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Development and comparison of bioanalytical methods to measure free analyte

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Abstract

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Free analyte is measured to be able to understand the pharmacological effects of a drug in the body, the binding to its ligand, and the effective drug level among other things. Thereby, it is important with correct measurements of free analyte, although it is not that easy to achieve since overestimations can occur. In this project, several immunoassays were developed for the bioanalytical methods Gyrolab and ELISA to measure free analyte, where the biotherapeutics Avastin® and Lucentis®, and the ligand VEGF were used as analytes. One difference between the methods is the short contact time of just a few seconds for Gyrolab compared to the sample incubation time of a couple of hours for ELISA. One difference between the antibodies is that Lucentis is an affinity-matured Fab region, and therefore, has a stronger affinity to VEGF compared to Avastin. When free Avastin was measured, the difference was significant, with ELISA estimating higher concentrations compared to Gyrolab. However, this was not the case for all assays. In some cases, this was probably due to differences between the methods that could not be seen. Otherwise, the results with no difference between the methods, when measuring free analyte with Lucentis as the drug, were expected due to the stronger affinity and longer halftime of dissociation. However, the difference with the longer sample incubation time for ELISA compared to the short contact time for Gyrolab seems to influence the measurement of free analyte, depending on the affinity of the components being measured.

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Populärvetenskaplig sammanfattning

Biologiska läkemedel är ett stort område inom den medicinska världen och används mot diverse olika sjukdomar. Ett exempel på en antikropp är bevacizumab, även känd under namnet Avastin® som ett godkänt biologiskt läkemedel. År 2018 såldes Avastin för 7 miljarder US$ världen över och tillsammans med kemoterapi används Avastin i behandling mot bland annat metastatisk bröstcancer, metastatisk kolorektalcancer och avancerad eller metastatisk njurcellscancer. Antikroppar binder till antigen, även kallade ligander, vilket leder till diverse immunresponser alternativt hindrande av bindning mellan liganden och dess receptor eller liknande. Avastin binder till vaskulär endotelial tillväxtfaktor (VEGF) som spelar en stor roll i den patologiska angiogenesen. Angiogenes innebär nybildning av blodkärl från befintliga blodkärl, som kan generera tumörtillväxt och metastatisk spridning då VEGF är överuttryckt. Bindningen mellan Avastin och VEGF leder till att bindningen mellan VEGF och dess receptor hindras och därmed inhiberas och neutraliseras de biologiska aktiviteterna som annars hade ägt rum, vilket därmed kan stoppa sjukdomsförlopp.

Antikropp och ligand (antigen) förekommer i flera olika former, såsom fri eller obunden antikropp, fri eller obunden ligand samt olika komplex av antikropp och ligand. Fri eller obunden antikropp är ofta den delen som bland annat beskriver omsättningen av läkemedlet i kroppen, toxiciteten och elimineringen. Det är även viktigt att kunna mäta fri ligand för att kunna bedöma och förstå ligandbindning, ockupering av ligand och den effektiva nivån av antikropp. Detta är några exempel på fördelen med att kunna mäta fri antikropp och ligand på ett korrekt sätt, för att få en bra bild över hur läkemedlet beter sig och behandlas i kroppen samt hur bindningen med liganden fungerar och hur mycket antikropp som behöver tillsättas. Det är däremot inte helt lätt att mäta fri antikropp och ligand på ett korrekt sätt, då det är lätt att en överestimering sker. Bland annat kan högre koncentrationer av fri antikropp eller ligand mätas om inkuberingstiden för provpåläggningen i metoden är lång, då jämvikten i provet kan påverkas så att antikropp eller ligand som är bundna i komplex släpper från dessa och istället binder till assayen och ger signal, vilket betyder att de mäts som fria eller obundna fast de egentligen inte är det.

Gyros Protein Technologies AB är ett företag i Uppsala som tillverkar immunoassay-plattformar, Gyrolab, som kvantifierar proteiner och antikroppar som är viktiga för utvecklingen och produktionen av biologiska läkemedel. En annan metod för att analysera biomolekyler är enzymkopplad immunadsorberande analys (ELISA) som har en hög känslighet och selektivitet, men jämfört med Gyrolab kräver mycket längre inkuberingstid för provpåläggningen och mycket mer manuellt arbete. Tack vare de små volymerna och därmed de korta kontakttiderna samt semi-automatiken för Gyrolab, är förhållandena bättre jämfört med ELISA för att kunna mäta fri antikropp och ligand på ett korrekt sätt. Det här examensarbetet gick ut på att utveckla och optimera immunoassays på både Gyrolab och ELISA för att kunna mäta fri antikropp och ligand, för att sedan jämföra dessa metoder sinsemellan.

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List of abbreviations

Avastin®	bevacizumab
α-VEGF mAb	α-VEGF VG76e monoclonal antibody
α-VEGF pAb (1)	α-VEGF (1) polyclonal antibody
α-VEGF pAb (2)	α-VEGF (2) polyclonal antibody
b	biotinylated
BSA	bovine serum albumin
CD	compact disc
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
H2	α-human IgG Fc monoclonal antibody H2
HRP	horseradish peroxidase
Ig	immunoglobulin
JDC-10	α-human IgG Fc monoclonal antibody JDC-10
K_D	equilibrium dissociation constant
kDa	kilodalton
kLC	α-human IgG Kappa light chain monoclonal antibody SB81a
k_{off}	dissociation constant
k_{on}	association constant
LLOQ	lower limit of quantification
Lucentis®	ranibizumab
M	molar (mol/L)
mAb	monoclonal antibody
pAb	polyclonal antibody
PBS	phosphate-buffered saline
PBS-T	PBS x1 + 0.01% Tween
PD	pharmacodynamic
PK	pharmacokinetic
TMB	tetramethylbenzidine
VEGF	vascular endothelial growth factor
4D2D9G8	α-human IgG Fc monoclonal antibody 4D2D9G8

1 Introduction

Development and comparison of bioanalytical methods to measure free analyte is a master thesis project in the master's program in Molecular Biotechnology Engineering at Uppsala University, proposed and performed at Gyros Protein Technologies AB. The company produces immunoassay platforms, called Gyrolab, giving information about ligand binding and quantification of proteins important for the biotherapeutic development and production. Biotherapeutics or antibodies have been and are of great importance for the treatment of different diseases, such as different types of cancer, by targeting specific parts in the body. This area is still growing fast and new technology is generated (Perez *et al.* 2014), thus in need of great bioanalytical methods. Two specific examples of antibodies, used in this project, are bevacizumab and ranibizumab, also known as the drugs Avastin® and Lucentis® respectively. These antibodies target vascular endothelial growth factor (VEGF) which is involved in tumour growth and metastatic spread when overexpressed (Wang *et al.* 2004). Avastin is, together with chemotherapy, approved for the treatment of metastatic colorectal cancer, metastatic breast cancer, and advanced or metastatic renal cell cancer among other things, acting by blocking the binding between VEGF and its receptor (Panoilia *et al.* 2015). In 2018, it was sold for over 7 billion US$ all over the world (Urquhart 2019). Lucentis is an approved drug in ophthalmology, in other words used for treatment of different sorts of eye disorders (Shahsuvaryan 2017).

Antibody and ligand (antigen) appear as both free antibody, free ligand, and in different complexes. Free or unbound antibody is often the part that determines the pharmacological effect of a biological drug. This is particularly true if the antibody (drug) is supposed to block the binding between the ligand and its receptor. It is also important to measure free analyte to be able to understand the pharmacokinetics and binding between antibody and ligand, occupation of ligand, and the effective antibody level (Lee *et al.* 2011). Therefore, it is of great interest to be able to measure free analyte in the correct way. One commonly used immunoassay method with high sensitivity and selectivity is enzyme-linked immunosorbent assay (ELISA), but compared to Gyrolabs short contact time, this method requires long sample incubation times and much more manual work (Aydin 2015). Due to the small volumes and semi-automatics that Gyrolab provides, the conditions to measure free analyte are better compared to using ELISA (Dysinger & Ma 2018). When a prolonged sample incubation is applied, overestimations of free analyte can occur depending on the affinity between the components, see an illustrated example in Figure 1.

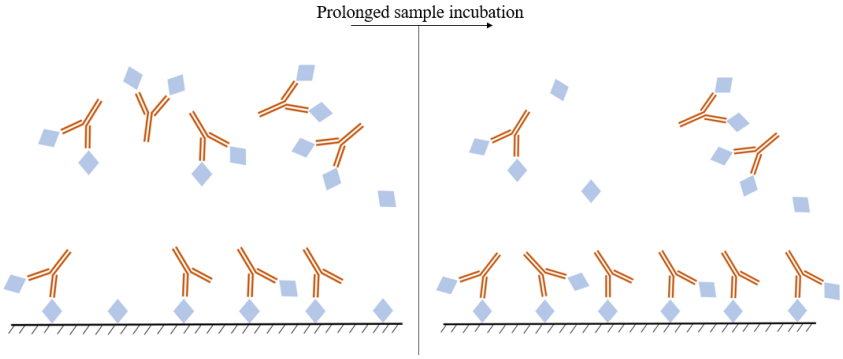

Figure 1. A potential situation where the equilibrium in the sample gets affected by a prolonged sample incubation. In the figure to the left, all antibodies in a sample that have at least one binding site available bind to the assay. In the figure to the right, dissociations of complex in the sample have occurred due to the prolonged sample incubation, leading to an overestimation of free analyte.

The purpose of this project was to develop and optimize assays to measure free analyte and examine the great potential of measuring it on Gyrolab, with the short contact time, by comparing it with ELISA, with the longer sample incubation time. This was done by optimizing eight different assays, four on Gyrolab and four on ELISA. The assays were optimized to achieve sensitive assays, generating reliable response measurements. Thereafter, experiments were performed in parallel on Gyrolab and ELISA to be able to compare them regarding the measurement of free analyte.

2 Background

2.1 What is an antibody?

Antibodies consist of two different regions, Fab and Fc regions (see Figure 2). Fab is the antigen-binding fragment and Fc is the crystallizable fragment, responsible for the biological activities. A full-length antibody consists of two Fab regions and one Fc region, and the size of the whole antibody is around 150 kDa. Antibodies can be both monoclonal, binding to one specific epitope, and polyclonal, binding to several epitopes. There are also different types of antibodies, named as immunoglobulins, with different effector functions. Immunoglobulin G (IgG) is the most common immunoglobulin in the human body, representing around 75% of all antibodies in the plasma of healthy individuals, and is very important for the humoral immune response as a major effector molecule. Generally, the most important effector functions are inactivation and removal of infectious agents and products. When it comes to IgG, the most important functions are the activation of complement in the immune system and binding to specific receptors (Nimmerjahn 2013). The complement system constitutes of proteins which, in different ways, activate inflammatory events when pathogens invade (Janeway *et al.* 2001). By activating complement, targets can be killed in several ways and binding to specific receptors generates different immune responses in the body (Nimmerjahn 2013).

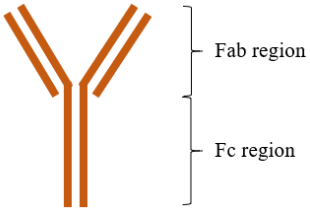

Figure 2. An illustrated antibody with marked Fab and Fc region.

Bevacizumab is a humanized monoclonal IgG, known by the name Avastin as a biotherapeutic (Wang *et al.* 2004). A humanized antibody is engineered to be more "human-like". In other words, parts of the antibody are substituted to make it more "human", to reduce the immune response against therapeutic antibodies, also called immunogenicity (Vaswani & Hamilton 1998). Bevacizumab is a full-length antibody that targets vascular endothelial growth factor (VEGF) which plays an important role in the pathological angiogenesis when overexpressed, generating tumour growth and metastatic spread. The antibody blocks the interaction between VEGF and its receptor, and thereby, inhibit and neutralize the biological activities that would otherwise have taken place (Wang *et al.* 2004). In combination with chemotherapy, Avastin is approved for the treatment of metastatic breast cancer, metastatic colorectal cancer, and advanced or metastatic renal cell cancer among other things (Panoilia *et al.* 2015). Another humanized monoclonal antibody that targets VEGF is ranibizumab, also known as Lucentis as an approved drug for use in ophthalmology. Lucentis is an affinity-matured Fab region derived from the same antibody as Avastin and has a molecular weight of 48 kDa (Shahsuvaryan 2017). Comparing a Fab region and a full-length antibody, the Fab region can be more diffusible, and penetrate tissues more rapid and complete compared to the full-length antibody (Ferrara *et al.* 2006). Also, using a Fab region compared to a full-length antibody may reduce the immunogenicity (Knight *et al.* 1995). Since Lucentis only consists of one Fab region, it only has one antigen-binding site (Shahsuvaryan 2017), compared to Avastin that has two. VEGF is a dimer and thereby, also has a bivalency, being able to bind two Avastin and two Lucentis molecules (Park *et al.* 2018).

2.2 Immunoassays

The basic concept of immunoassays as a technology is the binding between antibody and antigen (Gao *et al.* 2018) and there are many different methods dealing with this technology. Gyrolab and enzyme-linked immunosorbent assay (ELISA) are two examples of such methods.

2.2.1 What is Gyrolab and how does it work?

Gyros Protein Technologies AB produces immunoassay platforms called Gyrolab, giving information about ligand binding and quantification of proteins important for biotherapeutic development and production (Gyros Protein Technologies AB 2019). Due to the high throughput and small reagent volumes necessary, microfluidic devices like Gyrolab can be useful in the research and development of improved antibodies for diagnostic immunoassays

(Honda *et al.* 2005). The Gyrolab technique depends on capillary and centrifugal force, taking advantage of the microfluidic in which CDs are used to drive the flow of fluids, and the flow-through process is equivalent to the incubation time for ELISA. The disc consists of structures with hydrophobic barriers, volume defining chambers, overflow channels, and affinity capture columns on nanolitre scale (see Figure 3). The hydrophobic barriers contribute to a consequent addition of the liquids since the barriers break first when the discs are spun at a certain speed. Due to the volume defining chambers, the exact volume is added, and the excess fluid is removed by the overflow channels at a certain centrifugation speed. Therefore, the volume pipetted to the plate does not matter as long as it is more than the minimum volume (Gyros Protein Technologies AB 2019). The affinity columns in the Gyrolab CDs are filled with streptavidin-coated particles to which biotinylated capture reagents bind (Honda *et al.* 2005). By using controlled speed to spin the disc, an optimal binding and uniform conditions between assays can be obtained. Gyros Protein Technologies provides different CDs such as Bioaffy 20 HC, Bioaffy 200, Bioaffy 1000, and Bioaffy 1000 HC. The number stands for added volume in nanolitre and HC stands for high capacity. The columns in the Bioaffy 1000 HC CD is filled with a high capacity, solid porous, particle, compared to the Bioaffy 1000 CD (Gyros Protein Technologies AB 2019).

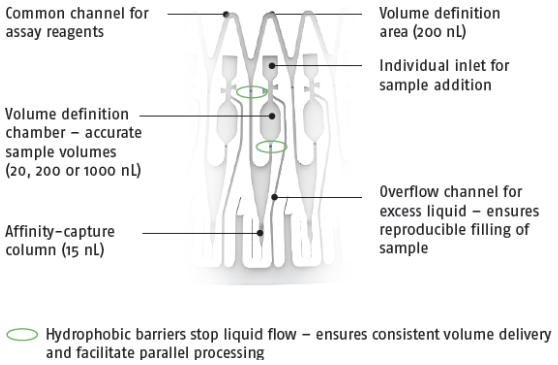

Figure 3. An illustration of one of the structures of the Gyrolab CDs. Reagents are added to the common channel and samples are added to the individual inlet. By using capillary and centrifugal force, the liquids are transferred to the volume definition chamber and then to the affinity column. The green areas mark the hydrophobic barriers which breaks at a certain centrifugation speed. The illustration is used with permission from Gyros Protein Technologies.

The Gyrolab Workstation has a laser-induced fluorescence detection, and a software generating column profiles with the distribution of the bound analyte. Thereby, an estimation of the affinity can be accomplished by viewing the column profiles, the narrower the distribution, the higher affinity (Honda *et al.* 2005). See Figure 4 for examples of how the column profiles may look like and how the difference in affinity can be seen. In addition to the column profiles, responses are generated, and by using a standard curve with known concentrations, the concentration of unknown samples can be measured.

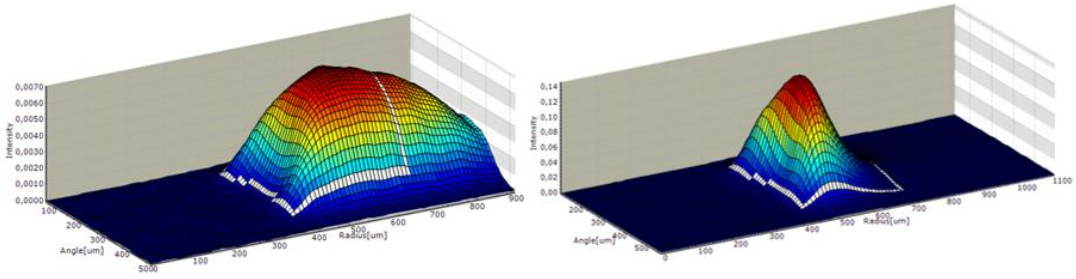

Figure 4. Two examples of how the column profiles may look like, with a broad peak to the left (low affinity) and sharp peak to the right (high affinity).

All assays ran on Gyrolab in this project were based on the following steps:

- Biotinylated capture reagent binding to the streptavidin-coated affinity column
- Sample with the analyte, binding to the biotinylated capture
- Alexa (fluorophore) labelled detecting antibody, binding to the analyte

Due to the small volumes, Gyrolab has a very short contact time, providing great conditions to measure free analyte in comparison to other methods where the equilibrium can be changed during the analysis (Dysinger & Ma 2018).

2.2.2 What is ELISA and how does it work?

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay in which quantitative analysis of antibody or antigen can be done by using an enzyme-linked conjugate and an enzyme-substrate. The capture reagent is adsorbed to a solid phase consisting of different types of plastic, such as polystyrene, polyvinyl, and polypropylene. A blocking solution is used to block the sites that are not occupied by the capture and thereafter, the analyte can be added. In the next step, a secondary antibody that binds to the analyte is added. This secondary antibody is either enzyme-conjugated or biotinylated so that streptavidin conjugated with the enzyme can bind to the antibody. By adding a substrate reacting with the enzyme, a specific colour is generated which can be read by a spectrophotometer at a specific wavelength (Aydin 2015).

There are different types of ELISA, such as indirect and sandwich. For indirect ELISA, the wells are coated with an antigen, the sample with the antibody is added and thereafter, an enzyme-conjugated secondary antibody is added. In other words, it is the antibody that is measured in an indirect ELISA. When the substrate is added, a signal is given, and the response can be measured. By using a standard curve with known concentrations, the concentration of unknown samples can be measured. The difference between the indirect and the sandwich ELISA is that the wells are coated with an antibody instead of an antigen in the latter method. So, in the sandwich ELISA, the antigen is bound to and in between two antibodies; the capture and the enzyme-conjugated antibody. In other words, the antigen is measured in a sandwich ELISA. This bioanalytical method, ELISA, is a sensitive and specific method but requires a lot of manual work and incubation time in between the different steps, such as coating, blocking, sample and substrate addition (Aydin 2015).

2.3 What is pharmacokinetics and pharmacodynamics?

Pharmacokinetics (PK) describes the behaviour of a drug in the body, such as metabolism, distribution, and elimination. Thereby, the drug is measured in a PK assay. In a pharmacodynamic (PD) assay, the target or ligand is measured, to describe the interaction between the drug and the target (Ratain & Plunkett 2003). In this project, both PK and PD assays were performed, in which the indirect assay on ELISA corresponds to the PK assay and the sandwich assay corresponds to the PD assay. Avastin (bevacizumab) and Lucentis (ranibizumab) were used as the drugs and VEGF as the ligand. For the PK assays on Gyrolab, biotinylated VEGF was bound to the streptavidin-coated column, Avastin or Lucentis was bound to VEGF and the detecting antibody was bound to Avastin or Lucentis (see Figure 5). For the samples, when measuring free analyte, a fixed concentration of Avastin or Lucentis was mixed with different concentrations of VEGF.

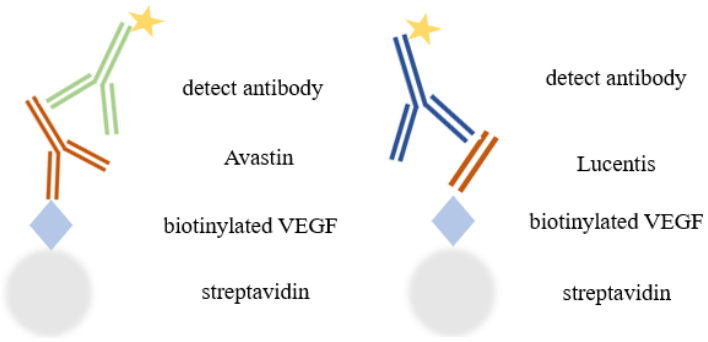

Figure 5. Schematic illustrations of the setup of the PK assays on Gyrolab, with Avastin (to the left) and Lucentis (to the right) as the drug.

For the PD assays on Gyrolab, biotinylated Avastin or Lucentis was bound to the column, VEGF was bound to Avastin or Lucentis and the detecting antibody was bound to VEGF (see Figure 6). For the samples, when measuring free analyte, a fixed concentration of VEGF was mixed with different concentrations of Avastin or Lucentis.

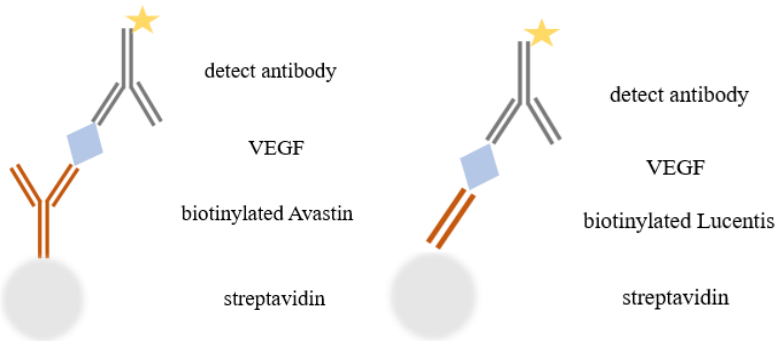

Figure 6. Schematic illustrations of the setup of the PD assays on Gyrolab, with Avastin (to the left) and Lucentis (to the right) as the drug.

For ELISA, the setup was the same as for Gyrolab except that the detecting antibody was biotinylated instead of the capture since the enzyme horseradish peroxidase (HRP) was

conjugated to streptavidin which binds to the biotinylated detecting antibody. The substrate, Tetramethylbenzidine (TMB) solution, reacts with HRP, generating a specific colour at a specific wavelength. In this project, a stop solution was added after the TMB solution, which stops the reaction between the enzyme and the substrate and changes the colour. The setup of the PK assays on ELISA with Avastin and Lucentis as the drug can be seen in Figure 7. In the same way as for Gyrolab, the samples, when measuring free analyte, consisted of a fixed concentration of Avastin or Lucentis and different concentrations of VEGF.

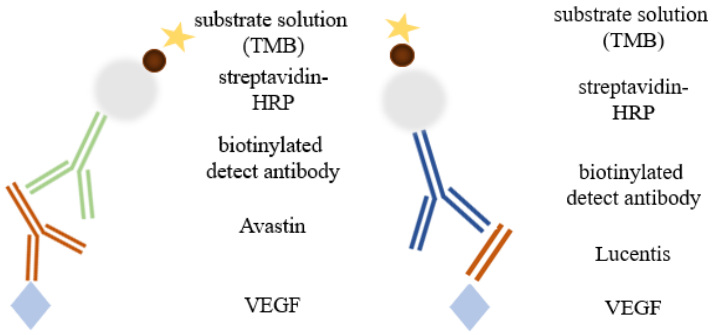

Figure 7. Schematic illustrations of the setup of the PK assays on ELISA, with Avastin to the left and Lucentis to the right.

The setup of the PD assays on ELISA with Avastin and Lucentis as the drug can be seen in Figure 8. In the same way as for Gyrolab, the samples, when measuring free analyte, consisted of a fixed concentration of VEGF and different concentrations of Avastin or Lucentis.

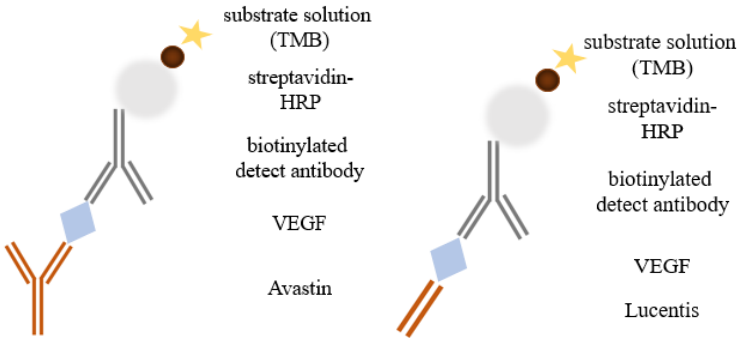

Figure 8. Schematic illustrations of the setup of the PD assays on ELISA, with Avastin to the left and Lucentis to the right.

2.4 Importance of measuring free analyte and general problems

Antibody and ligand (antigen) appear in many different forms, such as free antibody, free ligand, monovalent antibody-ligand complex, and bivalent antibody-ligand complex. By monovalent means that one of the antibodies Fab regions is bound to the ligand, while bivalent means that both Fab regions are bound to the ligand. Free or unbound antibody is often the part that determines the pharmacological effect of a biological drug, which is particularly true if the antibody (drug) is supposed to block the binding between the ligand

and its receptor. This is one example of the advantage of being able to measure free analyte in the correct way and it is important to get a good picture of the pharmacokinetics and to be able to understand the antibody and ligand binding, occupation of ligand, and the effective level of antibody. Since free or partly free analyte is considered being biologically active, the efficacy and toxicity would be better correlated with free and not total analyte (Lee *et al.* 2011).

One specific example is C5a which is a component in the complement system and binds to a specific receptor expressed on different immune cells in the body, leading to intracellular signalling. By blocking C5a with antibodies, complement based diseases can be treated (McGeer *et al.* 2017). Since specific antibodies can be used as drugs and block this component, C5a, it is important to be able to quantify the amount of free component to make it possible to get a good understanding of the interaction between the antibody (drug) and its ligand. However, some problems may arise when measuring free analyte. As an example, free analyte may be overestimated depending on the sample incubation for the method. In this specific case, C5a also has a shared epitope with another component, C5. Therefore, antibodies will bind both to C5a and C5, but to C5 with a lower affinity than to C5a (Dysinger & Ma 2018). Thanks to the short contact time for Gyrolab, the risk for overestimation of free analyte gets much smaller. Partly since the possibility of binding between the antibodies and C5 components gets very small, and that the general problem with the analyte creating signal giving complex due to dissociation during the incubation gets smaller (Lee *et al.* 2011).

2.5 Curve fitting

By using the response generated from immunoassays and compare it against a calibration curve, for example a standard curve, the concentration of an analyte in a sample can be determined. Ideally, there would be a standard for every existing concentration generating a "true curve", which means an infinite number of standards. Since that is not practically applicable, a curve must be estimated by interpolating between standards. The interpolation is performed by choosing a mathematical model that will make a good approximation and generate a curve model that will get close to the "true curve". The curve model is fitted to the data to obtain one curve that gives the best fit. If the curve fit is good, the concentrations of the unknown samples will be as close to the accurate values as possible. The curve fit will never be perfect due to random variation in data and the curve model will not be exactly as the "true curve". These problems can be reduced by increasing the number of replicates and standards, which provide a balance of what is practically applicable. To obtain curve models for immunoassays, many mathematical functions have been tried. For "true curves" of immunoassays, with data of a sigmoidal "S" shape, a straight-line curve model cannot fit the model. In that case, a logit-log model works better. However, the logit-log model is just capable of modelling symmetric data effectively. An example of a function that is related to a linear logit-log model and widely used is the four-parameter logistic (4PL) function. Another model is the five-parameter logistic (5PL) function, which is extended by adding a fifth

parameter and controls the degree of asymmetry. When the curve model is fitted against the data, the free parameters are adjusted until the approximation gets as close to the "true curve" as possible. By adding the fifth parameter, asymmetric data can be modelled effectively as well and the 5PL model has proven to give better curve fits data compared to the 4PL, being able to eliminate lack-of-fit errors that appear in the 4PL model (Gottschalk & Dunn 2005). In this project, the 5PL model has been used for the curve fits on both Gyrolab and ELISA.

2.6 Affinity and kinetics

Investigation of an eventual interaction between two molecules is a very common experiment in biochemistry and cellular and molecular biology, and the answer should be quantitative with a number describing the affinity. One basic, reversible, reaction can be seen below (see equation 1). A molecule D, say a drug, in this case, interacts with a molecule L, say a ligand, in this case, forming a complex DL (Pollard 2010).

$$[D] + [L] \Leftrightarrow [DL] \quad (1)$$

At equilibrium, a dissociation constant (K_D) can be obtained by using the following equation (see equation 2), where k_{off} is the dissociation rate and k_{on} the association rate. The stronger the reaction or higher the affinity between the components, i.e. the reactants D and L are more completely converted to the complex DL, the lower value of K_D (Pollard 2010).

$$K_D = \frac{[D][L]}{[DL]} = \frac{k_{off}}{k_{on}} \quad (2)$$

In this case, with the basic, reversible, reaction (see equation 1), the stoichiometry is assumed to be 1:1. As mentioned earlier, full-length antibodies like Avastin, are bivalent, meaning that one Avastin molecule can bind two VEGF molecules. Therefore, the stoichiometry is more complicated than a relation of 1:1 but is still a reasonable assumption to start with. Another value that can be estimated with the dissociation rate, using equation 3, is the halftime of dissociation between components (Pollard 2010).

$$t_{\frac{1}{2}} = \frac{ln2}{k_{off}} \quad (3)$$

This value represents the time it takes until 50% have dissociated and can be used to get an idea regarding the time needed to get a significant overestimation of free analyte. If the halftime of dissociation is estimated to be much longer than a specific sample incubation time, the equilibrium in a sample will not be affected that much, and significant overestimations should not be seen. Although, if the halftime of dissociation is in the range of a sample incubation time for a method, significant overestimations should be seen. Examples of k_{off} values from the literature, obtained with other techniques than used in this project but with the same components, and calculated $t_{1/2}$ values can be seen in Table 1. The k_{off} values for Avastin and VEGF are higher than for Lucentis and VEGF, leading to longer halftimes of

dissociation for Lucentis and VEGF compared to Avastin and VEGF. Due to the long halftime of dissociation for Lucentis and VEGF, of around one day, the experiments performed with these components can be seen as negative controls in this project. Even though the sample incubation time is much longer for ELISA compared to the short contact time for Gyrolab, the equilibrium in a sample with Lucentis and VEGF should not be that affected due to the strong affinity.

Table 1. k_{off} and $t_{1/2}$ values for Avastin and Lucentis, k_{off} values obtained from literature.

k_{off} Avastin	**$t_{1/2}$ Avastin**	**k_{off} Lucentis**	**$t_{1/2}$ Lucentis**
$k_{off}=3.1\cdot10^{-5}$ s^{-1} (Papadopoulos *et al.* 2012)	$t_{1/2}=\ln2/3.1\cdot10^{-5}=373$ min	$k_{off}=0.73\cdot10^{-5}$ s^{-1} (Papadopoulos *et al.* 2012)	$t_{1/2}=\ln2/0.73\cdot10^{-5}=1583$ min
$k_{off}=6.50\cdot10^{-10}$ $M\cdot1.75\cdot10^{5}$ $M^{-1}s^{-1}=11.375\cdot10^{-5}$ s^{-1} (Wang *et al.* 2014)	$t_{1/2}=\ln2/11.375\cdot10^{-5}=102$ min	$koff\leq10^{-5}$ s^{-1} (Lowe *et al.* 2007)	$t_{1/2}\geq\ln2/10^{-5}=1155$ min
$k_{off}=8.16\cdot10^{-5}$ s^{-1} (Khalili *et al.* 2012)	$t_{1/2}=\ln2/8.16\cdot10^{-5}=142$ min	$k_{off}=0.39\cdot10^{-5}$ s^{-1} (Yang *et al.* 2014)	$t_{1/2}=\ln2/0.39\cdot10^{-5}=2962$ min
$k_{off}=21.9\cdot10^{-5}$ s^{-1} (Yang *et al.* 2014)	$t_{1/2}=\ln2/21.9\cdot10^{-5}=53$ min	$k_{off}\leq10^{-5}$ s^{-1} (Lowe *et al.* 2007)	$t_{1/2}\geq\ln2/10^{-5}=1155$ min
$k_{off}=32.9\cdot10^{-5}$ s^{-1} (Yang *et al.* 2014)	$t_{1/2}=\ln2/32.9\cdot10^{-5}=35$ min		

By measuring the concentration of D_{free}, L_{free} or DL without disturbing the equilibration, the K_D value can be obtained from the shape of the curve when plotting, for example, the concentration of DL versus the concentration of L_{free}. In that case, the K_D value can also be obtained from the concentration of L_{free} that is required to convert half of D_{tot} into DL. Ideally, when measuring the binding between the components, the concentration of one of the components, say D, is fixed and lower than K_D while the other component, L, is used in a wide range of concentrations. If high concentrations of L are included, the concentration of free D will reach a plateau since L will saturate D. This plateau is necessary to estimate the equilibration constant (Pollard 2010). IC_{50} is a measure of the half-maximal inhibitory concentration. In other words, IC_{50} is the concentration of a component, in this case, Avastin/Lucentis or VEGF, that is required to inhibit half of the VEGF or Avastin/Lucentis. Therefore, the K_D value can be estimated to be the same as the IC_{50} value at equilibrium. GraphPad Prism is a software that can be used to calculate IC_{50} with obtained data of concentrations for, in this case, added VEGF or Avastin/Lucentis and free Avastin/Lucentis or VEGF in the samples. In this software, the data is fitted using a non-linear regression algorithm and a specific equation to get the IC_{50} value (Aykul & Martinez-Hackert 2016). One way to calculate the IC_{50} value in GraphPad Prism is by using *the logarithm of the inhibitor versus response curve*. The inhibitor is, in this case, added VEGF to the samples in

the PK assay and added Avastin or Lucentis to the samples in the PD assay. The response is the measure of free analyte. The equation can be seen below (equation 4), with the top being the top plateau, the bottom being the bottom plateau, and hillslope the steepness of the curve (GraphPad Prism 8, Curve Fitting Guide).

$$Y = Bottom + \frac{(Top - Bottom)}{\left(1+10^{\left((LogIC_{50}-X)HillSlope\right)}\right)} \quad (4)$$

3 Materials

The reagents, consumables, and instruments that were used in the project can be seen in Table 2, Table 3, and Table 4.

Table 2. Reagents used in the project.

Reagents	Supplier	Product number
α-human IgG Fc monoclonal antibody H2	Southern Biotech	9042-01
α-human IgG Fc monoclonal antibody JDC-10	Southern Biotech	9040-01
α-human IgG Kappa light chain monoclonal antibody SB81a	Abcam	ab99832
α-human IgG Fc monoclonal antibody 4D2D9G8	Abcam	ab31925
α-VEGF monoclonal antibody VG76e	Abcam	ab119
α-VEGF polyclonal antibody (1) ab106580	Abcam	ab106580
α-VEGF polyclonal antibody (2)	R&D Systems	AF-293-NA
Avastin®	Roche	Lot B8012H08
Lucentis®	Novartis	538757
Biotinylated VEGF	Acro Biosystems	VE5-H8210
VEGF	Acro Biosystems	VE5-H4210
Biotinylated BSA	BioNordica (Vector Laboratories)	B-2007
Streptavidin-HRP	Thermo Scientific	N504
Substrate ELISA (1xTMB)	Thermo Scientific	00-4201-56
Stop solution ELISA	Thermo Scientific	N600
EZ-Link Sulfo-NHS-LC-Biotin	Thermo Scientific	
Alexa Fluor 647 Monoclonal Antibody Labeling Kit	Thermo Scientific	

Table 3. Consumables used in the project.

Consumables	Supplier
ELISA plates Nunc Maxisorp Flat-Bottom Plate	Thermo Scientific
ELISA sealing film polyester	VWR
Microtiter plate 0.2 mL Skirtad 96-well PCR plate	Gyros Protein Technologies AB
Microtiter plate foil	Gyros Protein Technologies AB
Bioaffy 200 CD	Gyros Protein Technologies AB
Bioaffy 1000 CD	Gyros Protein Technologies AB
Bioaffy 1000 HC CD	Gyros Protein Technologies AB
Amicon Ultra-4 Ultracel 30K column	Amicon

Table 4. Instruments used in the project.

Instruments	Supplier
Gyrolab workstation	Gyros Protein Technologies AB
Cenrifuge 5810R	Eppendorf
Cenrifuge miniSpin plus	Eppendorf
Nanophotometer	LabVision
ELISA reader SpectraMax ABS	Molecular Devices

4 Methods

The general procedure that was performed during this project can be seen in Figure 9. There were eight assays in total: four assays on Gyrolab and four assays on ELISA. For those four assays, there were two PK and two PD assays with both Avastin and Lucentis as the drug. Therefore, this general procedure was performed four times, with the PK and the PD assay and with Avastin and Lucentis as the drug. The step with the comparison between Gyrolab and ELISA for measurement of free analyte was performed twice, with two sample incubation times investigated for ELISA the second time. So, first a comparison between Gyrolab and ELISA with two hours of sample incubation was performed. Then, a comparison between Gyrolab and ELISA with both two and four hours of sample incubation was performed. The calculation of K_D values was performed for all comparison runs for Gyrolab and ELISA.

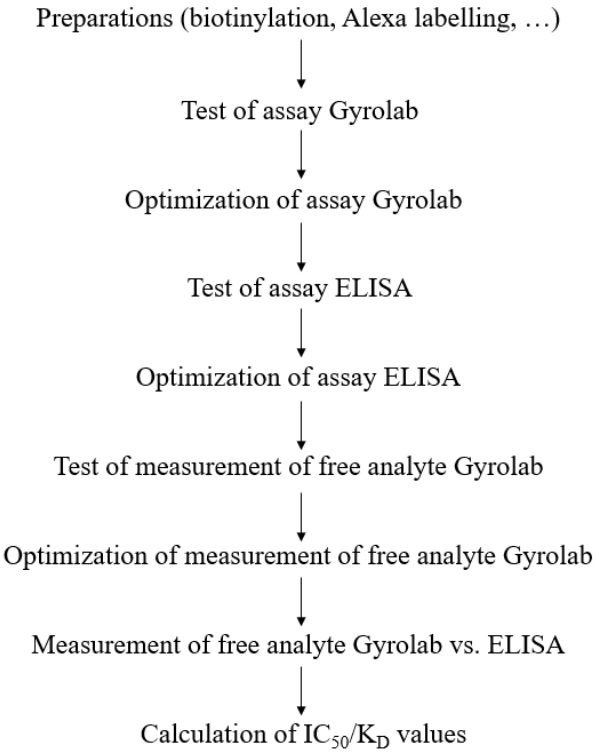

Figure 9. The general procedure during this project.

All the manual work for running Gyrolab can be seen in Figure 10. Since much work is automated, there are not that many manual steps. The instrument performs the capture, sample, and detecting reagent additions as in ELISA (see Figure 11), but with much smaller volumes leading to short contact times compared to ELISAs long incubation times. Therefore, a run on Gyrolab took approximately one hour, dilutions and preparations excluded, compared to ELISA, which took almost 24 hours in total. For Gyrolab, the capture reagent was diluted in PBS-T, the samples were diluted in RexxipA, and the detecting reagent was diluted in RexxipF. The dilutions were performed with maximum steps of 1:50.

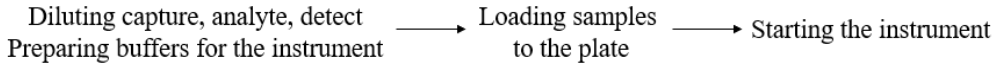

Figure 10. The general workflow for Gyrolab.

All steps that were performed in each ELISA run can be seen in the general workflow in Figure 11. The capture reagent was incubated overnight (16-16.5 hours) at +4 C˚, each well was coated with 50 µL. All the washing steps were performed by pipetting 300 µL washing buffer (see Table F3 in Appendix F) to each well and discard it, this was done four times for every washing step. The blocking buffer was incubated for one hour at room temperature, 200 µL was loaded to each well. The sample was incubated for two hours at room temperature, 100 µL was loaded to each well. When comparing the measurement of free analyte on Gyrolab and ELISA, four hours of sample incubation was also used to examine if there would be any differences between the sample incubation time of two and four hours. For the detecting antibody step, 100 µL was loaded to each well and was incubated for one hour at room temperature. The streptavidin-HRP was incubated for 20 minutes at room temperature and 100 µL was added to each well. For the substrate solution (TMB) incubation, 100 µL was added to each well and was incubated 20-30 minutes, covered with aluminum foil to avoid direct light. The stop solution was added after the substrate solution incubation, 50 µL was added to each run and the OD was measured at 450 nm within 30 minutes. All the incubation steps except the capture, streptavidin-HRP, and substrate additions were performed with gentle shaking. For ELISA, the capture reagent was diluted in PBS, and all the other reagents were diluted in blocking buffer (see Table F1 and Table F2 in Appendix F). All the dilutions were performed with maximum steps of 1:50.

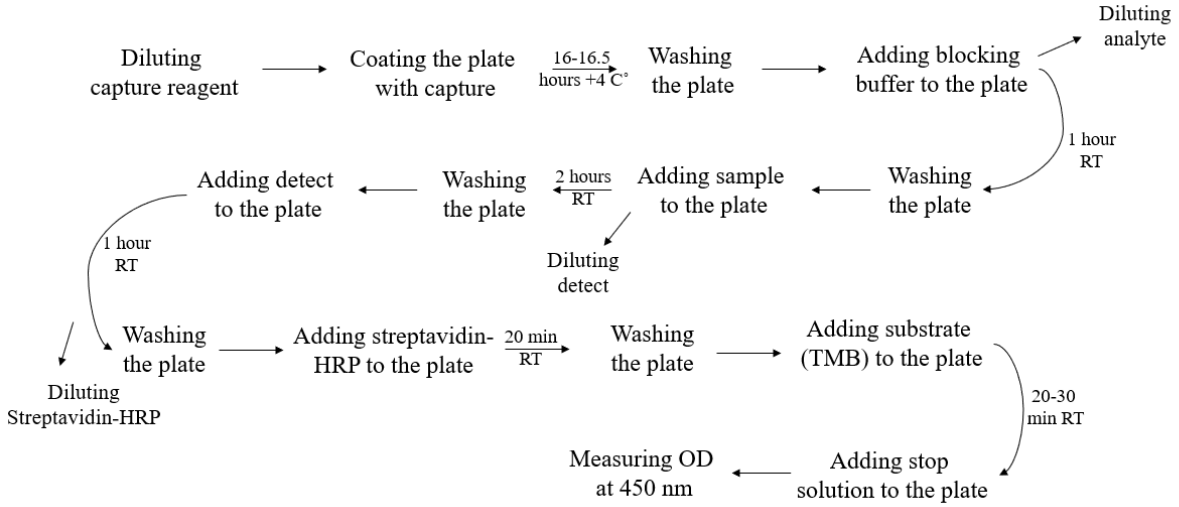

Figure 11. The general workflow for ELISA used in this project.

4.1 Biotinylation and Alexa labelling

For Gyrolab, the capture reagent needed to be biotinylated to attach to the streptavidin-coated column and for ELISA, the detecting reagent needed to be biotinylated to bind to the streptavidin that was conjugated with the HRP enzyme. The reagents were either diluted or concentrated to reach a concentration of 1 mg/mL if it was not the original concentration. If the sample contained >0.02% sodium azide, a buffer exchange was performed. The concentration and buffer exchange of the samples were performed in the same way with Amicon Ultra-4 Ultracel 30K columns. One vial with 1 mg biotinylation reagent from the EZ-Link Sulfo-NHS-LC-Biotin kit, stored at -20 ˚C, was dissolved in 1 mL MilliQ water. Biotinylation reagent and the capture or detecting antibody were mixed at a 12 times molar excess of biotin. The mixture was incubated for approximately one hour at room temperature while shaking gently. Thereafter, the biotinylated antibody was purified with a Protein Desalting Spin Column from the kit to remove unbound biotin. The protein concentration was measured at a nanophotometer at 280 nm. The biotinylation was performed by following the Gyrolab User Guide (Gyros Protein Technologies AB 2019), with recommendations regarding the excess of biotinylation reagent compared to the reagent that was going to be labelled. However, no measurements regarding successful biotinylation were performed, but it was first tested when experiments were performed. Therefore, if more of a specific biotinylated reagent was needed, the old and the new labelled reagent was compared to see if they performed in the same way and gave the same results.

The detecting reagent needed to be Alexa labelled for Gyrolab, to be able to get detected by the instrument. The antibodies were either diluted or concentrated to reach a concentration of 1 mg/mL if it was not the original concentration. If the sample contained >0.02% sodium azide, a buffer exchange was performed. The concentration and buffer exchange of the samples were performed in the same way with Amicon Ultra-4 Ultracel 30K columns. A 1:10 volume of 1 M Sodium bicarbonate buffer was added to the sample if the sample was not stored in a borate buffered saline, and therefore, had a slightly higher pH than 8. One vial containing the reactive dye from the Alexa Fluor™ 647 Monoclonal Antibody Labeling Kit was dissolved in 10 µL MilliQ water. 5 µL of the reactive dye was added to the sample and was incubated approximately one hour at room temperature with occasional shaking and covered in aluminum foil to avoid direct light. A purification column from the kit was packed with the purification resin and the labelled reagent was added to the column and centrifuged. Protein concentration and degree of labelling were measured on the nanophotometer. The sample was diluted in PBS + 0.2% BSA to reach a final concentration of 1000 nM. The Alexa labelled reagents were tested in the experiments to see if the response measurements were good, no matter what the degree of labelling was.

4.2 Optimization of PK and PD assays on Gyrolab and ELISA

For all assays, on both Gyrolab and ELISA, optimization work was performed to get a sensitive assay with high affinity and response. The assays were evaluated and optimized to reach as high signal/background values as possible, to be able to measure free analyte with reliable values. On Gyrolab, the sensitivity and affinity could be investigated by looking at the gradients of the standard curves, the responses, and the column profiles. The choice of reagents given from the optimization on Gyrolab was used on ELISA as well, but the different concentrations of these reagents required optimization specific for ELISA. Different checkerboards were performed on ELISA, for which different parameters were investigated by looking at the signal/background values. Eight different assays were developed and optimized: two PK and two PD assays on both Gyrolab and ELISA, with Avastin and Lucentis as the drug. For the optimization experiments, duplicates were performed.

4.2.1 Optimization of assays on Gyrolab

For the PK assay on Gyrolab with Avastin as the drug, the capture bVEGF concentration was evaluated by titrating the bVEGF concentration by diluting it with bBSA. Different detecting antibodies with different concentrations and different CD types were also evaluated. With Lucentis as the drug, different detecting antibody concentrations were evaluated. The detecting antibody kLC was the only one binding to the Fab region of the antibody, and therefore, this antibody was used for the PK assay with Lucentis as the drug since Lucentis only consists of one Fab region. The optimization experiments can be seen in Table 5.

Table 5. The optimization experiments for the PK assays on Gyrolab, with Avastin and Lucentis as the drug.

Exp.	Capture	Capture conc. (nM)	Analyte	Analyte conc. (ng/mL)	Detect	Detect conc. (nM)	CD
1	bVEGF	296	Avastin	0, 1, 50, 2500	H2, 4D2D9G8, kLC, JDC-10	5, 10, 20	Bioaffy 200
2	bVEGF diluted in bBSA	296 148, 74, 37	Avastin	0, 1, 50, 2500	H2, kLC, JDC-10	H2: 5, 10, 20, the rest: 10	Bioaffy 200
3	bVEGF diluted in bBSA	296 148, 74, 37	Avastin	0, 1, 50, 2500	H2, kLC, JDC-10	10	Bioaffy 1000 HC
4	bVEGF diluted in bBSA	296, 148	Avastin	0, 1, 50, 2500	H2, kLC, JDC-10	10, 20, 40	Bioaffy 1000 HC
5	bVEGF	296	Lucentis	0, 0.2, 1, 5, 25, 125, 625, 3125	kLC	5, 10, 20	Bioaffy 1000 HC

A lower limit of quantification (LLOQ) test was also performed for the PK assay with Avastin as the drug, with different quality control samples. The LLOQ was estimated by performing three runs with standard curves, newly prepared for each run, and QC samples. By following the “Guideline on bioanalytical method validation” (Committee for Medicinal Products for Human Use 2011), the QC sample that held the requirements of (CV concentration % + |average bias %|) < 40, (CV concentration %) < 25 and (|average bias %|) < 25 for two of the three runs was estimated as the LLOQ for the assay. The experiment performed for this test can be seen in Table 6 and was performed three times.

Table 6. The experimental setup for the test of the lower limit of quantification (LLOQ) for the PK assay with Avastin as the drug. This setup was performed three times. QC meaning quality control.

Capture	Capture conc. (nM)	Standard curve (ng/mL Avastin)	QC samples (ng/mL Avastin)	Detect	Detect conc. (nM)	CD
bVEGF diluted in bBSA	148	0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 4	0.025, 0.5, 0.1, 0.2	H2	20	Bioaffy 1000 HC

For the PD assay on Gyrolab with Avastin as the drug, different detecting antibodies with different concentrations, and different CD types were evaluated. The same capture reagent and concentration was used for all experiments. With Lucentis as the drug, different detecting antibody concentrations were evaluated. The same CD type, capture reagent, and concentration were used for all experiments. The optimization experiments for the PD assays on Gyrolab can be seen in Table 7.

Table 7. The optimization experiments for the PD assays on Gyrolab, with Avastin and Lucentis as the drug.

Exp.	Capture	Capture conc. (µg/mL)	Analyte	Analyte conc. (ng/mL)	Detect	Detect conc. (nM)	CD
1	bAvastin	100	VEGF	0, 0.01, 0.04, 0.16, 0.64, 2.56, 10.24, 40.96	α-VEGF mAb, α-VEGF pAb (1)	5, 10, 20	Bioaffy 1000
2	bAvastin	100	VEGF	0, 0.01, 0.04, 0.16, 0.64, 2.56, 10.24, 40.96	α-VEGF mAb, α-VEGF pAb (1)	5, 10, 20	Bioaffy 1000 HC
3	bAvastin	100	VEGF	0, 0.1, 0.4, 1.6, 6.4, 25.6, 102.4, 409.6	α-VEGF mAb, α-VEGF pAb (1)	5, 10, 20	Bioaffy 1000 HC
4	bAvastin	100	VEGF	0, 0.1, 0.4, 1.6, 6.4, 25.6, 102.4, 409.6	α-VEGF mAb, α-VEGF pAb (2)	5, 10, 20	Bioaffy 1000 HC
5	bLucentis	32	VEGF	0, 5, 20, 80, 320, 1280, 5120, 20480	α-VEGF pAb (2)	5, 10, 20	Bioaffy 1000 HC

For the PD assay with Avastin as the drug, a LLOQ test was performed with different quality control samples. The LLOQ was estimated by performing three runs with standard curves, newly prepared for each run, and QC samples. By following the "Guideline on bioanalytical method validation" (Committee for Medicinal Products for Human Use 2011), the QC sample that managed the requirements of (CV concentration % + |average bias %|) < 40, (CV concentration %) < 25 and (|average bias %|) < 25 for two of the three runs was estimated as

the LLOQ for the assay. The experiment performed for this test can be seen in Table 8 and was performed three times.

Table 8. The experimental setup for the test of the lower limit of quantification (LLOQ) for the PD assay with Avastin as the drug. This setup was performed three times. QC meaning quality control.

Capture	Capture conc. (nM)	Standard curve (pg/mL VEGF)	QC samples (pg/mL VEGF)	Detect	Detect conc. (nM)	CD
bAvastin	100	0, 2.5, 5, 10, 20, 60, 180, 360, 720	2.5, 5, 10, 20	α-VEGF pAb (2)	10	Bioaffy 1000 HC

Other parameters that were not changed during the optimization for the PK and PD assays on Gyrolab were different buffers to be seen in Table 9.

Table 9. Different buffers used for the assays on Gyrolab.

Capture buffer	Analyte buffer	Detect buffer
PBS-T (PBS + 0.01% Tween20)	RexxipA	RexxipF

4.2.2 Optimization of assays on ELISA

The optimization experiments for the PK assays on ELISA included different concentrations of the capture reagent, analyte concentrations, detect concentrations and streptavidin-HRP dilutions. Different checkerboards were performed to evaluate these concentrations, and can be seen in Table 10.

Table 10. The optimization experiments for the PK assays on ELISA, with Avastin and Lucentis as the drug.

Exp.	Capture	Capture conc. (µg/mL)	Analyte	Analyte conc. (ng/mL)	Detect	Detect conc. (ng/mL)	Streptavidin-HRP dilution
1	VEGF	0.2, 1, 5, 10	Avastin	0, 2, 20	bH2	200, 1000, 5000, 10 000	1:1000
2	VEGF	1	Avastin	0, 0.05, 0.2, 0.5, 1.25, 2	bH2	0, 12.5, 50, 200	1:1000, 1:2000
3	VEGF	1	Avastin	0, 0.02, 0.05, 0.2, 0.5, 1.25, 2, 5, 12.5, 25, 50, 100	bH2	20, 50	1:500, 1:1000
4	VEGF	0.2, 1, 5	Lucentis	0, 1.3, 13	bkLC	4, 20, 100	1:1000
5	VEGF	2.5, 5, 10	Lucentis	0, 1.5, 15	bkLC	100, 300, 900	1:1000

The optimization experiments for the PD assays on ELISA, with different checkerboards evaluating different capture concentrations, analyte concentrations, detect concentrations, and streptavidin-HRP dilutions, can be seen in Table 11.

Table 11. The optimization experiments for the PD assays on ELISA, with Avastin and Lucentis as the drug.

Exp.	Capture	Capture conc. (µg/mL)	Analyte	Analyte conc. (pg/mL)	Detect	Detect conc. (µg/mL)	Streptavidin-HRP dilution
1	Avastin	0.2, 1, 5, 10	VEGF	0, 5, 50	bα-VEGF pAb (2)	0.05, 0.5, 2.5, 5	1:1000
2	Avastin	5, 10, 20, 40	VEGF	0, 25, 250	bα-VEGF pAb (2)	0.25, 0.5, 1, 2	1:1000
3	Avastin	20, 40, 60, 80	VEGF	0, 2.5, 5, 10, 20, 40, 60, 90, 135, 200, 300, 450	bα-VEGF pAb (2)	1	1:1000
4	Lucentis	10, 20, 40	VEGF	0, 50, 500	bα-VEGF pAb (2)	0.5, 1, 2	1:1000

Other parameters that were not changed during the optimization for the PK and PD assays on ELISA were different buffers to be seen in Table 12. Recipes for the different buffers can be seen in Appendix F.

Table 12. Buffers used for the assays performed on ELISA.

Washing buffer	Blocking buffer	Capture buffer	Analyte buffer	Detect buffer
PBS + 0.05% Tween20	PBS + 1% BSA	PBS	Blocking buffer	Blocking buffer

4.3 Measuring free analyte

Both the PK and the PD assays were performed on Gyrolab and ELISA to measure free analyte. For the PK assays, this was done by preparing different samples with a fixed concentration of the drug (Avastin or Lucentis) and titration of the ligand (VEGF). For the PD assays, it was done by preparing different samples with a fixed concentration of the ligand (VEGF) and titration of the drug (Avastin or Lucentis). The samples were incubated a certain time to reach equilibrium and were then measured, with triplicates, on both Gyrolab and ELISA in parallel. The preparation and dilution of the samples was done in the same way

with the same dilutions and volumes for Gyrolab and ELISA, to achieve conditions that were as similar as possible.

4.3.1 Optimization measuring free analyte

For the measurements of free analyte on Gyrolab, optimization work was performed to investigate which concentrations of Avastin/Lucentis and VEGF that should be used to be able to measure free analyte with good signal. The incubation time for the samples (Avastin/Lucentis + VEGF) was also investigated to see for how long the samples needed to be incubated to reach equilibrium. The experiments from the optimization of measurement of free analyte with the PK assays on Gyrolab can be seen in Table 13. This optimization was only performed on Gyrolab, the chosen concentrations and incubation time was used in the same way on ELISA.

Table 13. The different optimization experiments to evaluate concentrations of Avastin/Lucentis and VEGF, and incubation time, for the measurement of free analyte with the PK assays on Gyrolab.

Exp.	**Capture**	**Avastin/ Lucentis**	**VEGF conc. (ng/mL)**	**Incubation time** (until start of the method)	**Detect**	**CD**
1	bVEGF diluted in bBSA (148 nM)	1, 3, 5 ng/mL Avastin	0, 0.01, 0.05, 0.2, 0.5, 2, 8, 20, 50	1-1.5 h at RT	H2 (20 nM)	Bioaffy 1000 HC
2	bVEGF diluted in bBSA (148 nM)	1, 4, 16 ng/mL Avastin	0, 0.5, 5, 15, 45, 90, 135, 202.5, 303.75	1 h 40 min at RT	H2 (20 nM)	Bioaffy 1000 HC
3	bVEGF diluted in bBSA (148 nM)	1, 4, 16 ng/mL Avastin	0, 0.5, 5, 15, 45, 90, 135, 202.5, 303.75	3 h 20 min at RT	H2 (20 nM)	Bioaffy 1000 HC
4	bVEGF diluted in bBSA (148 nM)	1, 4, 16 ng/mL Avastin	0, 0.5, 5, 15, 45, 90, 135, 202.5, 303.75	20 h 17 min (16 h at +4 C˚)	H2 (20 nM)	Bioaffy 1000 HC
5	bVEGF (296 nM)	4, 8 ng/mL Lucentis	0, 15.6, 46.9, 140.6, 281.3, 421.9, 633.75, 948.75, 1897.5, 3797	22 h 30 min at +4 C˚	kLC (10 nM)	Bioaffy 1000 HC

The experiments from the optimization of measurement of free analyte with the PD assays on Gyrolab, evaluating different concentrations of VEGF and Avastin/Lucentis and required incubation time for the samples to reach equilibrium, can be seen in Table 14.

Table 14. The different optimization experiments to evaluate concentrations of Avastin/Lucentis and VEGF, and incubation time, for the measurement of free analyte with the PD assays on Gyrolab.

Exp.	**Capture**	**VEGF conc. (pg/mL)**	**Avastin/ Lucentis**	**Incubation time** (until start of the method)	**Detect**	**CD**
1	bAvastin (100 µg/mL)	20, 60, 180	0, 1, 3, 9, 18, 27, 40.5, 60.75, 91.125 ng/mL Avastin	5 h at RT	α-VEGF pAb (2) (10 nM)	Bioaffy 1000 HC
2	bAvastin (100 µg/mL)	90, 180, 360	0, 0.5, 5, 15, 45, 90, 135, 202.5, 303.75 ng/mL Avastin	1 h 40 min at RT	α-VEGF pAb (2) (10 nM)	Bioaffy 1000 HC
3	bAvastin (100 µg/mL)	90, 180, 360	0, 0.5, 5, 15, 45, 90, 135, 202.5, 303.75 ng/mL Avastin	3 h 20 min at RT	α-VEGF pAb (2) (10 nM)	Bioaffy 1000 HC
4	bAvastin (100 µg/mL)	90, 180, 360	0, 0.5, 5, 15, 45, 90, 135, 202.5, 303.75 ng/mL Avastin	20 h 17 min (16 h at +4 C˚ degrees)	α-VEGF pAb (2) (10 nM)	Bioaffy 1000 HC
5	bLucentis (32 µg/mL)	360, 720	0, 5, 15, 45, 90, 135, 270, 540, 1080, 2160, 4320, 8640 ng/mL Lucentis	22 h 35 min at +4 C˚	α-VEGF pAb (2) (10 nM)	Bioaffy 1000 HC

4.4 Calculation of IC_{50}/K_D values

From the obtained concentrations of free analyte, IC_{50} values were calculated with GraphPad Prism 8 with a *logarithm of inhibitor versus response curve*. The inhibitor was, in this case, VEGF in the PK assay and Avastin/Lucentis in the PD assay. The response was the measure of free analyte, Avastin/Lucentis in the PK assay, and VEGF in the PD assay. The concentrations were transformed to the unit nM and the 10-logarithm was used on the inhibitor concentrations, on the x-axis. All the values that were included in the graphs were also included in these calculations, even though some of them were uncertain due to low signal/background values and/or values below estimated LLOQ. The IC_{50} values were estimated to be approximately the same as the K_D values in equilibrium.

5 Results

5.1 Optimization of PK and PD assays on Gyrolab

To be able to decide the setup of the PK and PD assays on Gyrolab, optimization work was performed with different capture concentrations, analyte concentrations, detecting antibodies and concentrations, and different CD types. Figures with the results can be seen in Appendix A and Appendix B, evaluating sensitivity, response, and affinity. The optimization on Gyrolab generated in one PK and one PD assay with both Avastin and Lucentis as the drug, which can be seen in Table 15.

Table 15. The optimized PK and PD assays on Gyrolab, with Avastin and Lucentis as the drug. LLOQ meaning lower limit of quantification.

	Capture	Capture conc.	Analyte	LLOQ analyte[1]	Detect	Detect conc. (nM)	CD
PK	bVEGF	148 nM	Avastin	0.05 ng/mL	H2	20	Bioaffy 1000 HC
PD	bAvastin	100 µg/mL	VEGF	20 pg/mL	α-VEGF pAb (2)	10	Bioaffy 1000 HC
PK	bVEGF	296 nM	Lucentis	-	kLC	10	Bioaffy 1000 HC
PD	bLucentis	32 µg/mL	VEGF	-	α-VEGF pAb (2)	10	Bioaffy 1000 HC

5.2 Optimization of PK and PD assays on ELISA

For ELISA, checkerboards were performed to be able to decide which concentrations that should be used with the same setup of reagents as for Gyrolab, by analysing the signal/background values (see Appendix C). This optimization work lead to one PK and one PD assay with both Avastin and Lucentis as the drug, which can be seen in Table 16.

[1] The lower limit of quantification (LLOQ) is estimated from three runs with standard curves, newly prepared for each run, and quality control samples. The requirements were set to a (CV concentration % + |average bias %|) < 40, (CV concentration %) < 25 and (|average bias %|) < 25. Estimating those values as LLOQ, two of three runs succeeded with the requirements.

Table 16. The optimized PK and PD assays on ELISA, with Avastin and Lucentis as the drug.

	Capture	Capture conc.	Analyte	Detect	Detect conc.	Streptavidin-HRP
PK	VEGF	1 µg/mL	Avastin	bH2	20 ng/mL	1:1000 dilution
PD	Avastin	60 µg/mL	VEGF	bα-VEGF pAb (2)	1 µg/mL	1:1000 dilution
PK	VEGF	2.5 µg/mL	Lucentis	bkLC	1 µg/mL	1:1000 dilution
PD	Lucentis	10 µg/mL	VEGF	bα-VEGF pAb (2)	0.5 µg/mL	1:1000 dilution

5.3 Optimization of PK and PD assays measuring free analyte on Gyrolab

Different concentrations and ratios of Avastin and VEGF, and different incubation times for these samples were evaluated to be able to measure free analyte in a good way. This was performed for both the PK and the PD assays. For the PK assay, this resulted in a range of VEGF:Avastin that was optimal to use, and in a required incubation time of 24 hours at +4 C˚, since there was no significant difference in the measurement of free analyte after 24 hours (see Figure D1 in Appendix D). An incubation time of 24 hours was used later in the project. For the PD assay, the optimization resulted in a required incubation time of 22 hours and 19 minutes at +4 C˚ for the VEGF:Avastin samples (see in Figure D2 in Appendix D). An incubation time of 23 hours was used later in the project. For the PK and the PD assay with Lucentis as the drug, different concentrations and ratios of the samples were evaluated, but the same incubation times as optimized for Avastin were used.

5.4 Gyrolab versus ELISA, measuring free analyte with the PK assay

To be able to investigate if the short contact time for Gyrolab will not generate an overestimation of free analyte compared to ELISA, the measurement of free analyte was performed on both Gyrolab and ELISA, with two different sample incubation times on ELISA. In a PK assay, the drug is measured, and therefore, free Avastin or Lucentis was measured.

5.4.1 Avastin as the drug

In Figure 12, the measurement of free Avastin for different ratios of Avastin:VEGF can be seen, with Gyrolab and ELISA with two hours of sample incubation. Overall, ELISA measured higher concentrations of free Avastin than Gyrolab did.

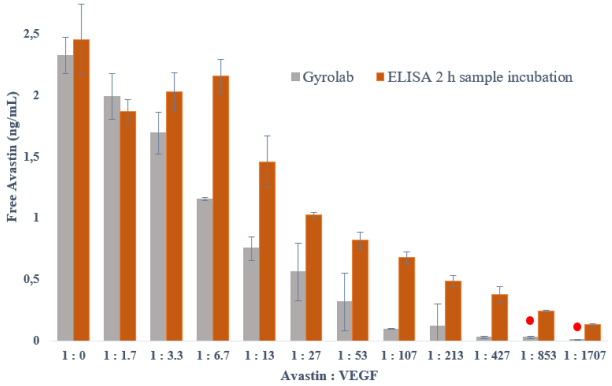

Figure 12. Free Avastin measured at different ratios of Avastin:VEGF, with a standard deviation for each mean value, Gyrolab in grey and ELISA with two hours of sample incubation in brown. The red dots indicate values below LLOQ for Gyrolab. Those marked values are uncertain.

The same experiment was performed with both two and four hours of sample incubation on ELISA (see Figure 13) where Gyrolab measured the lowest concentrations, ELISA with two hours of sample incubation measured higher concentrations and ELISA with four hours of sample incubation measured the highest concentrations of free Avastin.

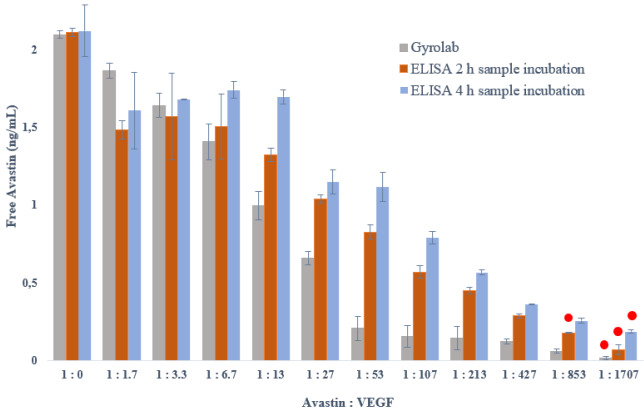

Figure 13. Free Avastin measured at different ratios of Avastin:VEGF, with a standard deviation for each mean value, Gyrolab in grey, ELISA with two hours of sample incubation in brown and ELISA with four hours of sample incubation in blue. The red dots indicate values below LLOQ for Gyrolab. For ELISA, the red dots indicate a signal/background value below 2 and a value below the lowest standard point. Those marked values are uncertain.

5.4.2 Lucentis as the drug

Free Lucentis was also measured and compared between Gyrolab and ELISA with different ratios of Lucentis:VEGF. ELISA with two hours of sample incubation measured slightly higher concentrations of free Lucentis than Gyrolab did, as can be seen in Figure 14a and b. However, there was almost no difference at all when comparing two and four hours of sample incubation on ELISA.

a) b)

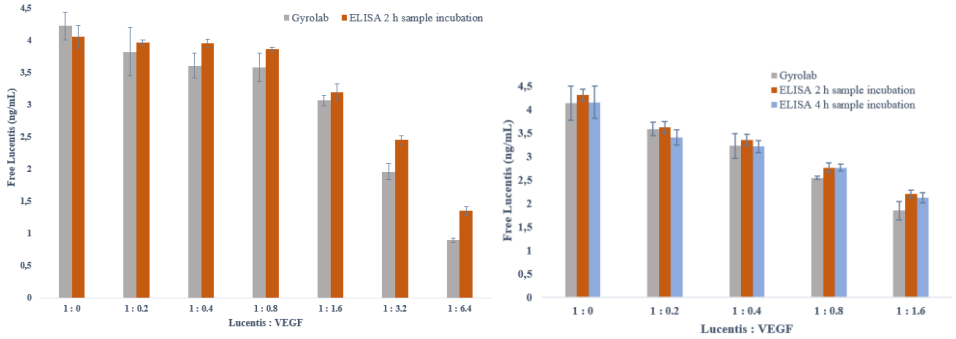

Figure 14. Free Lucentis measured at different ratios of Lucentis:VEGF, with a standard deviation for each mean value. a) Comparison between Gyrolab (grey) and ELISA with two hours of sample incubation (brown). b) Comparison between Gyrolab (grey), ELISA with two hours of sample incubation (brown) and ELISA with four hours of sample incubation (blue).

5.5 Gyrolab versus ELISA, measuring free analyte with the PD assay

The measurement of free analyte, and the difference between Gyrolab and ELISA, was also investigated with the PD assays. In a PD assay, the ligand is measured, and therefore, the free analyte was free VEGF. The experiments were performed with both Avastin and Lucentis as the drug, which in the PD assays work as capture reagents.

5.5.1 Avastin as the drug

The measurements with Avastin as the capture reagent were performed with a fixed concentration of VEGF and different concentrations of Avastin. In Figure 15, the comparison between Gyrolab and ELISA with two hours of sample incubation can be seen. From a molar ratio of 1:75 of VEGF:Avastin, ELISA measured higher or approximately the same concentrations of free VEGF compared to Gyrolab. For molar ratios below that, Gyrolab measured higher or approximately the same concentrations of free VEGF compared to ELISA.

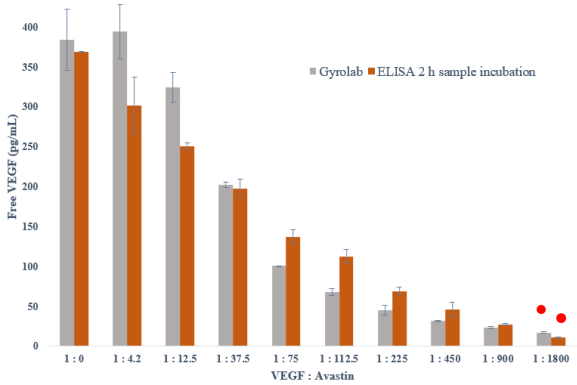

Figure 15. Concentration of free VEGF for different ratios of VEGF:Avastin with a standard deviation for each mean value, Gyrolab in grey and ELISA with two hours of sample incubation in brown. The red dots indicate values below LLOQ for Gyrolab. For ELISA, the red dots indicate a signal/background value below 2 and a value below the lowest standard point. Those marked values are uncertain.

The comparison between Gyrolab, ELISA with two hours of sample incubation, and ELISA with four hours of sample incubation can be seen in Figure 16, with the same pattern as the previous comparison between Gyrolab and ELISA with the PD assay and Avastin as the drug (see Figure 15). From a molar ratio of 1:75 of VEGF:Avastin, Gyrolab measured the lowest concentrations, ELISA with two hours of sample incubation measured higher concentrations and ELISA with four hours of sample incubation measured the highest concentrations of free VEGF. For molar ratios below that point, Gyrolab measured higher or approximately the same concentrations of free VEGF compared to ELISA.

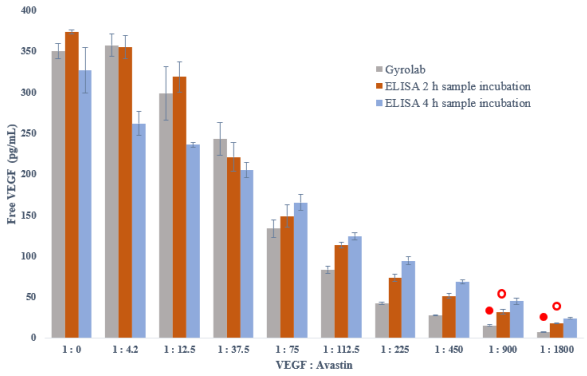

Figure 16. Concentration of free VEGF for different ratios of VEGF:Avastin with a standard deviation for each mean value, Gyrolab in grey, ELISA with two hours of sample incubation in brown and ELISA with four hours of sample incubation in blue. The red dots indicate values below LLOQ for Gyrolab. For ELISA, the red circles indicate a signal/background value below 2. Those marked values are uncertain.

5.5.2 Lucentis as the drug

The measurements of free VEGF with Lucentis as the capture reagent was performed with a fixed concentration of VEGF and different concentrations of Lucentis. The comparison between Gyrolab and ELISA with two hours of sample incubation can be seen in Figure 17a and the comparison between Gyrolab, ELISA with two hours of sample incubation and ELISA with four hours of sample incubation can be seen in Figure 17b. No obvious pattern could be seen regarding which method estimating free VEGF the highest. ELISA measured a slightly lower concentration of free VEGF compared to Gyrolab in the comparison between Gyrolab and ELISA with two hours of sample incubation, as can be seen in Figure 17a. For the comparison between Gyrolab and ELISA with both two and four hours of sample incubation (see Figure 17b), ELISA measured slightly higher concentrations of free VEGF from a molar ratio of 1:19 of VEGF:Lucentis. Although, the differences were not significant and there was almost no difference between ELISA with two and four hours of sample incubation.

a) b)

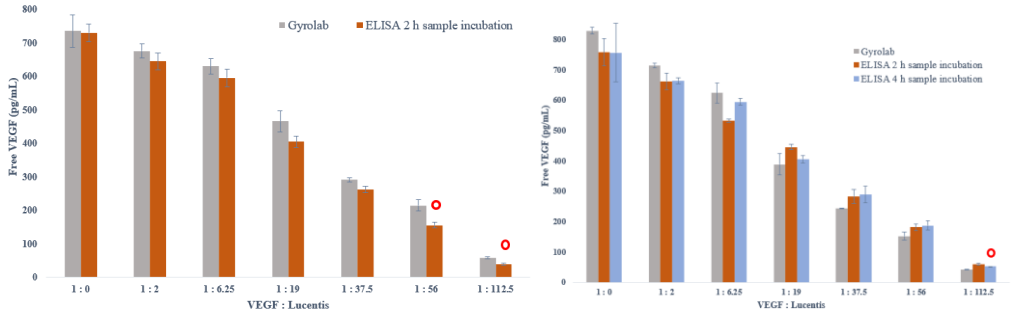

Figure 17. Concentration of free VEGF for different ratios of VEGF:Lucentis with a standard deviation for each mean value. a) Comparison between Gyrolab (grey) and ELISA with two hours of sample incubation (brown). b) Comparison between Gyrolab (grey), ELISA with two hours of sample incubation (brown) and ELISA with four hours of sample incubation (blue). The red circles indicate a signal/background value below 2. Those marked values are uncertain.

5.6 Gyrolab versus ELISA IC_{50}/K_D values

To be able to compare the measurements of free analyte on Gyrolab and ELISA with actual numbers, IC_{50} values were calculated and estimated to be approximately the same as the K_D values, which can be seen in Table 17. There was a significant difference between the K_D values for Gyrolab and ELISA with the PK assay and Avastin as the drug. There was also a huge difference in the second run with the PD assay and Lucentis as the drug. Although, there were no big differences for the other assays. Some of the runs did not reach a bottom plateau in detected concentrations of free analyte, and therefore, the IC_{50} values are not that reliable for all runs. The curve fits for the determination of the IC_{50} values performed in GraphPad Prism can be seen in Appendix E.

Table 17. IC_{50} values, estimated to be approximately the same as the K_D values, when measuring free analyte with the different assays and methods. An estimation of the curve fitting was done to be able to judge the reliability of the values.

Assay run	Drug	IC_{50}/K_D Gyrolab (nM)	IC_{50}/K_D ELISA 2 h sample incubation (nM)	IC_{50}/K_D ELISA 4 h sample incubation (nM)	Estimation of curve fitting generating IC_{50} values[2]
PK run 1	Avastin	0.09589	0.3185	-	Good – OK – -
PK run 2	Avastin	0.1564	0.5328	0.8944	Good – OK – OK
PD run 1	Avastin	0.2791	0.3353	-	Good – Good – -
PD run 2	Avastin	0.4329	0.3813	0.7250	Good – Good – OK
PK run 1	Lucentis	Ambiguous	0.1410	-	Bad – Bad – -
PK run 2	Lucentis	0.1688	0.1204	Ambiguous	OK – Bad – Bad
PD run 1	Lucentis	0.6871	0.5806	-	OK – OK – -
PD run 2	Lucentis	0.3973	2.015	0.8368	OK – OK – OK

6 Discussion

As described in the background, it is important but not that easy to measure free analyte in a good way with reliable results. A prolonged sample incubation can affect the equilibrium in the samples, due to the dissociation that occurs after some time, depending on the affinity. When components dissociate from complexes, they may bind to the assay instead and will, therefore, be measured as free, leading to an overestimation of free analyte. In Figure 18, an example of this situation is illustrated.

[2] The estimation of the curve fitting generating the different IC_{50} values was based on the looks of the curve fits. If the curve fitting curves had both a top and a bottom plateau they were estimated as “Good”. If they sort of reached a plateau or at least had points at low concentrations, they were estimated as “OK”. If they only had a few measured concentrations and did not have any distinct plateau, they were estimated as “Bad”. The curve fitting curves can be seen in Appendix E.

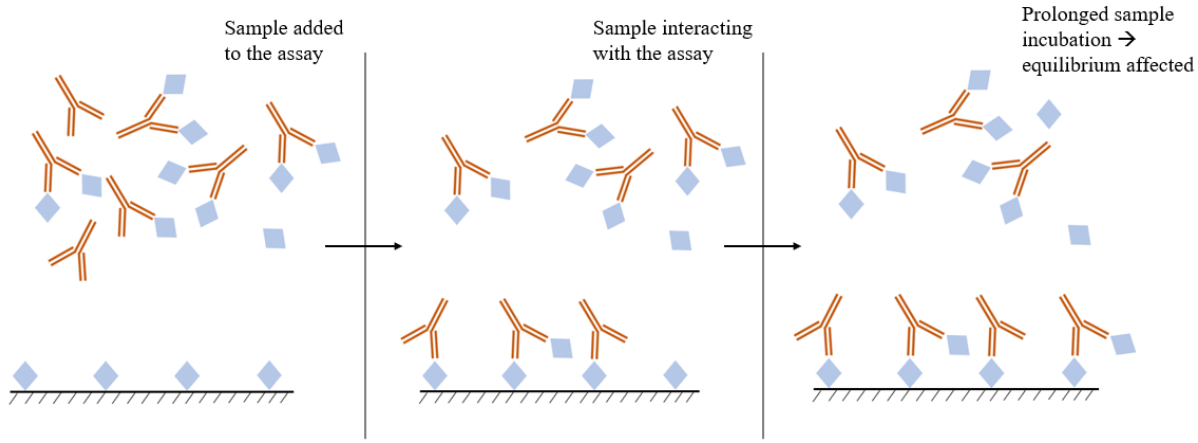

Figure 18. A potential situation where the equilibrium in the sample gets affected by the prolonged incubation. This example describes a PK assay in which free Avastin is measured. First, the sample is added to the assay. Thereafter, the sample interacts with the assay, Avastin molecules that have at least one VEGF binding site free will bind to the assay. Then, if the sample incubation is prolonged, the equilibrium in the sample can be affected when Avastin:VEGF complexes dissociate and Avastin can bind to the assay instead, leading to an overestimation of free Avastin.

The difference between Avastin and Lucentis is that Lucentis is an affinity-matured Fab region derived from Avastin, which means that Lucentis has a lower K_D value and higher affinity to VEGF than Avastin has (Papadopoulos *et al.* 2012). Therefore, the equilibrium should be affected easier when using Avastin as the drug compared to using Lucentis. Looking at k_{off} values in literature and calculating the halftime of dissociation for the components (see Table 1), it would take approximately 50-140 minutes for Avastin:VEGF (Khalili *et al.* 2012, Wang *et al.* 2014, Yang *et al.* 2014) and approximately 1500 minutes for Lucentis:VEGF (Lowe *et al.* 2007, Papadopoulos *et al.* 2012, Yang *et al.* 2014). Those numbers indicate that it requires a much longer time to affect the equilibrium for the samples with Lucentis and VEGF compared to the samples with Avastin and VEGF. For Avastin:VEGF, the halftime of dissociation probably happens within the sample incubation time on ELISA, while it does not for Lucentis:VEGF. Therefore, the experiments with Lucentis and VEGF can be seen as negative controls in this project.

6.1 How were the assays chosen?

The choice of the different parameters, such as capture concentration, detecting antibody and its concentration, and CD type was based on the sensitivity, affinity, background, and response values for the assays while evaluating different options. For Gyrolab, the sensitivity could be evaluated by looking at the concentration versus response curves, to see which detecting antibody managing to detect the lowest concentration of the analyte. By looking at those curves, the background and response values could also be evaluated, by comparing different curves with each other. As an example, one curve could represent a specific capture concentration, detecting antibody and concentration, and a specific CD, while another curve could represent all the same components except the detecting antibody. In that way, the choice of detecting antibody could be made, and so on. The affinity could also be evaluated

on Gyrolab by looking at the column profiles, as described in the background. The narrower the distribution of the column profiles, the higher affinity (Honda *et al.* 2005). With Gyrolab, the choice of CD type could be made both by looking at the concentration versus response curves, to investigate the background and response values etcetera, and by looking at the column profiles, which one giving the best precision data.

Overall, all assays could be evaluated by examining the coefficient of variation (CV), accepting a CV of ~10% and lower. On ELISA, the assays were evaluated by looking at the signal/background values and CV values. The evaluated concentration with the highest signal/background and not a huge variance was chosen. Although, only duplicates were performed during the optimization since there are a limited number of wells per ELISA plate and one plate requires much work time, so, therefore, the variances were not that reliable. Anyhow, the setup of the assays was decided based on the optimization work on Gyrolab, while the concentrations of the reagents required specific optimization for ELISA.

6.2 Measuring free analyte with the PK assay and Avastin as the drug

In the PK assay with Avastin as the drug, free Avastin was measured for different ratios of Avastin:VEGF. At low molar ratios of VEGF, 1:1 complex will be formed (Sumner *et al.* 2019), meaning that one binding site of Avastin will still be available. So, at those low molar ratios, all Avastin in the sample will bind to the assay since at least one binding site for VEGF is available. When the molar ratios are higher, 1:2 complexes of Avastin:VEGF will be formed and for even higher molar ratios, even higher orders of complexes may be formed (Sumner *et al.* 2019). This means that the equilibrium can be affected at a bit higher molar ratio of VEGF, when both binding sites of Avastin are occupied and not all Avastin molecules in the sample should bind to the assay.

6.2.1 How did Gyrolab and ELISA differ?

Looking at Figure 12 and Figure 13, it could be seen that ELISA with two hours of sample incubation measured higher concentrations of free Avastin compared to Gyrolab, and ELISA with four hours of sample incubation measured even higher concentration of free Avastin. This reinforces the theory that a prolonged sample incubation, with a couple of hours for ELISA compared to a contact time of a few seconds for Gyrolab, can affect the equilibrium in the samples. From the calculated K_D values (see Table 17) it could also be seen that there was a difference between Gyrolab and ELISA. So, both the visual results in the graphs and the actual numbers from estimating the K_D reinforces the theory with the possibility of an overestimation of free analyte when applying a longer sample incubation.

6.3 Measuring free analyte with the PD assay and Avastin as the drug

With the PD assay and Avastin as the drug, free VEGF was measured for different molar ratios of VEGF:Avastin. As for the PK assay, 1:1 complex will be formed at low molar ratios of Avastin (Sumner *et al.* 2019). This means that one binding site of Avastin and one binding site of VEGF will still be available. For higher molar ratios of Avastin, 1:2 complexes of VEGF:Avastin will be formed, and for even higher molar ratios, even higher orders of complexes may be formed (Sumner *et al.* 2019). If VEGF can bind to the assay, sterically, even though one Avastin is bound, it would mean that all VEGF molecules in the sample bind to the assay at low molar ratios of Avastin. At higher molar ratios, the equilibrium could be affected by the dissociation of at least one Avastin molecule, making it possible for the VEGF to bind to the assay instead.

6.3.1 How did Gyrolab and ELISA differ?

The results were a bit different between the PK and the PD assay with Avastin as the drug. For the PK assay, ELISA measured higher concentrations of free analyte at almost all points (except for a few where the molar ratio of VEGF was low). For the PD assay, ELISA measured higher concentrations of free analyte from a molar ratio of VEGF:Avastin of 1:75. From that specific molar ratio, the same pattern as for the PK assay appeared, with Gyrolab measuring free VEGF the lowest, ELISA with two hours sample incubation higher and ELISA with four hours sample incubation the highest (see Figure 12 and Figure 13). Before that point, both methods measured approximately the same concentrations, or Gyrolab measuring slightly higher concentrations than ELISA. Looking at the calculated K_D values (see Table 17), there was almost no difference between Gyrolab and ELISA, which makes sense since the pattern appeared from a molar ratio of VEGF:Avastin of 1:75.

One reason for this could be that it was less captured Avastin molecules per VEGF molecules in the samples for ELISA compared to Gyrolab. Even though the assays on Gyrolab and ELISA were performed as much alike as possible, it is very likely that there were some differences between them. Since the binding capacity of the ELISA plate could not be examined, it is unclear how many Avastin molecules that adsorbed to the assay. Therefore, one theory to the pattern seen for the PD assay with Avastin as the drug, is that there were enough binding sites available on the assay only from the specific molar excess of Avastin in the sample. If there would be more Avastin molecules captured on the plate, the possibilities for a VEGF molecule to bind to the assay would probably be higher. So, maybe the equilibrium was affected even before that specific molar ratio, but it did not give rise to any signal until there were enough Avastin molecules available on the assay. See an illustration of how the assay looks like step by step with a further addition of Avastin in the sample in Figure 19. If there were less Avastin molecules captured per VEGF molecules in the samples on ELISA compared to Gyrolab, it may have been necessary with a quite high molar excess of Avastin for the measurements on ELISA to be able to see the affected equilibrium.

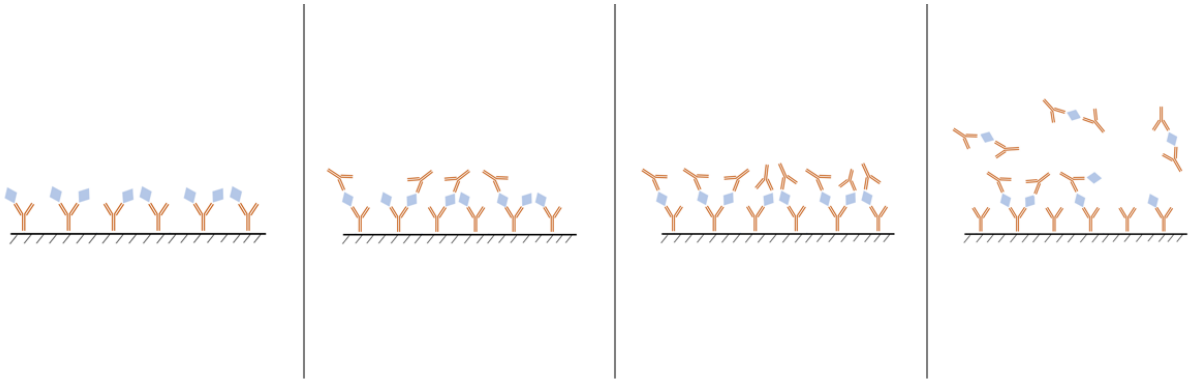

Figure 19. Each picture illustrate how it could have looked like on the PD assay with Avastin as the drug, for different samples with higher and higher concentration of Avastin. The VEGF concentration was kept the same for all samples. It can be seen in the picture to the right that there are more available Avastin molecules on the assay when more Avastin is added to the sample.

Since Avastin is a bigger molecule than VEGF, the difference should be seen at an earlier stage for the PK assay for which VEGF is the capture, which is true in this case. So, even though the equilibrium is affected, it may not be shown in the response due to the amount of capture reagent adsorbed to the assay. To examine this theory further, it may be possible to perform experiments on ELISA plates with different binding capacities to see if there would be any differences in the measurements. Something that strengthens the theory is that the results from Gyrolab and ELISA with Lucentis as the drug does not show those differences between the PK and the PD assay, where Lucentis has almost the same size as VEGF and around one third of the size of Avastin. Although, the measurements with Lucentis as the drug may not be that accurate when comparing the two methods, due to the strong affinity.

6.4 Measuring free analyte with the PK and the PD assay and Lucentis as the drug

Free Lucentis and free VEGF were also measured for different ratios of Lucentis:VEGF and VEGF:Lucentis respectively with the different assays, PK and PD. Since the antibody only consists of one Fab region, there is only one binding site for VEGF (Shahsuvaryan 2017). If one VEGF bind to one Lucentis molecule, Lucentis cannot bind to the assay. Anyhow, one VEGF molecule still has two binding sites for Lucentis, meaning that 2:1 complex can be formed with Lucentis:VEGF at higher concentrations of VEGF. So, for the PD assay, a VEGF molecule can still bind to the assay even though one Lucentis molecule is bound. Therefore, higher molar ratios of Lucentis would probably be required to push down the signals when measuring free VEGF. That theory holds for the results generated in this project (see Figure 14 and Figure 17).

Since the K_D and k_{off} value is so low for Lucentis and VEGF (Shahsuvaryan 2017), there are probably not that much that will happen regarding the equilibrium even in four hours during a sample incubation. This is also shown for the experiments performed in this project. For none of the assays, the PK and the PD, there was a significant difference between Gyrolab and

ELISA. Additionally, there was basically no difference between the measurements with two and four hours of sample incubation on ELISA either, which corresponds to the k_{off} and $t_{1/2}$ values obtained from the literature, indicating of a halftime of dissociation of around one day for Lucentis and VEGF (see Table 1). Differences between the methods and other errors in the measurements are probably the reason for the small differences that could be seen.

6.5 Comparison of K_D values

An overestimation of free analyte means that more inhibitor is required to push down the concentration of the free analyte. Therefore, the affinity between the two components is measured not to be as strong as it is when an overestimation occurs, and therefore, the K_D value should be higher. Due to this, it should be possible to compare the two methods numerically by computing the K_D values for the different experiments performed. Although, there are many ways to calculate these values and no method will give the same result as another.

In this project, GraphPad Prism 8 was used to determine the approximate K_D values by calculating the IC_{50} values. The obtained values were used to compare the two methods. If the difference was less than a 2-fold, the values were considered approximately the same, but if it was more than a 2-fold difference they were considered being different. The differences that could be seen between Gyrolab and ELISA was with the PK assay measuring free Avastin (see Table 17). This could be seen visually, looking at the graphs as well (see Figure 12 and Figure 13), in which Gyrolab estimated free Avastin lower than ELISA did. There was also a big difference between Gyrolab and ELISA with two hours of sample incubation with the PD assay and Lucentis as the drug. Although, the difference was not that big between Gyrolab and ELISA with four hours of sample incubation. Since the curve fit was not that good for those experiments, not reaching the bottom plateau, the K_D values are probably not reliable. Otherwise, there were no significant differences, which correspond to the visual results. The PD assay with Avastin as the drug showed the same pattern as the PK assay, but not until a certain molar ratio of VEGF:Avastin (see Figure 16), and therefore, the K_D values were probably not that different between Gyrolab and ELISA.

If the real K_D value is low, it is probably easier to measure the correct concentrations of free analyte even with a long sample incubation time, since the equilibrium is not that easily affected when the affinity is strong compared to when it is not as strong. This could be one reason for the K_D values (see Table 17) and the visual graphs (see Figure 14 and Figure 17) measuring free analyte with Lucentis being so similar when comparing Gyrolab and ELISA.

6.6 Limitations

6.6.1 Measurements on Gyrolab and ELISA

Even though the experiments that were performed on Gyrolab and ELISA to compare the methods were performed in parallel and as much as possible in the same way, there will always be some differences. Some of the unclear results could be due to those differences. One example is the capture, and in this project, it was impossible to know if the capture reagents had the same spacing and number of molecules for the two methods, which are likely different. Another obvious difference between the methods is the automatics, ELISA requires much more manual work compared to Gyrolab. The manual work can lead to differences in execution from time to time etcetera. For example, the washing steps could have been performed differently even though they were performed as much alike as possible. Since the comparison between the sample incubation time of two and four hours on ELISA was performed on two different plates, those results can differ a bit just due to the manual work. Another difference between the methods is the analysis of the data. When analysing results from Gyrolab, there are more than just numbers to analyse. By looking at the column profiles it can be very clear that something strange happened to some replicate, then that specific value can be excluded. When analysing the results from ELISA, there are just numbers from the spectrophotometer, and unnormal values can just be found by comparing the numbers for the replicates with each other.

There may also be different things affecting the results. One thing that was noticed when running ELISA was that the shaker generated quite much heat. Therefore, the incubation time was not performed at room temperature but slightly above. There may also have been varying temperatures different days. This could and could not have been affecting the results. The stability of the antibodies and VEGF may have been affected by the elevated temperature during a longer period and may have given rise to differences for the experiments with sample incubation times of two and four hours, as an example.

6.6.2 K_D values

Affinity constants such as K_D and k_{off} are generally hard to determine in a correct way and will never be the same when they are experimentally obtained. In literature, many different values can be found, much depending on which method that has been used but the values can differ even if practically everything is performed in the same way. Therefore, these K_D values are a bit unreliable when estimated and compared between experiments, but if there is a significant difference between experiments that have been performed as much alike as possible, it is probably an approved way of comparing the methods. As mentioned, GraphPad Prism 8 was used to determine the IC_{50} values in this project, estimated being approximately the same as the K_D values at equilibrium.

In GraphPad Prism, there is more than one way to calculate the IC_{50}, with different equations and different constraints. The equation chosen, *the logarithm of the inhibitor versus response curve*, seemed to be the best of choice for this project but has its weaknesses too. It probably

gave unreliable values for those experiments not managing to reach the bottom plateau in free analyte concentration, since the bottom value in the equation probably got higher than it was. Although, it seemed better to use that equation instead of the *Absolute IC_{50}* where a constrain for the baseline needs to be set. If that value had been set to zero, and the values with the lowest concentrations were far from zero, the probability of the curve fitting being wrong would be quite high. That would also, probably, lead to unreliable IC_{50}/K_D values. It is not sure that the values should reach zero either, they could be reaching a plateau at a value higher than zero as well, even if the results generated do not indicate of such situation.

The chosen equation, *the logarithm of the inhibitor versus response curve*, were evaluated by setting a constrain of the bottom value to zero, to be able to compare the obtained K_D values. When using all values that can be seen in the graphs, even the ones being unsure, there were almost no differences when setting the bottom value to zero or not. For some experiments, when only the certain values were included, there was a big difference in obtained K_D values when setting the bottom value to zero or not. This probably indicates that the bottom plateau was not reached for those experiments when all values were not included. Therefore, it was decided to include all values even though they had low signal/background values and/or were below the LLOQ. The estimation may not be correct due to those uncertain values, but it seemed more accurate to include those values to be able to reach the bottom plateau as good as possible. Anyhow, the obtained values were used to compare the methods by deciding that there was a difference if there was more than a 2-fold difference between K_D values. If there were less than a 2-fold difference, the values were considered approximately the same.

6.7 Conclusions

Depending on the affinity, the equilibrium in a sample will be affected after some time, and components that were bound will be unbound. Thereby, components that were not free will be detected as free, depending on the affinity and sample incubation for a specific method. From the dissociation rate k_{off}, halftime of dissociation can be calculated, estimating how long time it would take for half of the complexes in a sample to dissociate. The lower the dissociation rate, the longer the halftime of dissociation. One huge difference between the two bioanalytical methods examined in this project is that Gyrolab has a contact time of just a few seconds compared to the sample incubation time of a couple of hours for ELISA. Therefore, depending on the affinity, a difference between the measurement of free analyte with these methods should be seen. The equilibrium in a sample should not be able to be affected on Gyrolab while it might be affected on ELISA, due to the prolonged sample incubation time. This was clearly seen for the PK assay, measuring free Avastin, where the pattern with Gyrolab measuring the lowest concentration, ELISA with two hours of sample incubation measuring higher concentration, and ELISA with four hours of sample incubation measuring the highest concentration of free Avastin. These results agree with the theory of an affected equilibrium, where Avastin molecules that were bound get unbound and detected as free.

Although, all results did not agree with this theory. The results from the PD assay with Avastin as the drug showed the same pattern as for the PK assay, but not until a specific molar excess of Avastin in the sample was reached. One theory is that there may be differences between the capture on the assays on Gyrolab and ELISA. If the binding capacity on ELISA gives a lower limited number of Avastin molecules available to adsorb to the assay, which is probably the case, the probability probably gets lower that a VEGF molecule that has dissociate from a complex will bind to the assay instead. More Avastin molecules with binding sites that are captured on the assay will probably generate more possibilities for free VEGF molecules to bind to the assay. When measuring free analyte with Lucentis as the drug, there were almost no differences between Gyrolab and ELISA. That is probably due to the difference in affinity between Avastin and Lucentis, where the affinity is stronger for Lucentis, with a lower K_D and k_{off}. Since the halftime of dissociation for Lucentis and VEGF seems to be much longer than the sample incubation time for ELISA, it is reasonable that the results did not show any significant differences between the measurements on Gyrolab and ELISA. The small differences that could be seen was probably due to differences between the methods, performances, and errors in the measurements.

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Appendix A – Optimization of PK assays on Gyrolab

Optimization work was performed on Gyrolab to get a sensitive assay with reliable, stable response measurements. For the PK assay with Avastin as the drug, different capture bVEGF concentrations, detecting antibodies and concentrations, and different CD types were evaluated.

Capture concentration

In Figure A1, concentration versus response curves for different capture concentrations for the PK assay can be seen. Four different concentrations of bVEGF diluted in bBSA were evaluated, 37, 74, 148, and 296 nM. The two highest concentrations, 296 and 148 nM bVEGF, gave approximately the same sensitivity and response, while 74 and 37 nM generated worse sensitivity. Therefore, the capture concentration of 148 nM bVEGF diluted in bBSA was chosen for the PK assay on Gyrolab.

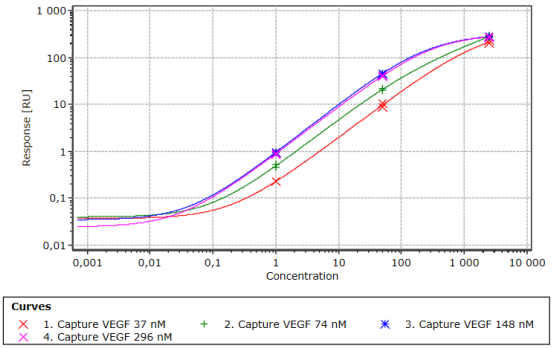

Figure A1. Test of different capture concentrations for the PK assay on Gyrolab. There are four standard curves with different capture concentrations, 296, 148, 74, and 37 nM bVEGF (diluted in bBSA). The concentration is measured in ng/mL.

Detecting antibody and concentration with Avastin as the drug

In Figure A2a and b, the evaluation of different detecting antibodies for the PK assay can be seen. All detecting antibodies except 4D2D9G8, which almost did not generate any signal at all (see Figure A2a), gave approximately the same response. The detecting antibody H2, red curve, gave the highest sensitivity since it was possible to detect Avastin at around 0.05 ng/mL using the detecting antibody H2, while it was possible to detect Avastin at around 0.1 ng/mL with the detecting antibodies kLC and JDC-10. The detecting antibody H2 gave the lowest background as well and was chosen for this PK assay on Gyrolab. Regarding the concentration, there was almost no difference between the concentrations that were evaluated, but 20 nM seemed to be the best of choice due to the low background and high responses (see Figure A2b).

a) b)

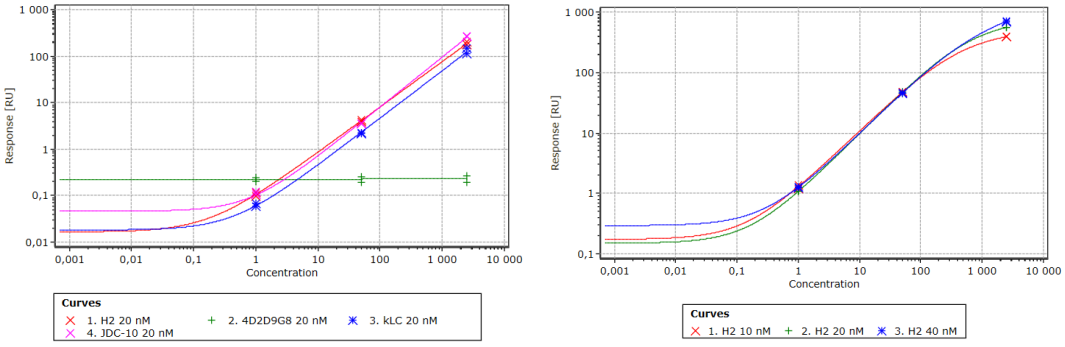

Figure A2. Test of different detecting antibodies and different concentrations for one of them, for the PK assay on Gyrolab. The concentration is measured in ng/mL. a) The detecting antibodies H2, 4D2D9G8, kLC, and JDC-10, evaluated at 20 nM. b) The detecting antibody H2, evaluated at 10, 20 and 40 nM.

Bioaffy 200 CD or Bioaffy 1000 HC CD?

Two different CD types were evaluated for the PK assay with Avastin as the drug, Bioaffy 200 CD, and Bioaffy 1000 HC CD. The first one mentioned gave a bit lower background (see Figure A3a and b), but the column profiles and precision of the data was much better with the Bioaffy 1000 HC CD (see Figure A4a and b), and therefore that one was chosen.

a) b)

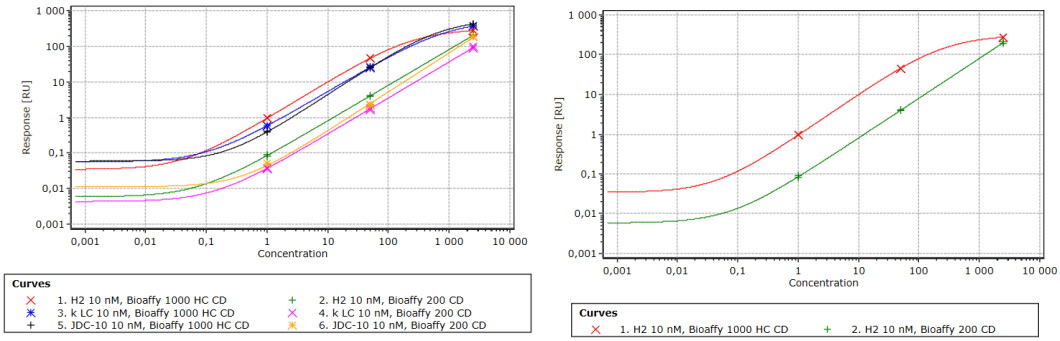

Figure A3. Comparison of Bioaffy 200 CD, and Bioaffy 1000 HC CD for the PK assay with Avastin as the drug. a) Comparison between the two CDs and three different detecting antibodies, H2, kLC and JDC-10. b) Comparison between the two CDs, with the detecting antibody H2.

a) b)

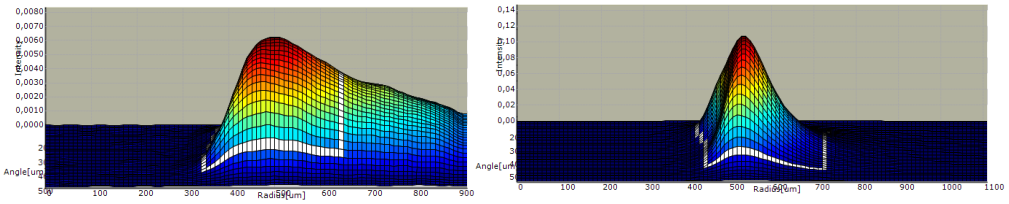

Figure A4. Comparison between column profiles generated from runs with the PK assay and Avastin as the drug. The left picture shows a column profile with Bioaffy 200 CD, the right picture shows a column profile with Bioaffy 1000 HC CD. Both pictures were generated from the same samples.

Detecting antibody concentration with Lucentis as the drug

For the PK assay with Lucentis as the drug, one detecting antibody with different concentrations was evaluated. When using the detecting antibody kLC, Lucentis could be measured from an approximate concentration of 0.5 ng/mL while Avastin could be measured from an approximate concentration of 0.05 ng/mL. Otherwise, the background and response values were approximately the same (see Figure A5b). There was almost no difference between the different concentrations, but 10 nM was chosen due to a bit lower background and still reaching high response values (see Figure A5a).

a) b)

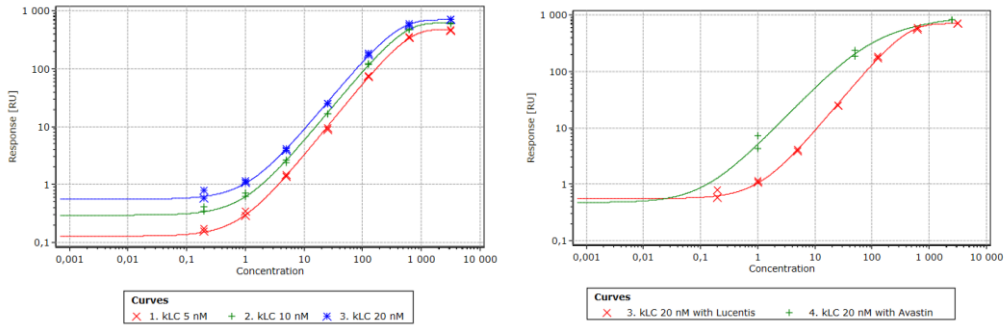

Figure A5. Comparisons with the detecting antibody kLC for the PK assay with Lucentis as the drug. a) Comparison of different concentrations, 5, 10, and 20 nM, of the detecting antibody kLC. b) Comparison between the detecting antibody kLC with Lucentis and Avastin as the analyte.

Appendix B – Optimization of PD assays on Gyrolab

For the PD assay with Avastin as the drug, different detecting antibodies and concentrations, and different CD types were evaluated on Gyrolab.

Detecting antibody (first) and CD type with Avastin as the drug

From the beginning, the detecting antibodies α-VEGF mAb and α-VEGF pAb (1) were evaluated for the PD assay. The results showed that the α-VEGF mAb was able to measure lower concentrations of VEGF than α-VEGF pAb (1) (see Figure B1a), and therefore, α-VEGF pAb (1) was eliminated. Two different CD types were evaluated as well, Bioaffy 1000 CD and Bioaffy 1000 HC CD. The first one mentioned gave a bit lower background value, while the Bioaffy 1000 HC CD gave better results in general (see Figure B1a and b), and therefore, was chosen for this assay.

a) b)

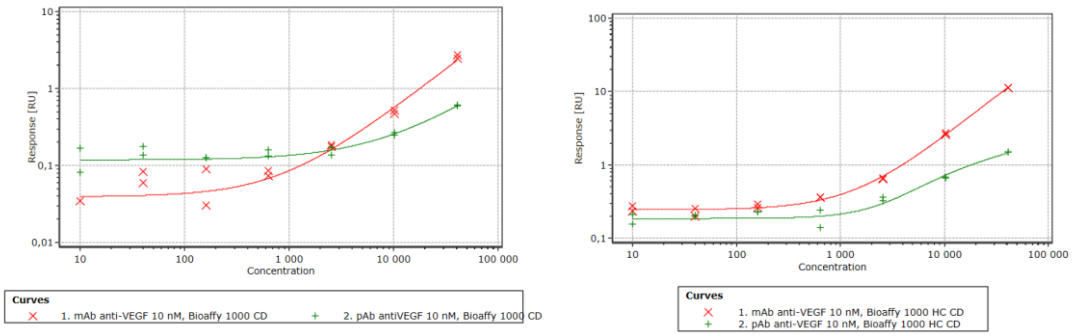

Figure B1. Comparison of different detecting antibodies, and two different CDs for the PD assay. a) Comparison of the detecting antibodies α-VEGF mAb and α-VEGF pAb (1), with the Bioaffy 1000 CD. b) Comparison of the α-VEGF mAb and α-VEGF pAb (1), with the Bioaffy 1000 HC CD.

Detecting antibody and concentration with Avastin as the drug

Another detecting antibody was evaluated for the PD assay with Avastin as the drug as well. The detecting antibody α-VEGF pAb (2) gave a much more sensitive assay compared to the α-VEGF mAb and α-VEGF pAb (1), being able to measure VEGF at around 5 pg/mL compared to the α-VEGF mAb being able to measure VEGF at around 500 pg/mL (see Figure B2a) and was therefore chosen for this assay. Three different concentrations, 5, 10 and 20 nM, were evaluated and the difference between these concentrations was small, with just a small difference in background and response values, but at 10 nM the signal/background was a little bit higher compared to the concentrations of 5 and 20 nM (see Figure B2b). Therefore, the detecting antibody α-VEGF pAb (2) with a concentration of 10 nM was chosen.

a) b)

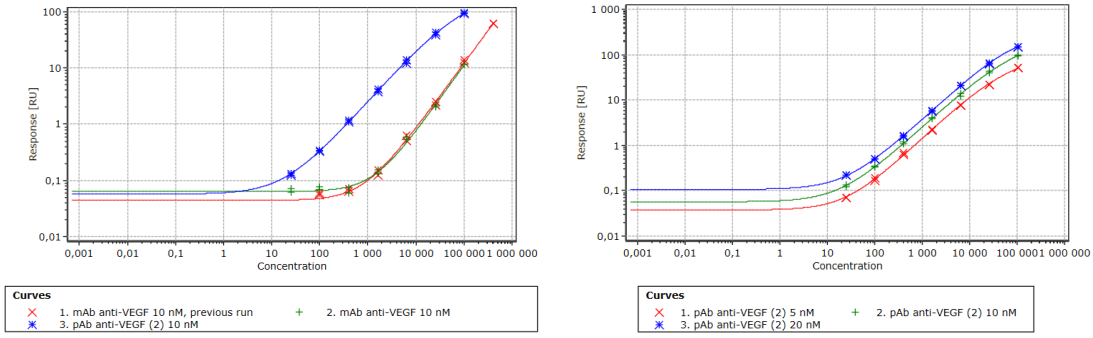

Figure B2. Test of different detecting antibodies and different concentrations for one of them, for the PD assay on Gyrolab. The concentration is measured in pg/mL. a) The detecting antibodies α-VEGF mAb and α-VEGF pAb (2), evaluated at 10 nM. b) The detecting antibody α-VEGF pAb (2), evaluated at 5, 10 and 20 nM.

Detecting antibody concentration with Lucentis as the drug

For the PD assay with Lucentis as the drug, one detecting antibody with different concentrations was evaluated. There were almost no differences for the different concentrations, but at 10 nM it seemed like the signal/background was a little bit higher compared to 5 and 20 nM (see Figure B3a), and therefore, that concentration was chosen. The detecting antibody was also compared when measured with Avastin as the drug, the background was a bit higher with Lucentis compared to Avastin, otherwise, the sensitivity and response values seemed to be approximately the same (see Figure B3b).

a) b)

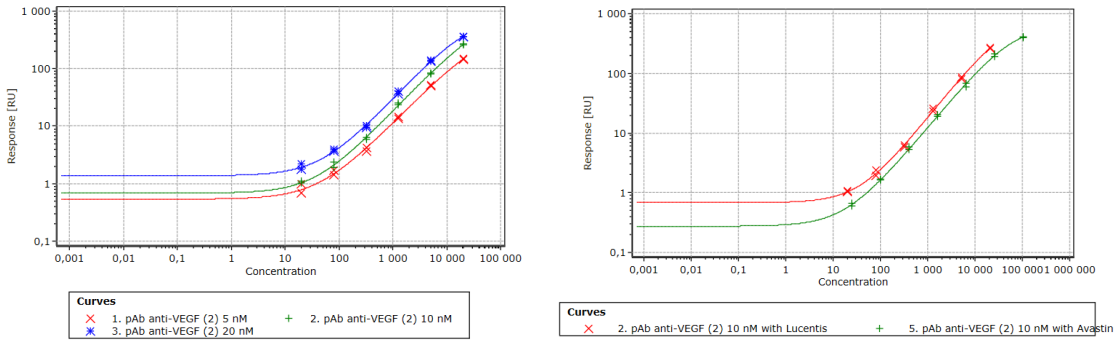

Figure B3. Comparisons of the detecting antibody α-VEGF pAb (2) for the PD assay with Lucentis as the drug. a) Comparison of different concentrations, 5, 10, and 20 nM, of the detecting antibody α-VEGF pAb (2). b) Comparison between the detecting antibody α-VEGF pAb (2) with Lucentis and Avastin as the analyte.

Appendix C – Optimization of PK and PD assays on ELISA

Optimization work was performed on ELISA as well, with different concentrations of the capture, analyte, and detecting reagent evaluated. Different streptavidin-HRP dilutions were also evaluated. The chosen concentrations were the ones giving the highest signal/background values without having a huge variation. The CV values were only calculated for duplicates, and therefore, not that reliable. Although, the average CV value for a certain concentration may indicate a too big variance.

PK assay with Avastin as the drug

In the first checkerboard, different capture VEGF and detect bH2 concentrations were evaluated. It could be seen (see Table C1) that the lowest detect bH2 concentration and 1 µg/mL capture VEGF gave the highest signal/background values.

Table C1. Average signal/background values for the first checkerboard on ELISA with the PK assay and Avastin as the drug. Different capture VEGF concentrations and detect bH2 concentrations were evaluated. Avastin was measured at 2 ng/mL. A streptavidin-HRP dilution of 1:1000 was used.

	0.2 µg/mL capture VEGF	**1 µg/mL capture VEGF**	**5 µg/mL capture VEGF**	**10 µg/mL capture VEGF**
0.2 µg/mL bH2	19.7	35.3	42.4	34.7
1 µg/mL bH2	15.3	32.8	29.6	26.3
5 µg/mL bH2	12.8	13.6	9.8	9.1
10 µg/mL bH2	9.0	10.3	6.9	5.4

There were no significant differences looking at the average CV values (see Table C2) for the different capture and detecting concentrations.

Table C2. CV values for the first checkerboard on ELISA with the PK assay and Avastin as the drug. Different capture VEGF concentrations and detect bH2 concentrations were evaluated. Avastin was measured at 2 ng/mL. A streptavidin-HRP dilution of 1:1000 was used.

	0.2 µg/mL capture VEGF	**1 µg/mL capture VEGF**	**5 µg/mL capture VEGF**	**10 µg/mL capture VEGF**
0.2 µg/mL bH2	7.0	12.5	8.0	13.7
1 µg/mL bH2	10.2	5.7	2.4	11.6
5 µg/mL bH2	5.8	2.4	1.0	10.9
10 µg/mL bH2	3.1	7.6	2.1	18.3

In the second checkerboard, different analyte Avastin concentrations, detecting bH2 concentrations and streptavidin-HRP dilutions were evaluated. These concentrations evaluated with a streptavidin-HRP dilution of 1:1000 and 1:2000 can be seen in Table C3 and Table C4 respectively. There were no significant differences between the streptavidin-HRP dilution of 1:1000 and 1:2000. The detecting concentration of 50 ng/mL gave the highest signal/background value and the analyte Avastin could be detected for the whole range from 0.05 ng/mL to 2 ng/mL.

Table C3. Average signal/background values for the second checkerboard on ELISA with Avastin as the drug. Different analyte Avastin concentrations, detecting bH2 concentrations and a streptavidin-HRP dilution of 1:1000 was evaluated. A capture concentration of 1 µg/mL VEGF was used.

Streptavidin-HRP 1:1000	0.05 ng/mL Avastin	0.2 ng/mL Avastin	0.5 ng/mL Avastin	1.25 ng/mL Avastin	2 ng/mL Avastin
12.5 ng/mL bH2	1.6	3.5	6.9	13.4	16.4
50 ng/mL bH2	1.7	3.9	7.6	13.4	16.4
200 ng/mL bH2	1.5	3.4	6.5	12.2	14.7

Table C4. Average signal/background values for the second checkerboard on ELISA with Avastin as the drug. Different analyte Avastin concentrations, detect bH2 concentrations and a streptavidin-HRP dilution of 1:2000 was evaluated. A capture concentration of 1 µg/mL VEGF was used.

Streptavidin-HRP 1:2000	0.05 ng/mL Avastin	0.2 ng/mL Avastin	0.5 ng/mL Avastin	1.25 ng/mL Avastin	2 ng/mL Avastin
12.5 ng/mL bH2	1.6	3.4	6.0	12.0	14.8
50 ng/mL bH2	1.6	3.4	6.6	13.3	16.4
200 ng/mL bH2	1.6	3.4	6.6	12.8	16.7

No huge variances could be seen for the evaluation of the different concentrations and dilutions, as can be seen in Table C5 and Table C6 with all the CV values for a streptavidin-HRP dilution of 1:1000 and 1:2000 respectively.

Table C5. CV values for the second checkerboard on ELISA with the PK assay and Avastin as the drug. Different analyte Avastin concentrations, detect bH2 concentrations and a streptavidin-HRP dilution of 1:1000 was evaluated. A capture concentration of 1 µg/mL VEGF was used.

Streptavidin-HRP 1:1000	0.05 ng/mL Avastin	0.2 ng/mL Avastin	0.5 ng/mL Avastin	1.25 ng/mL Avastin	2 ng/mL Avastin
12.5 ng/mL bH2	0.8	1.0	5.6	7.5	4.1
50 ng/mL bH2	3.2	1.5	0.5	6.3	5.0
200 ng/mL bH2	3.6	4.2	6.3	3.0	1.1

Table C6. CV values for the second checkerboard on ELISA with the PK assay and Avastin as the drug. Different analyte Avastin concentrations, detect bH2 concentrations and a streptavidin-HRP dilution of 1:2000 was evaluated. A capture concentration of 1 µg/mL VEGF was used.

Streptavidin-HRP 1:2000	0.05 ng/mL Avastin	0.2 ng/mL Avastin	0.5 ng/mL Avastin	1.25 ng/mL Avastin	2 ng/mL Avastin
12.5 ng/mL bH2	3.5	2.2	1.0	1.5	2.6
50 ng/mL bH2	1.5	3.3	0.5	3.7	0.9
200 ng/mL bH2	2.4	4.3	3.5	3.7	10.9

Different detect bH2 concentrations, analyte Avastin concentrations, and streptavidin-HRP dilutions were evaluated again in the third checkerboard. There were no big differences in the average signal background values, see Table C7, but a detect bH2 concentration of 20 ng/mL seemed to give a slightly higher value. The difference between the two streptavidin-HRP dilutions was not that high either.

Table C7. Average signal/background values for the third checkerboard on ELISA with the PK assay and Avastin as the drug. Different analyte concentrations, detect bH2 concentrations and streptavidin-HRP dilutions were evaluated. The capture VEGF concentration was 1 µg/mL.

Avastin	20 ng/mL bH2, streptavidin-HRP 1:500	50 ng/mL bH2, streptavidin-HRP 1:500	20 ng/mL bH2, streptavidin-HRP 1:1000	50 ng/mL bH2, streptavidin-HRP 1:1000
0.02 ng/mL	1.8	1.8	1.7	1.8
0.05 ng/mL	2.9	2.6	2.9	2.7
0.2 ng/mL	7.6	8.0	7.7	7.6
0.5 ng/mL	16.3	14.7	17.7	16.4
1.25 ng/mL	30.0	30.0	31.5	31.3
2 ng/mL	37.2	41.1	45.5	43.5

No huge variances could be seen for the evaluation of the different concentrations and dilutions, as can be seen in Table C8 with all the CV values.

Table C8. CV values for the third checkerboard on ELISA with the PK assay and Avastin as the drug. Different analyte concentrations, detect bH2 concentrations and streptavidin-HRP dilutions were evaluated. The capture VEGF concentration was 1 µg/mL.

Avastin	20 ng/mL bH2, streptavidin-HRP 1:500	50 ng/mL bH2, streptavidin-HRP 1:500	20 ng/mL bH2, streptavidin-HRP 1:1000	50 ng/mL bH2, streptavidin-HRP 1:1000
0.02 ng/mL	4.1	1.9	2.1	1.9
0.05 ng/mL	5.0	1.0	0.6	2.4
0.2 ng/mL	3.9	3.9	0.8	1.2
0.5 ng/mL	0.6	3.6	0.1	7.4
1.25 ng/mL	3.4	4.0	1.5	8.7
2 ng/mL		8.6	1.4	10.0

PK assay with Lucentis as the drug

Different concentrations of the reagents were also investigated with Lucentis as the drug. In the first checkerboard, different capture VEGF and detect bkLC concentrations were evaluated. The average signal/background values can be seen in Table C9, the values were low.

Table C9. Average signal/background values for the first checkerboard on ELISA with the PK assay and Lucentis as the drug. Different capture VEGF concentrations and detect bkLC concentrations were evaluated. Lucentis was measured at a concentration of 1.3 ng/mL. The streptavidin-HRP was diluted 1:1000.

	0.2 µg/mL capture VEGF	1 µg/mL capture VEGF	5 µg/mL capture VEGF
4 ng/mL bkLC	1.1	0.9	1.1
20 ng/mL bkLC	1.0	1.1	1.2
100 ng/mL bkLC	1.2	1.3	1.6

In the second checkerboard, higher concentrations of the capture VEGF and detect bkLC were evaluated for two different concentrations, 1.5 and 15 ng/mL, of the analyte Lucentis, as can be seen in Table C10 and Table C11 respectively. The lowest capture concentration, 2.5 µg/mL, and the highest concentration of detect bkLC, 0.9 µg/mL, gave the highest signal/background values.

Table C10. Average signal/background values for the second checkerboard on ELISA with the PK assay and Lucentis as the drug. Different capture VEGF concentrations and detect bkLC concentrations were evaluated. Lucentis was measured at a concentration of 1.5 ng/mL. The streptavidin-HRP was diluted 1:1000.

	2.5 µg/mL capture VEGF	**5 µg/mL capture VEGF**	**10 µg/mL capture VEGF**
0.1 µg/mL bkLC	1.9	1.9	1.9
0.3 µg/mL bkLC	3.0	3.1	2.6
0.9 µg/mL bkLC	4.4	4.6	4.2

Table C11. Average signal/background values for the second checkerboard on ELISA with the PK assay and Lucentis as the drug. Different capture VEGF concentrations and detect bkLC concentrations were evaluated. Lucentis was measured at a concentration of 15 ng/mL. The streptavidin-HRP was diluted 1:1000.

	2.5 µg/mL capture VEGF	**5 µg/mL capture VEGF**	**10 µg/mL capture VEGF**
0.1 µg/mL bkLC	38.1	35.9	37.5
0.3 µg/mL bkLC	48.3	51.5	42.3
0.9 µg/mL bkLC	53.9	51.0	47.9

Looking at the average of the CV values for the different concentrations, for 1.5 ng/mL and 15 ng/mL Lucentis in Table C12 and Table C13 respectively, no big variances could be seen.

Table C12. CV values for the second checkerboard on ELISA with the PK assay and Lucentis as the drug. Different capture VEGF concentrations and detect bkLC concentrations were evaluated. Lucentis was measured at a concentration of 1.5 ng/mL. The streptavidin-HRP was diluted 1:1000.

	2.5 µg/mL capture VEGF	**5 µg/mL capture VEGF**	**10 µg/mL capture VEGF**
0.1 µg/mL bkLC	1.6	0.4	5.4
0.3 µg/mL bkLC	15.8	12.7	0.6
0.9 µg/mL bkLC	3.0	2.8	4.8

Table C13. CV values for the second checkerboard on ELISA with the PK assay and Lucentis as the drug. Different capture VEGF concentrations and detect bkLC concentrations were evaluated. Lucentis was measured at a concentration of 15 ng/mL. The streptavidin-HRP was diluted 1:1000.

	2.5 µg/mL capture VEGF	**5 µg/mL capture VEGF**	**10 µg/mL capture VEGF**
0.1 µg/mL bkLC	5.4	1.6	3.8
0.3 µg/mL bkLC	8.3	7.0	8.1
0.9 µg/mL bkLC	6.2	0.1	9.9

PD assay with Avastin as the drug

In the first checkerboard with the PD assay and Avastin as the drug, different capture Avastin and detect bα-VEGF pAb (2) concentrations were evaluated, as can be seen in Table C14. The signal/background values were highest for the highest capture Avastin concentration, 10 µg/mL, and 0.5 µg/mL detect bα-VEGF pAb (2).

Table C14. Average signal/background values for the first checkerboard on ELISA with the PD assay and Avastin as the drug. Different capture Avastin concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 50 pg/mL and the streptavidin-HRP was diluted 1:1000.

	0.2 µg/mL capture Avastin	**1 µg/mL capture Avastin**	**5 µg/mL capture Avastin**	**10 µg/mL capture Avastin**
0.05 µg/mL bα-VEGF pAb (2)	1.1	1.1	1.9	2.3
0.5 µg/mL bα-VEGF pAb (2)	1.1	1.3	4.0	5.5
2.5 µg/mL bα-VEGF pAb (2)	1.1	1.4	3.4	4.4
5 µg/mL bα-VEGF pAb (2)	1.0	1.1	2.5	3.6

For the capture Avastin concentration of 5 µg/mL, the CV values were a bit higher. Otherwise, there were no huge differences looking at the average CV values for the different concentrations (see Table C15).

Table C15. CV values for the first checkerboard on ELISA with the PD assay and Avastin as the drug. Different capture Avastin concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 50 pg/mL and the streptavidin-HRP was diluted 1:1000.

	0.2 µg/mL capture Avastin	**1 µg/mL capture Avastin**	**5 µg/mL capture Avastin**	**10 µg/mL capture Avastin**
0.05 µg/mL bα-VEGF pAb (2)	0.2	1.0	16.1	3.3
0.5 µg/mL bα-VEGF pAb (2)	9.0	0.8	3.1	9.5
2.5 µg/mL bα-VEGF pAb (2)	0.1	2.9	14.1	1.3
5 µg/mL bα-VEGF pAb (2)	8.0	0.8	23.1	13.1

In the second checkerboard, different capture Avastin and detect bα-VEGF pAb (2) concentrations were evaluated at 25 and 250 pg/mL VEGF, as can be seen in Table C16 and Table C17 respectively. The signal/background values were highest for the highest capture Avastin concentration, 40 µg/mL, and 1 µg/mL detect bα-VEGF pAb (2).

Table C16. Average signal/background values for the second checkerboard on ELISA with the PD assay and Avastin as the drug. Different capture Avastin concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 25 pg/mL and the streptavidin-HRP was diluted 1:1000.

	5 µg/mL capture Avastin	**10 µg/mL capture Avastin**	**20 µg/mL capture Avastin**	**40 µg/mL capture Avastin**
0.25 µg/mL bα-VEGF pAb (2)	2.0	2.1	2.1	2.3
0.5 µg/mL bα-VEGF pAb (2)	2.0	2.5	2.3	3.1
1 µg/mL bα-VEGF pAb (2)	2.2	3.0	3.2	3.7
2 µg/mL bα-VEGF pAb (2)	2.1	2.9	2.8	3.3

Table C17. Average signal/background values for the second checkerboard on ELISA with the PD assay and Avastin as the drug. Different capture Avastin concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 250 pg/mL and the streptavidin-HRP was diluted 1:1000.

	5 µg/mL capture Avastin	**10 µg/mL capture Avastin**	**20 µg/mL capture Avastin**	**40 µg/mL capture Avastin**
0.25 µg/mL bα-VEGF pAb (2)	10.4	9.5	10.4	10.0
0.5 µg/mL bα-VEGF pAb (2)	10.3	12.0	11.5	14.7
1 µg/mL bα-VEGF pAb (2)	11.9	16.5	18.3	20.3
2 µg/mL bα-VEGF pAb (2)	9.7	15.4	15.1	17.5

As can be seen in Table C18 and Table C19, with 25 pg/mL and 250 pg/mL VEGF respectively, there were a bit higher CV values for the two highest concentrations of capture Avastin, but the values differed a lot when measured with different detect concentrations. Otherwise, there were no huge differences looking at the average CV values for the different concentrations.

Table C18. CV values for the second checkerboard on ELISA with the PD assay and Avastin as the drug. Different capture Avastin concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 25 pg/mL and the streptavidin-HRP was diluted 1:1000.

	5 µg/mL capture Avastin	**10 µg/mL capture Avastin**	**20 µg/mL capture Avastin**	**40 µg/mL capture Avastin**
0.25 µg/mL bα-VEGF pAb (2)	7.7	3.7	7.6	9.6
0.5 µg/mL bα-VEGF pAb (2)	6.8	7.0	7.9	16.2
1 µg/mL bα-VEGF pAb (2)	8.3	1.7	15.8	2.3
2 µg/mL bα-VEGF pAb (2)	4.7	1.8	1.1	

Table C19. CV values for the second checkerboard on ELISA with the PD assay and Avastin as the drug. Different capture Avastin concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 250 pg/mL and the streptavidin-HRP was diluted 1:1000.

	5 µg/mL capture Avastin	**10 µg/mL capture Avastin**	**20 µg/mL capture Avastin**	**40 µg/mL capture Avastin**
0.25 µg/mL bα-VEGF pAb (2)	8.2	5.7	2.6	23.1
0.5 µg/mL bα-VEGF pAb (2)	1.8	8.0	7.6	17.7
1 µg/mL bα-VEGF pAb (2)	7.2	7.3	11.4	2.2
2 µg/mL bα-VEGF pAb (2)	4.0	6.3	6.9	

In the third checkerboard, different capture Avastin concentrations were evaluated at different analyte VEGF concentrations, as can be seen in Table C20. The signal/background values were highest for the two highest capture Avastin concentration, 60 and 80 µg/mL.

Table C20. Average signal/background values for the third checkerboard on ELISA with the PD assay and Avastin as the drug. Different analyte VEGF concentrations and capture Avastin concentrations were evaluated. The streptavidin-HRP was diluted 1:1000.

	2.5 pg/mL VEGF	**5 pg/mL VEGF**	**10 pg/mL VEGF**	**20 pg/mL VEGF**	**40 pg/mL VEGF**	**60 pg/mL VEGF**	**90 pg/mL VEGF**	**135 pg/mL VEGF**	**200 pg/mL VEGF**	**300 pg/mL VEGF**	**450 pg/mL VEGF**
20 µg/mL capture Avastin	1.1	1.2	1.5	2.0	3.1	4.2	5.4	7.3	9.6	14.2	19.7
40 µg/mL capture Avastin	1.2	1.3	1.6	2.3	3.6	4.7	6.9	8.8	11.9	16.2	24.0
60 µg/mL capture Avastin	1.2	1.3	1.7	2.4	3.7	5.0	6.9	9.8	12.6	19.2	25.8
80 µg/mL capture Avastin	1.2	1.4	1.8	2.6	4.2	5.4	7.5	10.7	14.2	20.9	27.6

There were no huge differences looking at the average CV values for the different concentrations, as can be seen in Table C21.

Table C21. CV values for the third checkerboard on ELISA with the PD assay and Avastin as the drug. Different analyte VEGF concentrations and capture Avastin concentrations were evaluated. The streptavidin-HRP was diluted 1:1000.

	2.5 pg/mL VEGF	**5 pg/mL VEGF**	**10 pg/mL VEGF**	**20 pg/mL VEGF**	**40 pg/mL VEGF**	**60 pg/mL VEGF**	**90 pg/mL VEGF**	**135 pg/mL VEGF**	**200 pg/mL VEGF**	**300 pg/mL VEGF**	**450 pg/mL VEGF**
20 µg/mL capture Avastin	2.0	0.9	2.5	0.8	4.0	3.8	0.5	1.6	0.5	0.9	3.2
40 µg/mL capture Avastin	2.1	1.0	2.8	1.6	4.5	6.1	0.4	11.8	4.4	1.3	3.7
60 µg/mL capture Avastin	1.5	1.6	0.2	4.0	3.4	0.9	1.2	3.1	0.7	6.4	1.6
80 µg/mL capture Avastin	0.9	1.8	1.6	3.2	3.7	6.4	5.7	1.9	8.7	2.8	7.6

PD assay with Lucentis as the drug

In the first checkerboard with the PD assay and Lucentis as the drug, different capture Lucentis and detect bα-VEGF pAb (2) concentrations were evaluated, at 50 and 500 pg/mL VEGF, as can be seen in Table C22 and Table C23 respectively. The signal/background values were highest for the highest capture Lucentis concentration, 40 µg/mL, and were approximately the same for the detect bα-VEGF pAb (2) concentrations. Even though the capture concentration of 40 µg/mL Lucentis gave the highest signal/background values, 10 µg/mL was used for this assay due to the amount of reagent that was needed.

Table C22. Average signal/background values for the first checkerboard on ELISA with the PD assay and Lucentis as the drug. Different capture Lucentis concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 50 pg/mL and the streptavidin-HRP was diluted 1:1000.

	10 µg/mL capture Lucentis	**20 µg/mL capture Lucentis**	**40 µg/mL capture Lucentis**
0.5 µg/mL bα-VEGF pAb (2)	1.6	1.7	2.0
1 µg/mL bα-VEGF pAb (2)	1.6	1.9	2.0
2 µg/mL bα-VEGF pAb (2)	1.7	1.9	2.2

Table C23. Average signal/background values for the first checkerboard on ELISA with the PD assay and Lucentis as the drug. Different capture Lucentis concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 500 pg/mL and the streptavidin-HRP was diluted 1:1000.

	10 µg/mL capture Lucentis	**20 µg/mL capture Lucentis**	**40 µg/mL capture Lucentis**
0.5 µg/mL bα-VEGF pAb (2)	6.9	8.6	11.6
1 µg/mL bα-VEGF pAb (2)	6.9	9.9	11.0
2 µg/mL bα-VEGF pAb (2)	7.5	10.0	12.0

Looking at the CV values in Table C24 and Table C25, there were no big differences for the different concentrations evaluated.

Table C24. CV values for the first checkerboard on ELISA with the PD assay and Lucentis as the drug. Different capture Lucentis concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 50 pg/mL and the streptavidin-HRP was diluted 1:1000.

	10 µg/mL capture Lucentis	**20 µg/mL capture Lucentis**	**40 µg/mL capture Lucentis**
0.5 µg/mL bα-VEGF pAb (2)	1.2	3.0	0.9
1 µg/mL bα-VEGF pAb (2)	2.6	0.5	9.4
2 µg/mL bα-VEGF pAb (2)	2.4	3.3	0.7

Table C25. CV values for the first checkerboard on ELISA with the PD assay and Lucentis as the drug. Different capture Lucentis concentrations and detect bα-VEGF pAb (2) concentrations were evaluated. VEGF was measured at 500 pg/mL and the streptavidin-HRP was diluted 1:1000.

	10 µg/mL capture Lucentis	**20 µg/mL capture Lucentis**	**40 µg/mL capture Lucentis**
0.5 µg/mL bα-VEGF pAb (2)	2.1	5.3	2.5
1 µg/mL bα-VEGF pAb (2)	3.5	1.7	14.3
2 µg/mL bα-VEGF pAb (2)	1.8	5.6	6.5

Appendix D – Optimization of PK and PD assays measuring free analyte on Gyrolab

For the measurements of free analyte with the PK and PD assays, optimization experiments were performed to be able to decide for how long the samples with Avastin/Lucentis and VEGF should be incubated to reach equilibrium. In Figure D1a and b, a comparison of different incubation times for the PK assay can be seen, indicating of required incubation time of 24 hours to reach equilibrium since there were no differences after that point. The different graphs show different fixed concentrations of Avastin, 1, and 4 ng/mL.

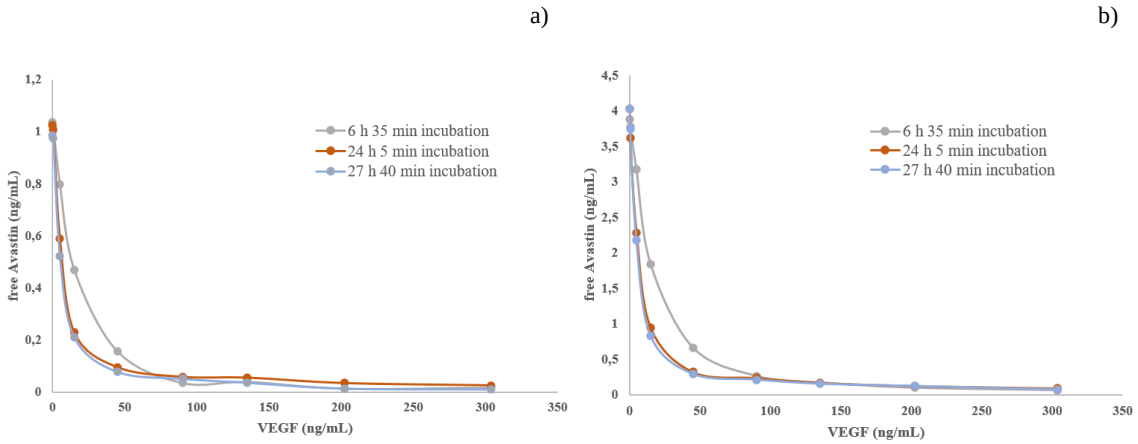

Figure D1. Comparison of three different incubation times for the Avastin:VEGF samples with the PK assay. a) Comparison with the fixed concentration of 1 ng/mL Avastin in the samples. b) Comparison with the fixed concentration of 4 ng/mL Avastin in the samples.

For the PD assay, the experiments gave a required incubation time of 22.5 hours to reach equilibrium, as can be seen in Figure D2a and b, with 180 and 360 pg/mL fixed VEGF respectively.

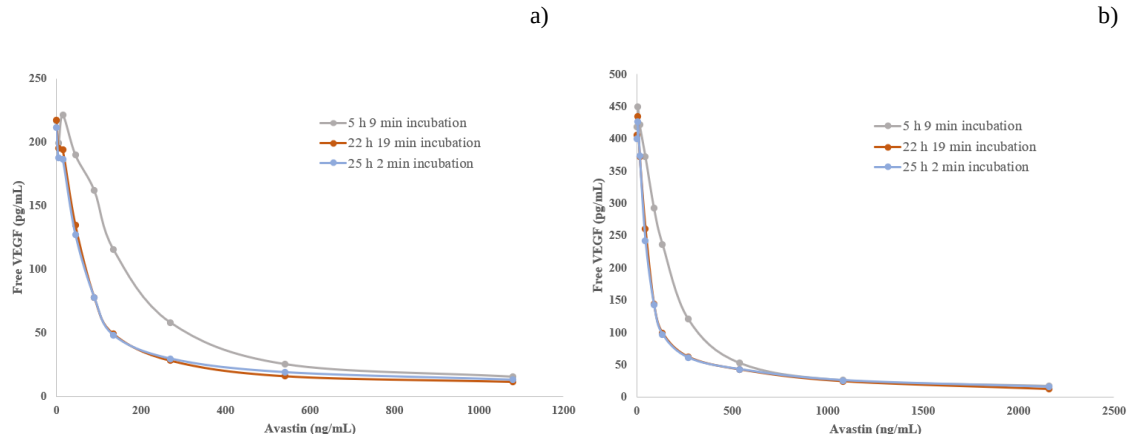

Figure D2. Comparison of three different incubation times for the VEGF:Avastin samples with the PD assay. a) Comparison with the fixed concentration of 180 pg/mL VEGF in the samples. b) Comparison with the fixed concentration of 360 pg/mL VEGF in the samples.

Appendix E – Curve fits for calculation of IC_{50} values

GraphPad Prism 8 was used to calculate IC_{50} values, estimated the same as the K_D values at equilibrium, in which *the logarithm of the inhibitor versus response* curve was used. Curve fits were generated, to be able to determine the IC_{50} values. For the PK assay measuring free Avastin, the curve fits for the experiments on Gyrolab and ELISA can be seen in Figure E1a and b. The curve fits for the experiments on Gyrolab were good and the ones for the experiments on ELISA were rather good, with the ones in Figure E1b being a bit worse.

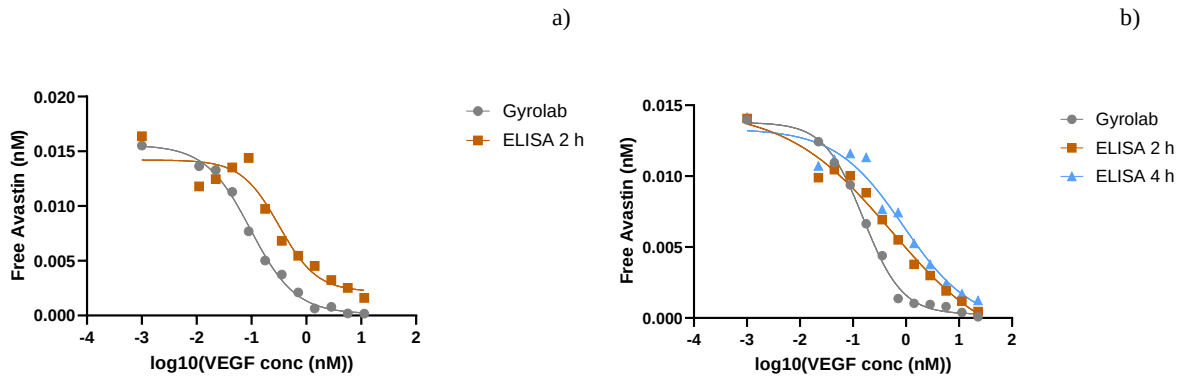

Figure E1. Curve fits for calculation of IC_{50} values with the PK assay and Avastin as the drug, for two experiments. Graphs are generated from GraphPad Prism 8. a) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, and ELISA with two hours of sample incubation. b) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, ELISA with two hours of sample incubation, and ELISA with four hours of sample incubation.

For the PD assay with Avastin as the drug, the curve fits for the experiments on Gyrolab and ELISA can be seen in Figure E2a and b. All curve fits, with the sigmoidal S curve, except the one for ELISA with four hours of sample incubation were rather good.

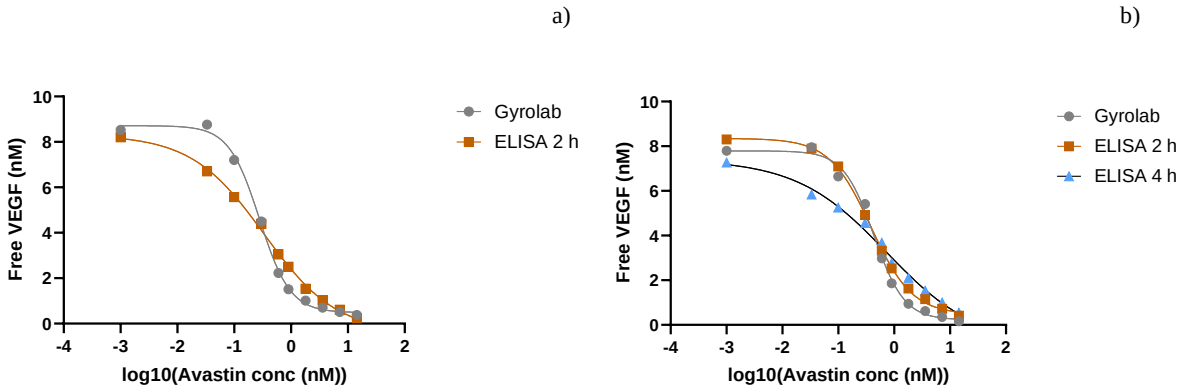

Figure E2. Curve fits for calculation of IC_{50} values with the PD assay and Avastin as the drug, for two experiments. Graphs are generated from GraphPad Prism 8. a) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, and ELISA with two hours of sample incubation. b) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, ELISA with two hours of sample incubation, and ELISA with four hours of sample incubation.

In Figure E3a and b, the curve fits for the PK assay measuring free Lucentis can be seen. The curve fit for the experiment performed on Gyrolab in Figure E3b was rather good, but the other ones were not that good, not reaching the bottom plateau.

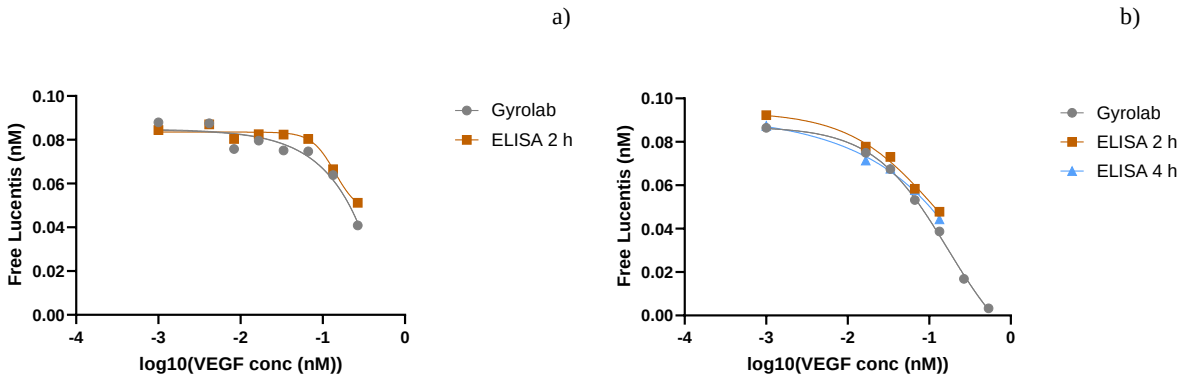

Figure E3. Curve fits for calculation of IC_{50} values with the PK assay and Lucentis as the drug, for two experiments. Graphs are generated from GraphPad Prism 8. a) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, and ELISA with two hours of sample incubation. b) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, ELISA with two hours of sample incubation, and ELISA with four hours of sample incubation.

For the PD assay with Lucentis as the drug, the curve fits for the experiments on Gyrolab and ELISA can be seen in Figure E4a and b. All curve fits were rather good, but not really reaching the bottom plateau.

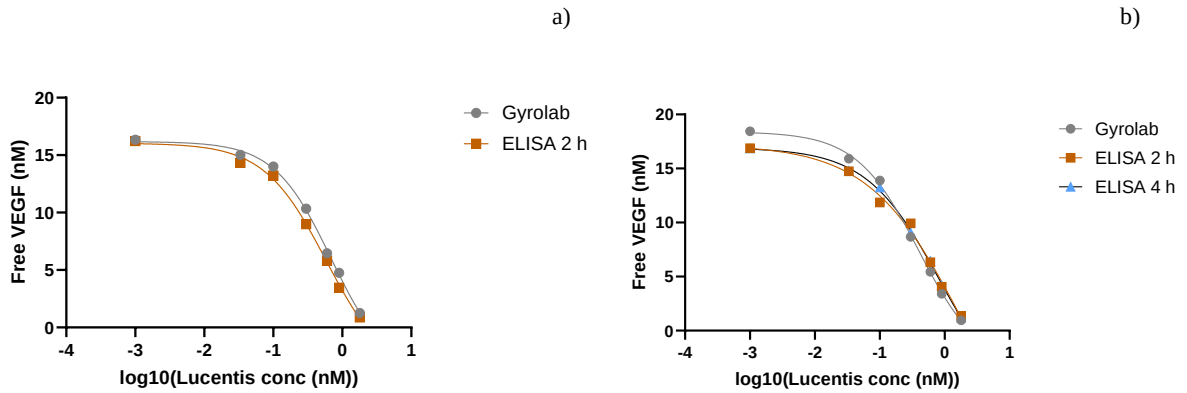

Figure E4. Curve fits for calculation of IC_{50} values with the PD assay and Lucentis as the drug, for two experiments. Graphs are generated from GraphPad Prism 8. a) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, and ELISA with two hours of sample incubation. b) Curve fit for the logarithm of inhibitor versus response, with values from measuring free analyte on Gyrolab, ELISA with two hours of sample incubation, and ELISA with four hours of sample incubation.

Appendix F – Buffers

All the buffers that were used for the experiments on ELISA can be seen in Table F1, Table F2, and Table F3.

Table F1. The PBS buffer used for the experiments on ELISA.

PBS	**10 mL**
MilliQ water	9 mL
PBS x10	1 mL

Table F2. The blocking buffer used for the dilutions of the samples, detecting reagents, and streptavidin-HRP for ELISA.

Blocking buffer	**100 mL**
MilliQ water	80 mL
PBS x10	10 mL
BSA 10%	10 mL

Table F3. The washing buffer used for the washing steps on ELISA.

Washing buffer	**1000 mL**
MilliQ water	895 mL
PBS x10	100 mL
Tween 10%	5 mL