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Optimization of a pharmacokinetic assay in a bridging assay format using the Gyrolab immunoassay platform

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Abstract

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Anti-TNF alpha antibodies were among the first approved antibody drugs and now belongs to the best-selling drugs. Today, several companies are developing biosimilars to those drugs which will increase the access of medications and potentially reduce health care costs. There is a great demand for pharmacokinetic assays for anti-TNF-alpha drugs and the bridging assay format is a potential tool, mostly due to its high serum tolerance.

This project at Gyros Protein Technologies AB aimed to investigate the properties of the solid phase on the Gyrolab and to utilize this to optimize the bridging assay to be used as a pharmacokinetic assay for a human antibody in the presence of serum. The solid phase was optimized by incorporating three reagents with increasing molecular weight and examining the column profiles generated. Furthermore, the capture reagent was titrated with b-BSA to avoid cross-binding of both arms of the antibody to the capture reagent. Since the background was relatively high, further optimization was done to reduce background and increase the signal to noise ratio. The performance of the optimized bridging assay was compared to alternative PK assay formats. The estimated sensitivity of the bridging assay was 5 ng/ml compared to 250 ng/ml for the indirect antibody assay and 2.5 ng/ml for the bridging assay using an anti-idiotypic antibody as detect.

The optimized bridging assay performed well without dilution in buffer and was therefore used for affinity determination of Humira in neat serum. Variable concentrations of TNF-alpha were added to a fix concentration of Humira to compete with the interaction. Calculated KD-values were similar regardless of whether the measurements were performed in neat serum or after dilution in Rexxip buffer.

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Populärvetenskaplig sammanfattning

Bioteknik är ett konstant växande område vars applikationer sträcker sig över många fält, såsom hållbar utveckling samt läkemedelsutveckling. Monoklonala antikroppar, alltså proteiner som binder till ett specifikt mål har visat sig vara fördelaktigt vid bekämpning av sjukdomar. De första godkända antikroppsläkemedlen och som nu tillhör de mest sålda är anti tumörnekrosfaktor (TNF)-alfaantikroppar, proteiner som alltså binder till TNF-alfa. TNF alfa är ett substans i kroppen som finns i en förhöjd mängd hos personer med olika typer av inflammatoriska sjukdomar (FASS 2019). Adalimumab är ett läkemedel som används vid behandling av ledgångsreumatism då den blockerar TNF-alfa och på så vis hämmas inflammationen.

Idag utvecklar många bolag så kallade biosimilars, vilka härmar tidigare godkända läkemedel. Dessa utvecklas i syfte att öka tillgången på mediciner samt potentiellt kunna sänka sjukvårdskostnaderna. En viktig del av läkemedelsutveckling är en farmakokinetisk analys, vilket beskriver hur koncentrationen av ett läkemedel ändras över tid. Detta säkerställer både dess säkerhet och effektivitet. Den vanligaste metoden för att kvantifiera ett läkemedel idag är enzyme-linked immunosorbent assay, ELISA, en relativt enkel metod som dessutom inte kräver någon speciell utrustning.

Gyros Protein Technologies AB tillhandahåller Gyrolab ™, en miniatyriserad automatiserad immunoassay plattform där analysen sker på en CD-skiva bestående av ca 100 kolonner. Varje kolonn är förpackade med streptavidinbelagda kulor där ett biotinylerat fångande reagens kan binda in. En analyt binder i sin tur in och mäts sedan med hjälp av detekterande reagens märkt med fluorescens. Fluorescensen i varje datapunkt visualiseras i en 3D-profil och den integrerande fluorescensen över kolonnen rapporteras som resulterande respons.

Syftet med detta projekt var att först undersöka egenskaperna hos den fasta fasen på Gyrolab™ plattformen samt optimera en bridging assay för att användas för farmakokinetiska analyser för humana antikroppar i närvaro av humant serum. En bridging assay är en assay som fångar antikroppen på ena armen och detekterar den på den andra och på så sätt brygger analyten (antikroppen) mellan de två reagensen. Den fasta fasen undersöktes genom att inkorporera tre reagens med ökande molekylvikt och undersöka resulterande kolonnprofiler. Resultaten visade på att ca 1000 nM krävdes för att mätta kolonnen med TNF-alfa molekylen, medan molekyler med betydligt högre molekylvikt krävde en mindre koncentration. En TNF-alfa bridging assay optimerades genom att först titrera biotinylerat TNF-alfa med biotinylerat BSA för att minska densiteten på den fasta fasen. Eftersom bakgrunden fortfarande var hög utfördes ytterligare optimering för att minska bakgrund och öka förhållandet mellan signal till bakgrund och på så vis öka känsligheten.

Prestandan hos den optimerade bridging assayen jämfördes med alternativa farmakokinetiska assayformat. Känsligheten hos bridging assayen uppskattades till 5 ng/ml jämfört med 250 ng/ml för den indirekta antikropps assayen och 2.5 ng/ml för bridging assayformatet med en anti-idiotypisk antikropp som detekterande.

Vid slutet av projektet användes den optimerade bridging assay för affinitetsbestämning av Humira i neat serum. Variabla koncentrationer av TNF-alfa tillsattes till en fix koncentration av Humira för att konkurrera med interaktionen. K_D, som används för att utvärdera bimolekylära interaktioner beräknades efter 2, 24 och 48 timmar av inkubering och uppvisade liknande resultat, oberoende av mätningarna utfördes i 100% serum eller utspätt i buffert.

Detta projekt visade en bridging assay med TNF alfa som fångande och en anti-idiotypisk antikropp med hög affinitet som detekterande är mest optimal för kvantifiering av Humira. Dessutom erhålls samma K_D oavsett om mätning sker i buffert eller 100% serum.

Contents

Abbreviations

Abs	antibodies
b	biotinylated
BSA	bovine serum albumin
CD	compact disk
DOL	degree of labelling
ELISA	enzyme-linked immunosorbent assay
f	fluorescently labelled
FDA	food and drug administration
HC	high capacity
Ig	immunoglobulin
kDa	kilodalton
LIF	laser induced fluorescence
LLOQ	lower limit of quantification
MW	molecular weight
PBS	15mM phosphate buffer, 150 mM NaCl, pH 7.4
PBST	15 mM phosphate buffer and 150 mM NaCl, pH 7.4, Tween 0,02%
PK	pharmacokinetic
PMT	photo multiplier tube
S/B	signal to background
TNF	tumour necrosis factor

1 Introduction

The master thesis *Optimization of a pharmacokinetic assay in a bridging assay format using the Gyrolab immunoassay platform* is done as a final project at the master's programme in molecular biotechnology engineering at Uppsala University. The master thesis is proposed by Gyros Protein Technologies AB.

Biotechnology is one of the fastest-growing fields in technology. It has become a crucial tool for sustainable development and provides products and technologies to feed the hungry and battle diseases. Millions of people around the world have been helped by biotechnology drugs and vaccines.

In drug development, a pharmacokinetic (PK) analysis is essential since it provides information about the distribution of a drug throughout the body. ELISA is the most commonly used format of immunoassay for evaluating a drug's concentration. However, the platform suffers from drawbacks, such as limited sensitivity, matrix intolerance, a narrow dynamic range as well as being time-consuming (Joyce *et al.* 2014). Furthermore, preclinical drug development is characterized by aggressive timelines and a large number of samples (Roman *et al.* 2011). This demonstrates the importance of a more efficient and cost-effective drug development process.

Gyros Protein Technologies AB is developing and producing Gyrolab^{TM}, which is an automated immunoassay platform. The company focus on providing tools for academia and industry to increase biomolecule performance and productivity from research to clinical development. (Williams *et al.* 2017).

This project aimed to investigate the properties of the solid phase on the Gyrolab ^{TM} platform. I evaluated three reagents with increasing molecular weight (MW) incorporated into the solid phase; TNF-alpha, BSA, and IgG. Furthermore, I utilized my knowledge regarding the solid phase to optimize a bridging assay as a PK assay for a human antibody in the presence of human serum.

A bridging assay has the advantage of a good serum tolerance which allows for a more sensitive assay. There is a great demand for anti-TNF alpha drugs where a bridging assay is a potential tool. My project at Gyros Protein Technologies contributed to knowledge that could be used in future assay developments.

2 Theory

2.1 Antibodies

Antibodies (Abs), or Immunoglobulins (Igs), are Y-shaped proteins that are used by the immune system to defend against foreign antigens. It is composed of two light chains and two heavy chains, each with a variable and a constant region. The variable region is the antigen-binding site and includes the ends of the light and heavy chains. The constant region, on the other hand, mediates effector functions as opsonization (Abcam 2019a). Figure 1 shows the general structure of an antibody, with the important regions presented.

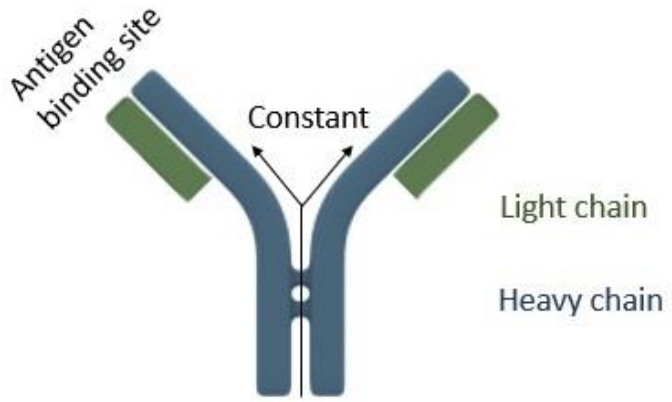

Figure 1. The general structure of an antibody. The variable region is where the antigen binds.

There are five major groups of Igs; IgG, IgA, IgM, IgD, and IgE, whereas IgG being the most abundant one in plasma (Diamandis & Christopoulos 1996). Antibodies can be produced as either monoclonal or polyclonal antibodies. Polyclonal antibodies originate from multiple B-cells whereas monoclonal antibodies are derived from a single B-cell (Abcam 2019b).

Adalimumab, with the brand name Humira, is a human monoclonal antibody primarily used as a treatment for rheumatoid arthritis and inflammatory bowel disease. It is directed against the cytokine TNF-alpha which is a part of the immune system and by binding to it, Adalimumab can decrease the inflammatory process (FASS 2019). In the report "EP Vantage 2018 Preview" released by Evaluate Pharma, adalimumab (Humira®) tops the world's top 10 best-selling drugs in 2017 (Brown *et al.* 2017).

2.2 Immunoassays

Immunoassays are methods that measure the presence or concentration of an analyte of interest. The quantitation of the analyte depends on the binding between the analyte and an

antibody. Immunoassays come in different variations and is widely used in many areas of pharmaceutical analysis (Darwish 2006).

2.2.1 Bridging assay

In a bridging assay, the target is used as both capture and detection reagent. A bridging assay consists of three components; a biotinylated capture reagent, an analyte of interest and an Alexa 647 labelled detection reagent. The analyte will form a bridge between the two labelled molecules (Gyros Protein Technologies AB 2019a). In this project, TNF alpha was used as both capture and detection reagent. The analyte of interest was Adalimumab (Humira). A schematic overview of the bridging assay described is presented in Figure 2.

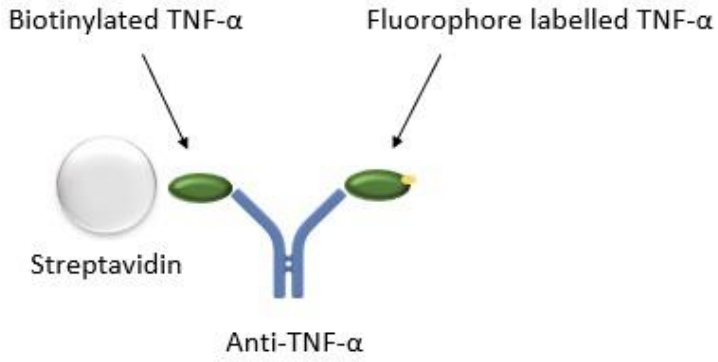

Figure 2. A schematic overview of the optimized bridging assay. A biotinylated antigen binds to the streptavidin-coated beads and an antibody binds to the antigen. Finally, a detection reagent is added.

Two formats of the bridging assay presented below are also used in this project. A schematic overview of the two assay formats are presented in Figure 3. The difference between the two assays presented is the capture reagent used; to the left, a biotinylated antigen is used and to the right an anti-idiotypic antibody, a monovalent Fab is used. In both assays, an anti-idiotypic antibody is used as a detection reagent.

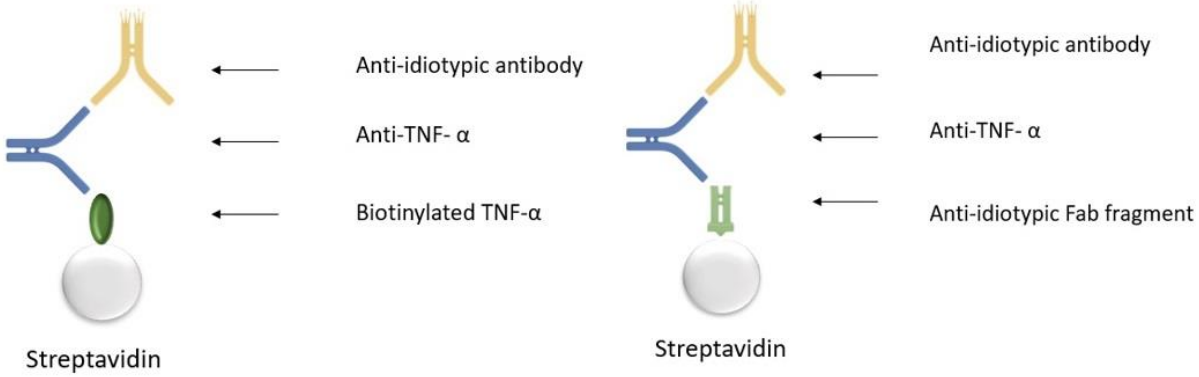

Figure 3. Schematic overview of two bridging assays. To the left, a biotinylated antigen binds to the solid phase and capture the monoclonal antibody Humira. The detect is an anti-idiotypic antibody. The assay to the right only differs in the capture reagent, in this assay a monovalent Fab is bound to the solid phase.

2.2.2 Indirect antibody assay

The target is used as the capture reagent in an indirect antibody assay. The detection reagent is often an anti-human IgG (Gyros Protein Technologies AB 2019b). The indirect antibody assay performed during this project includes a biotinylated TNF-alpha, Humira and at last a fluorescently labelled anti-human monoclonal antibody. A schematic overview of the indirect antibody assay described is presented in Figure 4.

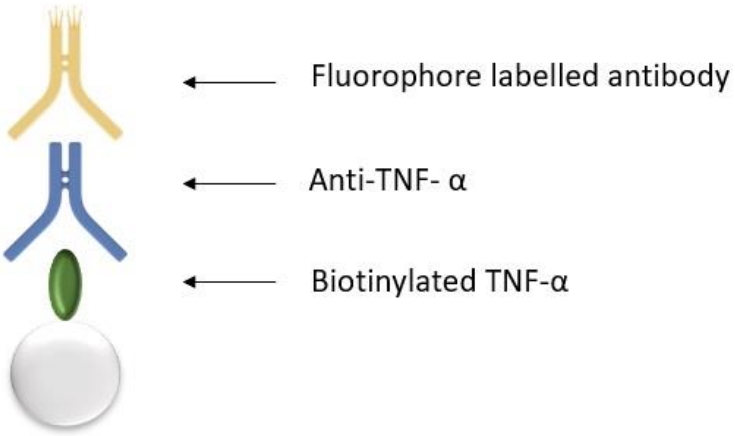

Figure 4. A schematic overview of an indirect antibody assay. A biotinylated antigen binds to the streptavidin-coated beads and an antibody binds to the antigen. Finally, a fluorophore labelled antibody is added.

2.3 PK analysis

A PK analysis examines the distribution of a drug over time once it is administered. Absorption, distribution, metabolism, and excretion are all important properties to be considered in the development of a new therapeutic drug (Fan & de Lannoy 2014). It is an essential part of the development since it ensures safety and efficacy (Williams *et al.* 2017).

Therapeutic antibodies may exist in three forms; unbound, partially bound and fully bound. If one ligand molecule is bound to the antibody, then it's said to be partially bound. A free PK assay can measure unbound and partially bound due to the bivalency of antibodies, whereas a total PK assay can measure all three forms (Talbot *et al.* 2015). The choice of assay will have an impact on the interpretation of the pharmacokinetics of a therapeutic drug. Therefore, the understanding of which form that is being measured has gained much attention (Kuang *et al.* 2010). An indirect antibody assay can detect either free Humira or Humira with only one TNF-alpha bound to it, whereas a bridging assay could detect only free drug since binding of both arms of the antibody are required.

According to an article published in AAPS Journal 2015 (Talbot *et al.* 2015) a free PK assay is preferred when examining target protein coverage, whereas a total PK assay is used to understand the relationship between total drug concentrations and adverse effects.

2.4 Affinity

Affinity can be described as the binding strength between a molecule and its ligand. It is typically measured by the equilibrium dissociation constant (K_D), which is the ratio of how quickly an antibody dissociates from its antigen (k_{off}) to how quickly it associates with the antigen (k_{on}), see equation 1. The dissociation and association of an antibody to its antigen is a reversible process, thus K_D and affinity are inversely related. A lower K_D equals a lower antibody concentration and a higher affinity (Abcam 2019c).

$$K_D = \frac{k_{off}}{k_{on}} \quad (1)$$

2.5 Gyrolab ™

2.5.1 The Gyrolab ™ platform

The Gyrolab ™ system is an open platform that allows for many application areas since the user can design its experimental set-up. The analysis takes place in a microfluidic CD device which has up to 112 affinity columns (Gyros Protein Technologies AB 2019a). Each column is pre-packed with SA coated beads which will immobilize a biotinylated capture reagent. Binding of analyte to capture reagent is measured by laser-induced fluorescence (LIF) using fluorescently labelled detection reagents. Gyrolab's ™ flow-through system that uses centrifugal and capillary forces to steer the fluidic through the SA beads enables short incubation times, which in turn minimize matrix effects (Williams *et al.* 2017).

2.5.2 Gyrolab CD

The analysis takes place in the microstructures of the CDs. The solid phase comprises SA coated beads and each SA can bind up to four biotins (Hytönen 2017). There are currently three different types of Bioaffy ™ CD's available which only differ in the sample volume applied over the column; 20, 200 and 1000 nl (Gyros Protein Technologies AB 2019a)

Figure 5 is showing the functional parts of Bioaffy ™ CD. The common channel is used for the addition of capture and detection reagent, whereas the individual inlet is used for the addition of -analyte (Gyros Protein Technologies AB 2019a). The capture reagent enters by capillary action and hydrophobic breaks stop the liquid from moving any further. Once loaded into all structures, centrifugal force moves capture reagent through the affinity column. Next, analyte enters by capillary action and are applied to the column by centrifugal force. Finally, detection reagent is added, and the CD is moved to a LIF station where every column is scanned.

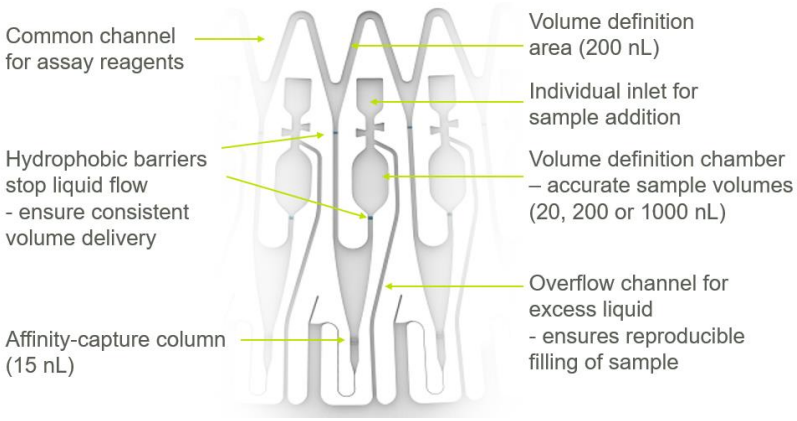

Figure 5. Schematic overview of an individual microstructure. Reagents are added to the common channel and samples in the individual inlet. Capillary action and centrifugal force are used to transfer liquids to the affinity-capture column. Illustration used with permission from Gyros Protein Technologies.

3 Material and methods

All runs performed on Gyrolab were executed according to Gyrolab User Guide P0020528 (Gyros Protein Technologies AB 2019a) using the software Gyrolab Control. Results are analysed with the Gyrolab Evaluator software and column profiles generated were examined using Gyrolab Viewer. Each unknown sample was quantified against a standard curve. A PMT-setting of 1% was in this case enough. If a too high PMT setting is used, the detector will be saturated, while a too low PMT setting results in weak signals being lost in the background scatter (Gyros Protein Technologies AB 2019a).

3.1 Material

All experiments in this project are made in vitro and human samples are handled in a laboratory only used for analysis of human serum. All consumables, reagents and instruments used during the project are stated in Table 1, Table 2 and Table 3.

Table 1. Consumables used in each run on the Gyrolab platform.

Product	Supplier
Gyrolab Bioaffy ™ 1000 CD	Gyros Protein Technologies AB (Uppsala, Sweden)
Gyrolab Bioaffy ™ 1000HC CD	Gyros Protein Technologies AB (Uppsala, Sweden)
Microplate	Gyros Protein Technologies AB (Uppsala, Sweden)
Microplate foil	Gyros Protein Technologies AB (Uppsala, Sweden)

Table 2. Bioreagents, buffers, labelling kits and serum used on the Gyrolab platform.

Reagent	Supplier
b-BSA	Vector Laboratories (BioNordika)
Recombinant TNF alpha	Prospec
Anti- Adalimumab. IgG2B.	R&D systems
Alexa 647 labelled Human IgG Fc	Southern Biotech
Human TNF alpha	Prospec
Anti-Adalimumab. Monovalent Fab	Bio-Rad
Rexxip buffers	Gyros Protein Technologies AB (Uppsala, Sweden)
PBST	Gyros Protein Technologies AB (Uppsala, Sweden)
PBS	Gyros Protein Technologies AB (Uppsala, Sweden)
PBS with 0.2% BSA	Gyros Protein Technologies AB (Uppsala, Sweden)
EZ-Link Sulfo-NHS-LC-LC-Biotin Labeling Kit	Thermo Scientific
Alexa Fluor 647 Monoclonal Antibody Labeling Kit (A-20186)	Molecular Probes
Alexa Fluor 647 Microscale Protein Labeling Kit (A30009)	Molecular Probes
Human serum pool	Seralab
Individual human serum	Serlab

Table 3. Instruments used during the project.

Instrument	Supplier
Nanophotometer	LabVision
Gyrolab workstation	Gyros Protein Technologies AB (Uppsala, Sweden)
Centrifuge 5804 R	Eppendorf

3.2 The solid phase

This section describes the optimization of the solid phase on the Gyrolab platform, with regards to capacity and surface density. The capture reagents used were TNF alpha, BSA and IgG. Two different CDs were used, Bioaffy 1000 CD and Bioaffy 1000HC CD. Humira was diluted in Rexxip H, which is a buffer designed for quantification in human serum. Rexxip H contains reagents to neutralize heterophilic antibodies, which are necessary for quantification in human or cynomolgus serum.

3.2.1 Biotinylation and Alexa 647 labelling

Biotinylation of TNF alpha, BSA and IgG were done according to protocol B1.1 Biotinylation of capture reagent in Gyrolab user guide P0020528 (Gyros Protein Technologies AB 2019a). The capture reagents were diluted to a final concentration of 1 mg/ml before biotinylation. Biotin was dissolved in Milli-Q water to a final concentration of 1 mg/ml. Capture reagent and biotinylation reagent were mixed at a 12 times molar excess of biotinylation reagent. For 100 µg of 1 mg/ml IgG, 4.5 µl of 1 mg/ml biotinylation reagent was added. The mixture incubated 1 hour at room temperature with occasional shaking and later separated on a Protein Desalting Spin Column. Protein concentration was determined by measuring the absorbance at 280nm using a nanophotometer.

Alexa 647 labelling of IgG were done according to protocol B1.2 Fluorophore labelling of detection reagent in Gyrolab user guide P0020528 (Gyros Protein Technologies AB 2019a). The detection reagent was diluted to a final concentration of 1 mg/ml before labelling and 1:10 volume of 1M Sodium bicarbonate buffer was added. The mix was transferred to a vial containing the reactive dye. The vial was wrapped in aluminium foil and incubated 1 hour at room temperature with occasional shaking. The purification column was packed with purification resin from the kit to a final volume of 1.5 ml. The labelled reagent was added onto the column. Protein concentration and degree of labelling (DOL) was determined by measuring absorbance at 280 and 650nM using a nanophotometer. DOL is expressed as a molar ratio of label/protein.

For smaller proteins, as TNF alpha and BSA, an Alexa Flour 647 Microscale Protein Labelling kit from Molecular Probes were used. Labelling of TNF alpha and BSA was done according to protocol (Molecular Probes 2006).

3.2.2 Investigation of column capacity

During this part of the project, the solid phase was optimized with regards to capacity, by examining the column profiles generated by Gyrolab Viewer. The column profiles are representations of the signal intensity in each CD microstructure and illustrate the position of the analyte (Gyros Protein Technologies AB 2019). The capture reagents were first biotinylated and then also Alexa 647 labelled. A new method was built in the Gyrolab client, that enabled for immediate reading after addition of the capture on the solid phase. The concentrations of each capture reagent used on both CDs are summarized in Table 4.

Table 4. Titration of capture reagents on Bioaffy 1000CD and Bioaffy 1000HC CD.

	Concentration TNF alpha [nM]	Concentration BSA [nM]	Concentration IgG [nM]
Bioaffy 1000 CD	50, 100, 200, 300, 500, 700, 1000	10, 50, 100, 200, 300, 500, 700, 1000	50, 100, 300
Bioaffy 1000HC CD	50, 100, 200, 300, 500, 700, 1000, 2000	300, 500, 700, 1000	10, 50, 100, 300, 766

3.2.3 Titrating capture reagent with BSA

In a bridging assay, saturation can cause binding of both arms of the drug antibody to the capture reagent. To reduce the density of the capture reagent on the solid phase, the capture reagent was titrated with BSA (Gyros Protein Technologies AB 2019b). The capture reagent was titrated using following proportions of biotinylated capture reagent and biotinylated (b)-BSA; 10% capture reagent/90% BSA, 5% capture regent/95 % BSA, 1% capture reagent/99% BSA, 0.5% capture reagent/99.5 BSA and 0.1% capture reagent/99.9% BSA.

A total concentration of 1000nM of capture reagent was used on Bioaffy 1000 CD. A stock of 1000nM was made for b-BSA and b-TNF-alpha and mixed according to proportions stated above. For example, for the capture with 10% TNF-alpha, 10µl of b-TNF-alpha were mixed with 90µl of b-BSA. For Bioaffy 1000HC CD, a total concentration of 2000nM of capture reagent was used. A stock of 2000nM was made for both b-BSA and TNF-alpha and mixed in the same way as for Bioaffy 1000 CD. Standard series corresponding to 5000-0.32 ng/ml Humira diluted in Rexxip H were used. The detect was diluted in Rexxip F to a final concentration of 25 nM. In the end, standard curves generated were compared to see what effect titration with BSA has.

3.3 Optimization of a bridging assay

This section describes the optimization of the bridging assay using Rexxip H as diluent. The goal was to minimize matrix effect and thereby maximize sensitivity. TNF-alpha was used as the capture and detection reagent. The standard 3-step method, including one capture

addition, one analyte addition and one detection addition was used. Standard series corresponding to 5000-0.32 ng/ml Humira were used.

3.3.1 Evaluation of Rexxip buffers

Two experiments were performed where Rexxip buffers with different properties were evaluated as diluents for capture and detection reagents. In a first experiment, three different buffers were used; Rexxip ccs, Rexxip H and Rexxip A. The exact content of the Rexxip buffers are a business secrete. However, Rexxip ccs contains a lower amount of blocking proteins which can be beneficial. A total of 6 experiments were run in parallel according to Table 5. A capture reagent concentration of 1% TNF-alpha was used to be able to see differences in the background. The detect concentration used was 25 nM.

Table 5. Summary of the buffers used in every experiment.

Experiment number	Capture buffer	Analyte buffer	Detect buffer
1	Rexxip ccs	Rexxip H	Rexxip ccs
2	Rexxip A	Rexxip H	Rexxip A
3	Rexxip ccs	Rexxip H	Rexxip A
4	Rexxip A	Rexxip H	Rexxip ccs
5	Rexxip ccs	Rexxip A	Rexxip ccs
6	Rexxip ccs	Rexxip ccs	Rexxip ccs

In a second experiment, PBST and PBS-BSA were tested as buffers for capture and detection reagents. PBS-BSA is a standard buffer that proved beneficial in a TNF-alpha assay several years ago. The dynamic range and the sensitivity were improved compared to having Rexxip F as detect buffer. Rexxip F contains a detergent which might not be beneficial in this case. A total of 4 experiments were run in parallel according to Table 6. As in 3.3.2, 1% TNF-alpha was used as capture reagent as well as a detect concentration of 25nM.

Table 6. Summary of the experimental setup. The buffers used in each experiment are listed.

Experiment number	Capture buffer	Analyte buffer	Detect buffer
1	PBST	Rexxip H	Rexxip A
2	PBS-BSA	Rexxip H	Rexxip A
3	PBS-BSA	Rexxip H	PBS-BSA
4	PBST	Rexxip H	PBS-BSA

3.3.2 Optimization of capture and detect concentration

A titration was performed to determine the optimal capture and detection concentration for the specific bridging assay. The experiment was run on both Bioaffy 1000 CD and Bioaffy 1000HC CD. By diluting the capture with b-BSA, the density of the capture reagent on the solid phase is reduced. As the capacity of the capture reagent is reduced, the column profiles will become more elongated. The theory was that by switching to a high capacity particle, using Bioaffy 1000HC CD, one could dilute the capture enough for the analyte not to cross-link with both arms while the capacity remains high enough so that the column profiles do not flow outside the integration area.

To further optimize the assay sensitivity the optimal concentration of detection reagent can be titrated with the aim to reduce background in relation to the specific signal (Gyros Protein Technologies AB 2019a). As mentioned in 3.2.3, the density of the capture reagent onto the solid phase can be reduced by titrating with BSA. In this experiment, PBS-BSA was used as a detection buffer. A summary of the experimental set up is presented in Table 7.

Table 7. Capture concentration and detect concentration for every experiment.

Experiment number	Capture concentration (% TNF-alpha)	Detect concentration (nM)
1	0.1	25, 12.5 and 6.25
2	0.5	25, 12.5 and 6.25
3	1	25, 12.5 and 6.25
4	5	25, 12.5 and 6.25

3.4 Optimization of alternative PK assays formats

An indirect antibody assay is a commonly used assay format on the Gyrolab platform and was therefore not further optimized in this project. A capture concentration of 10% TNF-alpha and a detect concentration of 12.5nM were used. A matrix of 1% serum was used.

The two bridging assay formats, one having TNF-alpha as capture and an anti-idiotype as detect and the other one having two different anti-idiotypes as capture and detect were diluted in a matrix of 10% serum during optimization. The detect reagent was diluted in Rexxip F to a final concentration of 12.5nM. Both assays were run on Bioaffy 1000CD and Bioaffy 1000HC CD. 1000nM capture reagent was used on Bioaffy 1000CD and 2000nM was used on Bioaffy 1000HC CD.

3.4.1 Anti-idiotypic bridging

The two bridging assay formats, one having an anti-idiotype as detect and the other one having an anti-idiotype as capture and detect were optimized by titration with BSA. The capture reagent was titrated using following proportions of biotinylated capture reagent and b-

BSA; 100:0, 50:50, 30:70, 20:80, 10:90, 5:95 and 1:99. Standard series corresponding to 50 000-3.2 ng/ml neat Humira were used. The standard curves were later compared to find the optimal amount of BSA, with regards to sensitivity, dynamic range, and background.

3.5 Quantify analyte using alternative PK assay formats

This section describes the quantification of the analyte using alternative PK assay formats. In total, four different assay formats were compared, which are all presented in chapter 2.2. The selectivity and the performance of the assays were evaluated. Selectivity is the ability of an analytical method to both differentiate and quantify the analytes in a sample consisting of several components (Andreasson *et al.* 2015).

3.5.1 Indirect vs Bridging assay in serum

The optimized bridging assay was compared to an indirect antibody assay in serum. The standard curves were prepared pooled serum followed by dilution in Rexxip H. The detect concentration was 12.5nM for both assays. In the indirect antibody assay, the detect was diluted in Rexxip F, while PBS-BSA was used in bridging assay. A summary of the experimental set up is presented in Table 8.

Table 8. Experimental set up for the two assays used. Capture concentration and serum amount are presented.

Assay	Capture concentration (% TNF-alpha)	Serum matrix (%)
Bridging assay	5%	0, 10, 50
Indirect antibody assay	10%	0, 1, 5

3.5.2 Quality controls

Standard curves were made in 100% pool serum and diluted in Rexxip H to the final serum concentrations stated in Table 9. The assay range stated is the back-calculated concentrations to neat serum. All quality controls (QCs) were prepared in a matrix comparable to the standard curve and were diluted in Rexxip H before analysis to contain the same amount of serum as the standard curve. In parallel to this, control experiments were run for all assays. The standard curves were diluted in four types serum, 1 pool, and 3 different individual serum. These were evaluated before continuing assigning lower limit of quantification (LLOQ).

LLOQ is the lowest analyte concentration that can be accurately measured in an assay (Gyros Protein Technologies AB 2019a). It was determined by analysing several QC samples at the lower part of the assay range, having four replicates for each concentration. The lowest analyte concentration that gave a % coefficient variation (CV) <10 and % Bias <20 was assigned as LLOQ. Another criteria is that the total error should be <30%, which is the sum

of absolute average bias and %CV (Liu *et al.* 2012). This gives just an approximate value of the LLOQ for the specific assay.

Table 9. Serum concentration used for each assay.

Assay	Serum concentration (%)	Assay range, neat (ng/ml) n=7
Bridging assay	50	100 000-0.64
Indirect antibody assay	1	500 000-32
Bridging assay (anti-idiotype as detect)	100	5000-0.32

The precision and accuracy were determined using spiked human serum samples from individual donors. The serum samples were provided by Seralab and collected from consented donors at FDA-licensed donor centres. QC-samples were made near LLOQ. Since the pooled serum available was found not to be representative of the performance from the individual serums available, two new serum pools were made, one for the optimized bridging assay, consisting of 8 individuals and one for indirect antibody assay and bridging assay format with anti-idiotype as detect, consisting of 4 individuals. This was due to limited amount of individual serum.

For the bridging assay, QC-samples of 10 and 5 ng/ml were prepared in individual serum with four replicates of each. The standard curve and the QC-samples were made in 100% serum. QC-samples of 500, 250, 125 and 62.5 ng/ml were made for the indirect antibody assay and run in 1% serum. For bridging assay format with TNF-alpha as capture and an anti-idiotype as detect QC-samples of 5, 2.5, 1.25 and 0.625 ng/ml were made in 50% serum.

3.6 Utilizing the optimized bridging assay for affinity determination of Humira in neat serum

The optimized bridging assay was used in combination with the Gyrolab Affinity software to perform K_D measurements of the Humira-TNF-alpha interaction in neat serum. Variable concentrations of TNF-alpha were added to a fix concentration of Humira to compete with the interaction.

In this experiment, two fixed concentrations of 500 pM (high) and 100 pM (low) Humira were used. The variable was TNF-alpha with the highest concentration of 100 nM, diluted 1:2 to make 12 sample points. The following stocks were made: 10 000 nM TNF-alpha in PBS-BSA, 100 nM Humira in Rexxip H, capture concentration of 5% TNF-alpha and finally a detect concentration of 12.5 nM. A total of 4 affinity series were made according to Table 10.

The variable TNF-alpha was also diluted in the same buffer as the one stated in the Table 10. Equal volumes of the variable interactant and the fixed interactant were mixed, and all samples incubated on a microtiter plate in the fridge for approximately 2 hours. The volume

needed for the run were transferred to a new microtiter plate and run on the Gyrolab platform. The samples on the microtiter plate were put in the fridge overnight and were run again after 24h respectively 48h of incubation. The Gyrolab Affinity Software was used to enable data analysis.

Table 10 Affinity series.

Experiment number	Buffer	Humira concentration (pM)
1	Rexxip H	500
2	100% serum	500
3	Rexxip H	100
4	100% serum	100

4 Results

4.1 The capacity of the solid phase

To investigate the capacity of the two different particles used in the Gyrolab platform, three different capture molecules of different sizes were both biotinylated, and fluorophore labelled. After flowing the reagents over the columns and detecting the fluorescence, the resulting column profiles were studied using Gyrolab Viewer, to ensure that the integration area was covered, and that the entire capacity was utilized. The concentrations needed for each capture molecule on Bioaffy 1000CD and Bioaffy 1000HC CD are listed in Table 11. The resulting concentration of IgG after biotinylation and fluorophore labelling was 766 nM, which is the number in the parenthesis. This resulted in an almost saturated column, therefore the concentration needed was set to approximately 1000 nM.

Table 11. Capture reagent concentration. The number in the parenthesis is the concentration used in the experiment, while the other one is the concentration needed.

	Concentration TNF alpha [nM]	Concentration BSA [nM]	Concentration IgG [nM]
Bioaffy 1000 CD	1000	200	300
Bioaffy 1000HC CD	2000	1000	1000(766)

TNF alpha is the smallest molecule in this experiment and a concentration of 1000nM is needed to saturate the column on the Bioaffy 1000 CD and at least 2000 nM to saturate the column on the Bioaffy 1000HC CD. Even though the MW of IgG is almost twice the size of BSA, an equal amount of them are needed to cover the integration area on both CDs. Resulting column profiles for TNF-alpha, BSA and IgG are presented in Figure 6 and Figure

7. The integration area (the area inside the white box) illustrates the part of the signal that is included in the calculation of the sample response value and this area must at least be covered. As the concentration increases, more of the integration area is covered and eventually resembles a loaf.

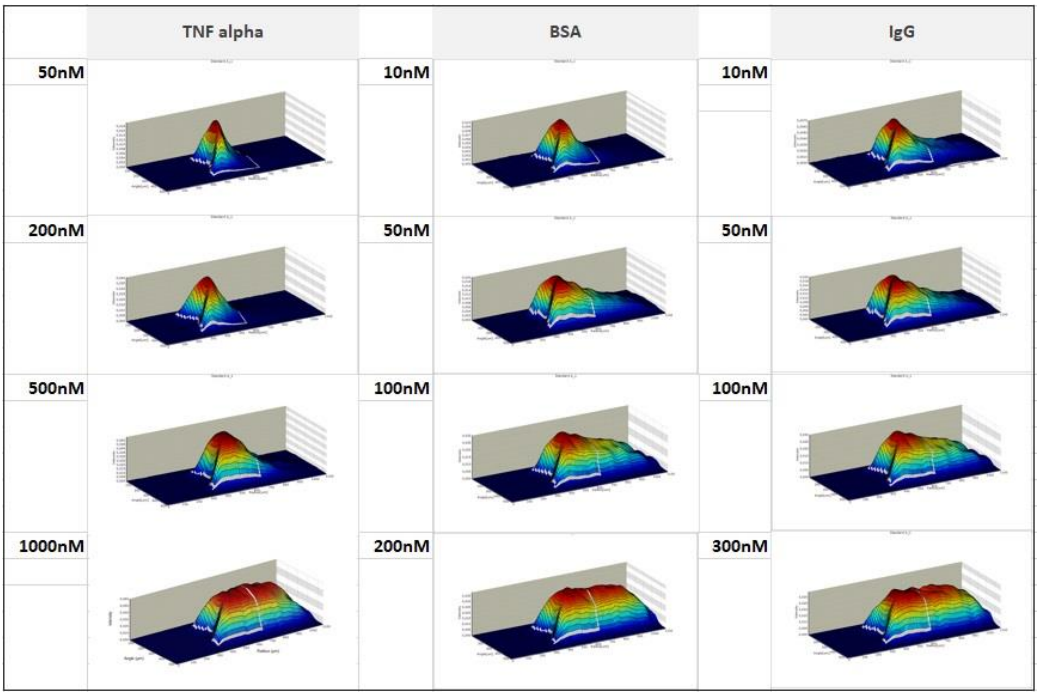

Figure 6. 3D view of column profiles for different concentrations of capture reagent giving intensity as peak height. Bioaffy 1000 CD was used.

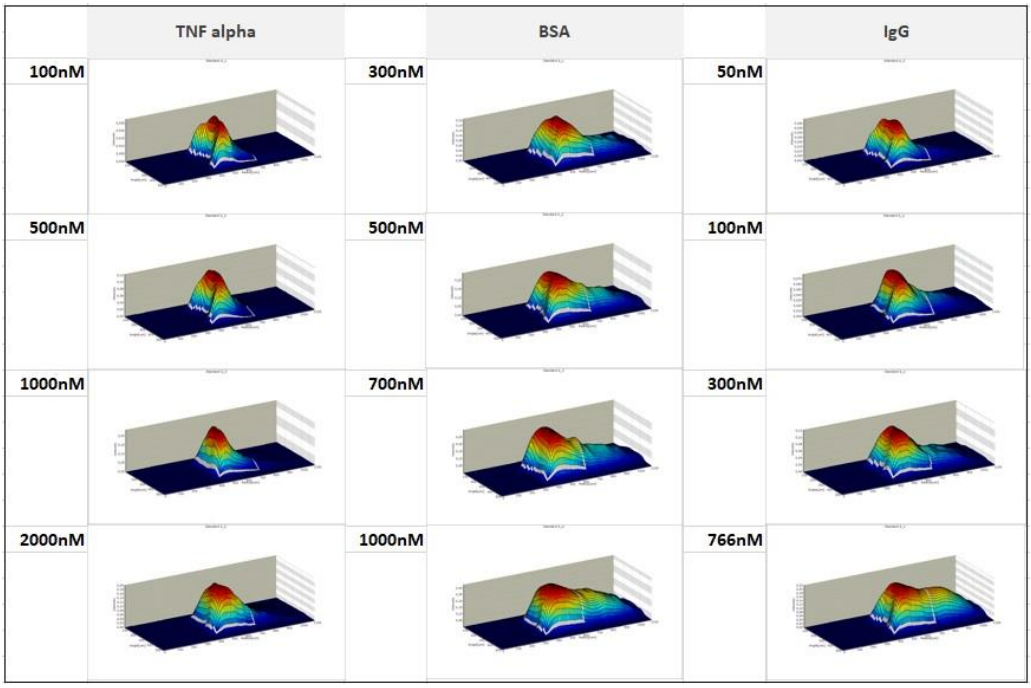

Figure 7. 3D view of column profiles for different concentrations of capture reagent giving intensity as peak height. Bioaffy 1000 HC CD was used.

4.2 Optimizing the surface density of the solid phase in a TNF-alpha bridging assay

To optimize the surface density of the two particles used in the Gyrolab platform, the capture reagent b-TNF-alpha was titrated with b-BSA to reduce the density of the capture reagent on the solid phase. Figure 8 and Figure 9 show the effect of titration on a 3-step bridging assay with fluorophore labelled TNF-alpha as detecting reagent. As can be seen in Figure 8, increasing from 90% BSA to 95% gave no distinct improvement on Bioaffy 1000 CD. However, increasing to 99% BSA improved the assay sensitivity by reducing the background while maintaining a good dynamic range. The background was further decreased with increased amount of BSA, without improving the performance since the entire curve is shifted. On Bioaffy 1000HC CD (see Figure 9) a similar pattern is visualized; however, a narrower dynamic range is obtained. Furthermore, a higher background is obtained. Further optimization work must be done.

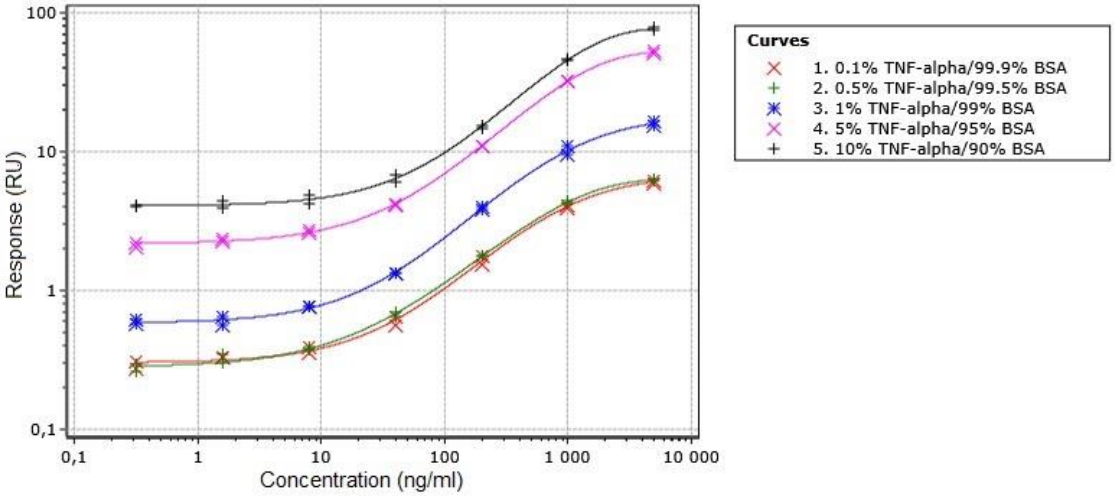

Figure 8. Standard curves using different proportions of TNF-alpha and BSA. These were run on Bioaffy 1000 CD.

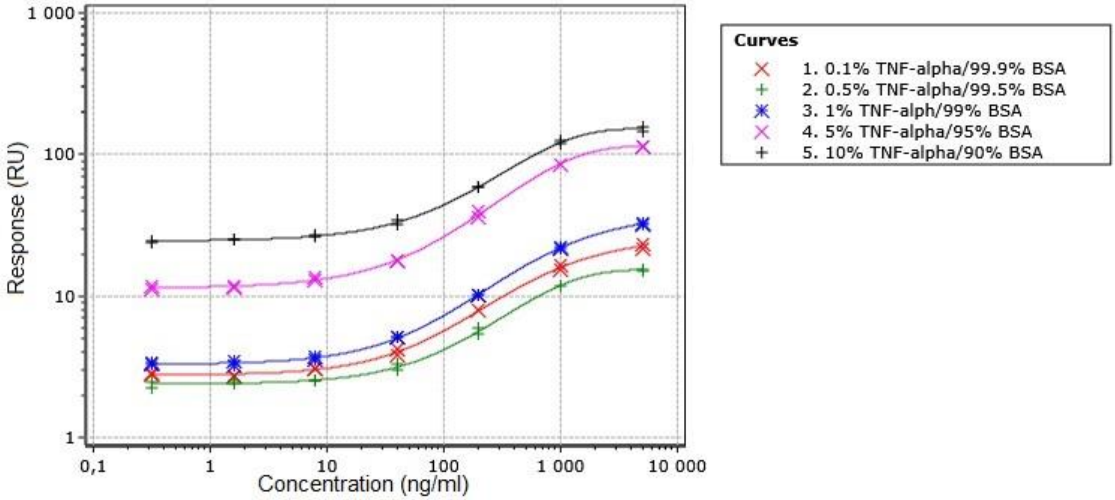

Figure 9. Standard curves using different proportions of TNF-alpha and BSA. These were run on Bioaffy 1000HC CD.

4.3 Further optimization of the TNF-alpha bridging assay

Since the background of the TNF-alpha bridging assay was relatively high, further optimization work was performed with the aim to reduce the background and increase the signal to noise ratio, thereby increasing the sensitivity and the working range of the assay. This section includes the results of all the optimization work.

4.3.1 Evaluation of buffers

In this experiment, different buffers were tested for capture reagent, analyte, and detection reagent. The results are presented in Figure 10, where the yellow curve corresponds to the

optimal curve achieved in section 4.2 and acts as a reference. As one can see, the background is decreased for all curves compared to the reference. Furthermore, Rexxip A as diluent for detect compared to Rexxip ccs lowers the background for this specific assay (compare red and blue curve). This is also the case when comparing the green and pink curve, which also differs in detection buffer. The sensitivity is increased compared to the reference as well. For this specific bridging assay, Rexxip A seems to be more beneficial than Rexxip F as detection diluent.

Signal to background (S/B) is the ratio between the signal and the absolute value of the mean of the blank's signal (Gyros Protein Technologies AB 2019a). A higher value at a certain analyte concentration means that the detected analyte is more significantly separated to the background level indicating a higher sensitivity of the assay. The S/B ratio at 40 ng/ml for the blue, red and the yellow curve is presented in Table 12 and shows that the sensitivity is almost twice as high with Rexxip A as detect diluent.

Table 12. S/B ratio at 40 ng/ml for five standard curves.

Capture, analyte, and detection diluent	S/B ratio at 40 ng/ml
PBST – Rexxip H – Rexxip F	2,25
Rexxip ccs – Rexxip H – Rexxip A	4,21
Rexxip ccs – Rexxip H – Rexxip ccs	2,44
PBST - Rexxip H – Rexxip A	3,7
PBST – Rexxip H – PBS_BSA	5,2

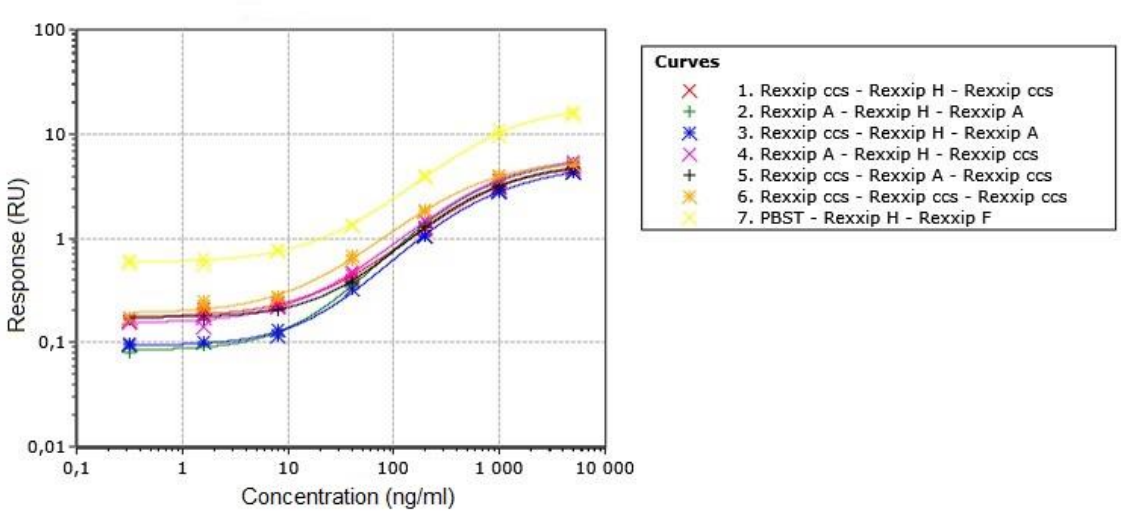

Figure 10. Diluting capture reagent, analyte, and detection reagent in different buffers. The yellow curve is the reference where capture reagent is diluted in PBST, analyte in Rexxip H and detection reagent in Rexxip F.

In a second experiment, different buffers were tested for capture reagent and detection reagent. PBST and PBS-BSA were used as capture reagent buffer, whereas Rexxip A and PBS-BSA were used as detection reagent buffers. PBS-BSA contained 0.2% BSA. The black curve (see Figure 11) is used as the reference. The purple curve shows a broad dynamic range, as well as sensitivity, though still a relatively high background. As can visualized in Table 12, the sensitivity is much higher for the standard curve having PBS-BSA as detect buffer. The results show that the combination using PBST for capture, Rexxip H for the analyte and PBS-BSA for detect is the most optimal.

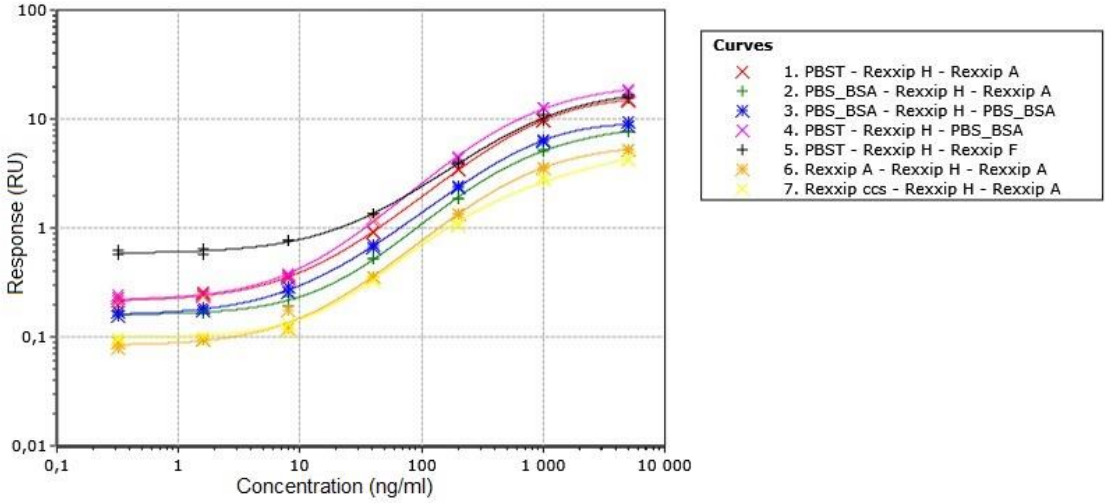

Figure 11. Diluting capture reagent, analyte, and detection reagent in different buffers. The black curve is the reference where capture reagent is diluted in PBST, analyte in Rexxip H and detection reagent in Rexxip F.

4.3.2 Titrating concentration of detect for optimal sensitivity

The concentration of the detect was titrated with the aim to achieve the optimal S/B ratio without affecting the dynamics in the upper end of the curve.

Decreasing the detect concentration increases the S/B ratio when 5 and 1% TNF-alpha is used as capture (see Table 13). The background is lower for 1% TNF-alpha, compared to 5% TNF-alpha, however, using 5% TNF-alpha results in a greater sensitivity and larger dynamic range (see Figure 12). The optimal curve is the black one with a capture concentration of 5% TNF-alpha/95% BSA and a detect concentration of 12.5nM.

Similar experiments were performed using the Bioaffy 1000HC CD. The column profiles were not as broad as for Bioaffy 1000CD, however, the background was increased (data not shown). The HC-particle was therefore not proved beneficial in this specific model system.

Table 13. S/B values for a specific amount of TNF-alpha at a certain detect concentration. S/B values presented are a mean value between two replicates. At 40 ng/ml Humira, the standard curve had a great slope.

Amount TNF-alpha (%)	Detect concentration (nM)	S/B at 40 ng/ml Humira
5	25	4,12
5	12.5	8,27
5	6.25	10,10
1	25	0,96
1	12.5	6,35
1	6.25	9,19
0.5	25	0,32
0.5	12	0,16
0.5	6.25	0,14
0.1	25	0,28
0.1	12.5	0,14
0.1	6.25	0,14

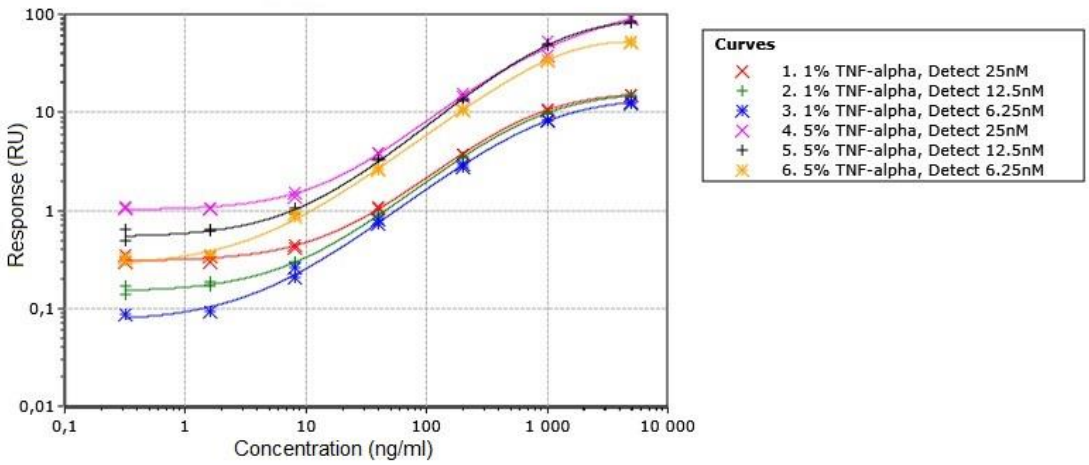

Figure 12. Changing capture and detect concentration. 5% TNF-alpha/ 95% BSA and 1% TNF-alpha/99% BSA were used as capture reagent.

4.4 Optimizing alternative PK assays formats

Three alternative PK assay formats for Humira were evaluated:

- Indirect antibody assay using TNF-alpha as capture and a general anti-human IgG as detect
- Bridging assay using TNF-alpha as capture and an anti-idiotypic antibody towards Humira as detect
- Bridging assay using an anti-idiotypic Fab fragment as capture and an anti-idiotypic antibody as detect.

The indirect antibody assay is well established at the Gyrolab platform, so no further optimization work was performed on this assay.

In the same way as the bridging assay, the alternative PK assays had to be optimized. The optimization was made by titrating the capture reagent with BSA.

4.4.1 Optimizing a bridging assay using TNF-alpha as capture and an anti-idiotypic antibody towards Humira as detect

The results for the bridging assay format with an anti-idiotype as detect is presented in Figure 13. Increasing the concentration of b-BSA in the capture results in increased sensitivity and dynamic range for the standard curves until it reached a concentration of 90% BSA. Further increase of BSA results in a decreased dynamic range since the curves loses capacity in the upper end of the curve with virtually no effect of the background (refer to the green and the red curve in Figure 13). The optimal composition of the capture reagent is, therefore, the blue curve, which has a capture reagent concentration of 10% TNF-alpha and 90% BSA (10:90).

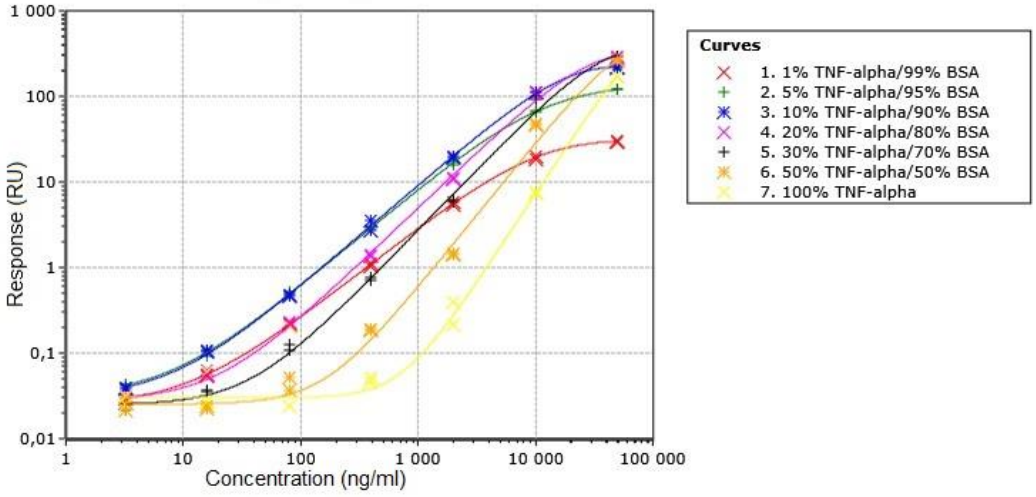

Figure 13. Titrating capture reagent with BSA for the bridging assay format with an anti-idiotype as detect.

4.4.2 Optimizing a bridging assay using an anti-idiotypic Fab fragment as capture and an anti-idiotypic antibody as detect

The results for the bridging assay format with anti-idiotype as capture and detection reagent is presented in Figure 14. In this case, both the dynamic range and the sensitivity got worse with increasing amount of BSA. The most optimal curve is the yellow one, having a capture concentration of 100% anti-idiotype.

Results for Bioaffy 1000HC CD are not shown since it was not proven beneficial for either model system. The column profiles were more enriched, but the background also went up (see appendix 8.1).

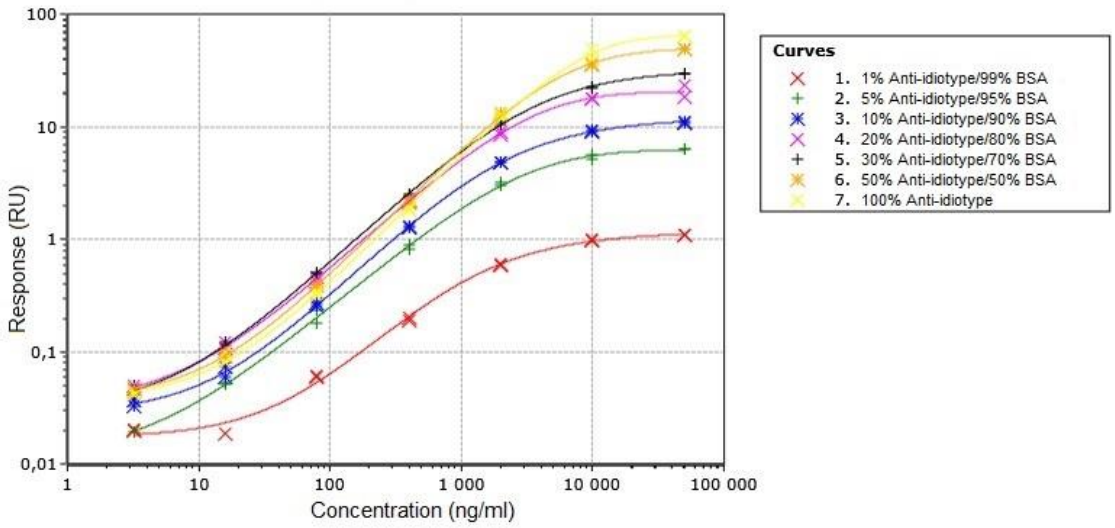

Figure 14. Titrating capture reagent with BSA for the bridging assay format with two different anti-idiotypes as capture and detect.

4.5 Evaluating the different PK assay formats in presence of serum

4.5.1 Evaluating the serum sensitivity

Presented in Figure 15 are the performance of three different assay formats diluted in Rexxip H. The optimized bridging assay had a significantly higher background than the other two assays. Also, the dynamic range was less since the curve loses capacity in the upper end of the curve. The indirect antibody assay shows a low background, high sensitivity, as well as a large dynamic range.

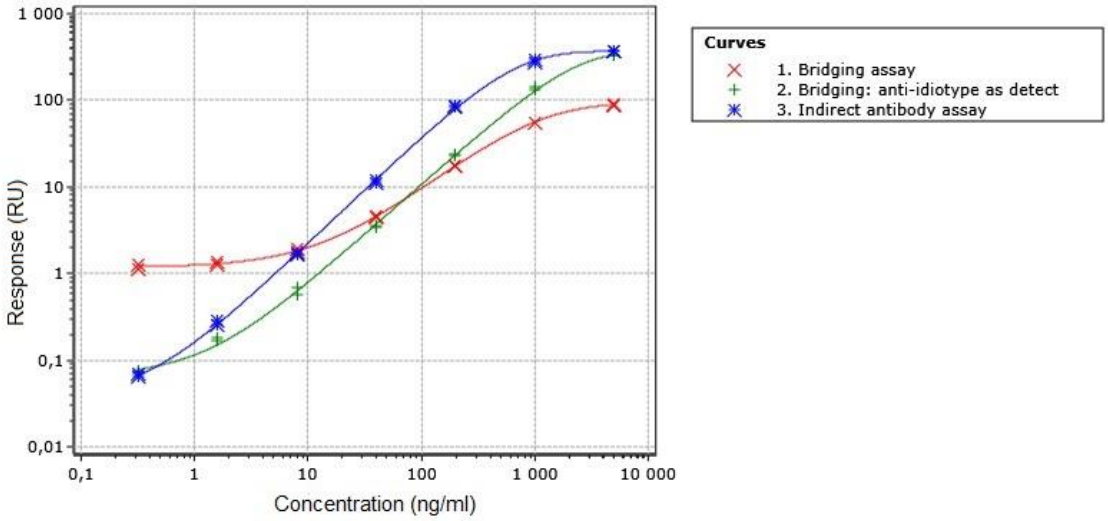

Figure 15. Comparison of three assay formats diluted in Rexxip H.

Visualized in Figure 16 are the performance of the four different assay formats in presence of human serum in assay concentrations. For the indirect antibody assay, 0, 1 and 5% serum were tested and for the bridging assays 0, 10 and 50% serum were tested. The indirect antibody assay is sensitive to the serum matrix with significant background already at low serum concentrations. The bridging assay formats, on the other hand, is virtually unaffected by the serum matrix. In Figure 17, the back-calculated concentrations to neat serum is visualized for the different assays visualising the resulting sensitivities.

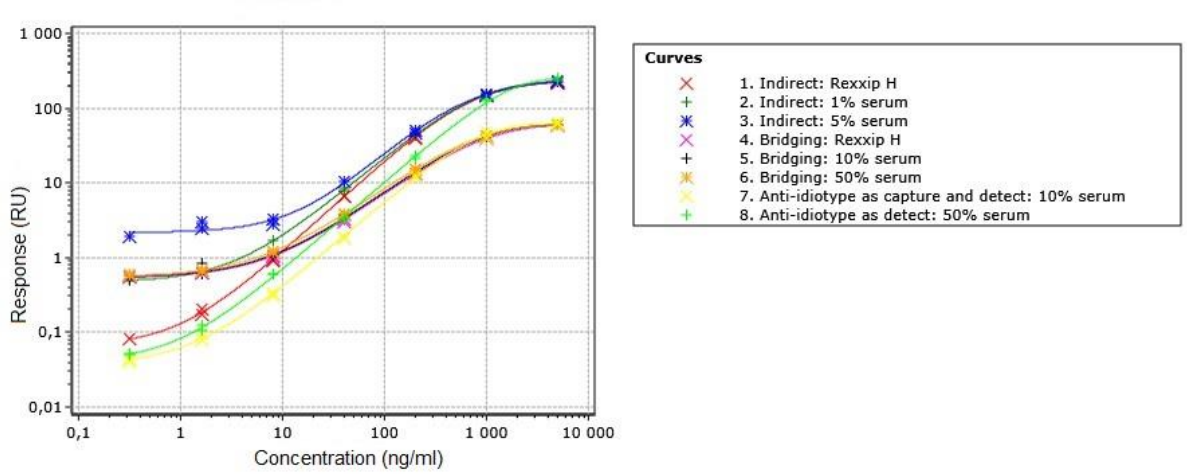

Figure 16. Performance of the four assays formats in presence of human serum.

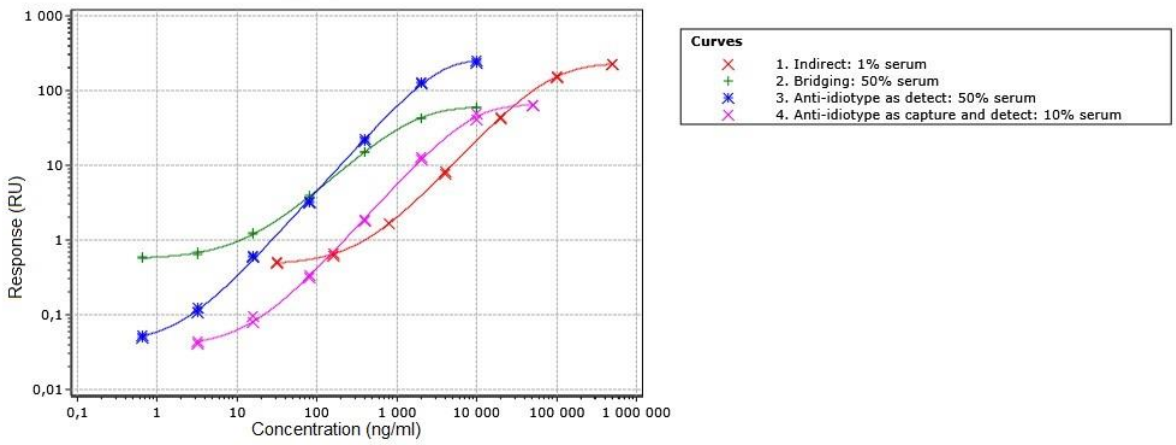

Figure 17. Sensitivity for the four assay formats presented in neat concentration.

QC samples were quantified in the presence of pooled serum. The total error was examined for all assays and the analyte concentration that gave a total error below 30% was assigned as an estimated LLOQ. By examining the total error in Table 14 one can see that for 10 ng/ml it's clearly above 30 and at half that concentration the total error reaches almost a hundred. The bridging assay using TNF-alpha as capture and detect gave an LLOQ in the range of 20 ng/ml (see Table 14). Similar reasoning was used to determine the LLOQ for the other two assays. The LLOQ was 125 ng/ml for the indirect antibody assay (see Table 15). The bridging assay with an anti-idiotype as detect gave an even lower LLOQ, of 1.25 ng/ml (see Table 16). All these concentrations are presented as back-calculated to neat serum.

Table 14. Accuracy and precision for bridging assay. Matrix: 50% serum pool.

QC (ng/ml) n=4	Average conc. (ng/ml)	CV conc. (%)	|Avg. Bias| (%)	Total error
40	37	1,6	7,5	8,9
20	22,8	10,6	13,8	24,4
10	12,3	13,7	23,0	36,7
5	9,09	13,1	81,7	94,8

Table 15. Accuracy and precision for indirect antibody assay. Matrix: 1% serum pool.

QC (ng/ml) n=4	Average conc. (ng/ml)	CV conc. (%)	|Avg. Bias| (%)	Total error
500	519	10,6	3,8	14,4
200	195	7,6	2,8	9,4
125	145	5,9	16,3	22,8
100	78,7	20,5	21,3	41,8

Table 16. Accuracy and precision for the bridging assay with an anti-idiotype as detect. Matrix: 100% serum pool.

QC (ng/ml) n=4	Average conc. (ng/ml)	CV conc. (%)	|Avg. Bias| (%)	Total error
5	4,9	10,7	1,7	12,4
2,5	3,0	4,5	20,3	24,8
1,25	1,4	6,0	13,7	19,7
0,625	0,79	14,1	26,3	40,4

4.5.2 Evaluating the selectivity of the different assay formats

Before examining the performance in the presence of serum from multiple individuals for all assays, control experiments were run for all assays. The results for the bridging assay using TNF-alpha as capture and detect are presented in Figure 18. It is showing that the standard curve that was diluted in pool serum lies on top of the standard curves that were diluted in different individuals. However, by examining Figure 19, which represents the indirect antibody assay, one can see that the pool has a larger background than all the tested individuals. A similar result is shown for the bridging assay format with an anti-idiotype as detect in Figure 20. For this reason, two new pools were made, one consisting of 8 individuals and one consisting of 4 individuals.

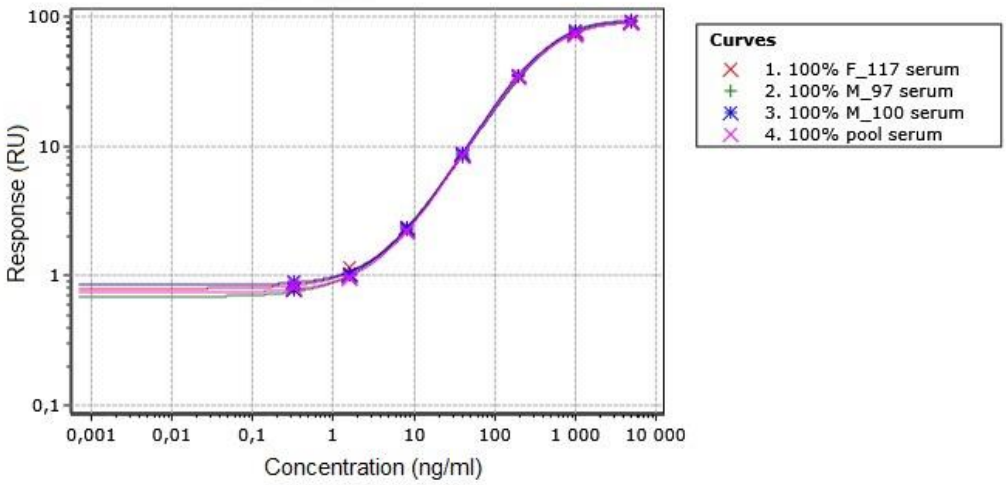

Figure 18. Standard curves for bridging assay diluted in either pooled or individual serum

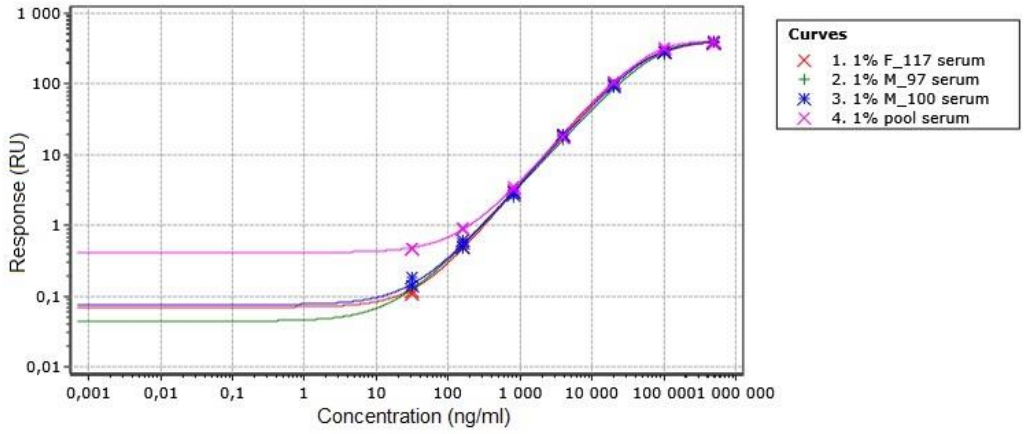

Figure 19. Standard curves for indirect antibody assay diluted in either pooled or individual serum.

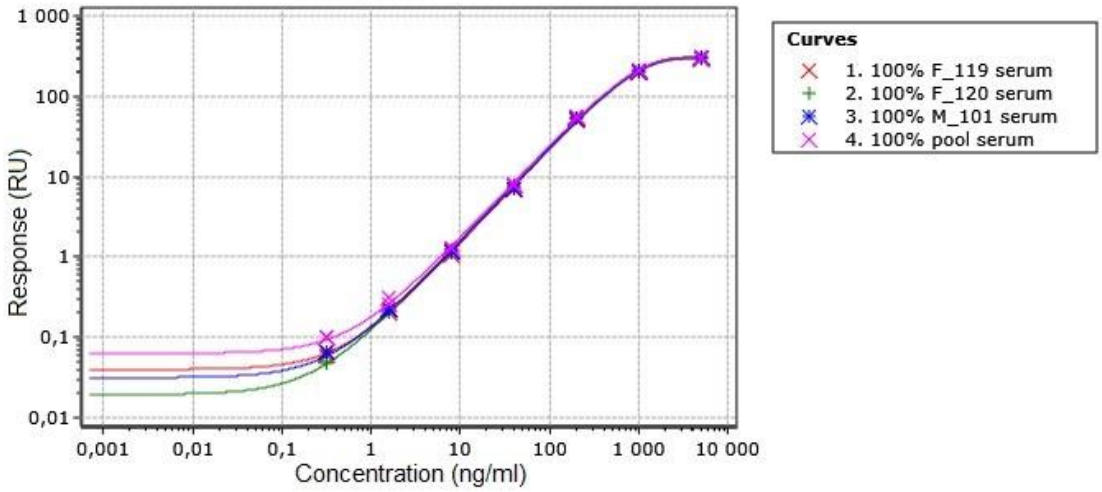

Figure 20. Standard curves for bridging assay with an anti-idiotype as detect diluted in either pooled or individual serum.

As shown previously in the report, the bridging assays were insensitive to interference from the serum matrix in comparison to the indirect antibody assay. Standard curves prepared in 0, 10 or 50 % serum almost overlay. However, for both the bridging assay using TNF-alpha as capture and detect and the bridging assay using TNF-alpha as capture and anti-idiotypic antibody as detect a phenomenon was noticed when the assay was performed in 100 % serum. The standard curves were significantly shifted to the left as visualised in Figure 21 and Figure 22.

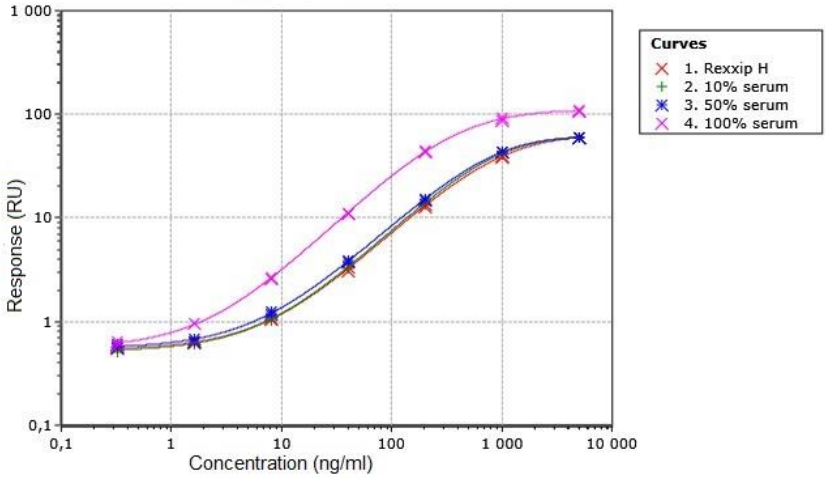

Figure 21. Standard curves for the optimized bridging assay diluted in Rexxip H or serum.

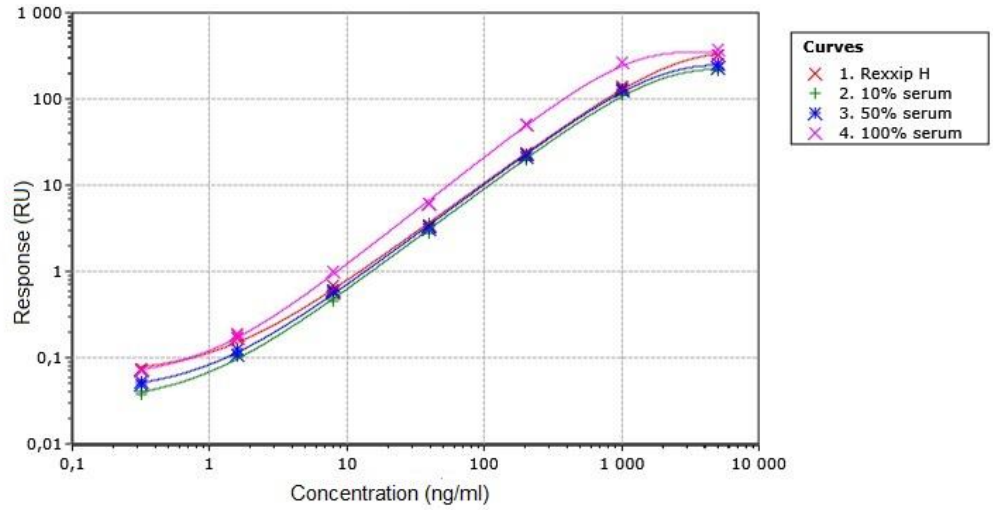

Figure 22. Standard curves for the bridging assay with anti-idiotype as detect diluted in Rexxip H or serum.

Precision and accuracy in individuals for all assays were determined using spiked human serum samples near LLOQ. The LLOQ for the optimized bridging assay was previously in this report assigned to 20 ng/ml, however that was in a matrix of 50% serum. Since the sensitivity were improved for the standard curve diluted in 100% serum compared to the one diluted in 50% serum (see Figure 21), lower concentrations of Humira were tested, 10 ng/ml respectively 5 ng/ml in 100% serum. Standard curves were diluted in 1% serum for the indirect antibody assay and 50% serum were used for the indirect antibody assay respectively the bridging assay format with an anti-idiotype as detect. QC-samples were diluted separately in individual serum and quantified against a standard curve prepared in the pool of all individual serum.

Selectivity data for the bridging assay are presented in Table 17. CV and average bias are lower than 10% respectively 20% for all QC-samples at both concentrations, except one. These results show that the optimized bridging assay is robust and have an estimated LLOQ of approximately 5 ng/ml. The precision and accuracy data for the indirect antibody assay is presented in Table 18 and shows that the assigned LLOQ of 125 ng/ml was not enough, since one individual shows a higher average bias. The LLOQ was corrected to 250 ng/ml. For the bridging assay with an anti-idiotype as detect, CV and bias are increased when the concentration is decreased (see Table 19). In this case, a higher concentration, around 2.5 ng/ml is probably necessary to meet the criteria for CV and bias in all individuals.

Table 17. CV concentration and absolute average bias presented for 8 individual QC-samples. This is the bridging assay

Sample name (ng/ml)		Average conc. (ng/ml)	CV conc. (%)	|Avg. Bias| (%)	Total error
M_97	10	8,3	4,3	11,8	16,1
	5	4,4	7,2	12,9	20,1
M_98	10	9,4	4,9	5,8	10,7
	5	5,3	6,0	5,8	11,9
M_100	10	8,8	1,3	12,3	13,6
	5	4,4	7,5	12,3	19,8
M_101	10	8,8	3,9	11,6	15,5
	5	4,3	1,7	15,0	16,7
F_117	10	8,6	1,0	13,6	14,6
	5	4,3	4,5	13,1	17,6
F_119	10	8,7	2,5	12,7	15,2
	5	4,2	9,5	16,5	26,0
F_120	10	8,2	9,0	17	26,0
	5	4,3	14,4	14,8	29,2
F_121	10	10	5,5	0,11	5,6
	5	5,6	6,9	12	18,9

Table 18. CV concentration and absolute average bias presented for 4 individual QC-samples. This is the indirect antibody assay.

Sample name (ng/ml)		Average conc. (ng/ml)	CV conc. (%)	|Avg. Bias| (%)	Total error
M_97	500	515	3,0	3,1	6,1
	250	266	2,3	6,6	8,9
	125	124	7,8	4,6	12,4
	62.5	62	18,1	0,8	18,9
M_98	500	524	8,6	4,9	13,5
	250	265	7,7	5,9	13,6
	125	149	3,1	18,9	22,0
	62.5	84,9	9,9	35,8	45,7
F_117	500	491	4,7	1,8	6,4
	250	237	1,9	5,3	7,1
	125	128	7,3	2,7	10,0
	62.5	82,4	39,2	31,8	71,0
F_121	500	594	5,4	18,8	24,2
	250	294	7,3	17,4	24,7
	125	158	8,3	26,3	34,6
	62.5	83,9	1,2	34,3	35,5

Table 19. CV concentration and absolute average bias presented for 4 individual QC-samples. This is the bridging assay format with an anti-idiotype as detect.

Sample name (ng/ml)		Average conc. (ng/ml)	CV conc. (%)	|Avg. Bias| (%)	Total error
M_97	5	3,86	5,5	22,7	28,2
	2,5	2,3	15,4	8,0	23,4
	1,25	1,35	14,4	8,4	22,8
	0,625	0,97	20,0	55,3	75,3
M_98	5	3,94	7,5	21,2	28,7
	2,5	2,35	0,60	6,2	6,8
	1,25	1,78	12,3	42,5	54,8
	0,625	<LLOQ	-	-	-
F_117	5	3,99	1,7	20,2	21,9
	2,5	2,47	4,5	1,4	5,8
	1,25	1,17	14,5	6,6	21,1
	0,625	0,936	30,3	49,8	80,1
F_121	5	4,08	6,0	18,4	24,4
	2,5	2,27	13,4	9,3	22,7
	1,25	1,34	10,1	7,3	17,4
	0,625	0,771	10,2	23,4	33,6

4.6 Affinity analysis

Humira was titrated with TNF-alpha using two fixed concentrations. The samples were incubated on a microtiter plate for 2 hours before the first run. Thereafter, the plate was put in the fridge overnight and were run again after 24h respectively 48h of incubation. A total of four affinity series were made with Rexxip H or 100% serum as buffers.

The Gyrolab affinity software uses a basic model consisting of four parameters which estimates both K_D and F_{tot} from the affinity series where V_{tot} is varied and F_{tot} is kept fixed. V_{tot} is the interactant with variable concentration, TNF-alpha in this case, and F_{tot} is the interactant with fixed concentration, which is Humira.

Figure 23 show K_D and F_{tot} in buffer and 100% serum after 2, 24, and 48 hours of incubation. The chart shows the concentration of non-bound fixed interactant on the y-axis and the user-entered V_{tot} on the x-axis. The calculated K_D and F_{tot} are very similar regardless if the measurement is performed in buffer or in 100% serum. Most calculated K_D-values lie near 50 pM and these are presented in Table 20 along with $F_{tot.}$ The F_{tot} Conc represents the total active concentration of Humira, while F_{tot} is the calculated concentration of Humira. The antibody has a low K_D value in the range of 8-70 pM, however, after 48h of incubation, K_D-values tend to stabilize around 30 pM. All F_{tot}-values are gathered and reaches approximately 1.9 nM after 48h of incubation.

As earlier mentioned, two fixed concentrations of Humira were titrated with TNF-alpha, 100 pM and 500 pM. The two concentrations of TNF-alpha results in similar K_D-values as can be seen in Figure 24. The affinity series presented are diluted in 100% serum. The calculated F_{tot} is roughly 4 times higher than the theoretical concentration of Humira in both cases, see Table 21.

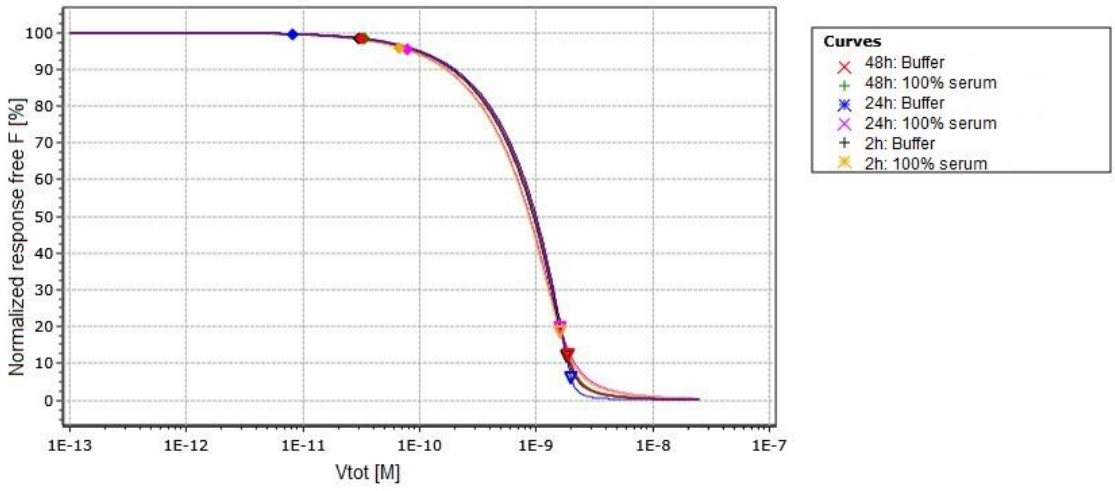

Figure 23. Affinity series diluted in Rexxip H or 100% serum. Calculated K_D (diamonds) and F_{tot} (triangles) are shown after 2, 24 and 48 hours of incubation.

Table 20. Calculated K_{D-} and F_{tot}-values.

Sample name	F_{tot} Conc [M]	K_D [M]	F_{tot} [M]
Buffer: after 48h	5,00E-10	3,16E-11	1,88E-09
100% serum: after 48h	5,00E-10	3,34E-11	1,88E-09
Buffer: after 24h	5,00E-10	8,11E-12	1,99E-09
100% serum: after 24h	5,00E-10	7,76E-11	1,60E-09
Buffer: after 2h	5,00E-10	3,00E-11	1,83E-09
100% serum: after 2h	5,00E-10	6,78E-11	1,59E-09

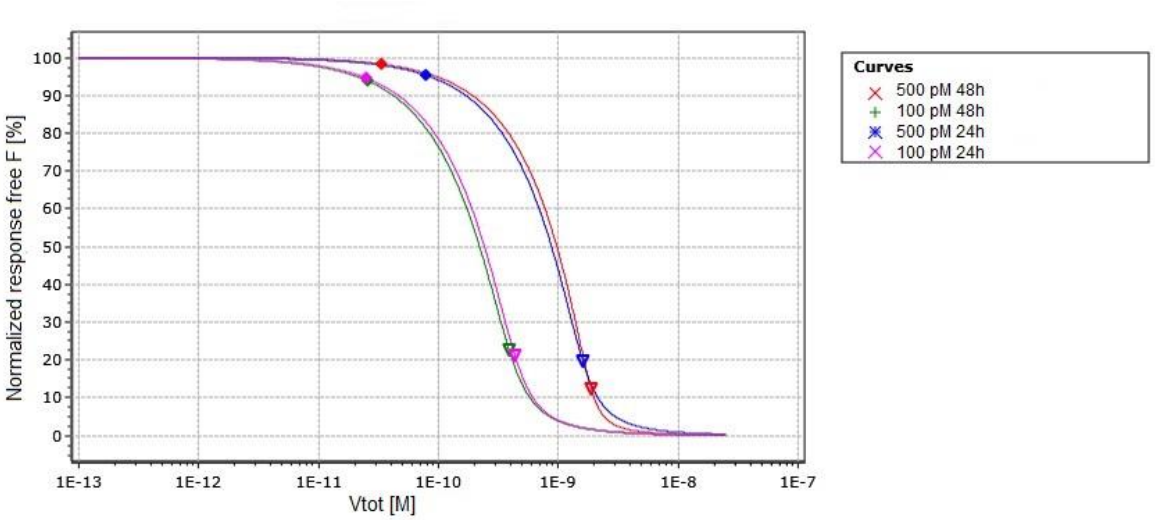

Figure 24. Affinity series diluted in 100% serum. Two fixed concentrations of Humira of 500 pM respectively 100 pM. K_D (diamonds) and F_{tot} (triangles) are shown after 24h and 48h of incubation

Table 21. Calculated K_D- and F_{tot} values.

Sample name	Ftot Conc [M]	Kd [M]	Ftot [M]
100% serum: after 48h	5,00E-10	3,34E-11	1,88E-9
100% serum: after 48h	1,00E-10	2,55E-11	3,90E-10
100% serum: after 24h	5,00E-10	7,76E-11	1,60E-9
100% serum: after 24h	1,00E-10	2,49E-11	4,34E-10

5 Discussion

This master thesis aimed to investigate the properties of the solid phase on the Gyrolab platform and utilize this to optimize bridging assay to be used as a pharmacokinetic assay for a human antibody in the presence of serum. The optimized bridging assay were compared with three alternative PK assay formats.

5.1.1 Comparison of all assays

The results from the investigation of the column capacity showed that for TNF-alpha as capture reagent, up to 1000 nM was needed to saturate the column using the Bioaffy 1000 CD, whereas 2000 nM was needed for Bioaffy 1000HC CD. The high capacity particle requires a higher concentration of capture reagent to fill the entire integration area, thus use the entire capacity.

The theory for investigating the HC-particle was that the capture could be diluted enough for the analyte to not cross-link with both arms, while the capacity is so high that the column profiles are not broadened outside the integration area and thereby show improved performance in a bridging assay. The results showed that although the column profiles were more enriched in the top of the column at the HC CD, the background increased. Thus, the HC-particle was not proven beneficial for any of the model system tested, however, it may be beneficial for others.

The indirect antibody assay appeared to have low background, high sensitivity as well as a large dynamic range when diluted in Rexxip H. However, it had a significant background already at low serum concentrations, whereas the bridging assay formats were virtually unaffected by the serum matrix. There is a great excess of human antibodies in serum and PK assays, therefore, require detection reagent that specifically binds to the drug (Bio-Rad 2019). The indirect antibody uses a general anti human IgG antibody which seems to cross-react with proteins left on the column. Since the bridging assay binds to the drug more specifically the matrix interference is overcome. However, using a bridging assay with the target in this case results in a narrower dynamic range.

The estimated sensitivity of the bridging assay format with an anti-idiotype as detect was 2.5 ng/ml compared to 5 ng/ml for the optimize bridging assay and 250 ng/ml for the indirect antibody assay. However, the optimized bridging assay appeared to be much more robust when diluting the standard curves in four types of serum, 1 pool and 3 different individual serum.

The standard curves for the bridging assay format having two different anti-idiotypes as capture and detect showed no improvement when titrating with BSA, instead the dynamic range decreased. The distance between two capturing anti-idiotypic fragments is probably too far for cross-linking of both arms of the drug, thus BSA having no effect. In addition to this, the assay did not show as good performance as the bridging assay having TNF-alpha as

capture and an anti-idiotype as detect since the performance of this type of assays is highly dependent on the properties of the anti-idiotypic reagent used as capture. In other words, the affinity of the capturing anti-idiotype is not as good as the affinity of Humira for target TNF-alpha.

In conclusion, findings from this project indicate that the combination of having TNF-alpha as capture reagent and a high affinity anti-idiotypic antibody as detection reagent seems to be the most optimal choice for quantification of Humira. Since production of an anti-idiotypic antibody is costly and time consuming, the bridging assay using the antibody target as capture and the detect might be a viable alternative for drugs earlier in development.

5.1.2 Affinity measurements

The calculated K_D-values after 2, 24 and 48 hours of incubation were similar regardless of whether the measurements were performed in neat serum or after dilution in Rexxip buffer. The K_D tend to stabilize around 30 pM after 48 hours of incubation. The K_D for Humira has previously been determined with the Gyrolab platform to 20 pM (Gyros Protein Technologies AB 2016), where incubation was made up to 72 hours.

F_{tot} was 4 times higher than the theoretical concentration of Humira for both concentrations. This is probably due to two things; the bivalency of Humira and the uncertainty of the active concentration of TNF-alpha. Humira has two binding sites for TNF-alpha, so the theoretical concentration of binding sites is1000 pM instead of 500 pM. Furthermore, there is an uncertainty of the active concentration of TNF-alpha which may contribute to the discrepancy between the theoretical and measured F_{tot}.

Modern antibody drugs in development are becoming more potent which allows for lower dosing. This requires more sensitive PK assays, especially for first in human trails. Due to the low serum interference of the bridging assay optimized in this project, the resulting sensitivity is improved compared to the indirect antibody format. This also enables affinity measurements in 100% serum which in some cases may be more biologically relevant. Furthermore, less dilutions are needed.

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References

Abcam. 2019a. Antibody structure and isotypes. online 2019: https://www.abcam.com/protocols/antibody-structure-and-isotypes. Accessed June 19, 2019.

Abcam. 2019b. What are polyclonal, monoclonal and recombinant antibodies? online 2019: https://www.abcam.com/protocols/a-comparison-between-polyclonal-and-monoclonal. Accessed June 25, 2019.

Abcam. 2019c. KD value: a quantitative measurement of antibody affinity | Abcam. online 2019: https://www.abcam.cn/primary-antibodies/kd-value-a-quantitive-measurement-of-antibody-affinity. Accessed September 26, 2019.

Andreasson U, Perret-Liaudet A, Waalwijk van Doorn LLC van, Blennow K, Chiasserini D, Engelborghs S, Fladby T, Genc S, Kruse N, Kuiperij HB, Kulic L, Lewczuk P, Mollenhauer B, Mroczko B, Parnetti L, Vanmechelen E, Verbeek MM, Winblad B, Zetterberg H, Koel-Simmelink M, Teunissen CE, Sahlgrenska akademin, Institutionen för neurovetenskap och fysiologi sektionen för psykiatri och neurokemi, Göteborgs universitet, Gothenburg University, Institute of Neuroscience and Physiology D of P and N, Sahlgrenska Academy. 2015. A Practical Guide to Immunoassay Method Validation. Frontiers in Neurology 6: 179–179.

Bio-Rad. 2019. Effective Tools for Drug Monitoring Assays. online 2019: https://www.bio-rad-antibodies.com/effective-tools-for-drug-monitoring-assays.html. Accessed October 1, 2019.

Brown A, Elmhirst E, Gardner J. 2017. EP Vantage 2018 Preview. online December 6, 2017: http://info.evaluategroup.com/rs/607-YGS-364/images/EPV18Prev.pdf. Accessed June 14, 2019.

Darwish IA. 2006. Immunoassay Methods and their Applications in Pharmaceutical Analysis: Basic Methodology and Recent Advances. International journal of biomedical science : IJBS 2: 217.

Diamandis EP, Christopoulos TK. 1996. Immunoassays. Academic Press, San Diego.

Fan J, de Lannoy IAM. 2014. Pharmacokinetics. Biochemical Pharmacology 87: 93–120.

FASS. 2019. Humira®. online 2019: https://www.fass.se/LIF/product?nplId=20150901000020. Accessed June 25, 2019.

Gyros Protein Technologies AB. 2019a. Gyrolab User Guide P0020528.

Gyros Protein Technologies AB. 2019b. Assay Development Guidelines – Pharmacokinetics. online 2019: http://user.gyros.com/user-zone/assay-development-guidelines-pharmacokinetics/. Accessed April 15, 2019.

Gyros Protein Technologies AB. 2016. Affinity determinationlGyrolab Immunoassays|Gyros Protein Technologies. online 2016: https://www.gyrosproteintechnologies.com/gyrolab-affinity-determination. Accessed October 1, 2019.

Hytönen VP. 2017. Optimized Streptavidin for Fluorescent Labeling of Biotinylated Targets. Cell Chemical Biology 24: 921–922.

Joyce AP, Wang M, Lawrence-Henderson R, Filliettaz C, Leung SS, Xu X, O'Hara DM. 2014. One Mouse, One Pharmacokinetic Profile: Quantitative Whole Blood Serial Sampling for Biotherapeutics. Pharmaceutical Research 31: 1823–1833.

Kuang B, King L, Wang HF. 2010. Therapeutic monoclonal antibody concentration monitoring: free or total? Bioanalysis 2: 1125–1140.

Liu XF, Wang X, Weaver RJ, Calliste L, Xia C, He YJ, Chen L. 2012. Validation of a gyrolab^{TM} assay for quantification of rituximab in human serum. Journal of Pharmacological and Toxicological Methods 65: 107–114.

Molecular Probes. 2006. Alexa Fluor 647 Microscale Protein Labeling Kit.

Roman J, Qiu J, Dornadula G, Hamuro L, Bakhtiar R, Verch T. 2011. Application of miniaturized immunoassays to discovery pharmacokinetic bioanalysis. Journal of Pharmacological and Toxicological Methods 63: 227–235.

Talbot JJ, Calamba D, Pai M, Ma M, Thway TM. 2015. Measurement of Free Versus Total Therapeutic Monoclonal Antibody in Pharmacokinetic Assessment is Modulated by Affinity, Incubation Time, and Bioanalytical Platform. The AAPS Journal 17: 1446–1454.

Williams K, Erickson R, Fischer SK. 2017. Overcoming disease-specific matrix effect in a clinical pharmacokinetic assay using a microfluidic immunoassay technology. Bioanalysis 9: 1207–1216.

7 Appendix

7.1 Bridging with anti-idiotype as capture and detect

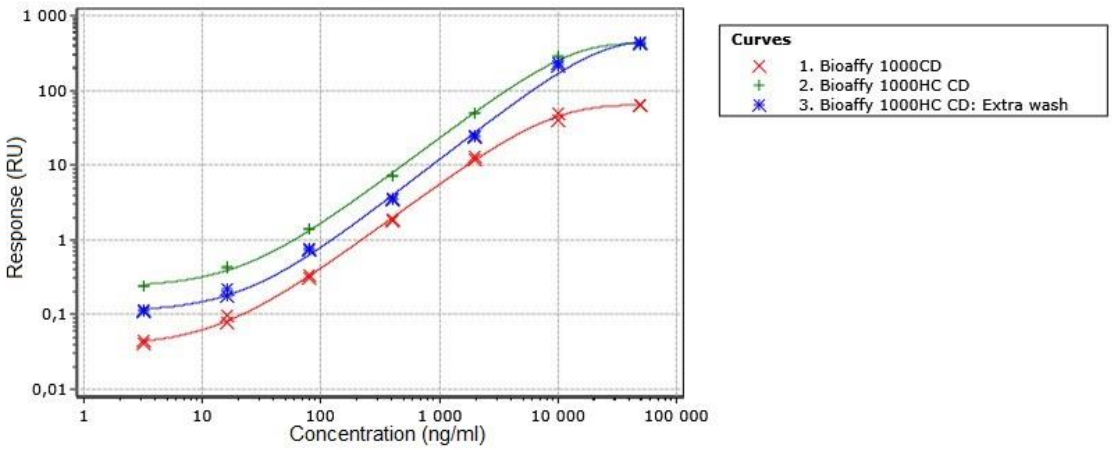

Figure 25. Comparison between Bioaffy 1000CD and Bioaffy 1000HC CD. As well as the effect of adding an extra wash.

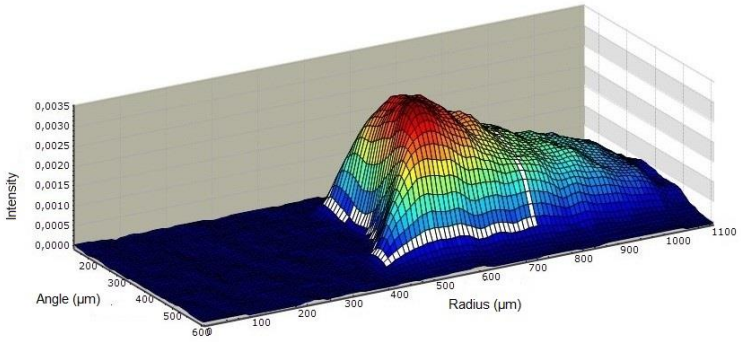

Figure 26. 3D view of a column profile giving intensity as peak height. Run on Bioaffy 1000 CD.

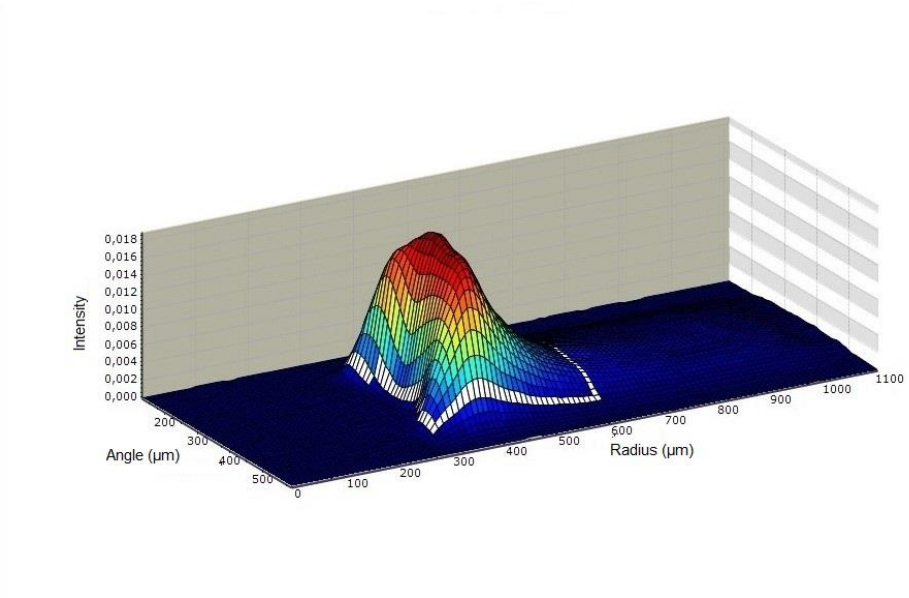

Figure 27. 3D view of a column profile giving intensity as peak height. Run on Bioaffy 1000HC CD.