×10^{-4}	Biacore

This reagent system has previously been used for affinity studies and is optimized with respect to certain areas in Bioaffy CD's [17]. In this thesis the Bioaffy CD's are used for further optimization and assay development and the mixing CD for kinetic characterization.

3. AIM

The aim of this Master's thesis is to design a method that characterizes kinetic binding properties for therapeutic monoclonal antibodies in Gyrolab. The method aims to have high precision, be robust, user-friendly and a competitive alternative to existing technologies. It will be automated, solution-based and consumes small sample and reagent volumes. The method will allow for a complete characterization of biomolecular interactions. In addition, using the flexibility and variety of immunoassay formats in Gyrolab, the question of antibody bivalence potentially can be evaluated.

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 CONSUMABLES

Product	Supplier
Gyrolab Bioaffy 200 (P0004180)	Gyros AB (Uppsala, Sweden)
Gyrolab Mixing CD	Gyros AB (Uppsala, Sweden)
Micro plate (P0004861)	Gyros AB (Uppsala, Sweden)
Micro plate foil (P0003160)	Gyros AB (Uppsala, Sweden)
Nanosep 30 K membrane (OD030C35)	Pall Corporation (Port Washington, USA)

4.1.2 REAGENTS

Bioreagent	Supplier
h-TSH	Immunometrics (London, UK)
Anti-TSH IgG 5401	Medix Biochemica (Kauniainen, Finland)
Anti-TSH IgG 5404	Medix Biochemica (Kauniainen, Finland)
Anti-TSH IgG 5407	Medix Biochemica (Kauniainen, Finland)
Anti-TSH IgG 5409	Medix Biochemica (Kauniainen, Finland)
Goat anti-mouse IgG	Jackson ImmunoResearch (West Grove, USA)
BSA	Calbiochem (San Diego, USA)

Labeling kits	Supplier
EZ- Link Sulfo NHS-LC-Biotin	Calbiochem (San Diego, USA)
Alexa Fluor 647 Monoclonal Antibody Labeling Kit	Molecular Probes (Carlsbad, USA)

Buffers	Supplier
Rexxip A (P0004820)	Gyros AB (Uppsala, Sweden)
Rexxip F (P0004825)	Gyros AB (Uppsala, Sweden)
PBST: 15 mM phosphate buffer and 150 mM NaCl, pH 7.4 (PBS), Tween 0,02%	Merck Eurolab (Darmstadt, Germany)

4.1.3 INSTRUMENTS

Instrument	Supplier
Gyrolab workstation	Gyros AB (Uppsala, Sweden)
Spectrophotometer 2100 pro	GE Healthcare (Uppsala, Sweden)

4.2 METHODS

The two different assay formats used in this thesis were BIA and IAA. As capture reagent biotinylated TSH (TSH-b) was used for both formats. The detecting reagent was Alexa-labeled goat anti-mouse IgG (Alexa-mAb) for the IAA and Alexa-labeled TSH (Alexa-TSH) for the BIA. The concentrations of the detecting reagents were 25 nM for all experiments.

4.2.1 CAPTURE AND DETECTION REAGENTS

Both the fluorescence labeling of mAbs and TSH as well as the biotinylation of TSH was performed according to the Gyrolab User Guide [22].

4.2.2 TEST OF ASSAY FORMATS

To test the two assay formats a standard curve was produced. A standard curve shows response as a function of analyte concentration [6]. To produce the standard curve a concentration series of [mAb] was generated. This was accomplished by diluting Anti-TSH IgG 5407 (mAb-5407) in Rexxip A to concentrations between 0 and 10 000 pM in 4 times dilution steps. Standard curves were produced for both the IAA and the BIA.

A mixture of TSH-b and biotinylated bovine serum albumin (BSA-b) was used as a capturing reagent. BSA-b was included to control the density of THS-b on the column. This is to reduce the background signal and to decrease the probability of mAbs binding two TSH-b bivalently; an action that would inhibit the BIA from working by preventing Alexa-TSH to react with the mAb. A ratio of 1:9 of [TSH-b]:[BSA-b], with a total concentration of 1500 nM, was used as suggested in [17].

4.2.3 AFFINITY MEASUREMENTS

One way of evaluating the two assay formats is to determine the dissociation constant K_D. This is accomplished by allowing a concentration series of antigen to react with a constant concentration of mAb until equilibrium is reached. For different concentrations of antigen, different amounts of antibody will be bound and therefore different responses will be detected. For low concentrations of antigen, a high amount of antibody will be free to interact with the capture and a high response will

be detected and vice versa. Previously K_D has been determined using the IAA format [17], which measures the dissociation constant for both the monovalent and the bivalent mAb. The BIA on the other hand measures the bivalent form only. This is visualized in equation E1 by the binding of only the free Ab and the AbAg-complex and not the Ag_2Ab-complex.

The TSH concentration series ranged from 200 nM to 12 pM with two times dilution steps for the IAA as well as for the BIA. The concentration of mAb-5407 was 500 pM. The samples were incubated over night for 14 hours. The same capture and detect concentration were used as in chapter 4.2.2. The experiment was performed in Bioaffy 200.

4.2.4 KINETIC CHARACTERIZATION

There are theoretically two ways of determining k_{ass} on Gyrolab using the pre-mixing technique. The first is based on a concentration series analogous to the one made in the affinity experiment. The difference being that the reaction is not allowed to reach equilibrium. The second way is to use a time series. This is done by having a constant amount of both antigen and antibody and letting them react for different time periods. Both setups yield the decreasing exponential curve for the monoclonal antibody, described in equations E6 and E7. The one chosen in this Master's thesis was the time series. This was due to two reasons; it is intuitively easier to comprehend and practically easier to work with, requiring only a few wells on the microtiter plate, one with antigen and one with the antibody.

The kinetic experiments were all performed with the IAA format. To ensure the equivalence of the mixing CD and the Bioaffy 200, initial experiments were performed on both CDs. The concentration of mAb-5407 was 500 pM and TSH 4 nM. Capture concentration remained 1:9 [TSH-b]:[BSA-b]. The time series ranged from 0s to 4560s in Bioaffy 200 and from 0s to 2093s in the mixing CD with 12 different mixing times.

4.2.5 KINETIC METHOD EVALUATION

To evaluate the method and find the optimal conditions a wide range of parameters were investigated. These included different capture concentrations, different total concentrations, different column contact times and different mixing times. The optimized conditions were chosen with respect to detection limits, precision, curve profiles, sample variation and assay curve.

4.2.5.1 VARYING CONCENTRATION OF ACTIVE CAPTURE LIGAND

By diluting the capturing THS-b with BSA-b a more sensitive assay is obtained. However, the dilution reduces the column capacity, which in turn lowers the response. To find the optimal conditions, four different percentages of TSH-b:BSA-b were examined; 100:0, 50:50, 30:70 and 10:90. This was done using a standard curve in the same manner as in chapter 4.2.2 with mAb-5401 in Bioaffy 200.

Table 4.1 – Capture composition. Four different combinations of TSH-b and BSA-b were investigated with a total concentration of 1500 nM in order to optimize capture conditions.

[TSH-b]	[BSA-b]	Total concentration	Ratio [TSH-b]:[BSA-b]
1500 nM	0 nM	1500 nM	100:0
750 nM	750 nM	1500 nM	50:50
450 nM	1050 nM	1500 nM	30:70
150 nM	1350 nM	1500 nM	10:90

4.2.5.2 VARYING COLUMN CONTACT TIME

The time it takes for the mixed solution to flow through the column (flowrate) can be varied in Gyrolab by adjusting the spin of the CD. A faster spin increases the flow rate and reduces the contact time whereas a slower spin results in a decreased flowrate and an increased contact time. To examine the influence of column contact time yet another standard curve was produced with the same concentration range as in chapter 4.2.1 in Bioaffy 200. Three different column contact times were investigated; 1, 2 and 10 minutes. The capture ratio 50:50 TSH-b:BSA-b was used in this and all further experiments.

4.2.5.3 VARYING TOTAL CONCENTRATION

To investigate the influence of the total concentration of TSH and mAb three different total concentrations were studied. The ratio of [TSH] and [mAb] were kept constant at 8 to 1. The three total concentrations of mAb-5401 and TSH used are shown in table 4.3.

Table 4.3 - Total concentrations. Three different total concentrations of TSH and mAb-5401 were used.

Experiment no	[TSH]	[mAb]
1	400 nM	50 nM
2	40 nM	5 nM
3	4 nM	0.5 nM

4.2.5.4 VARYING MIXING TIME

The final parameter examined was the mixing time. A time series with shorter mixing times ranging from 0s to 515s was tested with two different reagent concentrations in the mixing CD; one with 400 nM TSH together with 50 nM mAb-5401 and the other one with 40 nM TSH together with 5 nM mAb-5401.

4.2.6 KINETIC METHOD APPLICATION

4.2.6.2 ANTIBODY SCREENING

The two kinetic time series obtained from the evaluation were used in two antibody screening runs. The same batch capture and detect was used for the long and the short time series and the experiments were performed in room temperature (≈25°C). The time series were designed in such a way that 4 antibodies could be screened in duplicates with 6 mixing times in one mixing CD. Table 4.4 show the antibodies and concentrations used in the screening experiment.

Table 4.4 - Antibody screening. The left column show the four different antibodies used. The concentrations used for the long times series are show in the middle column and the concentrations for the short time series in the right column.

mAb no	Long time series		Short time series	
5401	[TSH]	4 nM	[TSH]	20 nM
5404	[mAb]	0.5 nM	[mAb]	2.5 nM
5407				
5409				

4.2.6.3 pH OPTIMIZATION

Even though most therapeutic antibodies are developed and used in around pH 7 it is not certain that this is their optimal pH for maximal interaction with antigen. In some pH conditions the antibody will display high performance with high k_{ass} values and in some it will perform poorly with low k_{ass} values. At some point the pH will be too high or too low for the antibody to react at all.

The short time series was used in order to investigate the optimum pH-conditions for mAb-5401. Experiments were performed in Rexxip A adjusted to pH 7, 6, 5 and 4 respectively. The optimum pH-conditions was examined using TSH concentrations of 40 nM and mAb-5401 concentrations of 5 nM.

When performing kinetic experiments it is important to ensure that the differences between two antibodies actually were due to kinetics and not to other outside factors. In the case of the pH optimization it was important to increase the pH to 7 for all buffers before flowing the analytes over the column, ensuring that the conditions were equal for the solid phase in all microstructures. This was obtained by adding a neutralization buffer to the mixing chamber after all reactions had taken place.

4.2.7 REPRODUCIBILITY

Reproducibility studies were made in order to evaluate the repeatability of the designed methods. The short time series was used for the robustness studies and four experiments were made within the course of three days. New reagent dilutions were made for all experiments with the concentrations described in chapter 4.2.6.2.

5. RESULTS

5.1 TEST OF ASSAY FORMATS

Figure 5.1 displays an overlay of the two standard curves obtained when testing the reagents and the two different assay formats. A standard curve gives information regarding the performance of the assay including sensitivity and dynamic measurement range [6].

It is clear from figure 5.1 that the IAA is more sensitive than the BIA since it allows for quantification in concentrations <10 pM. For the BIA on the other hand only concentrations above 100 pM is quantifiable. This has to be considered when planning the affinity measurement experiment. To generate a more sensitive BIA further optimization is needed.

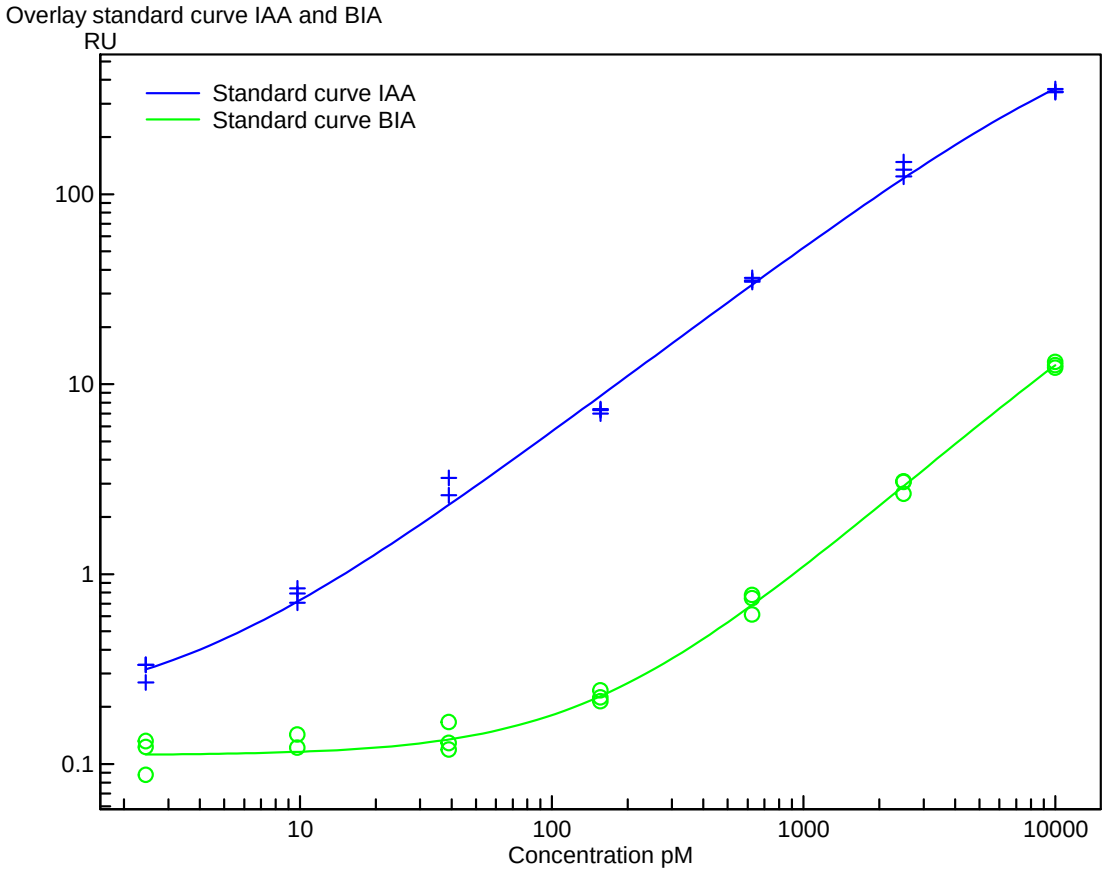

Figure 5.1 – Standard series. An overlay of the standard curves obtained from the IAA and the BIA for mAb-5407. The IAA assay displays linearity for the entire concentration range whereas the BIA shows linearity between 100 pM and 10 000 pM. The values are fitted to the equation in appendix 1.

5.2 AFFINITY MEASUREMENTS

Since the two assays give different responses, the values had to be normalized against the highest response value in order to make a comparison. Figure 5.2 shows an overlay of the normalized graphs obtained from the affinity experiment. The bridging immunoassay shows a faster decrease than the indirect antibody assay. One possible reason for this is due to the bivalence of the mAb-5407. For the IAA both the free mAb as well as the mAb bound to one TSH protein will be detected. For the BIA only the free mAb will be detected. As a consequence more TSH is needed for the IAA compared to the BIA before a decrease in response is obtained.

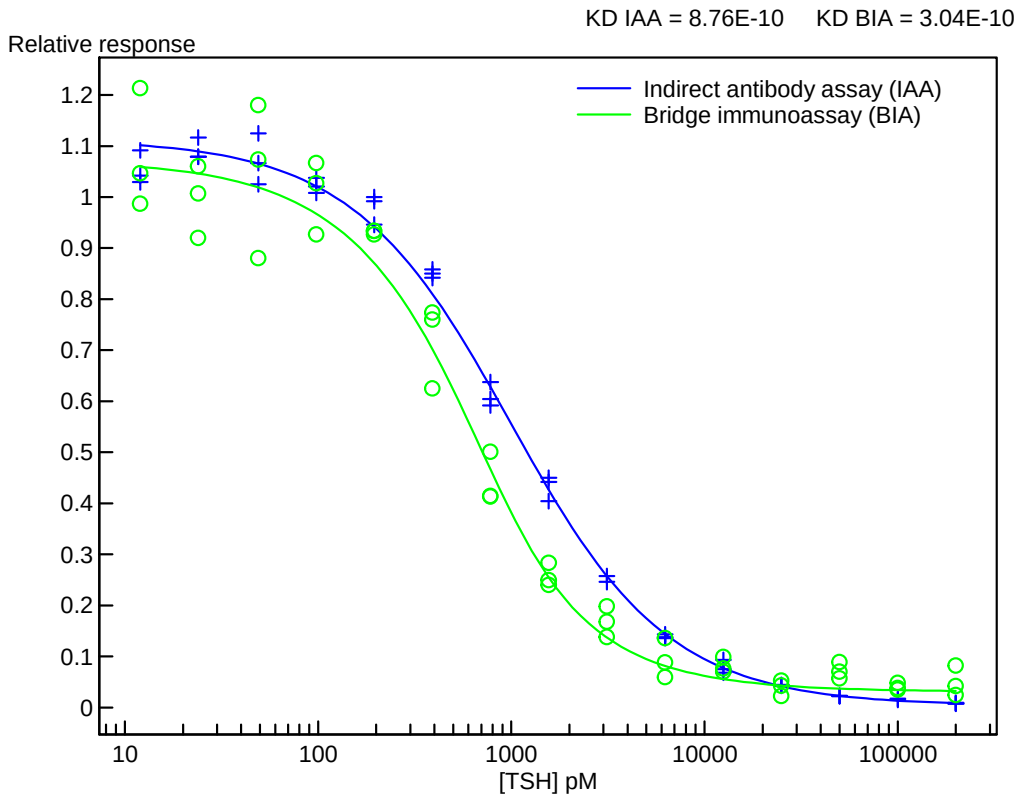

Figure 5.2 – Affinity measurements. The figure illustrates the graphs obtained from the affinity measurements for the competitive IAA and BIA assays. The TSH concentration on the x-axis increases from 12 pM to 200 nM. The y-axis shows the relative response values. The concentration of mAb-5407 was 500 pM and the experiment was performed in Bioaffy 200. The values are fitted to equation E5.

Table 5.1 show the K_D values obtained from the affinity experiment together with the 95% confidence limits. The IAA coincides fairly well with previous studies made with the IAA format with K_D values around 5.5 x 10^{-10} M [17]. The K_D values for the BIA are approximately half of the IAA K_D values.

Table 5.1 – Dissociation constants. The K_D values determined in the affinity experiment are shown with 95% confidence limits for the IAA and the BIA assays.

Assay format	K_D (M)
IAA	$8.8 \times 10^{-10} \pm 0.3 \times 10^{-10}$
BIA	$3.0 \times 10^{-10} \pm 0.2 \times 10^{-10}$

As mentioned in chapter 2.2.1, a necessary condition for using the curve fit in equation E5 is that there is a linear relation between the response value and the [mAb] for the concentration chosen. Figure 5.1 show that an antibody concentration of 500 pM is well within the linear range for both assays. Due to the low sensitivity of the BIA, the IAA was chosen for all further experiments.

5.3 KINETIC CHARACTERIZATION

The graphs obtained from the kinetic characterization are shown in figure 5.3. The two graphs display large variations between the replicates, something that increases the risk of a poor curve fit.

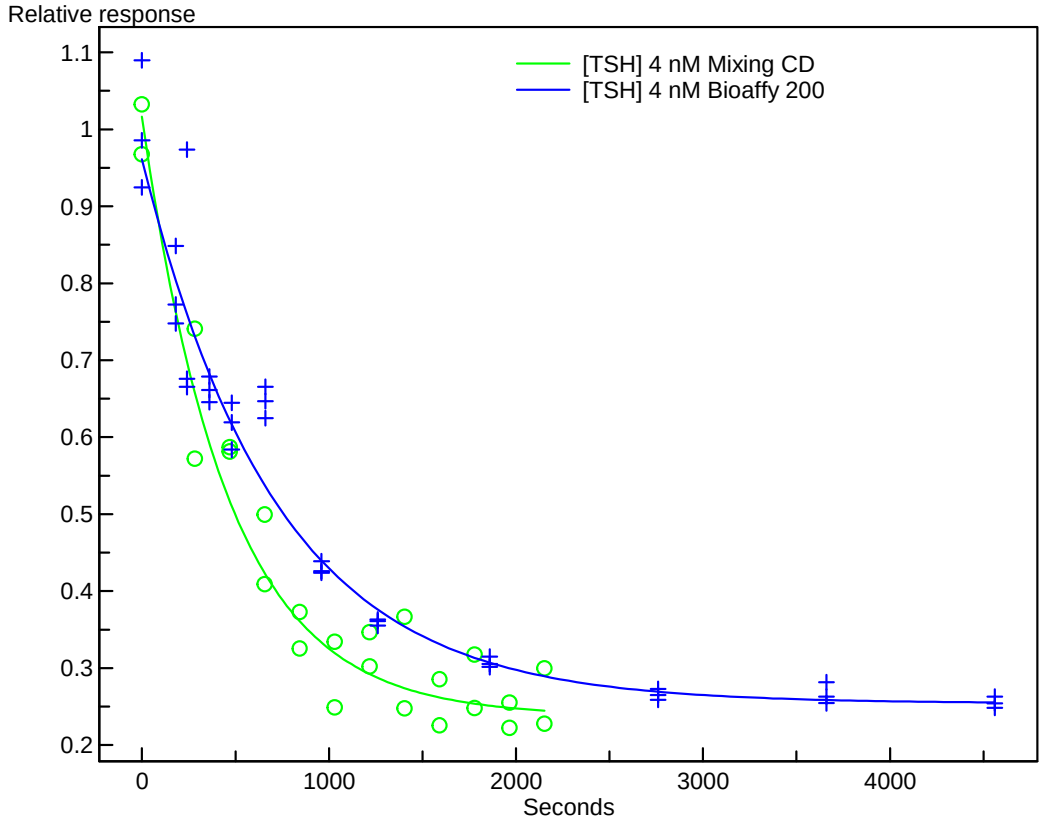

Figure 5.3 – Initial kinetic characterization experiment. The figure visualizes an overlay of the kinetic characterizations made in both the Bioaffy 200 and in the mixing CD. [mAb]: 0.5 nM and [TSH]: 4 nM. The values are fitted to the exponential decay model in equation E7.

The mixing CD used in this experiment was an early prototype of the final mixing CD, which might be one explanation to why the two graphs differ. Unfortunately different detection settings were used, resulting in different response levels. Therefore, the normalized curves are not comparable. Irrespective of the reason, it is clear that further optimization is needed in order to effectively perform kinetic characterizations.

The k_{ass} values calculated from the curve fits are shown in table 5.2. The value provided by Medix (see table 2.1) for mAb-5407 is 2.3 x10^5 $M^{-1}s^{-1}$. Even though there is a difference between the three values it is likely that is because of experimental errors and the fact that the method is not optimized. Since the k_{ass} values are calculated from the curve fit this could be one reason to why the values differ between the different CD formats and the different concentrations.

Table 5.2 – Association rate constants obtained from the kinetic characterization.

CD-type	k_{ass} (x 10^5 $M^{-1}s^{-1}$)
Bioaffy 200	3.4
Mixing CD	6.0

Determining k_{ass} in Bioaffy 200 is cumbersome and a time consuming process coupled with a high degree of uncertainty. An extensive experimental planning is required with manual pipetting at predetermined time points. In the mixing CD this is handled automatically which allows faster runs with higher precision and lower uncertainty. Therefore it is important to optimize the method developed for kinetic characterization in the mixing CD.

5.4 KINETIC METHOD EVALUATION

5.4.1 VARYING CONCENTRATION OF ACTIVE CAPTURE LIGAND

The standard curves for the four different capture percentages tested are displayed in figure 5.4. From the figure it is clear that the background signal is lowered with increasing [BSA-b]. However, a low column capacity leads to lowered response. The capture with only TSH-b presents a relatively modest working range compared to the ones diluted with BSA-b.

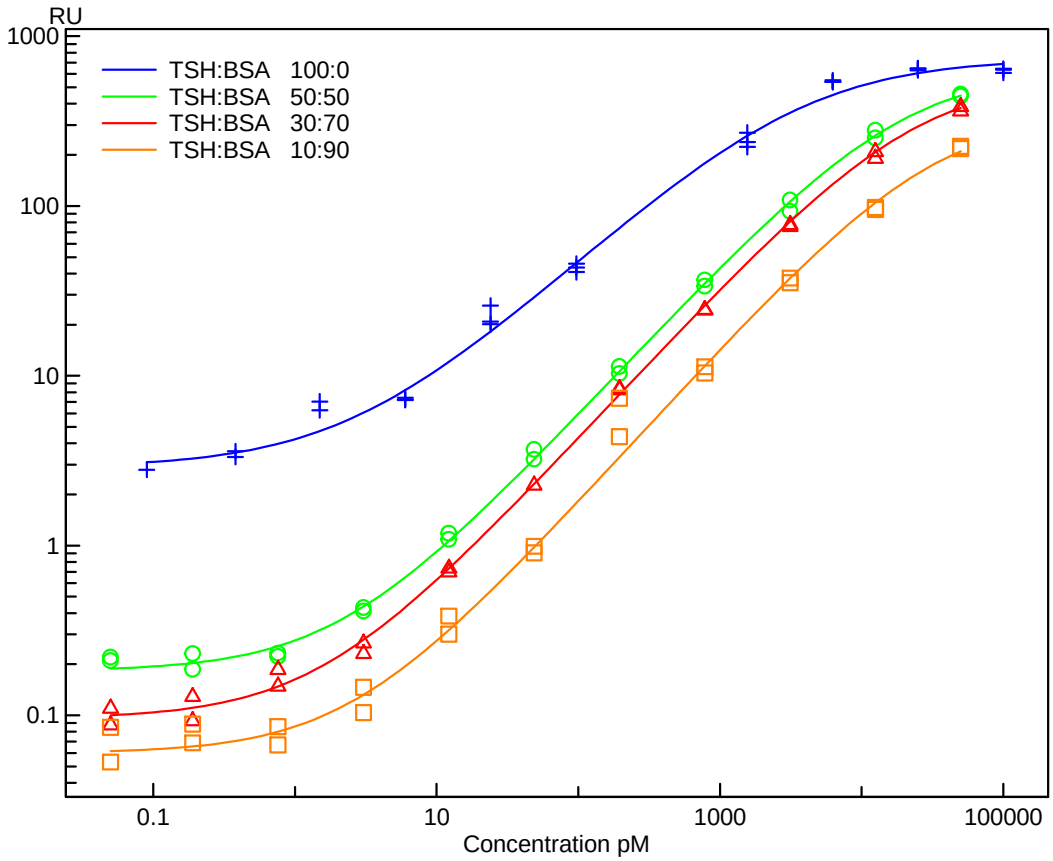

Figure 5.4 – Variation of active capture ligand. Standard curves are illustrated for four captures with different [TSH-b] and [BSA-b]: 100% TSH-b, 50% TSH-b and 50% BSA-b, 30% TSH-b and 70% BSA-b, and finally 10% TSH-b and 90% BSA-b. The final concentration was 1500 nM for all capture combinations. The experiments were performed in Bioaffy 200. The values are fitted to the equation in appendix 1.

In contrast to ELISA, Gyrolab presents 3D images of the column profiles in which valuable information can be obtained. These images can be used to remove outliers and faulty responses and also give indications regarding the molecular interactions. A high and narrow peak indicates high affinity whereas a wide and low peak indicates low affinity. The concentration of the capture biomolecule on the solid phase also influences the peak shape. Figure 5.5 show column profiles from the two capture combinations; 50:50 and 10:90 [TSH-b]:[BSA-b] at 49 pM mAb-5407.

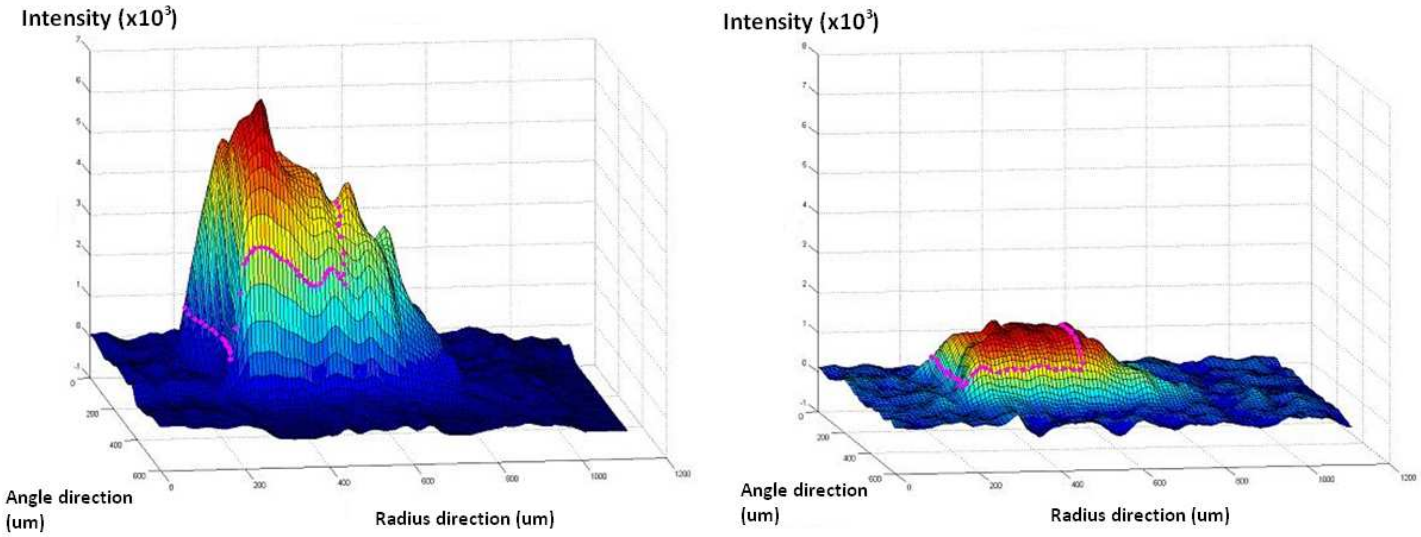

Figure 5.5 – A 3D view of the columns profiles. The reagents enter the column from left to right. The intensity of the area inside the pink lines is integrated and is returned as the response value. The capture with 50:50 [TSH-b]:[BSA-b] (left) gives a higher response than the 10:90 [TSH-b]:[BSA-b] (right). The standard series were performed in Bioaffy 200.

The optimal capture properties would be a capture with high sensitivity and sharp narrow peaks. However this is not the case with the reagent system used. The capture conditions with the highest sensitivity yields the widest peaks. Therefore a trade off had to be made and the 50:50 [TSH-b]:[BSA-b] was considered most suitable.

5.4.2 VARYING COLUMN CONTACT TIME

Lower flowrates give the mAbs more time to interact with the capture, which results in higher responses. On the other hand a low flow rate gives an increased difference in mixing time between the very first portion of sample passing through the column and the very last portion, which obviously has been mixed for a longer period of time. The time values for the x-axis in the decreasing exponential curve is decided by the actual mixing time in the mixing chamber plus the average time it takes to flow through the column. A column contact time of 10 minutes, with an average of 5 minutes, will have a great influence on the time values on the x-axis. As a result a short column contact time, thus a high flowrate, is desired. Figure 5.6 show three different 2D overlays of the column profiles for the three column contact times at three different antibody concentrations.

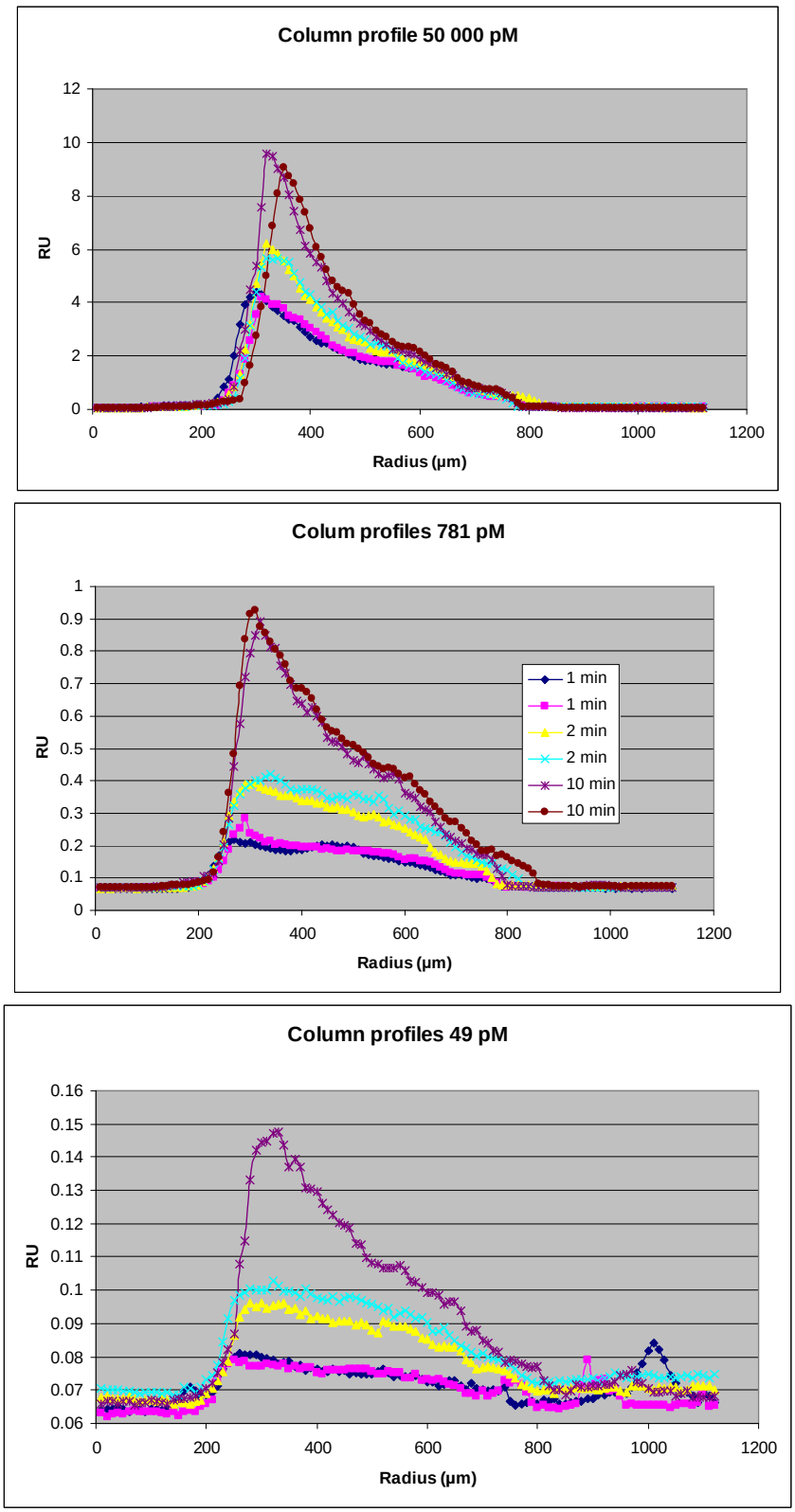

Figure 5.6 – 2D curve profiles for different column contact times. The sum of the intensities on each radii on the column is shown for three different antibody concentrations at three different column contact times for mAb-5401.

The three graphs in figure 5.6 show that 10 minutes has the highest column capacity. By decreasing the column contact time the response is lowered. For the highest [mAb], the concentration is enough for all three contact times to yield narrow peaks. For lower [mAb], the short contact times don't have the capacity to capture enough antibodies in order to yield a narrow peak.

To minimize the uncertainty contributions associated with contact time, and still maintain a high response value, 2 minutes was chosen.

5.4.3 VARYING TOTAL CONCENTRATION

An overlay of the three different total concentrations tested for the interactants (TSH and mAb), in the mixing CD, is shown in figure 5.7. The two highest concentrations appeared to be too high with regard to the mixing times used since they decreased to background response at the shortest mixing time (300 seconds). To determine k_{ass} for these, even shorter mixing times are needed. The lowest concentration appeared most promising with a slow decreasing exponential curve.

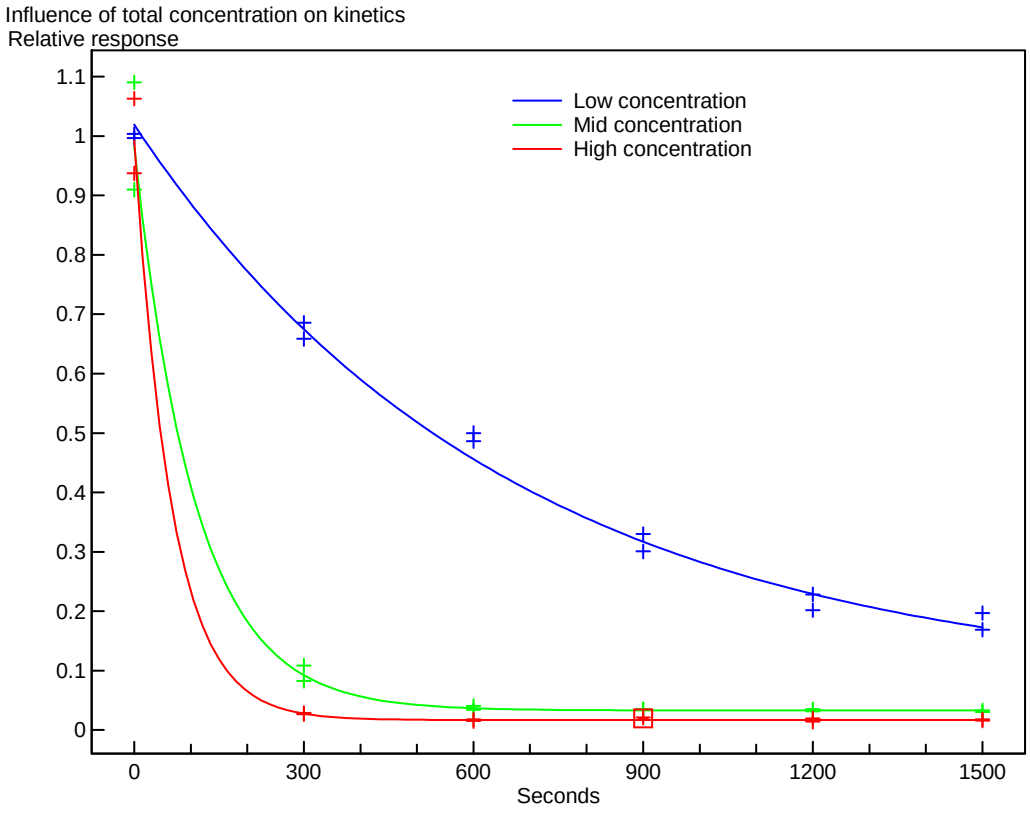

Figure 5.7 – The influence of total concentration on k_{ass} determination. Three different total concentrations of TSH and mAb-5401 were tested. Low: 4nM TSH, 0.5nM mAb; Mid: 40nM TSH, 5 nM mAb; High: 400 nM TSH, 50nM mAb. The values are fitted to the exponential decay model in equation E7.

5.4.4 VARYING MIXING TIME

Figure 5.8 show an overlay of the two different total concentrations tested using shorter mixing times. In the short time series, a TSH concentration of 40 nM together with a mAb concentration of 5 nM displayed a slow decreasing exponential curve. The high concentrations of 400 nM [TSH] and 50 nM [mAb] were too high for the shorter time series as well, decreasing to background response at the first mixing time (80 seconds). Even shorter mixing times than 80 seconds are needed in order to obtain a good curve fit and determine k_{ass} for such high concentrations. The results show that a shorter time series with shorter mixing times allows for higher concentrations than the long time series.

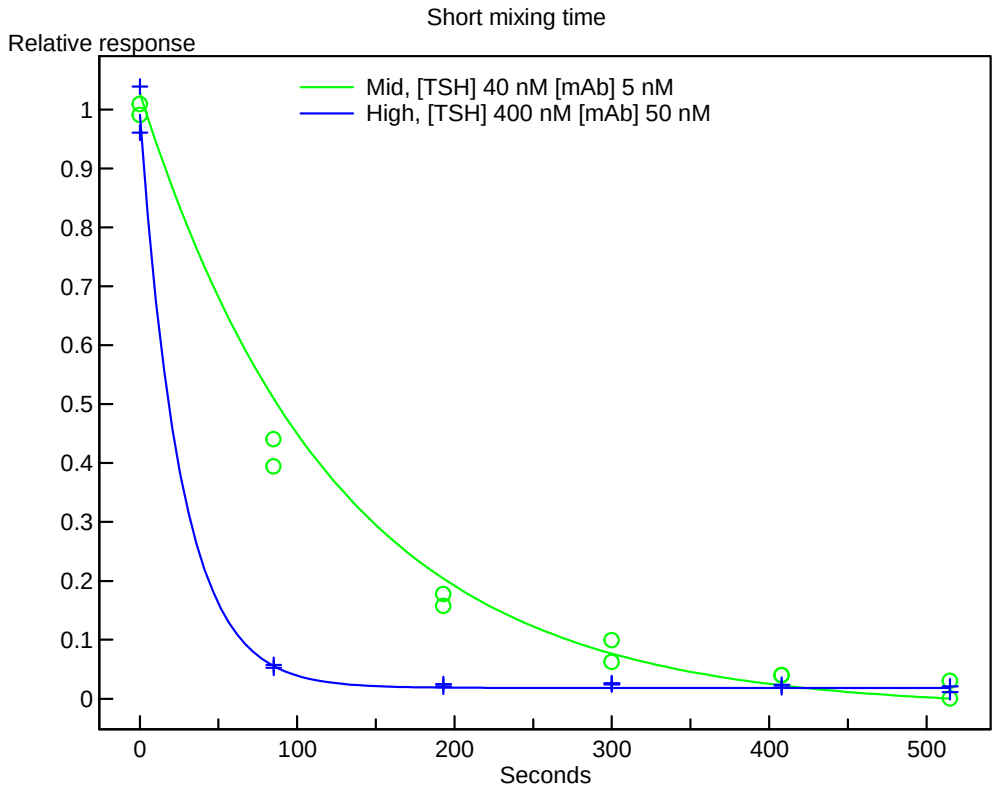

Figure 5.8 – The influence of mixing time on k_{ass} determination. A shorter time series with shorter mixing times were investigated. Two different total concentrations were used. *Mid* with 40nM [TSH] and 5nM [mAb] and *High* with 400nM [TSH] and 50nM [mAb]. The values are fitted to the exponential decay model in equation E7.

5.4.5 SUMMARY

The kinetic characterization resulted in two time series: one with short mixing times and higher total concentrations of the interactants and one with long mixing times and lower total concentrations of the interactants. Table 5.3 shows the conditions chosen for the two time series.

Table 5.3 – The optimal conditions chosen for the two time series.

	Capture (TSH-b:BSA-b)				Contact contact time (min)			Total concentration		
Condition	100:0	50:50	30:70	10:90	1	2	10	High	Mid	Low
Short mixing time		**X**				**X**			**X**	
Long mixing time		**X**				**X**				**X**

5.6 KINETIC METHOD APPLICATION

5.6.1 ANTIBODY SCREENING

Both screening runs revealed interesting information regarding the disparity in affinity between the four anti-TSH IgG mAbs. From figure 5.9 it is possible to distinguish the antibodies from each other with regard to k_{ass}. The faster the response is reduced the higher k_{ass}. The figure shows that mAb-5409 has the fastest decrease while mAb-5401 has the slowest.

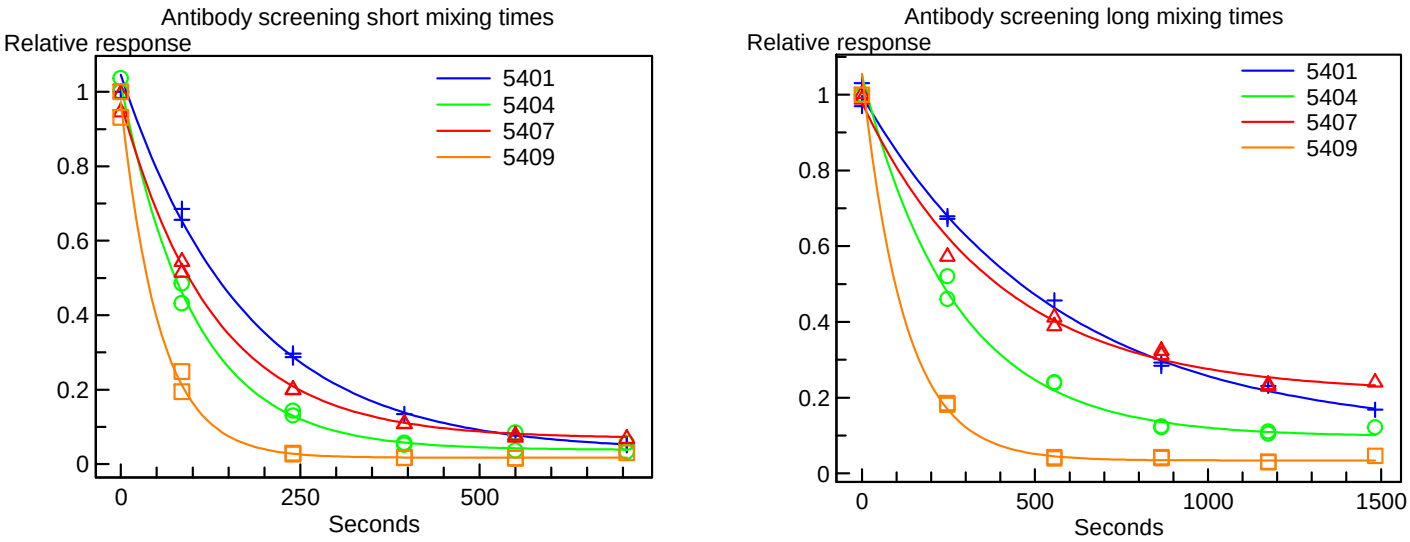

Figure 5.9 – Graphical overlays of the screening results. The short time series is shown in the left graph the long time series in the right graph. Relative response is plotted against time. Both series gave the same ranking: mAb-5409 (highest k_{ass}), mAb-5404, mAb-5407 and finally mAb-5401 (lowest k_{ass}).

Table 5.4 show the k_{ass} values obtained from the curve fit for the short and long time series compared to the Medix data. The table also shows the k_{ass} ratio for the four antibodies normalized against clone 5401 and the ranking between them.

Table 5.4 – Screening data calculated from the two screening runs. The k_{ass} values obtained for the long and short times series is shown for each clone. In addition k_{ass} ratios normalized against clone 5401 are shown together with the k_{ass} ranking. The data is compared to the values provided from Medix, using the Biacore technique.

	k_{ass} (x 10^5 M^{-1} s^{-1})			**k_{ass} ratio** (normalized against clone 5401)			**Ranking** (highest to lowest)		
	Biacore	Long	Short	Biacore	Long	Short	Biacore	Long	Short
5401	0.52	7.9	2.9	1.0	1.0	1.0	4	4	4
5404	1.4	13.7	5	2.7	1.7	1.7	3	2	2
5407	2.3	9	3.9	4.4	1.1	1.3	2	3	3
5409	3.2	26.9	9.4	6.2	3.4	3.2	1	1	1

When comparing the k_{ass} values obtained from the two time series with the Biacore data, provided by Medix, the results are similar. mAb-5409 is determined as the clone with highest k_{ass} and mAb-5401 as the clone with lowest. The k_{ass} ratio is relatively constant for the long and short time series even though the absolute k_{ass} values are somewhat higher for the long time series. The differences in absolute k_{ass} values between the long and short series are well within the range of variation between two different characterization techniques [23]. Though there are most likely refinements to be made regarding the time set on the x-axis that could explain the differences between the two time series.

In both time series developed in the mixing CD the ranking for mAb-5407 is lower than mAb-5404. This contrasts the Biacore data, in which mAb-5407 has higher k_{ass} than mAb-5404. When kinetic characterizations are performed on Biacore either the antibody or the antigen is used as capture. If the Biacore characterization used antibodies as capture, it might explain the differences in ranking between the Biacore data and the k_{ass} values calculated in the characterization experiment.

This has to do with the possible steric effects associated with the IAA format. When using a biotinylated TSH as capture there is a possibility of biotin binding the capturing TSH in the vicinity of the mAb-5407 epitope (see figure 5.10). If this occurs, the affinity may be reduced, giving lower k_{ass} values.

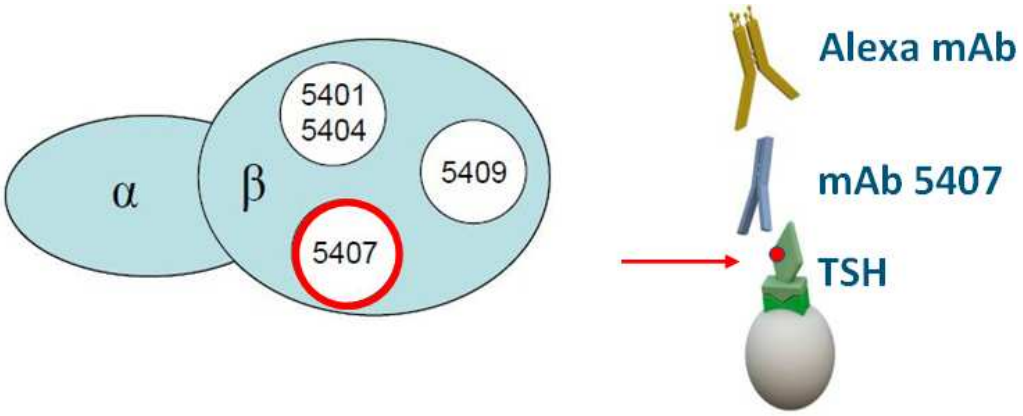

Figure 5.10 – A schematic epitope map of TSH. The figure illustrates the binding sites for each of the four antibody clones (left). If biotin had bound TSH in the vicinity of the mAb-5407 epitope the affinity may have been influenced by steric effects.

5.6.2 pH OPTIMIZATION

An alternative application of the time series is to investigate interactions under different pH-conditions with respect of association kinetics. Figure 5.11 show the resulting graph obtained from the experiment with short time series.

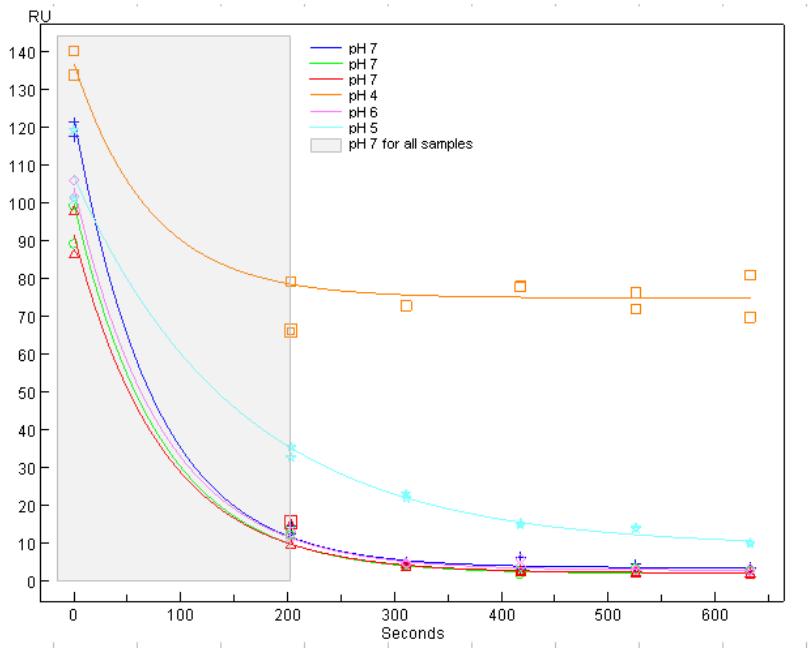

Figure 5.11 – pH-optimization results. Optimization experiments were performed for the short time series in the mixing CD. For pH 6 and 7 the affinity appeared unaffected whereas pH 4 and 5 reduced the affinity significantly. The grey area indicates the time in which the neutralization buffer was added to ensure equivalent conditions for the capture interactions. The values are fitted to the exponential decay model in equation E7.

The experiment shows that both pH 7 as well as pH 6 is favorable among the pHs tested. At pH 5 the affinity is noticeably affected with a lower association rate. In pH 4 the TSH/mAb interaction appears to be inactivated in the mixing chamber. The reduction in response is most likely an effect of the neutralization buffer added before flowing the mixture over the column. The neutralization buffer raises the pH to 7 and makes the analysis of the remaining antibody after the premixing stage once again possible. Since the column contact time is two minutes the same amount of TSH/mAb react in all structures, which explains the fact that the response is of similar values irrespective of mixing time.

5.7 REPRODUCIBILITY

To test reproducibility of the k_{ass} determination, the short time series developed was repeated four times. Figure 5.12 show an overlay of each experiment for each of the four antibodies and the k_{ass} values calculated from the curve fit in each run.

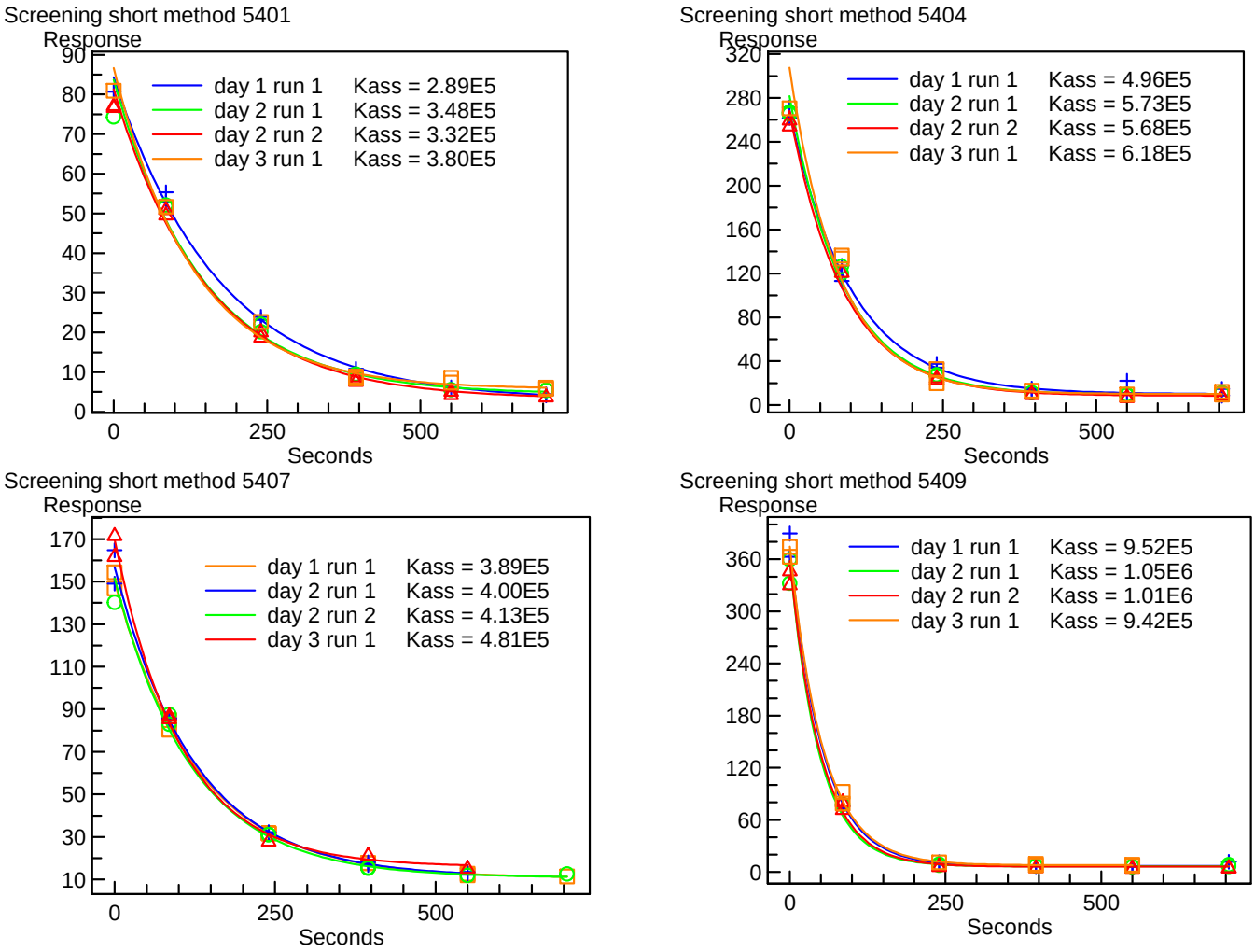

Figure 5.12 – Results for the reproducibility tests. Four experiments were performed with same experimental conditions over the course of three days. The overlays show that there are very small variations in k_{ass}, indicating that the kinetic characterization method is robust and reproducible. The values are fitted to the exponential decay model in equation E7.

Each experiment presented the same ranking with regard to k_{ass}. Table 5.5 show the 4-day average k_{ass} together with a comparable ranking. It also shows the standard deviation (STD) and the coefficient of variation (CV) for the k_{ass} values for each antibody.

Table 5.5 – Data calculated from the reproducibility tests. The average k_{ass} values were calculated for the four experiments for each clone as well as the standard deviation and the CV. In addition the ranking between the different clones are shown.

mAb no.	Average k_{ass} (x10^5 $M^{-1}s^{-1}$)	STD	CV%	Ranking (high k_{ass} to low)
5401	3.4	0.38	11.2	4
5404	5.6	0.51	9.0	2
5407	4.2	0.42	9.8	3
5409	9.9	0.53	5.3	1

Figure 5.13 display a diagram showing the k_{ass} values for each experiment for each clone together with the average k_{ass} for that clone. The standard deviation is indicated with error bars on the k_{ass} average.

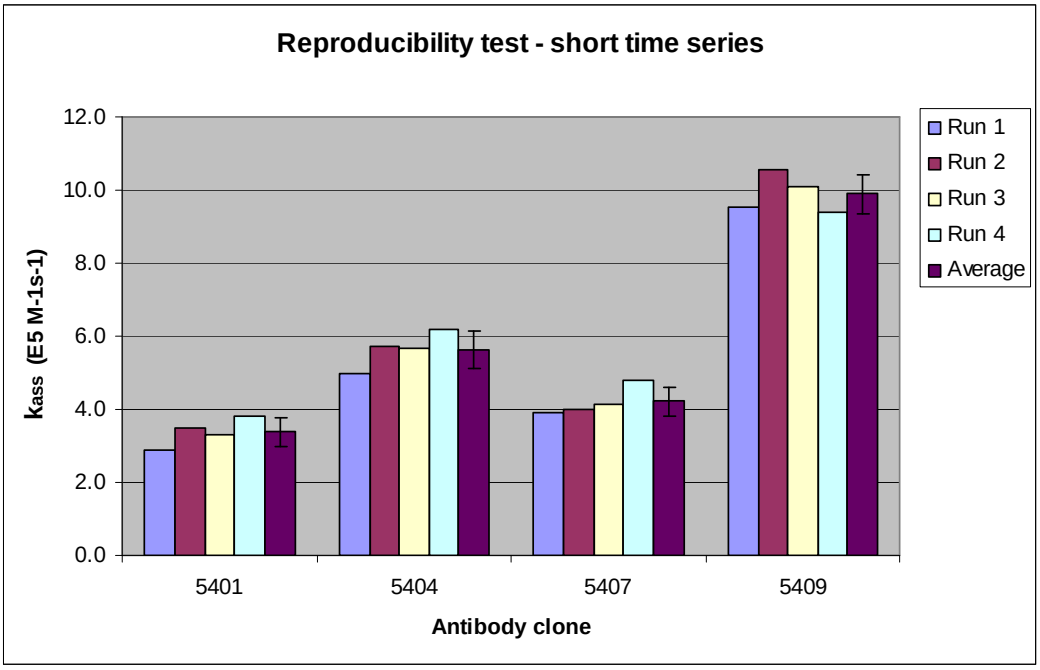

Figure 5.13 – Graphical illustration of the k_{ass} values from the reproducibility experiment. The variations in k_{ass} between each run for each clone are shown in the figure. Moreover, the calculated average k_{ass} for each clone is visualized together with the standard deviation.

The fact that the four consecutive experiments, all performed with new dilutions, presented identical k_{ass} rankings with low standard deviations and CV's indicate that the characterization method developed is reproducible and robust.

6. DISCUSSION

6.1 BIA AND IAA

When comparing the two assay formats used it is clear that the IAA is the more sensitive of the two (see chapter 5.1). One possible explanation is that some of the antibodies had bound to two capturing TSH on the solid phase, thus preventing the detecting TSH to bind in the BIA format (see figure 6.1). As a countermeasure the capture was diluted with BSA-b, though the dilution may not have been sufficient. Another explanation is due to the kinetics involved. When the antibody had bound the capturing TSH at one binding site it might have experienced steric effects, affecting the affinity at the other binding site. If the second binding site were less accessible than the first, fewer antibodies would have been detected. This means that $k_{ass,1} > k_{ass,2}$ in equation E1.

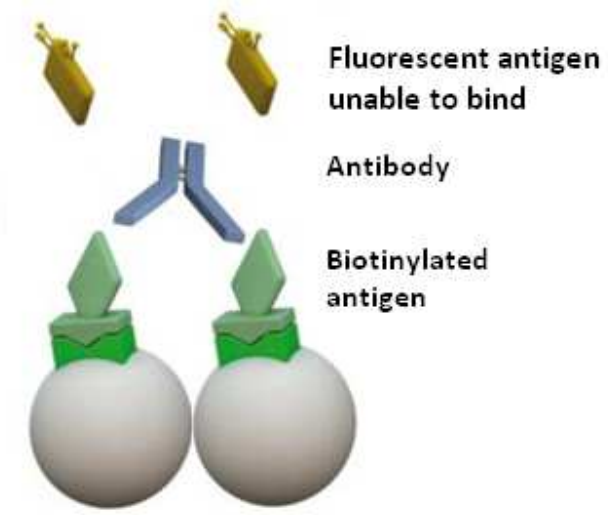

Figure 6.1 – A schematic hypothesis to why the BIA is less sensitive than the IAA. In the BIA the antibody might have bound two adjacent antigens thus preventing access for the detecting fluorescent antigen.

6.2 NORMALIZATION FOR GRAPHICAL COMPARISONS

To be able to compare the antibodies graphically the response values were normalized against the highest response value. This made the background seem higher for antibodies with low initial response values compared to antibodies with high initial response values (see figure 5.3 and 5.9). This could have been avoided by subtracting the background from all response values. This was not done for any of the graphs. However, all constants were determined using the curve fits from the non-normalized response values and hence did not affect the result in any way.

6.3 CURVE FITS

The k_{ass} values obtained from the kinetic characterizations were determined using the exponential decay model with baseline as curve fit (see equation E7). This curve fit was chosen because it is a conceptually simple model and is easily related to theoretical expressions (see equation E6). When developing a proof-of-concept method it is usually good to start with simple models. However, the model assumes that certain conditions are fulfilled and other, more complex, models or simulations may describe the system better.

6.4 REDUNDANT MIXING TIMES

The screening runs were all performed in duplicates with six mixing times. Since the mixing CD has 48 structures it is important to use each structure in the best possible way. Therefore it is interesting to investigate if there are any redundant mixing times that could be eliminated while still obtaining satisfactory k_{ass} values. Using only three mixing times for instance would double the number of antibodies to be screened simultaneously in one CD.

6.5 ADDITIONAL APPLICATIONS

It has been found that the response of the antibody, without addition of antigen, is closely related to its affinity properties. For antibodies with high k_{ass} values, more will bind to the column, which results in a higher response. If a collection of antibodies are kinetically characterized in the mixing CD in a certain reagent system and under predetermined conditions, k_{ass} can be plotted against the initial response. Figure 6.2 illustrates such a plot where the initial response values from the reproducibility screening are plotted against the calculated k_{ass} values. This plot can potentially be used as a reference curve for a larger screening run for antibodies with unknown kinetic constants. Using the same reagent system and the same experimental conditions in Bioaffy 200, a large pool of antibodies can easily be evaluated for k_{ass}. Antibodies showing higher response values than the antibodies in the reference curve will most likely have higher k_{ass} values. Antibodies with lower response will consequently have lower k_{ass} values. Using monoplicate sample points and a 5-CD run in Bioaffy 200, approximately 560 antibodies can be characterized in 5 hours (or 20 000 in a month). The antibodies presenting the highest initial response can subsequently be chosen for more precise characterizations in the mixing CD using the method developed in this thesis.

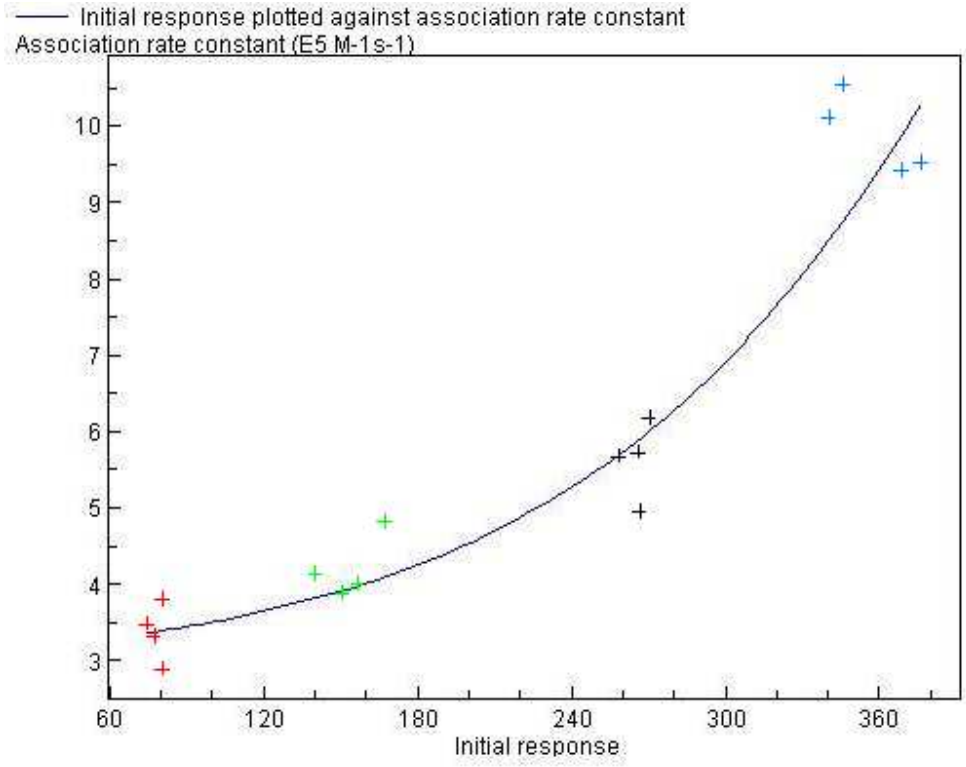

Figure 6.2 – An illustration of how Gyrolab may be used for large scale mAb-screenings. The k_{ass} values obtained from the reproducibility screenings are plotted against the initial response (containing only antibody). This graph can potentially be used as a reference curve for antibody screenings with unknown k_{ass} values. The red dots indicate the 5401-cluster, the green the 5407-cluster, the black the 5404-cluster and the blue the 5409 cluster.

The kinetic method developed can also be used for other formats. One example is to use the antibody as a capture and instead measure the free antigen concentration. This assay format can also be used for epitope mapping. The epitope map can be accomplished by using one of the antibodies as capturing reagent, mixing antigen and one of the other antibodies and use the Alexa-mAb for detection (see figure 6.3). By varying the capturing and mixing antibodies it is possible to create a map of the antibodies that share the same epitope.

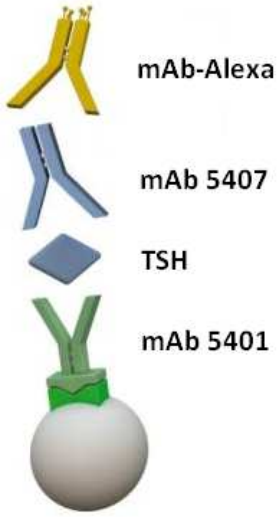

Figure 6.3 – Schematic illustration of a sandwich assay. This assay format could be used to create an epitope map for an antigen. By applying the kinetic method developed, and using different antibodies as capture, a map can be generated showing different binding epitopes.

6.6 ADVANTAGES WITH KINETIC MEASUREMENT USING GYROLAB

Several of the uncertainties associated with the kinetic measurements are removed by the automatic handling of all sample transfers and mixing steps, which is highly favorable. The integration of a mixing chamber in the CD makes it possible to evaluate biomolecular interactions for 48 samples in parallel.

In the label-free biosensor methods one of the reactants is immobilized to the solid phase. This could potentially affect the reactant on the solid phase and, in worst case, give faulty kinetic values. In the mixing CD all reactions occur freely in solution inside the mixing chamber, removing these potential issues. Moreover, it should also be possible to measure affinity in different matrices using this method. For solid phase interactions using label-free biosensors the background from bulk proteins may hide the specific signal for the antibody-antigen interaction.

As mentioned in chapter 2.2, the label-free biosensor methods derive K_D by measuring k_{diss}/k_{ass}. For antibodies with very low dissociation rates, k_{diss} cannot be obtained and therefore neither K_D. In Gyrolab, K_D is measured through equilibrium experiments, k_{ass} through the kinetic method and k_{diss} is calculated. Consequently kinetic constants for antibodies with low dissociation rates can be characterized.

6.7 THE "TRUE" VALUE OF A KINETIC CONSTANT

The reproducibility experiments displayed variations between the k_{ass} values for all antibodies. However, the variations were comparatively small to the variations experienced with other techniques. The values often vary depending on the technique, the experimental protocol and the individual performing the experiments. Studies show that even with a detailed protocol the variability between the kinetic constants were approximately 20% for the label-free biosensor techniques [23]. Therefore, it is very difficult to determine a "true" k_{ass} value for any antibody without experiencing variations.

Medix Biochemica has provided three different sets of K_D values for the four anti TSH-clones. They have been determined with either radioimmunoassay (RIA) or with Biacore and display a large variation between the two techniques (see appendix 2). As a consequence, in order to perform kinetic characterizations and be able to compare antibodies, it is important to perform the experiments in the same reagent system under similar conditions.

7. CONCLUSION

A method was developed for characterization of kinetic binding properties in Gyrolab with automated sample transfers. The characterization was performed in solution in a mixing CD, containing an integrated mixing chamber with nanoliter mixing volumes and with affinity binding columns. Association rate constants were determined for four anti-TSH antibodies, with values ranging from $3x10^5$ $M^{-1}s^{-1}$ to $10x10^5$ $M^{-1}s^{-1}$.

The antibodies were ranked according to k_{ass}. Reproducibility tests indicated that the method was robust and reproducible with CV-values around 10%. From the previously determined K_D-values [17] together with the k_{ass}-values determined through the kinetic method, k_{diss} can be calculated. This allows for kinetic characterizations of antibodies with low dissociations rate constants, which has proven to be problematic for label-free methods.

The method is applicable for precise characterization of kinetic constants and potentially also for large scale antibody screenings. Additionally, the method can be used for epitope mapping and pH-optimization.

8. FUTURE RESEARCH

With the method developed in this thesis, combined with previous work [17], Gyrolab can be applied to perform kinetic characterizations. However, there are still optimizations to be made:

- The method need to be compared against other existing label-based as well as label-free techniques. Differences in both k_{ass} values and clone ranking between the different techniques need to be investigated.

- The method needs to be evaluated in other reagent systems and with antibodies with lower/higher association rate constants. This will give information regarding the possible limitations of the method with respect to high and low k_{ass} values.

- Additional robustness studies need in order to investigate other parameters such as temperature influence, humidity, sample preparation and variations between instruments as well as between laboratories.

- The reference curve obtained when plotting the initial response against the k_{ass} value (see figure 6.2) is potentially an interesting application for future large-scale screenings. However, more research is needed in order to make such a curve reliable.

- The assay format has to be reevaluated. In order to perform future large scale screenings, the sandwich assay format might be more suitable than the IAA since is doesn't require known antibody concentrations.

- The length of the incubation time in the mixing chamber has to be studied further. Gyrolab offers the potential for longer as well as shorter mixing times than the ones designed in this thesis. Even though the ones used showed promising results, others might be more suitable.

- The number of antibodies to be analyzed in the kinetic method has to be investigated in order to optimize the use of the mixing CD. With three different mixing times, instead of six, characterization of up to eight antibodies can be made in one mixing CD. Though, fewer mixing times increases the risk of a poor curve fit, which imposes uncertainty in the k_{ass} determination. Therefore, further studies have to be made.

- Other, more complex, curve fits and assay formats need to be considered to be able to make more accurate k_{ass} determinations. Especially for understanding the effect of the bivalence of the antibody.

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11. APPENDICES

APPENDIX 1

The *5-Parameter Logistic Equation* used for the standard series curve fit using XLfit from IDBS.

$$y(x) = A + \frac{B - A}{\left[1 + \left(\frac{x}{C}\right)^{D}\right]^{E}}$$

Parameter	Description
A	The bottom plateau of the curve ie. The final minimum value of y.
B	The top plateau of the curve ie. The final maximum value of y.
C	The EC50 value represents the x value at which the middle y value is attained.
D	The slope factor. A positive value is returned when y is decreasing as x increases and a negative value returned when y is decreasing as x decreases.
E	The symmetry factor.

APPENDIX 2

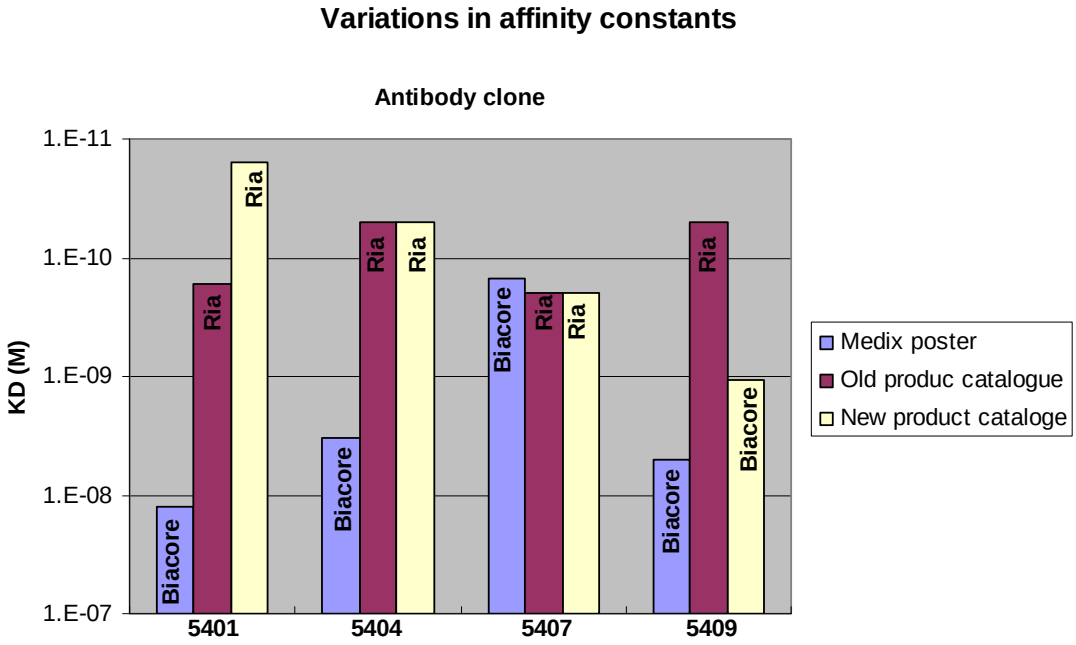

Figure 11.1 – The diagram show the variations of the K_D-values in the data provided by Medix Biochemica. Two different techniques were used to determine K_D: Radioimmunoassay and Biacore.