Degree Project in MSc Biotechnology in Engineering

CUSTOMIZED QUANTIFICATION OF HOST CELL PROTEIN WITH BIO-LAYER INTERFEROMETRY

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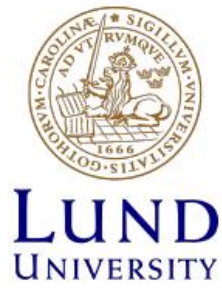

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Abstract

Host cell protein (HCP) is quantified in the purification steps of biopharmaceutical production and is part of ensuring the purity of the final drug product. The gold standard method for HCP quantification is enzyme-linked immunosorbent assay (ELISA), but a new approach is proposed here with fully customized bio-layer interferometry assays for yeast and chinese hamster ovary (CHO) cells.

The new method has been developed by proceeding from a kit assay, but exchanging and optimizing each step of the assay to ensure a fully customized assay with the maximum binding rates possible to maximize the sensitivity. Originally, 3,3'-diaminobenzidine (DAB) was used as a signal enhancer, but exchanging it to the less hazardous substrate 3-amino-9-ethylcarbazole (AEC) gave higher assay signals and better sensitivity.

The developed yeast HCP quantification assay showed the ability to quantify HCP levels in samples of different concentrations. The estimated precision and lower limit of quantification (LLOQ) were of promising values, comparable to the analytical parameters of the currently used ELISA. The bio-layer interferometry (BLI) approach has the ability to reduce assay time from ELISA's usual 2 days down to 2 hours and can be almost fully automized together with a liquid handler.

For pharmaceutical development, a faster HCP quantification could result in a faster feedback-loop allowing earlier adjustments to the purification process, and could be a great advantage for the aggressive deadlines that the biopharmaceutical discovery space experience.

Populärvetenskaplig sammanfattning

Visste du att många läkemedel tillverkas av genmodifierade bakterier? Också ofta i genmodifierade celler från däggdjur, till exempel från kinesiska hamstrars äggstockar. Läkemedel producerade i celler är de snabbast växande på marknaden, och kan till exempel användas för att producera insulin eller ett COVID-19 vaccin. I denna studie har en ny metod utvecklats för att säkerställa att sådana läkemedel är rena och ofarliga.

Generna i en cell innehåller instruktioner för hur cellen ska bilda proteiner och andra molekyler. Den aktiva substansen i läkemedel är ofta proteiner, och för att cellen ska bilda ett visst läkemedelsprotein läggs en extra gen in i DNAt - till exempel med gensaxen CRISPR/Cas9 som tilldelades Nobelpriset i kemi 2020. Cellen producerar då läkemedelsproteinet tillsammans med alla andra proteiner och molekyler den behöver för att leva, vilka kallas för värdcellsproteiner eller host cell proteins på engelska, förkortat HCP. De anses vara orenheter, och är oönskade i den slutgiltiga läkemedelsprodukten.

Under produktionen blandas cellerna, dess proteiner och läkemedlet i en enda sörja, vilket gör att allt som inte är läkemedelsmolekylen måste tvättas bort innan den kan användas. Men det är inte tillräckligt att rena upp läkemedlet - man måste också bevisa att det är så rent som man hävdar. Det gör man genom att analysera läkemedlet i olika delar av uppreningsprocessen för att se hur mycket orenheter det finns kvar, exempelvis HCP:er. Det är en sådan analys vi har fokuserat på i denna studien. Uppreningen är alltså väldigt viktig, och det ställs stora krav globalt på ett läkemedels renhet från myndigheter i exempelvis EU och USA.

Traditionellt används en metod som kallas ELISA (Enzyme-Linked ImmunoSorbent Assay), vilken tar upp till två dagar och kräver mycket manuellt arbete. Det är en så kallad immunanalys vilket betyder att man använder antikroppar för att mäta ett visst ämne. Vi har istället utvecklat en annan immunanalysmetod som tar två timmar och kan göras nästan helt automatiserad. Den är baserad på Bio-Layer Interferometry-tekniken, eller förkortat BLI, och har antagligen aldrig tidigare använts för att mäta HCP från ett specifikt läkemedels produktion. Vi har visat att man kan utveckla helt skräddarsydda analyser för olika organismer såsom kinesiska hamsterceller och jäst. Resultaten i denna studie talar för att den kan mäta HCP:er med samma precision och noggrannhet som dagens ELISA. En snabbare och automatiserad analysmetod skapar en effektivare feedback loop i uppreningsprocessen, vilket i sin tur gör att nya läkemedel kan utvecklas snabbare.

Läkemedel producerade i celler är livsviktiga mediciner för många människor, men det är minst lika viktigt att läkemedlena vi tar är rena och ofarliga. Därför behöver vi snabbt och automatisk kunna analysera orenheter i läkemedel för att se till att det inte finns kvar några bakteriebitar i tabletterna du tar varje morgon.

Popular Summary

Did you know that many pharmaceuticals today are produced using genetically modified bacteria? Also often in genetically modified mammalian cells, such as from the Chinese hamster's ovaries. This branch of pharmaceuticals is the fastest growing on the market and could for example be used for developing a vaccine candidate against COVID-19 or for producing insulin. In this study, a method for ensuring the purity of such pharmaceuticals has been developed.

The genes in a cell contain the instructions for how the cell should build proteins and other molecules. The active substance in pharmaceuticals is often a protein, and to make a cell produce a certain drug protein an extra gene is inserted into the DNA, for example by using the Nobel Prize awarded technology CRISP/Cas9. The cell produces the drug protein together with the rest of the molecules and proteins the cell needs to stay alive, which are called Host Cell Proteins or HCPs. They are considered impurities and are unwanted in the final drug product.

During production, the cells form a complex mixture with the HCPs and the drug protein. Before the drug can be used, it must go through a thorough purification process in multiple steps to ensure a high level of purity. But it is not enough to just purify the drug - its purity must also be proven. Throughout the purification process, the levels of remaining impurities are therefore closely monitored. This is strictly regulated by authorities all over the world, for example from the EU and the US. In this study, we have developed a new analytical method for quantifying one such group of impurities, the already mentioned HCPs.

Traditionally, a method called ELISA (Enzyme-Linked ImmunoSorbent Assay) is used, which can take up to 2 days and require several manual steps. ELISA is an immunoassay, meaning that antibodies are used for detecting a certain substance. Our new method is also antibody based but takes only 2 hours to perform and can be almost fully automized. The method is based on the Bio-Layer Interferometry technology, or BLI for short, which has probably never before been used for quantifying HCP from a customized drug production process. We have shown that customized HCP quantification BLI assays can be developed for different organisms, in particular yeast and Chinese hamster ovary cells. The precision and accuracy show promising results, almost reaching the goal of spanning as low as for the current ELISA. A faster and automized analysis creates a quicker feedback loop which can enable faster development of new pharmaceuticals.

Drugs produced in cells are vital for many people, but it is just as important that the drugs are pure and not hazardous. This new method allows us to analyze the purity of drugs in a quick and automatic way, and make sure that there is nothing left from that hamster ovary in the pills you take every morning.

Preface and Acknowledgement

This work has been performed at Novo Nordisk A/S in Målöv at the department 318 Bioanalysis. I would like to thank my supervisors Karen Duus and Jan Amstrup who have always discussed and moved my work forward with great interest, and who kept me going during the Corona lockdown. I am also grateful to my supervisor at Lund University, Jenny Schelin, who has supported me during the whole process, despite the distance, and to my examinator Marie Wahlgren who took her time to examine the report and support me with legal inquiries. I also want to thank all my colleagues at Novo Nordisk who have contributed with their scientific and laboratory knowledge and always helped me with all thinkable matters, and the Antibody Technology department who lended us their Octet® HTX almost everyday for six months.

Additionally I want to thank my friends, family and studying peers who have supported, laughed and spent time with me during this work.

Contents

Abbreviations

4PL curve	Four Parameter Logistic curve
α-HCP	anti-HCP
α-FITC	anti-FITC
Ab	Antibody
AEC	3-Amino-9-Ethylcarbazole
b-Ab	Biotinylated Antibody
BLI	Bio-Layer Interferometry
BSA	Bovine Serum Albumin
CHO	Chinese Hamster Ovary
CD	Compact Disc
CFR	Code of Federal Regulations
CV	Coefficient of Variation
DAB	3,3'-Diaminobenzidine
DMSO	Dimethyl Sulfoxide
DOE	Design of Experiments
ELISA	Enzyme-Linked ImmunoSorbent Assay
EU	European Union
FITC	Fluorescein Isothiocyanate
GMP	Good Manufacturing Practice
HCP	Host Cell Protein
HRP	Horseradish Peroxidase
LLOQ	Lower Limit Of Quantitation
MCR	Molar Coupling Ratio
MS	Mass Spectrometry
NN	Novo Nordisk A/S
OVAT	One Variable At a Time
PBS	Phosphate-Buffered Saline
RPM	Revolutions Per Minute
SAX	High Precision Streptavidin
SAX2	High Precision Streptavidin 2.0
SPR	Surface Plasmon Resonance
TBS	Tris-Buffered Saline
WSA	Working Standard A

1 Introduction

Biopharmaceuticals are today the most produced medicines in the pharma sector as compared to totally synthesized pharmaceuticals, and the market is growing quickly [1]. A biological medicine, or biopharmaceutical, is per definition a medicine whose active substance is made by a living organism [2]. It means that the drug is produced by a living cell, such as a bacterial, yeast, mammalian or other cell [1]. The drug is recombinantly produced, meaning that it is not normally produced by the cell, but the cell has been engineered to produce it. As described by Bracewell et al. [3], a cell produces a large range of endogenous proteins as well as the drug molecule. Endogenous molecules are internal and mostly all the molecules that a cell normally needs to survive. They are called host cell proteins, HCPs, and are unwanted in the final drug product as they can be hazardous for the patient, for example by causing immunogenicity or breaking down the active substance. Because of its hazardousness, the amount of HCP in a produced drug substance must be monitored during the whole production.

Novo Nordisk A/S (NN) is a healthcare company that produces biopharmaceuticals. The production processes are, according to regulations and guidelines, systematically monitored to follow, among other impurities, the amount of HCP in a drug production environment, both in preclinical development and in the everyday large-scale commercial production. This project has been carried out at a bioanalysis department at NN in the Greater Copenhagen Area.

Quantitation of HCP is traditionally measured using enzyme-linked immunosorbent assays, ELISAs [3], [4], and NN uses the same approach. It is an immunoassay that uses antibody-antigen interactions to detect and quantify analytes. It is cheap, has high throughput and is easy to perform [4]. Furthermore, it doesn't require special equipment that is not normally already found in a standard laboratory. The drawbacks, however, are mainly long incubation times, several manual handling steps and that an ELISA takes between 2-3 days for a laboratory technician to perform.

Besides the use of ELISA, other bioanalytical methods can be used for HCP measurements, for example Surface Plasmon Resonance (SPR), Gyrolab® technologies and bio-layer interferometry (BLI).

BLI is an optical technique that monitors molecular interactions and is among other things used for determination of protein concentrations such

as HCP. Compared to a traditional ELISA, BLI is a fast method and the machine time can be as short as a few minutes up to hours from start to result. BLI is a walk-away assay, meaning that it is partly automized, and after the assay is transferred to the machine, no more manual interventions are needed. This not only liberates time and resources from laboratory technicians who can focus on other work but also enables to have the equipment run the assay overnight. Automation makes the assay more consistent and reduces human errors. Furthermore, reducing manual pipetting for ergonomic reasons is a way to reduce monotonous repetitive work.

Using BLI instead of ELISA would save time and resources because, although ELISA is a well-known method at NN and the department, the already mentioned drawbacks such as long assay times give room for improvements. Moreover, the laboratories are continuously looking for possibilities for automation, and the implementation of BLI would be another step in the implementation of laboratory automation.

Based on these existing challenges and advantages, this master thesis project will focus on investigating the possibility to develop fully customized BLI assays for quantification of HCP that are comparable to existing ELISAs.

1.1 Aim and Objectives

The goal and purpose of this study has been to evaluate if it is possible to use BLI instead of ELISA for already existing assays for HCP quantification.

The objectives are to:

- Investigate and evaluate the commercial BLI kits for HCP quantitation.
- Investigate the possibility of using the same reagents (NN antibodies and HCP standard) as are used in the existing ELISAs.
- Investigate possible alternatives to the signal enhancing substrate that are less hazardous.
- Investigate and optimize the assay for different cell types such as production in Chinese Hamster Ovary cells (CHO) and yeast.
- Determine the analytical parameters such as LLOQ and precision for developed BLI assays.
- Automize the manual steps of the BLI assay.

1.2 Limitations

It is not within the scope of the project to implement the developed BLI method at the bioanalysis department at NN, as the laboratories at the department are working in a GMP regulated environment. An implementation therefore requires qualification and validation by an employee.

As an elaborated automation of the assay will first be relevant for routine analysis, automation of the assays will not be realized in this project. Furthermore, setup of an automated assay needs to be performed together with a programmer, and programming is out of scope in this project.

1.3 Good Manufacturing Practice

Good Manufacturing Practice, GMP, is a regulatory framework that states the minimal requirements that a pharmaceutical manufacturer has to follow [5]. There are legal instruments that regulate GMP, and in the European Union (EU) it is the regulation no. 1252/2014 and directive 2003/94/EC, that treat active substances and medicines for human use. The European Medicines Agency is responsible for controlling that the guidelines are followed by all medicinal manufacturers that are present and sell drugs in the EU.

At NN, drugs are produced and analyzed under GMP regulation. If the Octet® machine is to be used during drug production it has to be GMP compliant. The Sartorius' Octet® HTX needs to be GMP classified and the software fulfil 21 CFR part 11 [6] (Code of Federal Regulations). Furthermore, the biosensors also needs to be GMP classified [7].

1.4 The Company Novo Nordisk

Novo Nordisk is a global healthcare company primarily working within diabetes, haemophilia and growth disorders. It was founded in 1923, and have the headquarter in Denmark and have around 43 000 employees in 80 countries, with a little below half in Denmark. The company produces biopharmaceuticals recombinantly using both eukaryotes as well an prokaryotes, i.e. yeast, mammalian cells and *E. coli*. The production is highly regulated and performed under GMP rules with high demands for safety, quality and control. As stated on the NN homepage [8], NN is, and aims to stay, a leading healthcare company why the pipeline includes several new products. Fur-

thermore, investments are continuously made in novel treatments and technologies. The Novo Nordisk Foundation is the major stakeholder in NN, and each year the foundation supports academia as well as public projects with parts of the profit from NN.

In the NN bioanalysis department where this project was conducted, one of the tasks is to test samples from drug production development for process related impurities, including analysis for HCP. This is done at different steps of the downstream purification process. The samples are routinely analyzed using ELISA techniques.

2 Background and Theory

2.1 Antibodies in Brief

As described by Murphy and Weaver in Chapter 12 of Janeway's Immunobiology [9], antibodies are proteins that are produced by the immune system to help protect against foreign substances that enter the body, such as bacteria, viruses or other harmful particles. Not only humans, but also all vertebrates such as rabbits, goats and hamsters have antibodies as part of their immune systems. Antibodies are specialized, meaning that each antibody only binds to one other molecule. They are Y-shaped and have two important sections: the variable region and the constant region, see figure 1. The variability is what gives the antibodies their specificity, i.e. defines which antigen the antibody can bind to, while the constant region decides what group the antibody belongs to.

Antigens are the molecules that antibodies bind to and recognize, and are normally the foreign substances that the immune system sees as a threat, meaning they can mediate an immune response. It can be any protein or peptide that an antibody binds to, for example HCP, as long as it is able to trigger an immune response. Different antibodies can bind specifically to different parts of the antigen if it is big enough. Foreign substances can also be so small that raising an antibody against it is impossible.

There are two categories of antibodies, monoclonal and polyclonal antibodies. Monoclonal antibodies are produced by the same B cell against a certain antigen and are identical, while polyclonal antibodies are a mix of antibodies produced by different B cells against different antigens.

2.2 Immunoassays

Antibodies can be used *in vitro* – outside of the body - for a range of applications, for instance immunoassays. An immunoassay is when the antibody/antigen interaction is used to make a biochemical test [10], for example detecting a protein in a solution. This creates an ability to detect small proteins in complex matrices, for example the detection of a drug in a bloodstream.

Immunoassays are widely used for diagnostic purposes in the healthcare system. The antibodies used for immunoassays come from either animals such as rabbit, goat, hamster or are made by cells. One way to use the

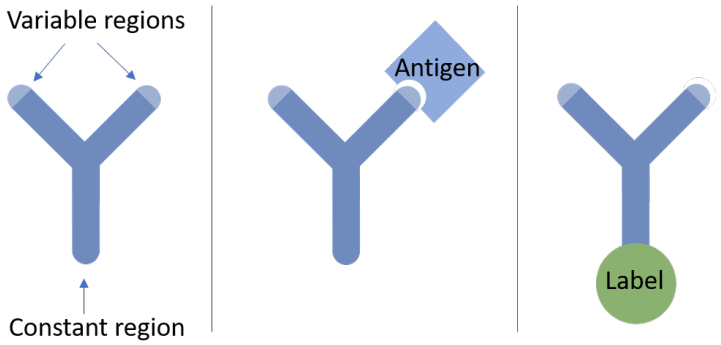


Figure 1: ***Antibody regions and bindings.*** *Antibodies are proteins produced by the immune system and have two main parts, the variable region and the constant region. The variable regions are specialized, which means that they only bind one other molecule. That molecule is called an antigen. The constant part is often used for labeling, i.e. for adding a desired molecule, for example an enzyme or a fluorophore which is then called a labeling molecule.*

antibodies in an immunoassay is to immobilize them to a surface from which the binding assay can take place. There are many different ways of detection, for example by a color shift or with optical sensors. Several layers of molecules can be used, for example in sandwich assays where two antibodies bind the same antigen, and where a secondary labeled antibody is added to be able to detect and enhance an output signal.

The immobilization of antibodies to a surface is possible with the help of coupling reactions, where molecules are added to the constant region of the antibody, or just by surface-protein interaction. These molecules used for a coupling can for example be biotin or amines, which bind strongly to other molecules that are immobilized on a surface.

Challenges with Measuring HCP HCPs are a mixture of endogenous proteins from a host cell line in which a recombinant drug is produced. They are unwanted in the final product as they can affect the drug's efficacy or be harmful for the patient, for example if the patient has an immune response against the HCPs, or if the HCPs work as adjuvants and activate the immune system. They can also have a proteolytic activity which can mediate the breakdown of the drug and effect efficacy, stability and safety [11].

The HCP consists of several different proteins, depending on the host and manufacturing processes [11]. The proteins are of different size, concentration

and immunogenicity, and the composition can vary greatly between different batches of drug substance and culturing conditions.

Because of this, it is advantageous to use immunoassays with polyclonal antibodies when measuring HCP. With this approach, a broad range of proteins can be detected - even proteins in very low concentration as long as they are able to mediate an immune response, i.e. generate antibodies. Sandwich immunoassays give, because of their signal enhancing mechanism, high sensitivity, automation potential and are low cost assays which are hard to match by other assay technologies, and their advantages are described in the United States Pharmacopoeia [11]. The polyclonal antibodies are produced by certain animals which are immunized with HCP from an upstream process, why the antibody pool is dependent on the immunoreactivity of the HCP's as well as the individual host animal [11].

It can be discussed if immunoassays actually are a good way of measuring HCP, as they are completely dependent on the antibody serum pool [3], and because all proteins will probably never be detected using immunoassays, since some of the HCP's are probably not mediating an immune response meaning that no antibodies are generated against them. That means that different proteins in the HCPs triggers antibodies with different affinities and the ones that don't trigger a creation of antibodies within the host animal will not be detected [12]. Furthermore, HCPs with low immunogenicity will only be detected with very low sensitivity [3]. Although, these proteins are probably less important to eliminate, because if they do not have an immunogenicity they should not be harmful for the patient. There is also a risk that HCPs "hide" in the purification process, for example by binding an coeluting with the product in the purification process [12]. Reducing and analysing the HCP content is a challenge for the pharmaceutical industry, and one of the reasons there are many steps in the developmental process of a drug. HCP measurements should be made not only in preclinical and clinical studies and during process validation [11] but also when the product gets to the market.

Due to the limitations of immunoassays, orthogonal techniques are preferably used in combination with immunoassays to ensure drug product purity [11]. Examples of orthogonal methods for HCP quantification are protein gel electrophoreses and mass spectrometry (MS). The main advantages of using an immunoassay instead of characterizing the HCP directly as in MS is that anything that triggers an immune response can be measured, and that specific molecules can be distinguished even in very complex matrices.

2.3 Bio-Layer Interferometry

BLI is an immunoassay which both can detect and quantify antigens as well as measure kinetics and affinities between e.g. antibodies and antigens, as described by for example Concepcion et al. [13] and Do et al [14]. The technique uses optical biosensors which can measure the amount and rate of molecular binding by detecting a wavelength shift in light, see figure 2. The binding assay takes place at the biocompatible sensor tip, which is dipped into a microtiter well containing the reagents. Inside the sensor, white light is sent towards the sensor tip. When the reagents bind they form a molecular layer on the sensor tip, which is detected by the sensor. The white light is reflected on two layers - on a reference layer inside the sensor, and at the molecular layer on the tip. The reflections create a shift in the interference pattern, which is read by a spectrometer and reported as a wavelength shift [13].

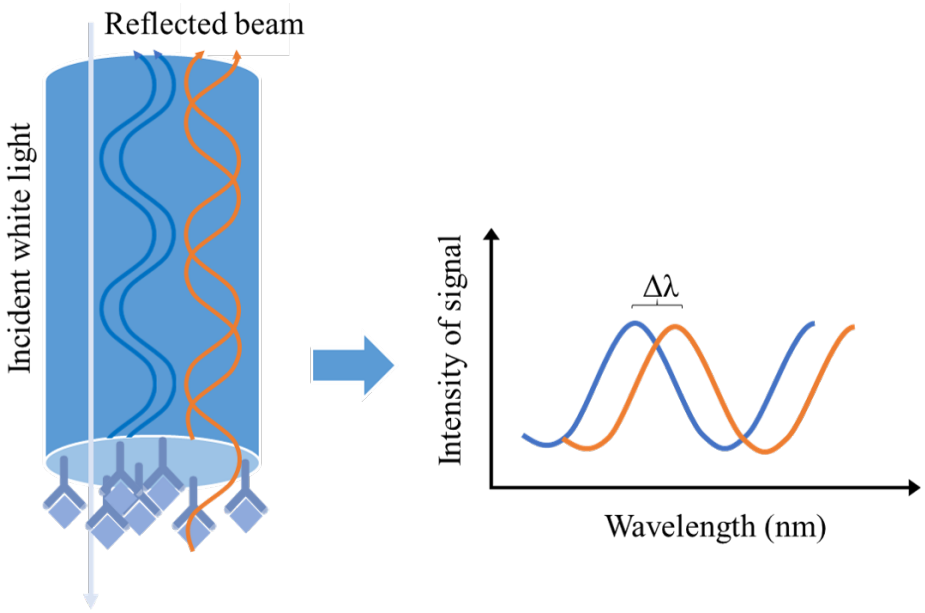


Figure 2: ***The optical properties of a bio-layer interferometry (BLI) sensor.*** *On the light-sensitive biosensor to the left a molecular layer forms on the tip when the binding assay takes place. Inside the sensors, white light is sent to the sensor tip, where it is reflected on an internal reference layer, and on the molecular surface. The reflected beams become dislocated to each other wich causes a wavelength shift ($\Delta\lambda$) between the reflected beams, which can be plotted as the graph to the right. The $\Delta\lambda$ will be the output of the assay and can detect, quantify of measure kinetics of binding events.*

Because of this functionality of dipping the sensor in the analyte solution, the technique is branded as a dip and read technique. It is a real time measurement of the optical thickness at the sensor tip. As the surrounding media's refractive index does not affect the signal, BLI works well in very crude matrices.

The BLI measurements have been conducted with a BLI instrument from Sartorius, named Octet® HTX. It is a high throughput system that allows measurements of up to 96 data points simultaneously. Sartorius also provides ready-to-use kits with different molecules already immobilized on the sensor tips, for example a CHO HCP kit and binding molecules like streptavidin or amines. Two software programs are connected to the Octet® HTX - one to manually plan and setup the experiments and one to analyze the data. To protect the sensors, the tips are sugar coated, meaning there is a thin layer of sucrose on the tips when they are stored, which needs to be washed away before use.

BLI has many fields of application and have been used for example for antibody kinetics screening [15], quantification of hormones [16] and for detection of biowarfares [17]. It is also used in vaccine research [18], and among others for quick detection of COVID-19 antibodies [19] in human blood samples.

Advantages and Drawbacks of BLI BLI based assays are semi-automated, walk-away assays, which work well in crude matrices and gives results in real time during measurement [18]. An assay can take from minutes up to hours depending on the assay setup, and can measure up to 96 sample data points in parallel dependent on the type of equipment used.

BLI is a flexible assay that can be used to measure many different types of molecules in different setups. Exchanging antibodies and adding or eliminating steps can easily be made. Assays can be very simple and use only one binding molecule, i.e. an antibody, which would only take a few minutes. More complex assays with several steps and signal enhancing substrates can also be used. The assays do not require any labeling as the detection system is based on optical biosensors, why the assays can be designed to be very simple. Labeling and signal enhancing substrates are primarily used for obtaining more sensitive assays.

The microvolumes also enables for reducing incubation times. As the assay takes place on the sensor tip, it can be enough with 50 μl of reagent

in each well when using 96 well microtiter plates with small wells. As the BLI also has real time monitoring of the signal, the incubation times can be minimized to the moment where the signal has been saturated. This gives opportunities for short assay times without long safety margins. Furthermore, several species' samples can be run simultaneously in one plate since each biosensor is coated individually, which eliminates the need for dividing different species' assays between different assay runs.

Using BLI for HCP quantification is still a method in development, and is not as well-known or optimized as the gold standard method ELISA. ELISA is very robust, reliable and well elaborated. It is not sure that the same precision and accuracy can be obtained with BLI as with ELISA, and different papers with different assay setups suggest different answers to this ([20],[4],[21],[22]). There are still challenges to solve when developing customized HCP assays on the BLI platform as it has not been made before. The equipment and sensors are still expensive compared to ELISA, but if sensors can be reused the cost per sample measured can be reduced.

Working with BLI The manual work of BLI includes planning the assay using the software, diluting reagents and preparing the sensors by incubating them in a buffer for 20 minutes. The assay as such - incubations, shaking and detection - is all carried out automatically in the machine. The workflow for BLI is seen in figure 3. Both 96 and 384 well plates can be used in any combination. As discussed later, the manual dilution steps can be automated with a liquid handler.

The output consists of binding rates in nanometer from each sensor, and is given graphically as a signal over time by the data analysis program as in figure 7b. Data for each well, like calculated concentration, residuals and r^2-values are also obtained, and a standard curve can be plotted with different equation types of own choice, for example a four parameter logistic (4PL) curve. Standard material with a known concentration of antigen can for example be purchased from Sartorius, but companies such as NN usually have their own HCP standard materials as they need very specific antibodies and antigens for phase 3 evaluations.

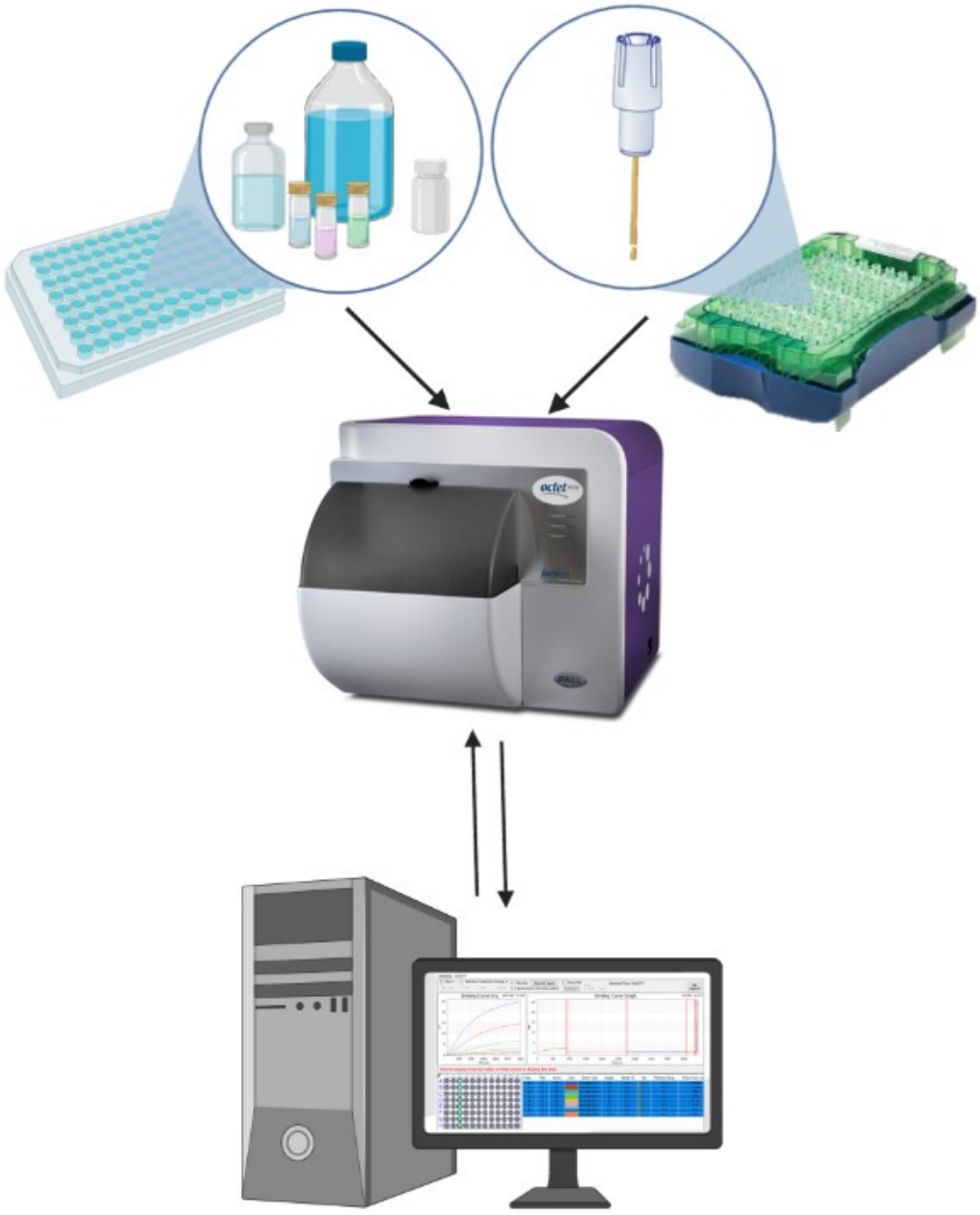


Figure 3: ***Workflow for the BLI Octet®.*** *The Octet® HTX (middle) needs pre-diluted reagents in microplates (top left) together with the biosensors in the biosensor tray (top right). The assay protocol is designed using a software to set incubation times, shake speeds and more. The output is binding rates which are visualised and interpreted in a data analysis software (bottom). Created with BioRender.com*

BLI Biosensor Types and Use There are several types of biosensors available which are pre-loaded with different binding molecules and are aimed at different assay types. The only sensors presently available elaborated for HCP assays are aimed at CHO HCPs, and are already coated with Anti-CHO HCP antibodies. Other biosensors, for example several different types coated with streptavidin, are available, for loading with biotinylated antibodies as described in Sartorius' Tech Note 28 [23] and Tech Note 24 [24].

For simple assays with few interacting molecules on streptavidin biosensors regeneration is possible, according to the Technical Note 14 from Sartorius [25]. The CHO HCP sensors are not recommended to be regenerated. Regeneration is aiming at breaking the antibody antigen interaction, and is therefore made with salt or a low pH. Regeneration should work up to 9 times [26] before losing more than 25% of binding by regenerating with an acid solution (10 mM glycine HCl, pH 1.7). However, in the assay used in this project a signal enhancing substrate was used which sticks all over the biosensor surface. The hypothesis is that it is rather the precipitated material that has to be washed away, at least before the antibody-antigen interaction can be broken. It would be very beneficial to be able to regenerate sensors, both for saving time and reagents of biotinylation and loading of sensors, and for enabling running several assays directly after each other. Additionally, the assay cost could be greatly reduced if the sensors could be reused.

HCP Assays on BLI Platforms Sartorius claims that no other companies are developing customized HCP assays on the BLI platforms. Instead, if HCP measurements are made the ready-to-use Anti-CHO HCP kit is used. Why a customized assay with specific antibodies was developed in this study is because the kit is insufficient in measuring the HCPs from the cells used at NN. Firstly, it is only available for CHO HCP analysis and, secondly, it uses antibodies which are not generated against the exact same HCP mixture or by the exact same host cell as in the actual drug developmental processes at NN.

Because HCP consists of such a complex mixture of proteins of different concentrations and immunogenicity, the analysis of HCP demands a high sensitivity. To obtain this, high signals are required, as a smaller change of HCP concentration then gives a bigger signal change. Because of this, the challenge has been to increase the signals as much as possible, why the signal enhancing step and substrate has been thoroughly investigated.

BLI is, in contrast to ELISA, normally a label free method, as the detection method is not dependent on the labels but on the amount of proteins attached to the biosensors. Nevertheless, several labeled molecules have been used in this assay setup to increase the signals and the sensitivity. As each binding step has an impact on the final assay signal, optimization of every step has been crucial.

2.4 Assay Setup

A sandwich assay has been setup with a primary, polyclonal antibody against HCPs, together with a secondary antibody for signal amplification, see table 1 and figure 4. The first α-HCP antibody (Ab1) is loaded onto the sensor by a biotin-streptavidin binding. The second antibody (Ab2) is also α-HCP, and is labeled with fluorescein isothiocyanate (FITC). The third antibody (Ab3) is a secondary antibody targeting FITC, and is labeled with a horse radish peroxidase enzyme (HRP). As the signal is amplified by a thicker molecular layer, a precipitating HRP substrate have been used.

The 3,3'-diaminobenzidine (DAB) was developed to be used as a signal enhancing substrate, but is classified as an hazardous and acute toxic substance. However, in this assay DAB has increased signals up to eight times, which increases the assay sensitivity greatly.

Table 1: ***Characteristics of antibodies used in the BLI assay.*** *Three antibodies are used in the general setup and correspond to figure 4. Ab1 and Ab2 are generated against yeast and Chinese Hamster Ovary (CHO) cell HCPs.*

Reagent	Type	Label
Ab1	Polyclonal α-HCP	Biotin
Ab2	Polyclonal α-HCP	FITC
Ab3	Monoclonal α-FITC	HRP

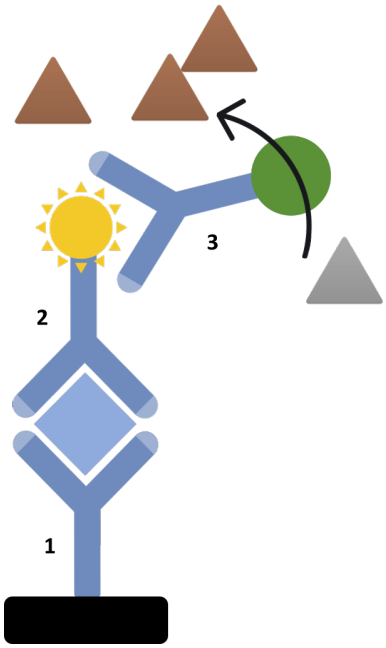


Figure 4: ***The molecular assay setup on the BLI platform.*** *The antibodies were loaded on the biosensor tip using a biotin-streptavidin binding. Black = biosensor, blue (Y-shape) = antibodies, light blue square = host cell protein, yellow = fluorescein isothiocyanate (FITC), green = horseradish peroxidase (HRP), gray = 3,3'-diaminobenzidine (DAB), brown = precipitated DAB. The position of the antibodies (Ab) referred to as Ab1, Ab2 and Ab3 can be seen and are specified in table 1.*

2.4.1 Biotinylation

The antibodies loaded onto the streptavidin surface are immobilized with a biotin-streptavidin binding. It is a strong binding where the streptavidin is immobilized on the biosensor surface while the biotin is bound to the antibodies, see figure 5. Preferably the antibody should be biotinylated on its constant part, so it can bind the surface with the constant part downwards, leaving the specific variable part free for binding the antigen. The biotin binds the primary amine groups on the antibody which are primarily found on lysine residues, and antibodies normally have one or many free amine groups that can form an amine coupling [27]. The level of biotinylation will affect how the antibody binds to the streptavidin coated surface. A high level of biotinylation means that several antibodies will bind, but there is a risk that the antibodies end up mostly in the wrong directions, blocking the surface for more antibodies to bind, see figure 5. That is called over-biotinylation in this report. A low level of biotinylation leads to fewer biotin-streptavidin bindings, and a bigger risk of more antibodies not binding the surface at all. Different assays require different levels of biotinylation, why it has to be investigated how much is needed for individual assays.

The level of biotinylation can be expressed as the molar coupling ratio (MCR), which means how many molecules of antibodies respectively biotin that are present in the solution. 1 mole of biotin and 1 mole of antibodies gives an MCR of 1:1.

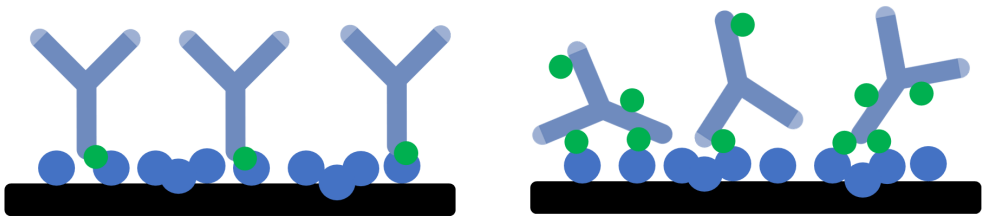


Figure 5: ***Biotinylated antibodies binding to surface.*** *On the black biosensor tip surfaces, streptavidin is immobilized (blue dots). Biotin (green dots), bound to the blue Y-shaped antibodies, bind the streptavidin and load the antibodies onto the biosensor tip surface. To the left, the optimal idea of how biotinylated antibodies bind to a surface. To the right the actual way biotinylated antibodies will end up on a surface.*

2.4.2 Horseradish Peroxidase Enzyme Reactions

The HRP is an enzyme which oxidizes its substrates using hydrogen peroxide as oxidizing agent [28]. It is widely used in immunoassays, as it can catalyse color changing and/or precipitating reactions. The HRP is conjugated to the constant part of an antibody, where it is used for signal enhancing reactions. Several substrates are available for HRP, but in the described work, DAB is used.

2.5 BLI versus Other Immunoassays

At the bioanalysis department at NN, several immunoassay techniques are used: ELISA, surface plasmon resonance (SPR) and Gyrolab®. SPR and Gyrolab® are both semi-automated immunoassay techniques, just like BLI, and could also have been used for this project with the aim of automating, speeding up, reducing manual handling and improving sensitivity as compared to ELISA. However, BLI has advantages over the two others such as high throughput, quick assay time, ease of use, and its availability to measure in complex matrices. The NN department was new to BLI and wanted to investigate the technique due to its many advantages.

As Roman et al. writes about the need for fast bioanalytical assays, "The biopharmaceutical discovery space on the other hand is characterized by aggressive timelines, large sample numbers, a variety of animal species and sample matrices, and limited available critical reagent and sample volumes." [29]. This summarizes quite well the need for speeding up assay times and throughput, which will both benefit research pace but also patients who will get newly developed medicines faster. The need for quick research of pharma and vaccines has not least been demonstrated during the COVID-19 pandemic.

The worldwide mostly used immunoassay is the ELISA assay. The method is widely used as it is simple, cheap and does not require any special laboratory equipment. It is a reliable technique that has been used and optimized for decades at NN, and although it is normally conducted manually, has been more and more automized with the help of liquid handlers. Nevertheless, running 4 samples takes around 2 to 3 days and the results are end-point. As smaller setups such as 384 well plates can be hard to handle manually, ELISA is normally performed in 96 well plates with sample volumes of approximately 200 μl [29]. As discussed, there is a need for more automated assays which

are faster, have greater throughput and give real time information while at the same time use smaller sample volumes.

SPR is another optical, label-free, walk away immunoassay that, just as BLI, gives real time information during measurements. It can be used as well for quantitation as for kinetic assays, and uses light diffraction to measure binding events. For example when coming to antibody discovery and development, BLI has several times shown to be a more suitable method than SPR as it can work well in very crude matrices and conditioned media, and has higher throughput than SPR due to the parallel manner the samples can be measured in [30], [15]. The BLI dip and read technique, which eliminates the need of tubing, is an advantage when it comes to HCP measurements [13], [30], as HCP solutions can be very crude when coming from early stages of the purification process. SPR has a vast need of maintenance and can be sensitive to crude matrices as the optical technique is influenced by the surrounding media, and as tubings can clog when working with complex mixtures.

Gyrolab® is sometimes called "ELISA on a disc", because it uses a CD-like (compact disc) platform with small capillary tubings where the samples and reagents move with help from centrifugal forces as the CD rotates [29]. It is not label free as the other two semi-automated assays discussed above, but Gyrolab® is a fast method that uses small reagent volumes. It can measure samples in parallel and the short incubation times (compared to ELISA) minimize matrix effects and the need for sample pre-treatment [31].

The smaller sample volumes and reaction surfaces in SPR, Gyrolab® and BLI together with the real time results are some of the reasons the assays can be performed much faster than ELISAs, as it allows incubation times to be reduced. As said, both SPR and Gyros can be used for reaching the aims of this thesis, however the BLI's ability to handle crude matrixes and to analyze up to 96 sample data points in parallel in a run that takes approximately an hour makes the platform very interesting and advantageous.

2.6 Automation of Pipetting

One objective of this study, and a current topic for many laboratories today, is to develop more automized assays. The BLI Octet® machine itself automizes a major part of the assay, but the reagents still need to be added to the microtiter plate wells, which takes around an hour for a laboratory technician. At the NN bioanalysis department, most ELISAs are today pipetted using

liquid handlers, why a setup for the BLI has also been designed to be used in the liquid handler to obtain an almost fully-automated assay.

Using a liquid handler saves time, eliminates repetitive manual actions and gives, together with the BLI machine, the possibility to make an almost fully automated assay end to end, which could possibly be used as an *at line* analysis. It also opens up for greater flexibility and higher throughput with the possibility of using 384 well plates, which can be difficult to work with manually due to small sample volumes and wells. Using the kinetic software would make it possible to use 8 different reagents in each run in two 384 well plates, which would give space for 96 sensors or data reading points in each run for the here developed method.

2.7 Design of Experiments

Part of this thesis project has been to learn and apply Design of Experiments, DOE, and it has been used for planning and executing a part of the experiments. As described by for example Leardi [32] and Brereton [33], DOE is when a multivariate approach is followed instead of a one variable at a time-approach (OVAT), meaning that several variables are altered in the same time when planning experiments. This can reduce the number of experiments while at the same time yield data with higher quality. Furthermore, all experiments can be planned on beforehand, instead of having to wait for the result of one experiment before being able to plan the next. Before performing a DOE, a certain experience with the assay is needed.

The idea is to define the intervals of the parameters which impact should be evaluated and make experiments at the extremes the intervals. For a three parameter experiment it is often illustrated as the corners of a cube, see figure 6. When results are plotted, this will indicate what will happen in the "room" in between the conducted experiments, and assumptions can be made about what the results supposedly will be also at the non-tested parameter values. DOE also allows to make a statistical analysis of obtained results for example by plotting the confidence interval in each point, depending on how many experiments that have been conducted, which is taken into account when designing the experiments.

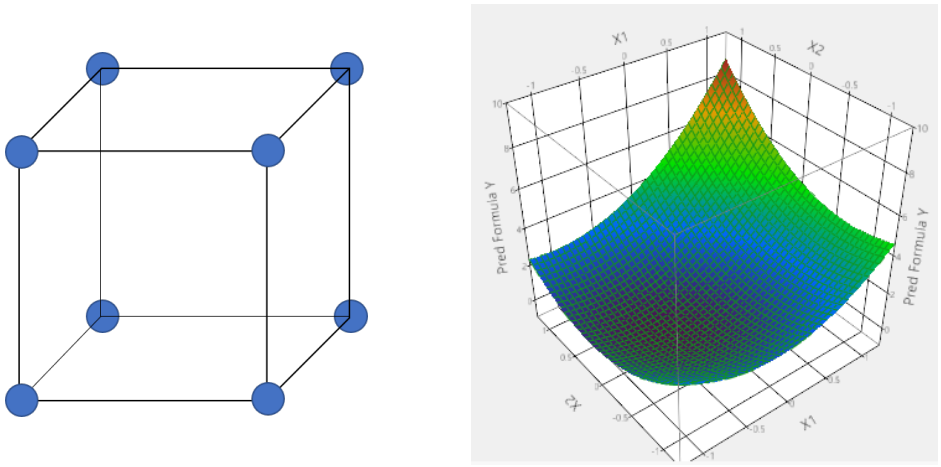


Figure 6: ***The idea of DOE.*** *Each corner of the cube represents an experiment with parameters at the extremes of their predetermined intervals. To the right, the response surface depending on the variables is shown, produced using SAS JMP.*

2.8 Statistical Analysis

To be able to compare BLI and ELISA, the analytical parameters must be compared. As working with bioassays, precision, accuracy and lower limit of quantification (LLOQ) were considered the most important parameters to investigate. For general bioanalytical methods a precision, or coefficient of variation, %CV, of less than 20% is expected [21] and at NN the same number is normally considered sufficient for ELISAs. Sartorius claims the Octet® gives a %CV<10% [34].

The HCP standard curve will be plotted as a 4PL curve. It is a sigmoidal curve that is symmetrical around the inflection point. A reliable 4PL curve shows the full sigmoidal shape with platforms at the top and the bottom. The middle part of the curve is used for concentration measurements - where it is assumed to be near linear. The concentrations are logged and plotted on the x-axis against binding rate signal on the y-axis. Measurements are read at equilibrium state of the binding. All data will be analyzed in the statistical data analysis software SAS JMP with the help of its built-in statistical tools.

3 Material and Methods

The original methods before customization are presented in this section, together with all material used during the project. The method development approaches are presented in the Results section. Antibodies can hereafter be written as Abs.

3.1 Materials

- BLI machinery, software and reagents from Sartorius
  - Octet® HTX
  - Software programs Octet Data Acquisition Software 11.0 and Octet Data Analysis Software 11.0
  - Ready-to-use kits from *Sartorious*
    - Dip and Read™Anti-CHO HCP Detection Kit, part no. 18-5123, containing:
      - α-CHO HCP biosensors
      - Fluorescein-α-CHO Abs, 100X concentrate
      - Sample diluent buffer with Kathon, azide-free
      - HRP-α-FITC Abs, 50X concentrate
      - Stable Peroxide buffer
      - Metal enhanced DAB, 10X concentrate
    - High Precision Streptavidin 2.0 (SAX2) Biosensors, part no. 18-5118
    - High Precision Streptavidin (SAX) Biosensors, part no. 18-5117
- NanoDrop® ND-1000 from Thermo Scientific
- DeNovix® DS-11 FX+ Spectrophotometer / Fluorometer from AH Diagnostics
- NN Abs:
  - α-CHO-HCP, 8.0 mg/ml
  - biotinylated α-CHO-HCP Abs
  - α-yeast-HCP 4.9 mg/ml,
  - biotinylated α-yeast-HCP Abs
- Standard material from NN:
  - NN Working standard A (WSA) for CHO, 100 μg/ml

  - NN WSA for yeast, 15360 ng/ml
- Fluorescein labeling
  - Molecular Probes® Fluorescein-EX Protein Labeling Kit, catalog number F10240
- Buffer exchange
  - Zeba™ Spin Desalting Column, 7K MWCO from Thermo Fisher Scientific, catalog number 89882
- Biotinylation
  - EZ-Link™ NHS-PEG4-Biotin from Thermo Fisher Scientific, catalog number 21362
- Sugar coating
  - Sucrose, ex Sigma part no. S0389
  - Nanopore water
- Alternative HRP substrates
  - AEC Substrate Kit (ab64252) from Abcam
  - StayYellow/HRP (ab169561) from Abcam
- Other reagents
  - Phosphate-buffered saline (PBS) buffer (0.010 M phosphate, 0.14 M NaCl, pH 7.4)
  - Tris-buffered saline (TBS) buffer (0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, 1.0% Bovine Serum Albumin (BSA), 0.05 % v/v Tween, 0.024 % thiomersal, pH 8.0)
  - Polyclonal Rabbit Anti-FITC/HRP (affinity isolated) F'(ab) from Agilent Dako, part number P510050-8

3.2 BLI Kit General Protocol

The general, basic protocol for running the BLI assay in the Octet® HTX:

1. Plan the experiment in the Data Analysis 11.0 software. Use the settings in table 2.
2. Equilibrate all reagents except DAB to room temperature and prepare a 96 well washing plate with sample buffer. Prepare the biosensor tray by putting the hydration plate in the biosensor box, putting the biosensor tray on top, and add the biosensors and let hydrate for at least 10 minutes.
3. Prepare the reagent plates in 96 or 384 well microplates. Use 200 μl in each well in the 96 well plates and 80 μl in each well in the 384 well plates.
   3.1. Prepare the standard curve by diluting the HCP standard material in sample buffer.
   3.2. Prepare the samples by diluting them into the analytical range of the standard curve in sample buffer.
   3.3. Add the standard and the samples to the sample plate.
4. Prepare the reagents by diluting them and place them in the assigned wells on the reagent plate/s:
   - FITC-α-HCP Ab diluted 1:100 in sample buffer (Ab2)
   - Sample buffer
   - HRP-α-FITC Ab diluted 1:50 in sample buffer (Ab3)
   - Peroxide buffer
   - metal enhanced DAB (diluted 1:10 in peroxide buffer)
     The DAB should stay in -20°C until right before use.
5. Place the plates in the Octet® HTX instrument at the assigned places according to the software.
6. Launch and run the Octet Data Acquisition Software with the settings found in table 2. Also use:
   - Read Head: X channels (high throughput), where X corresponds to the number of sensors used
   - Acquisition Rate: Standard (0.6 Hz, averaging by 5)
   - Under Run Setting, set plate temperature to 30°C and tick off the Delayed experiment start.
   - Under Advanced settings, set the sensor offset to 3 mm.

Table 2: ***General settings in the Octet Data Acquisition Software.*** *On the α-CHO sensors the Ab1 is already immobilized, why the first two steps Ab1 and Buffer 1 are excluded when working with the CHO sensors. Buffer 1, 2 and 3 are the same buffer type if nothing else is stated. Ab1 is the biotinylated α-HCP Ab for loading, Ab2 is the FITC-α-HCP Ab and Ab3 it the HRP-α-FITC Ab. They correspond to the Abs in figure 4 and table 1.*

	Time (s)	**Shake speed (rpm)**
Ab1	120	1000
Buffer 1	30	400
Sample (HCP)	1800	1000
Buffer 2	30	400
Ab2	1800	1000
Buffer 3	30	400
Ab3	240	1000
Peroxide buffer	30	400
DAB	60	1000

3.3 Buffer Exchange

Buffer exchange was carried out with a Zeba™ Spin Desalting Column according to the manufacturer's instructions.

3.4 Biotinylation of Antibodies

NN α-HCP Abs were biotinylated with a water soluble biotin using a biotinylation kit, EZ-Link™ NHS-PEG4-Biotin (Thermo Scientific). Biotinylation was carried out according to Sartorius' recommendations in Tech Note 28 [23] and Tech Note 24 [24] for CHO and yeast Abs. Biotinylation stopping was carried out with a Zeba™ Spin Desalting Column according to the manufacturer's instructions (Thermo Scientific). Concentrations were measured before and after biotinylation with a NanoDrop® or DeNovix®. When lower volumes than 30 μl were biotinylated, extra 15 μl of buffer were added to ensure full recovery of protein.

3.5 FITC-Labeling of Antibodies

Before FITC-labeling of the Ab2, the buffer was changed using a Zeba™ Spin Desalting Column according to the manufacturer's instructions. The Abs

were then FITC-labeled with the Molecular Probes "Fluorescein-EX Protein Labeling Kit (F10240)".

3.6 ELISA

ELISAs were conducted according to NN Standard Operating Procedures, but have not been performed in this project. The data from ELISAs in this report comes from routinely run assays. The CHO ELISA uses PBS and the yeast ELISA uses TBS as dilution buffer, but they have the same assay setup. The ELISAs do not use the same signal enhancing system, but instead uses tetramethylbenzidine (TMB) and spectrometry for signal detection. α-HCP Abs are coated to the surface by a surface-protein interaction, and the surface is then blocked with BSA. HCP samples and standards are added, and then another α-HCP Ab labeled with biotin. A streptavidin-coupled HRP enzyme binds the biotin, and lastly the TMB substrate is added.

3.7 Analytical Parameters

As the analytical parameters mostly are needed for comparing BLI results with ELISA results, it was decided that estimated values sufficed for this study.

The LLOQ was determined according to its definition

$$\frac{10\sigma}{m},$$

where σ is the standard deviation of a low concentration sample and m is the slope of the calibration curve, which in other words mean that the signal should be 10 times higher than the noise to be detectable, as the standard deviation is a measurement of the noise [35].

Precision explains how well replicate measurements correspond to each other [35]. The "precision estimate" was here determined from measuring the same samples two times, and then calculating the relative standard deviation as

$$\frac{\sigma}{\mu} \cdot 100,$$

where σ is the standard deviation and the μ is the average of the measurements. This is also called the coefficient of variation (%CV). The accuracy has not been calculated due to time and reagents' constraints.

4 Results

The development and optimization of the new method has contained several steps: a kit implementation and comparison to existing assays, a change of antibodies and, at last, a change of all reagents.

A proof of concept of the kit assay have been shown in the pre-study, presented in the Introductory Experiments. When the commercial kit assay had been tested and found suitable for the purpose, all reagents from the kit was exchanged to form a fully customized assay with in-house reagents and antibodies. Each reagent and assay step had to be optimized, why each step is described below. The assay was setup for CHO and for yeast. Furthermore, the HRP substrate has been exchanged to a new substrate yielding higher signals and a safer assay, and an automation of the manual pipetting work have been elaborated.

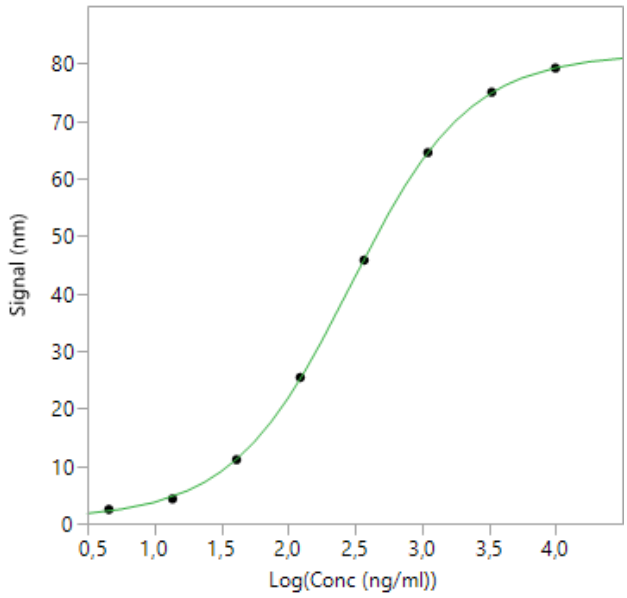
4.1 General BLI Protocol Results

The full kit assay used a three fold dilution of NN CHO WSA (HCP) in a standard curve consisting of seven points, starting at 10 000 ng/ml. The top concentration gave signals from the full assay of around 80 nm. The full standard curve and full assay signals can be seen in figure 7 and will be used as a reference for the project.

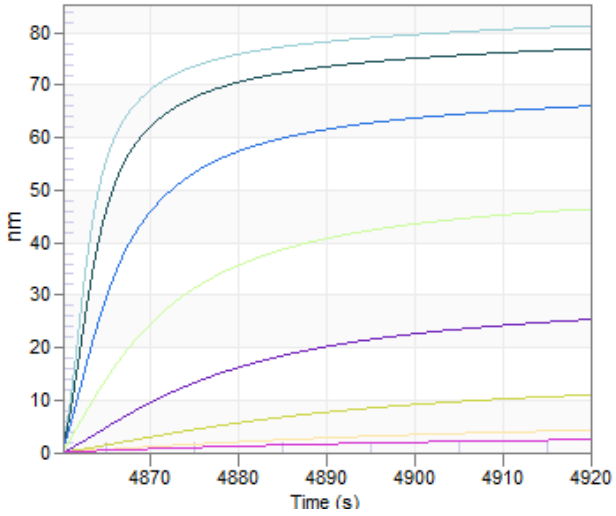
4.2 Customizing the NN BLI Assay

Each step of the assay was investigated and optimized to obtain the highest output signals possible. Full assay signal refers to the signal enhancing step's signal, while loading signals is the binding rate after the biotinylated antibodies have bound the sensor.

The graphics on the left side of the page visualizes the exchange of reagents step by step. The reagent discussed in the corresponding section is highlighted in green. The full assay setup can be seen in figure 4.



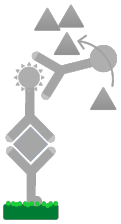
(a)



(b)

Figure 7: ***Outputs from the kit assay with HCP standard material from NN.*** *a) The standard curve for the kit assay showing a 4PL curve fit. Signals were read at equilibrium state. The highest concentration reached signals around 80 nm. b) The full assay signals from the DAB step for the different standard concentrations as presented by the Data Analysis Software.*

4.2.1 Exchange of Biosensors Reduces Signals



To be able to load sensors with biotinylated NN Abs, streptavidin coated sensors were used. SAX and SAX2 biosensors were used as they are specially developed for quantitation, and both be used for quality control production release [36], [37].

Both CHO and yeast assays were tested using both types of sensors, and both types of streptavidin sensors gave lower signals than the kit CHO sensors in all cases. As can be seen in table 3, the SAX sensors gave higher signals for yeast compared to the SAX2 sensors, while the SAX2 sensors gave higher signals for CHO. Due to this, different sensors were used for the two organisms further on. The CHO sensors could not be used for yeast, since the antibodies used are raised against CHO HCP.

Table 3: ***BLI biosensor signals obtained for different organisms.*** *The CHO and the yeast full assay signals when using different sensor types. Signals obtained for the highest concentrations of CHO and yeast HCP standard, 10 000 ng/ml and 900 ng/ml respectively.*

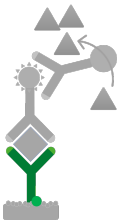
	SAX sensors	**SAX2 sensors**	**CHO sensors**
CHO (nm)	14	27	80
Yeast (nm)	54	41	-

4.2.2 Changing Storage Buffer to PBS Favors Loading

All NN antibodies used are stored in a liquid of 50% glycerol and 50% PBS. The glycerol impedes the biotin-streptavidin binding and the antibody-antigen binding, why the buffer was changed for all antibodies to sample buffer or PBS before biotinylation of the antibody, FITC marking or any other use in the BLI assay.

The exchange of the buffer gave clear results that a buffer exchange was needed: for NN yeast Abs MCR 1:100 Ab:biotin the loading signals before buffer change reached around 0.5 nm and full assay signals around 1.23 nm, while after buffer exchange the signal increased to 1.6 nm and 14 nm respectively.

4.2.3 Biotinylation of α-HCP Abs Affects Loading



Biotinylation and loading was investigated using an OVAT approach as there was no previous knowledge about affecting parameters. The investigated parameters were

- Biotinylation protocol and storage buffer
- b-Ab concentration
- Loading time
- Molar coupling ratio
- Dilution buffer

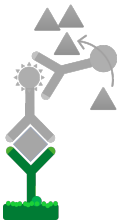
Biotinylation of Abs in Different Molar Coupling Ratios and Protocols The MCR is the amount of biotin molecules compared to the amount of antibody molecules in a solution, as discussed in section 2.4.1. The loading of the sensors lays the foundation for the rest of the assay, why four different MCRs for antibody:biotin were tested: 1:0.5, 1:1, 1:3 and 1:100 to scout if it made a difference and find the optimal MCR.

NN are using biotinylated antibodies for both CHO and yeast HCP detection in their HCP ELISA. These antibodies are biotinylated in a dimethylsulfoxid (DMSO) reagent with an MCR of 1:100. NN CHO α-HCP Abs were biotinylated with a water soluble biotin with MCRs of 1:0.5, 1:1 and 1:3 using a biotinylation kit.

Concentration of antibodies were measured before and after biotinylation with a NanoDrop or a DeNovix. The outcome of the biotinylation was first seen after loading the b-Abs on the sensors.

Biotinylation Protocol Impact on Assay Signals The difference in the three biotinylation protocols showed that the protocol from the Technical Note 28 [23] gave slightly higher loading signals when using the CHO MCR 1:0.5. This may be because the antibodies are not diluted before being mixed with the biotin. The protocol from Tech note 24 [24] gave about double the loading signal than the protocol in Tech note 28 [23] when running yeast b-Ab MCR 1:0.5 in a concentration of 25 μg/ml. However, the full assay signal was the same when using both protocols, indicating that it has no impact which protocol was used. The loading of Abs MCR 1:100, biotinylated in DMSO, gave significantly lower loading signals.

4.2.4 Loading of b-Abs Crucial for Full Assay Signal



The amount of antibody loaded on the sensor was important as it forms the foundation of the assay. The more antibodies loaded correctly, the more HCP can bind, which makes the assay more sensitive.

Loading was carried out online on the Octet® HTX by adding a loading step where sensors were dipped in a solution of b-Abs for a fixed time at 1000 rpm (revolutions per minute), and then dipped in buffer for 30 seconds and 400 rpm. For the loading investigation, only the b-Ab was changed and the rest of the reagents were from the kit. Buffer type impact was investigated, and loading time and b-Ab concentration was scouted for the different molar ratios of b-Abs.

When loading biotinylated antibodies a signal of at least 2 nm is desired according to the Tech Note 24 from Sartorius [24]. All full assays were performed according to the BLI Kit General Protocol, for the respective organism and antibodies. Overall it was assumed that the same settings would work similarly for both yeast and CHO, hence if a setting seemed optimal for one organism the same was assumed to also be optimal for the other.

The loading of biotinylated antibodies onto the streptavidin biosensors showed, surprisingly, that a lower MCR of antibody:biotin, lower concentration of b-Ab and shorter loading time gave higher full assay signals. Because of this, it was suspected that the sensors became over-biotinylated, indicating that antibodies bound in the wrong direction and thereby blocking the sensor tip. Because of this, testing mostly focused on reducing parameters such as concentration, MCR and loading time as much as possible. Reducing these parameters would also be beneficial for reducing the total assay time and for saving reagents. The approaches are described below.

Molar Coupling Ratio Scouting From introductory loading experiments with MCR 1:100, the MCR was suspected to impact the full assay signal greatly, why it was further investigated with several MCRs.

The impact of the b-Ab MCR was firstly explored by performing full assays with α-CHO-HCP Abs in MCRs of 1:100 and 1:1 with 15 minutes loading time. After showing no large difference in loading signal or full signal and time had been lowered to 2 minutes, CHO b-Abs in MCRs 3:1, 1:1 and 1:0.5 in a concentration of 12.5 μg/ml were run with a loading time

of 2 minutes according to the BLI Kit General Protocol.

When loading with b-Ab 1:100 and 1:1 for 15 minutes, both loading signals reached around 2.5 nm and full assay signals for the highest concentration (10 000 ng/ml) reached around 10 nm for both assays.

After lowering loading time to 2 minutes the MCR did still not seem to make a large difference, see table 4. However, the lowest MCR of 1:0,5 gave the highest full assay signals obtained with the CHO NN Abs, 27 nm.

Table 4: ***Effect on signal outcome of loading different biotinylated antibody molar coupling ratios.*** *Loading different MCRs with the same time and concentration showed that lower MCRs can give higher full assay signals, but shows no clear trend.*

MCR	**Time (mins)**	**Ab conc (μg/ml)**	**Load (nm)**	**Full assay (nm)**
1:0.5	2	12.5	2.8	27
1:1	2	12.5	2.3	19.6
1:3	2	12.5	2.8	25.4

Concentration Scouting of b-Ab Loading Concentration dependency was tested with the MCR 1:100 on SAX2 sensors. Biotinylated α-yeast-HCP Abs were loaded for 60 minutes in three different concentrations: 12.5 μg/ml, 24.5 μg/ml and 49 μg/ml according to loading description above. The first concentration study with yeast b-Abs MCR 1:100 can be seen in figure 8 and shows that independently of concentration, the loading signal is saturated at around 1.5 nm. Because of this, the lowest concentration of 12.5 ng/ml was chosen for further investigation.

The results were confirmed when the experiments were repeated with CHO b-Abs MCR 1:0.5 with the same three concentrations, when also measuring the full assay signal. The lower MCR slightly increased the loading signals, although the full assay signals showed to barely be affected by the b-Ab concentration as shown in table 5.

Table 5: ***Concentration scouting for loading.*** *Loading CHO b-Abs MCR 1:0.5 for 2 mins did not show any notable difference for the full assay signal.*

Ab conc (μg/ml)	**Max loading signal (nm)**	**Full assay signal (nm)**
12.5	2.4	25.4
25	3.3	27.8
50	3.4	26.9

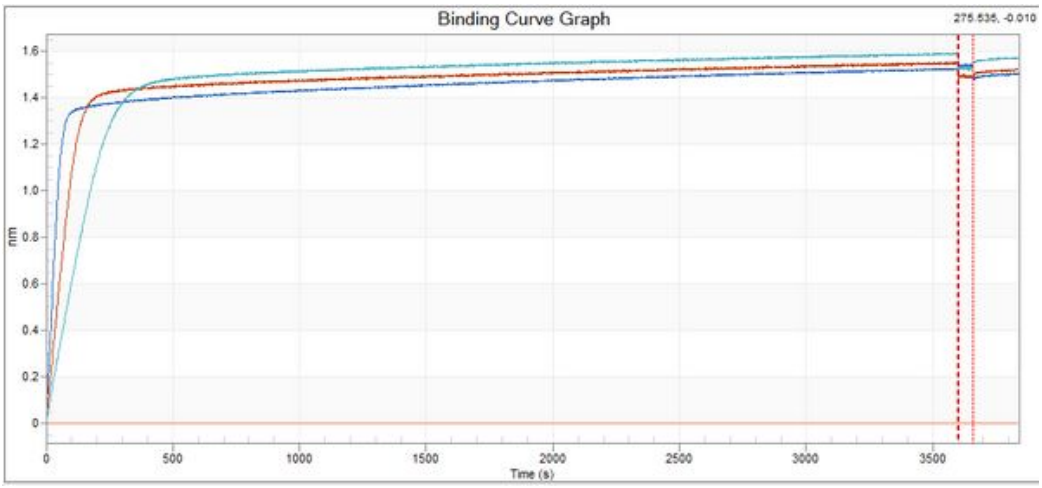


Figure 8: ***Effect of yeast b-Abs' concentration and loading time on loading signal.*** *Loading yeast b-Ab for 3600 seconds (60 minutes) onto the SAX2 sensors with three different concentrations. Dark blue = 49 µg/ml, red = 24.5 µg/ml and light blue = 12.5 µg/ml.*

Because of the low trends seen from the concentration scoutings, it was concluded that concentration did not to have any particular impact on the full assay signal. As a consequence of these results, it was decided to continue with the 25 μg/ml due to slightly higher signals than obtained with 12.5 μg/ml both for maximum concentration but especially for the lower concentrations in the standard curve, and due to more stable signals when using 25 μg/ml than 12.5 μg/ml in some experiments.

Loading Time Scouting Because MCR and concentration did not seem to have an impact on the full signal and since lower full assay signals than desired was still obtained, a shorter loading time was investigated.

Three concentrations of yeast b-Ab of 12.5 μg/ml, 24.5 μg/ml and 49 μg/ml, were loaded for 60 minutes on the SAX2 sensors to investigate the effect of time on loading signals. Loading signals can be read at each time point and visualized by the Data Analysis program.

The initial loading time experiment can be seen in figure 8, where the loading seemed to be saturated after about 500 seconds (approximately 8.3 minutes), after which it only increased slightly.

Then the full assay signal was also included into the assay, which was performed with CHO b-Abs MCR 1:0.5 in a concentration of 12.5 μg/ml with

a loading time of 2 and 15 minutes. It showed that loading for 2 minutes still saturated the loading signal, but the full assay signal in the end gave a higher value than when loading for 15 minutes, see table 6. Less than 2 minutes loading time would not give a saturated binding, why it was assumed to be wasting reagents to investigate. A better approach would then be to lower the concentration of bAb even more, which from concentration experiments did not seem beneficial (see section 4.2.4).

Table 6: ***Effect on full assay signal of loading time for biotinylated antibodies.*** *Loading the CHO b-Abs MCR 1:0.5 for 2 minutes increased the full assay signal compared to a 15 minutes loading time.*

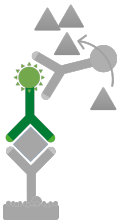
Loading time (mins)	**Max loading (nm)**	**Full assay (nm)**
2	2.2	19.4
15	2.6	10.3

Dilution Buffer To investigate whether the buffer impacted the assay, the loading was tested in both sample buffer and PBS. When loading CHO b-Abs MCR 1:0.5 in concentration of 25 μg/ml the loading signals were similar when diluting in sample buffer and PBS. Sample buffer gave a loading signal of around 1.3 nm while PBS gave a signal around 1.0 nm. It implied PBS could be used for further studies in the customized assays.

4.2.5 Sugar Coating Preserves the Loaded Biosensors

It was desirable to be able to load sensors and then store them, so they were ready to use when needed. For storage, the loaded biosensor tips needed to be preserved. Storage conditions were accomplished by sugar coating the sensors by dipping the sensors in a solution of 15% (w/v) sucrose in nanopore water. The sensors were then dried for 5 minutes at 37°C and stored in the original bag with the provided desiccant. Sugar coated sensors were compared to freshly loaded sensors. The sugar coated and non-coated sensors gave the same results, which implies that sugar coating did not have an effect on the stability of loaded sensors (data not shown).

4.2.6 Exchanging FITC Labeled Ab Affects Assay Minimally



To investigate whether FITC labeled NN-Abs could be used, the FITC-labeled Abs from the Anti-CHO HCP kit were replaced by FITC labeled NN Abs. Although the initial concentration of the kit FITC labeled Ab was not known, the FITC-labeled NN-Abs were diluted 1:100, as the kit FITC-labeled-Abs.

When a full kit assay was compared to an assay where the FITC-labeled antibody was exchanged, a parallel shift was seen when comparing the signal to concentration ratios, see figure 9. The shift means that the NN FITC Ab give slightly lower signals for corresponsing concentrations, but the reduction was considered small snough to keep using the NN FITC Ab.

It has to be kept in mind that the two compared FITC-labeled antibodies' specificity probably are different, which also should affect the signal. Furthermore, the optimal concentration of the NN FITC α-HCP Ab was not further investigated, which might have impacted the outcome.

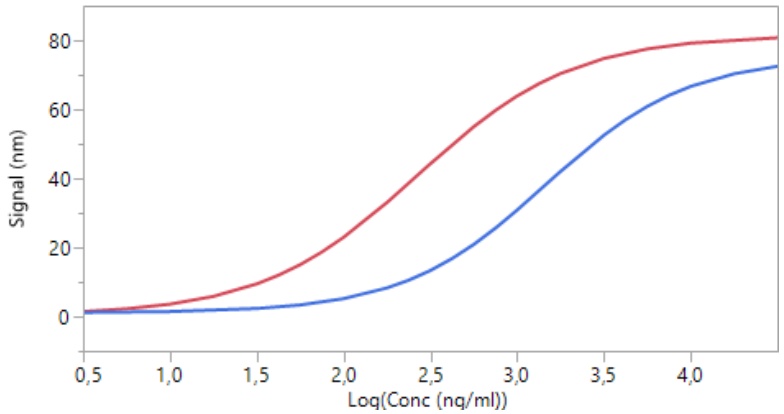
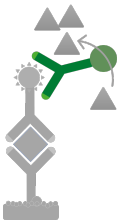


Figure 9: ***Difference in signal between kit FITC Abs and NN FITC labeled Abs standard curves.*** *The red curve obtained using the kit FITC Ab, and the blue using the NN FITC Ab. There is a difference in signal, but the curve more or less keeps its shape and the linear interval, why the NN FITC was assessed acceptable to use in further assays.*

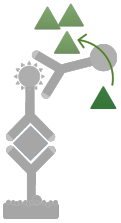
4.2.7 Exchanging HRP-α-FITC Ab Affects Assay Minimally



To show that all reagents could be exchanged form the kit, HRP-α-FITC Abs were ordered from Agilent Dako and a concentration scouting was run on the CHO kit using the Dako HRP-α-FITC Abs. The antibody was used in 7 dilutions, with a 2 fold dilution from 50 to 32 000 times dilution. The initial concentration of the HRP-α-FITC Ab was not known. The concentration scouting showed that a dilution of 100 and 200 gave comparable results to when the kit assay reagent was used. Lower concentrations gave lower signals, while a higher concentration gave saturated sensors and unreliable signals.

A 200 times dilution was the lowest dilution that could be used obtaining approximately the same signals as the kit assay, and was hence used for all fully customized assays.

4.2.8 New HRP Substrate Gives Higher Signals and Safer Assay



As the DAB is classified as a hazardous substance, it has to be handled in a fume hood. Furthermore, DAB has a short stability time, making it desirable to exchange it to another signal enhancing substrate. Two substrates were tested: AEC and StayYellow, which both are typically used for histochemical staining.

A full Anti-CHO HCP kit assay was run according to the BLI Kit General Protocol with 600 seconds for the signal enhancing step with the three substrates AEC, DAB and StayYellow. Three dilutions from the standard curve was used: 10 000 ng/ml, 370.4 ng/ml and 13.7 ng/ml NN CHO WSA in sample buffer. AEC and DAB were diluted in peroxide buffer, and StayYellow was diluted in the buffer provided with the StayYellow kit. AEC and StayYellow were diluted in two concentrations each.

Both substrates showed promising results with a concentration dependency in assay signal at all concentrations tested, see figure 10. This was very promising for solving the problems with the low full assay signals obtained from the loading of the SAX2 sensors.

As the StayYellow gave signals with irregular patterns, it was decided to continue with the AEC in a DOE to investigate the optimal parameters. The Anti-CHO HCP kit assay was used in the DOE and four parameters

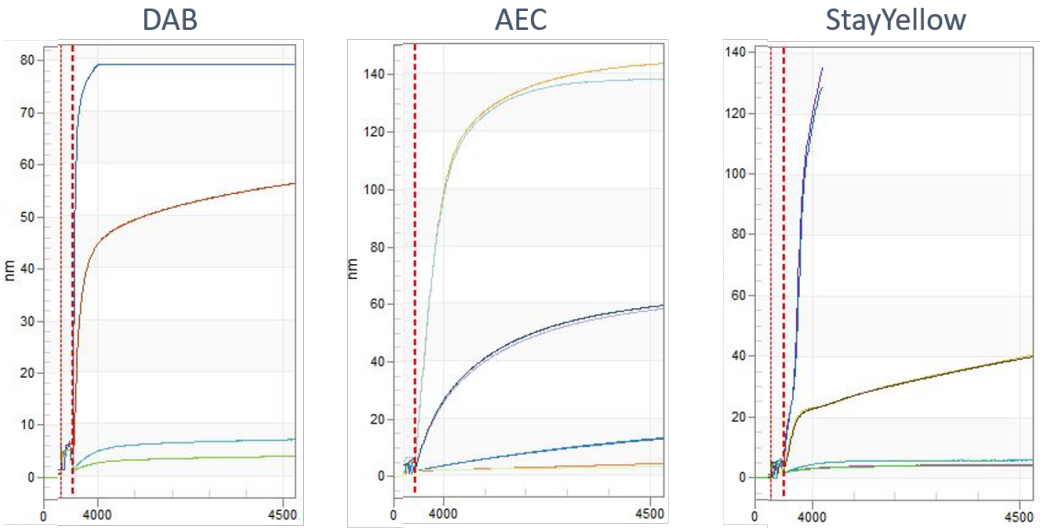


Figure 10: ***Signals obtained by use of either DAB, AEC or StayYellow as HRP substrate.*** *A full assay using the three signal enhancing substrates on three standard concentrations. The x-axes show the reading time in seconds and the y-axis the binding rate signal in nm. AEC and DAB are diluted in peroxide buffer and StayYellow in its provided buffer. AEC 1:1, DAB 1:10 and StayYellow 1:25. AEC and StayYellow both show higher signals than DAB for all the three HCP concentrations.*

were chosen for investigation: type of buffer, detection time, shake speed and concentration of signal enhancing substrate. The buffer was used for dilution of AEC and instead of peroxide buffer according to table 2. The DOE setup is shown in table 9 that can be found in Appendix C. All experiments for 400 rpm respectively 1000 rpm were run at the same time, all in all two runs were performed. The signals that "broke" before the reading time were read at the last time point where a signal was detected. In figure C.1 in Appendix C an example of "broken" signals can be seen.

The small DOE gave clear implications that concentration was the most affecting factor (see figure 11): the lower AEC concentration the higher the assay signal and the lowest risk for "broken" signals. It was also clear that peroxide buffer should not be used, while the use of either sample buffer was shown to be the best to use. Detection time gave higher signals at 600 seconds, but at the same time higher risk for "broken" signals. 60 seconds gave low but consistent signals. The shake speed did not seem to have an impact on the signal. No interactions were seen between the four parameters.

After the DOE a run with the AEC optimal parameters was carried out

with the Anti-CHO HCP kit to obtain a standard curve from an at the time optimal run. It showed a well-fitted 4PL standard curve with signals up to around 100 nm, as shown in figure 12. A reading time of 100 seconds gave the highest but yet stable signals. The short reading time, 100 seconds compared to the initial 600 seconds, explain why signals did not reach as high as in the initial tests. This shows that AEC can substitute DAB for BLI assays with signal enhancing substrates.

A comparison of the AEC and the DAB signals for the same concentrations can be seen in figure C.2 in Appendix C.

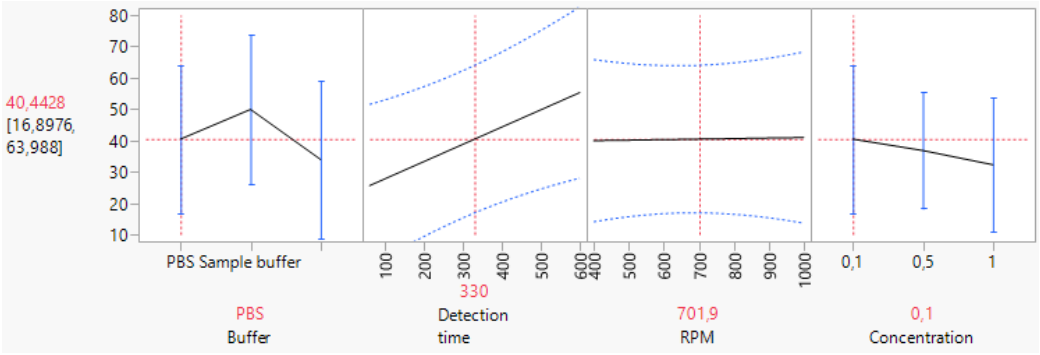
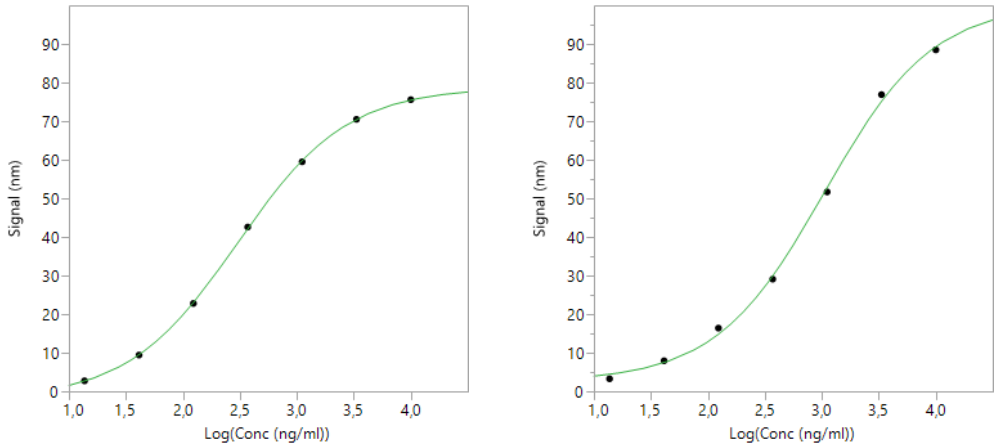


Figure 11: ***The trends for AEC environment shown from the DOE.*** *The impact of the different parameters for the full assay signal shown on the y-axis in nm. With the parameters chosen in red the full assay signal is predicted to be 40.44 nm for a concentration of 370.4 ng/ml NN CHO HCP WSA. The experiments have been carried out with the Anti-CHO HCP commercial kit. The blue lines are the confidence intervals which arre also described with the black numbers within brackets to the right. The y-axis shows the full assay signal in nm. The third buffer point on the x-axis is peroxide buffer. Detection time is measured in seconds and concentration is the volume percentage of AEC in buffer. Rpm is the unit for shake speed.*

AEC in TBS As TBS was used as standard buffer for the yeast assay, it was also investigated if AEC was affected by TBS. A full yeast standard curve was run with AEC in TBS. It showed significantly reduced signals with the signal for the highest HCP concentration of 900 ng/ml reduced from approximately 100 nm to approximately 50 nm (data not shown). It was not considered suitable to use AEC in TBS, and PBS was used as dilution buffer for AEC for all fully customized assays.



(a) ***DAB*** *(b)* ***AEC***

Figure 12: ***AEC vs DAB standard curve with the kit CHO assay on the BLI.*** *The same assay run with DAB (to the right) and with AEC (to the left) in 10% (v/v) in PBS with 100 seconds reading time. A 4PL curve is fitted to the standard curve points and show a good fit for both substrates with r^2-values of 0.999. The slightly higher signals of AEC yield a higher sensitivity of the assay.*

4.2.9 Buffer Type Impacts on Full Assay Signals

Antibodies can be very sensitive to the buffers they are stored or diluted in, and the buffer composition can greatly affect their binding characteristics and ability. Because of this, the full assay dilution buffer's impact on the full assay signal was investigated. Three different buffers were tested, sample buffer, PBS and TBS. The kit sample buffer was exchanged to PBS for CHO and TBS for yeast. TBS could not be used for loading as it contains BSA, which would block the sensors and hinder the biotinylated antibodies from binding. The assays were run in a comparison assay with the different buffers for the two organisms.

Changing to PBS seems to have a slight influence on the signal for the CHO assay, especially on the signals obtained around the linear part/inflection point of the standard curve, as can be seen in figure 13. Anyhow, the difference was not considered to discard the use of PBS, as it is very desirable to have a common buffer as is used for the ELISA, to facilitate the customization and comparison between the assays. For the yeast assay, using TBS was shown very beneficial compared to PBS, as can be seen in figure 14.

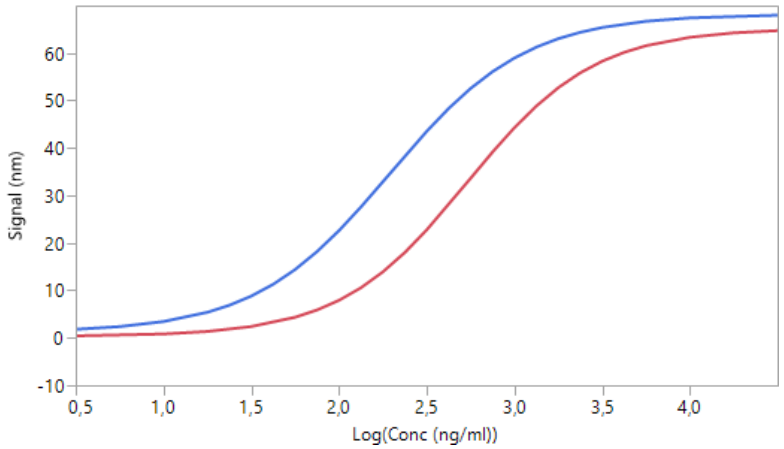


Figure 13: ***Standard curves for kit assay with sample buffer from kit and PBS.*** *The blue curve is the standard curve for the full kit assay with the sample buffer and the red is the full kit assay mixed in PBS. The PBS renders lower signals on the linear part, but not sufficiently to discard the use of PBS.*

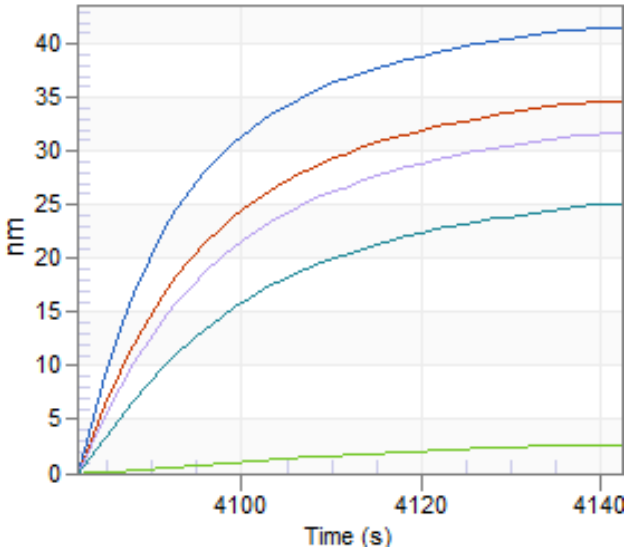


Figure 14: ***Signals for yeast full assay in different buffers.*** *The top two lines are yeast in sample buffer from the kit, the second two are yeast in TBS while the lowest is yeast in PBS. The same standard concentrations were used for all buffers: 900 and 300 ng/ml.*

4.2.10 Other Improvements of BLI Assay Increase Flexibility

To further enhance the assay, other improvements like buffer reuse and loading in 384 well plates further increased the flexibility of the assay.

Reuse of Washing Buffer To save buffer and pipetting time it was desirable to dip the sensors in the same buffer wells several times. As the surrounding matrix should not affect the signal obtained, and since the different reagents should not interact and interfere with other steps, it should not affect the assay signal to dip the sensors in the same buffer wells several times. The kinetic software in the Data Acquisition Software allows the sensors to dip in the same wells several times. The two top concentrations of the CHO standard curve were run according to the BLI Kit General Protocol, but run with the kinetic software to test the dipping in the same buffer. All sample buffer steps were dipped in the same buffer.

The reuse of buffer gave approximately the same signals as when dipping sensors in different buffer wells. The signals are normally around 80 nm and 75 nm when dipping in new buffer, but varies slightly in between runs. When dipping in the same buffer several times, the signals were 83.5 nm and 76.4 nm. No reference sensors were run enabling subtraction of background signal, so the difference could also be specific for this run. This was considered an acceptable deviation, and this approach was used for example in the planned automation assay.

Loading in 384 Well Plates Loading in 384 well plates would increase flexibility, save reagents and open up for opportunities to run more samples at the same time and not having to first load, preserve and store. It would enable loading and running directly after each other withing the same run, without affecting number of samples, reagents or microplates.

Loading CHO and yeast b-Abs in 384 well plates was tested after loading signals had stabilized, and did not show any difference from loading in 96 well plates. 384 wells were used as standard measure for loading in further experiments.

Premixing of FITC-α-HCP and HRP-α-FITC To save space in the microplates and time in the assay, a mixing of FITC-α-HCP (Ab1) and HRP-α-FITC (Ab2) prior to addition to the microplate was investigated. Antibodies were mixed without shaking in room temperature for around 30

minutes before the machine assay, and incubated for 30 minutes at 1000 rpm in the BLI machine. The premixing of the two antibodies yielded very low full assay signals at first try, why it was not further investigated.

4.3 Customized Assays

The yeast and CHO assays were performed as fully customized assays both with AEC and with DAB, and samples were run on the yeast assay.

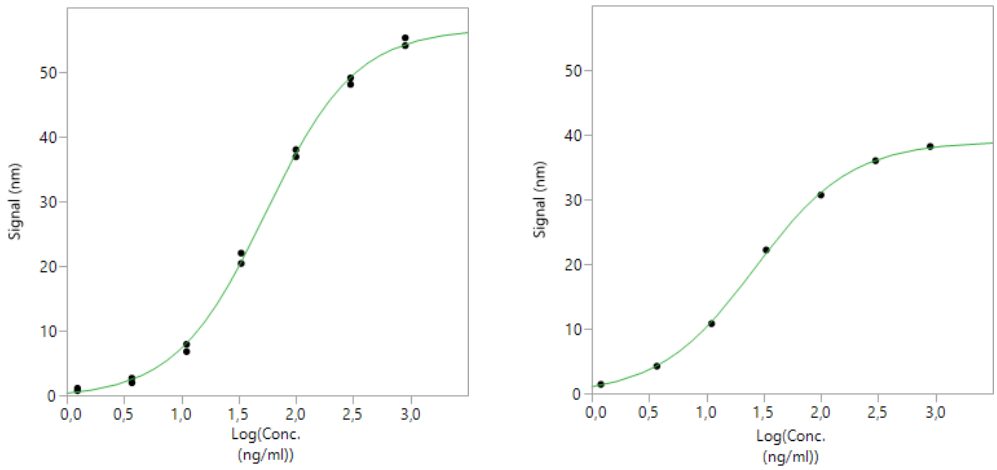
4.3.1 Customized Yeast BLI Assay Give Well-Fitted 4PL-Curves

The final customized yeast assay had the following settings:

Biosensor	SAX sensors
Biotin	Water soluble EZ-Link™ NHS-PEG4-Biotin, MCR 1:0.5, protocol Tech Note 24 [24]
Load	25 μg/ml b-Ab MCR 1:0.5 diluted in PBS
Standard	NN yeast HCP in TBS, 7 points 3 fold dilution from 900 ng/ml + reference (TBS buffer)
FITC-α-HCP Ab	NN-α-HCP Abs FITC labeled with Molecular Probes® Fluorescein-EX Protein Labeling Kit, diluted 100 times in TBS from unknown initial concentration
HRP-α-FITC Ab	Polyclonal Rabbit α-FITC/HRP diluted 200 times in TBS
HRP substrate	DAB diluted 10 times in peroxide buffer/AEC diluted 10 times in PBS
Wash buffers	1 & 4 PBS, 2 & 3 TBS

The yeast assay with DAB renders a standard curve with an acceptable 4PL fit, using the exact same standard concentrations as in the yeast ELISA, see figure 15a. This shows that a fully customized assay can be made with yeast. The customized yeast assay using DAB overall gave higher signals for corresponding concentrations than the CHO kit assay, see figure 16.

Running with AEC with the same standard curve also give a nice 4PL fit, however with lower signals than the DAB assay when using a detection time of 100 seconds, see figure 15b.



(a) ***DAB*** *(b)* ***AEC***

Figure 15: ***Standard curves from customized yeast assay with DAB and AEC.*** *Left, yeast on SAX sensors using DAB as precipitating substrate with 60 seconds reading time. Two runs fitted with 4PL giving an r^2-value of 0.999. The highest concentration of 900 ng/ml reached signals around 55 nm. Right, yeast using SAX sensors with AEC as precipitating substrate with 100 seconds reading time.*

4.3.2 Customized CHO BLI Assay Challenges

The final customized CHO assay had the following settings:

Biosensor	SAX2 sensors
Biotin	Water soluble EZ-Link™ NHS-PEG4-Biotin, MCR 1:0.5, protocol Tech Note 24 [24]
Load	25 μg/ml b-Ab MCR 1:0.5 diluted in PBS
Standard	NN CHO HCP in PBS, 7 points 3 fold dilution from 10 000 ng/ml + reference (PBS buffer)
FITC-α-HCP Ab	NN-α-HCP Abs FITC labeled with Molecular Probes® Fluorescein-EX Protein Labeling Kit, diluted 100 times in PBS from unknown initial concentration
HRP-α-FITC Ab	Polyclonal Rabbit Anti-FITC/HRP diluted 200 times in PBS
HRP substrate	DAB diluted 10 times in peroxide buffer/AEC diluted 10 times in PBS
Wash buffers	PBS

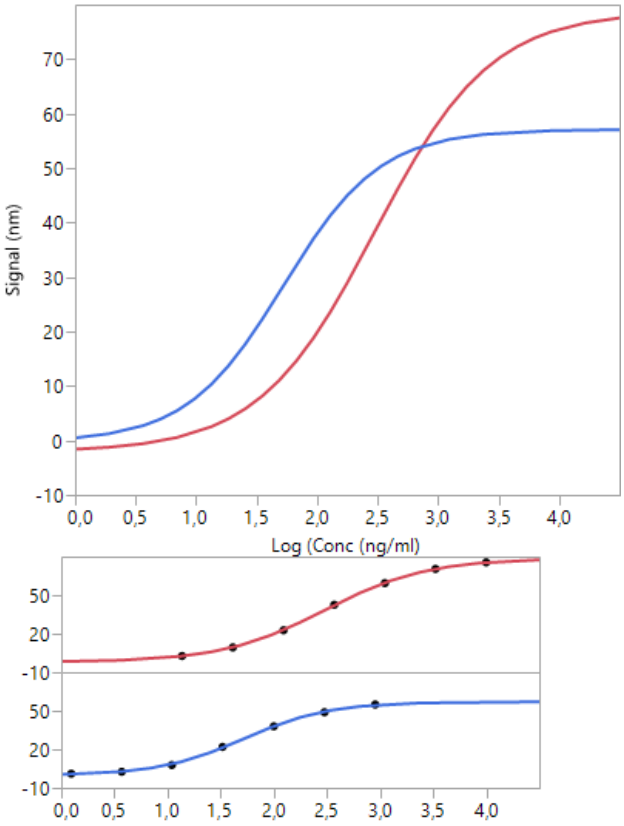


Figure 16: ***Standard curves from yeast assay with all reagents exchanged compared to CHO kit.*** *Blue curve is customized yeast using DAB, red curve is Anti-CHO HCP Kit. The y-axis and x-axis units in the lower figure are the same as in the upper figure. The customized yeast assay with DAB actually gives higher signals for the same concentrations as the CHO kit assay for the concentrations that are measured with both assays.*

The CHO BLI assay did not work well with the customized assay with PBS buffer, and gave standard curves with reversed pattern and very high background noise of 3.18 nm for DAB and 19 nm for AEC assay (see figure C.3 in Appendix C). However, running with sample buffer instead of PBS gave better results as shown in figure 17. From this setup, an upper asymptote were not achieved. The highest reached signal for the highest concentration of standard (10 000 ng/ml) was 27 nm as compared to 80 nm with the Anti-CHO HCP kit.

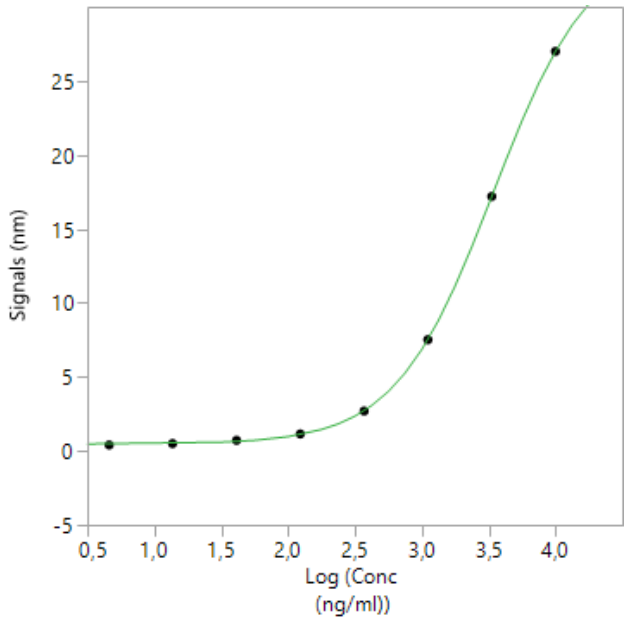


Figure 17: ***Standard curve from CHO assay with SAX2 sensors and NN antibodies.*** *The half-customized CHO assay with SAX2 sensors with NN standard and primary antibodies, but sample buffer, HRP-α-FITC Abs from kit and DAB as precipitating substrate. The curve shows the lower part of the sigmoidal curve from the 4PL fit. The fully customized assay gave reversed shape of the standard curve, see figure C.3.*

4.4 Quantitative Comparison to ELISA Assays

To ensure the BLI assay was usable and measured HCP concentrations in the same range as the ELISA based assays, samples that had previously been analyzed with ELISA were also measured with the BLI yeast HCP quantification assay. A comparison of the BLI CHO kit and the CHO ELISA can be found in Appendix A.

4.4.1 Yeast Assay with DAB Yields Comparable HCP Quantifications to ELISA

The analyzed samples were in different concentrations and taken from different parts of the drug purification process. They were diluted in TBS buffer within the analytical range of the standard curve. Six samples of varying concentration were analyzed, whereof three samples were run two times on two different days to investigate repeatability.

The sample quantification results were in the same order of magnitude as ELISA, see figure 18. For the higher concentrations the BLI/ELISA quota was around 1.17 and for the lower around 0.52. The analytical parameters are described in section 4.4.3.

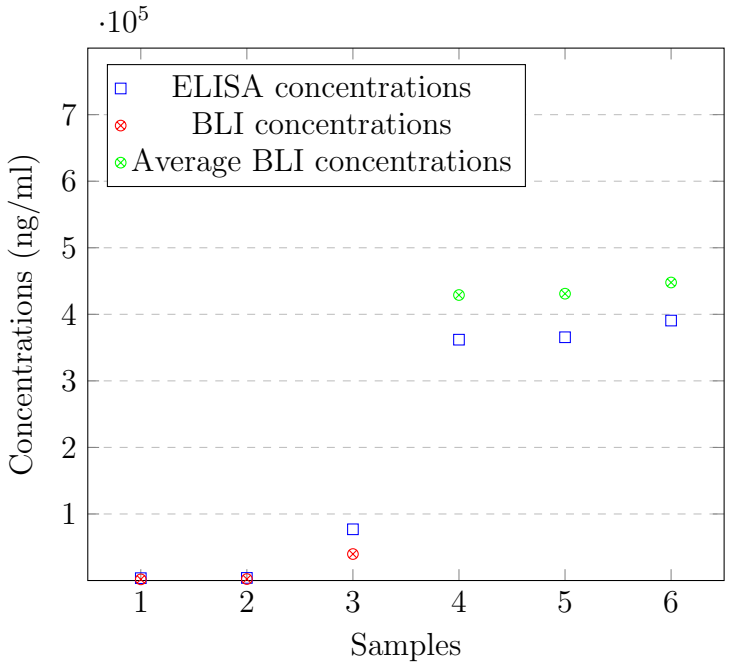


Figure 18: ***Comparison of concentration measurements of yeast samples analyzed using BLI and ELISA with DAB.*** *Six samples were measured on the two platforms, only using in-house reagents except for the signal enhancing substrate. All samples were diluted into three concentrations, and the back calculations are visualized. The red BLI concentrations were only measured once, while the average BLI concentrations were measured two times, why the mean value has been plotted as green dots. ELISA samples were only measured once with a 7 points dilution curve.*

4.4.2 Yeast HCP Quantification Assay with AEC Differs from ELISA

The quantification of samples was more challenging with AEC than with DAB, but are in the same order of magnitude even if they do not correspond fully as shown in figure 19. The assay was run with two samples in duplicates once due to limited amount of sensors, why variation between BLI measurements is not known.

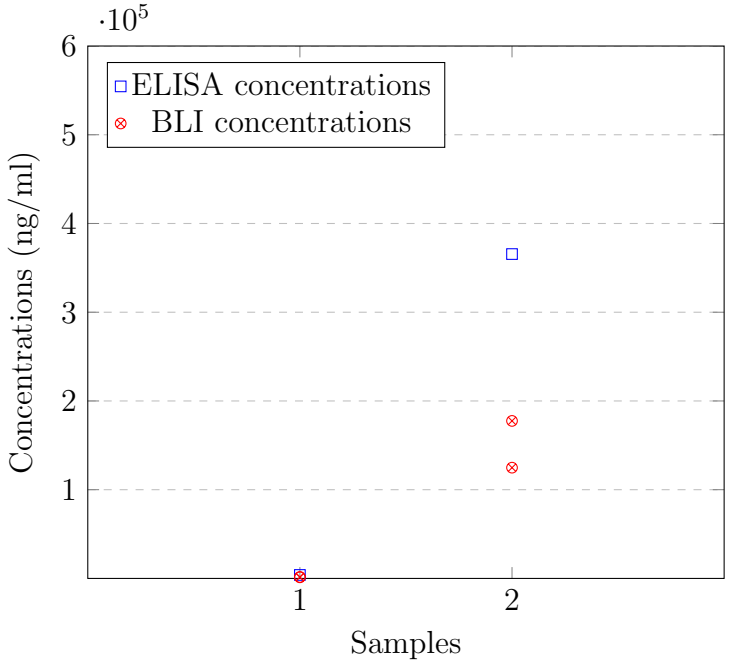


Figure 19: ***Comparison of concentration measurements of yeast samples analyzed with BLI and ELISA with AEC.*** *Two samples have been measured on the two platforms with a fully customized yeast BLI assay. The BLI samples were measured in duplicates and diluted into three concentrations, and the back calculations' averages are visualized. ELISA samples were measured once with 7 points of dilution.*

4.4.3 Analytical Parameters Promising for Yeast DAB Assay

As only the yeast customized assay with DAB gave a full and useful standard curve, the analytical parameters were only discussed for this assay. As this is only an initial study whish will not be used for clinical testing at this stage, the estimated analytical parameters discussed were few and based on a small amount of data.

Three HCP samples were measured two times on separate days with the customized yeast DAB assay. To only have measurements in duplicates is not optimal for calculating statistical parameters - it does not give significant answers. Nevertheless, it gives an estimate of the parameters that, for this study, can be good enough and implicate results.

The relative precision estimate was calculated to 25.6% with the formula from 3.7, which is lower than the desired<20%. Because the measurements are only made twice analysing more samples would give a more accurate estimate.

The LLOQ can be argued in different ways. The standard curve made from measurements in duplicate and fitted to a 4PL sigmoid curve in SAS JMP have an r^2 value of 0.999, meaning the curve is very well defined. The parallelism F test gives a number of 0.7505, meaning the curves are highly parallel and that the experiment is repeatable. The curve also shows a dilutional linearity all the way down to the concentrations of the most diluted standard points at 1.24 ng/ml. The residuals of the standard curve concentration points are consistently lower than the acceptable range of 20%, even at the lowest standard concentration point of 1.24 ng/ml, why it could be argued that LLOQ can be as low as that. However, if calculating LLOQ as ten times the background, it would land around 2.6 ng/ml, which is also close to the LLOQ of the corresponding ELISAs.

The accuracy has not been determined, but the linearity and precision together give an estimate of the accuracy as it could be argued that accuracy is a measurements of the precision in the outer ranges of the linear range. This argues that the accuracy, just as the precision, would be estimated to be of decent values.

4.5 Regeneration of Sensors Needs More Investigation

As the regeneration experiments did not show promising results in the introductive experiments (see Appendix A) it was not further investigated.

4.6 Automation Increases Flexibility of Assay

To automate the assay would reduce hands on time and almost create an end-to-end automated assay, where the sensors and reagents would only need to be transferred to the BLI machine. It was designed theoretically in two ways.

The automation of the manual pipetting steps was designed to be performed using a liquid handler such as Biomek 7 or similar. Due to time restrictions this part was only setup theoretically and never run using a liquid handler. The idea was to make one 384 wells plate with reagents that are the same for all assays, which means all buffers, Ab2 and Ab3 and the HRP substrate, that could be prepared in advance and kept at 4°C for several days, see figure 20.

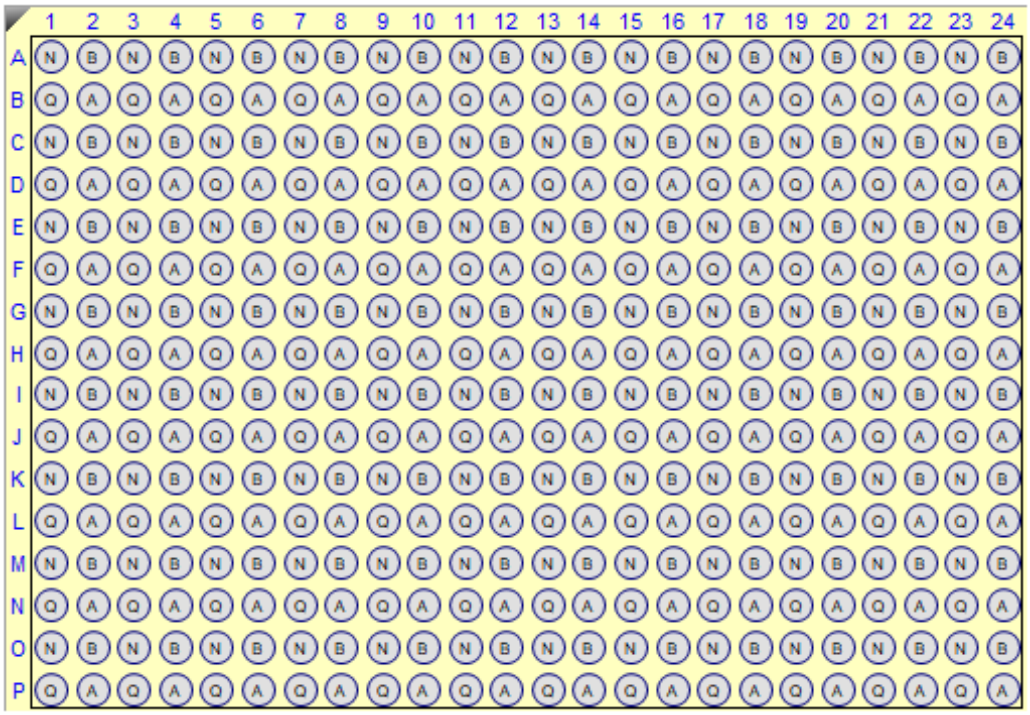
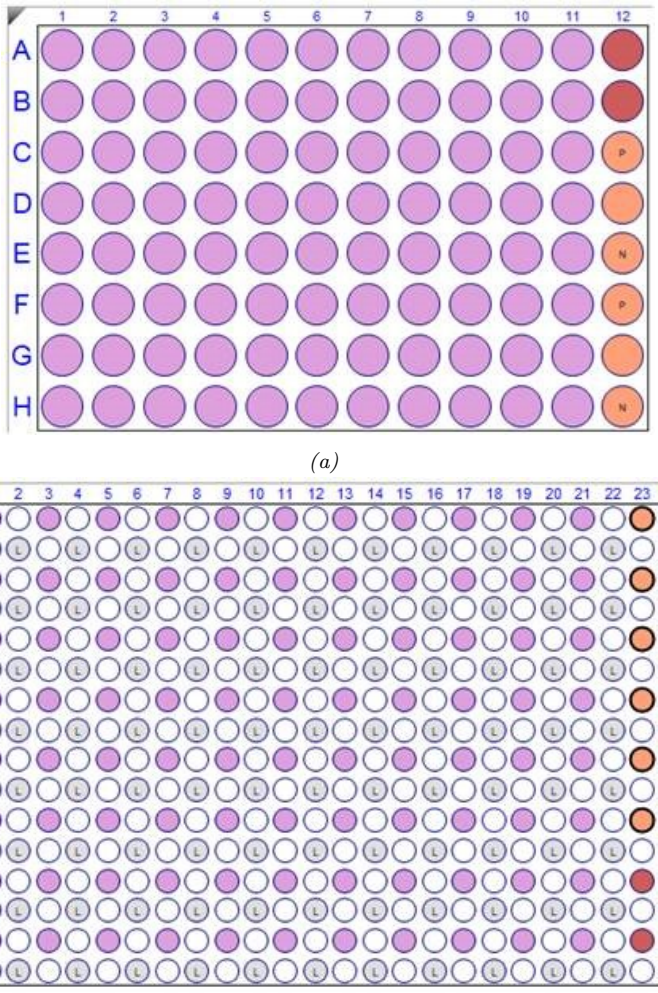


Figure 20: ***The setup design for the reagent plate for the automated BLI assay.*** *The second plate contains the reagents that can be prepared in advance and stored at 4° C for several days. N=Ab2, B=buffer, Q=Ab3, A=signal enhancing substrate (AEC).*

The other plate would be prepared just prior to performing the assay and would contain the standard, the samples, the controls and the references.

Two setups of the first plate were considered. As this plate might have to be mixed manually and needs to be very reliable, it was primarily designed to be a 96 well plate. The plate would have space for 10 samples in full dilution series if using 96 sensors, see figure 21. This design allowed for 5 dipping steps with 96 sensors - 1 in the 96 well plate and 4 in the 384 plate. To have enough space it was assumed that dipping in the same buffer did not affect the assay, that no regeneration was required and that AEC worked in the same buffer as the rest of the reagents (which would be challenging for the yeast assay in TBS). The sensors could be loaded with b-Ab, sugar coated and stored in advance, so preloaded sensors would be available when needed. This would reduce assay time by a few minutes.

The other considered setup was to have the samples transferred into 384 well plates after being diluted and mixed in bigger volumes. There would still be space for 10 samples. With two 384 well plates there is room for 8 different reagents, which would allow both loading and assay in the same run. There would also be space for a second buffer, for example for the yeast assay where PBS is needed for loading and AEC, while the other steps should be diluted and washed in TBS. It was still designed for dipping the sensors in the same buffer wells several times, and that no regeneration was possible or needed.



(a)

(b)

Figure 21: ***The sample plate setup for automation of BLI assay using a liquid handler.*** *The two ideas for the sample plates in 96 (a) respectively 384 (b) well format have both space for 10 samples and would be prepared right before analyzing samples. The 96 well plate is used if manual interventions are needed, while the more flexible 384 plate can be used for a fully automated dilution assay. a) Column 1=standard curve, column 2-11=samples, red=reference (buffer), orange/pink=controls with low, medium and high concentration. b) Pink column 1=standard, pink column 3-21= samples, red=reference (buffer), orange=controls; low, medium, high, grey L=biotinylated antibody.*

5 Discussion

HCP quantifications are at present most commonly made by ELISAs, which often require long incubation times and manual interventions. In early phases of drug product development, kits can be used, but when moving into the later phases of the clinical drug development, fully customized assays are needed. In this study, a method development based on a kit assay evolved to a fully customized HCP quantification assay which could be used in all stages of clinical development. The opportunity to use a fast and fully automated assay with small reagent volumes should be of great interest for the pharmaceutical industry which is always fighting tight deadlines and needs fast development of new potential drugs.

5.1 Customization of Assay

The method development is dependent of the optimization of each step, why each part has been discussed separately.

5.1.1 Kit Assay

The commercially available Anti-CHO HCP kit assay works well for estimating HCP concentrations also using the NN standard, which was expected as several companies use the kit for early phases of clinical studies, either in ELISA assays or by the use of BLI. Sartorius claims 80 nm is around the maximum signals normally reached with the kit, why the NN HCP seem to work well with the kit. If NN would want to use the kit assay for initial studies that would be suitable, especially as the BLI equipment can be used in a GMP environment. Although, to get a specific assay to use in all phases of clinical studies, an assay with in-house antibodies is required.

5.1.2 Sensors

The biosensor surface seems to have a major impact on the assay. The full assay signals, decreased almost 8 times compared to the kit assay when using SAX2 sensors instead of ready-to-use CHO sensors, see table 3. It was worrisome, as the signals greatly affects the sensitivity of the assay. The reason it is important is probably because the streptavidin-biotin binding is

the first step of the binding assay - if it does not work well the rest of the assay will not work either.

As can be seen in table 3, the signals can be almost doubled when choosing the right streptavidin sensors. Overall the loading and the parameters affecting it (sensor type, biotinylation, loading, buffer) seem to have an impact on the final signals, as this lays the foundation of the assay. The different streptavidin sensors probably have different degrees of streptavidin on their surface, which can match better or worse with the biotinylation and thereby binding of the antibodies. The sensors used for kinetics probably have a lower level of streptavidin to facilitate the measurement of on and off rate, while sensors for quantification should have more streptavidin as high signals are wanted.

It could be interesting to see if another binding type would give higher signals, for example protein A or anti-histidine sensors, which would require another coupling chemistry of the antibodies. Also in the kit assay, streptavidin biosensors are used for coupling chemistry to the biotinylated antibodies. Sartorius probably have optimized buffers, reagents and possibly specialized streptavidin sensors which make the kit assay render such high signals.

5.1.3 Biotinylation

It was first assumed that the lower amount of biotin, the higher the full assay signal, but it showed that neither the biotinylation level (MCR) nor the biotinylation protocol actually showed a notable impact, and neither did the loading concentration, see tables 4 and 5. Instead, it seemed like the loading and storage buffers were the most affecting parameters. This could be due to that the biotin-streptavidin binding is strong and binding happens fast, and the equilibrium constant is strongly shifted towards binding. Then, for example, the concentration does not affect the binding much.

Differences in the antibody pools and the amount of primary amine groups could also be part of why yeast and CHO antibodies responded differently to the biotinylation and get different loading signals. An MCR of 1:1 or 1:100 should not make a difference, as in both cases the binding reaction should be saturated, so it should result in the same amount of biotins per antibody. The MCR 1:1 could assure that the biotin only binds to the most reactive primary amines, but since we don't know where those amines are situated on the polyclonal antibodies, it might not matter. The washing of

the antibody storage solution becomes more important as the MCR increases, as the risk for having free biotin in the solution which would compete with the antibodies, blocking streptavidin from binding actual antibodies.

It could be considered a waste of antibodies when biotinylating in an MCR of 1:0.5, as only half of them will bind an biotin on average. In practice, it just corresponds to a decreased concentration of b-Abs in the solution, which showed to not have an impact in the loading concentration experiments. Because of the low difference in full assay signal, we would recommend using MCR 1:1 in further assays ensuring not wasting valuable antibodies.

That an MCR of 1:100 can be used in ELISA without causing similar kinds of questions is because the biotin is not used for immobilizing the molecules, but as a labelling molecule on the second antibody for binding a streptavidin labelled HRP enzyme. The differences in assay setup is also one of the reasons why the whole assay can not just be transferred onto the BLI platform.

5.1.4 Loading

The most crucial parameters for loading showed to be biosensor type, loading time and loading buffer. Individual biosensors and buffers have to be found for each assay, together with a short loading time. It is not surprising that buffers have an impact as antibodies' binding ability can be very sensitive to the surrounding environment. The short loading time could actually also be expected due to the strong affinity and fast binding characteristics between biotin and streptavidin in a buffer like PBS. That shorter loading time gave around the same loading signal but almost doubled the full assay signal compared to a longer loading time, see table 6, could be because the biotin-streptavidin binding happens fast, but the extra time during longer loading time gives room for unspecific binding events. It could for example be free biotin in the solution, or other proteins in the standard or sample that interacts with the sensor surface. This could possibly block the bound α-HCP antibodies' antigen binding sites and impede the loading of HCP. Adding BSA or loading in TBS containing BSA and tween could be an approach of investigation to find a solution to the low full assay signals. Although, it seems like as long as the loading time is kept down, the need for such work arounds is not needed.

Another possibility is that the antibodies themselves bind to the surface in an unspecific way or with less exposed biotins and end up in the wrong

direction. They could then block the tip surface and impede the binding of more antibodies binding.

The whole biotinylation and loading section would benefit from a DOE where the parameters' impact can be investigated simultaneously.

5.1.5 Sugar Coating

The possibility of sugar coating is useful and opens up for more flexible assay planning. The sensors are also sugar coated by the producer and it is a very easy and cheap way of storing sensors. Also, if sensors have been regenerated, they could probably again be sugar coated and stored. Kol et al. [38] have stored loaded regenerated streptavidin sensors for up to two weeks without sugar coating and without losing sensitivity, why coating sensors probably could prolong the storage time of regenerated sensors even more.

5.1.6 FITC- and HRP-Labeled Abs

The two antibodies for signal enhancing does not seem to impact the assay much. As long as the Abs are in excess to the binding sites, it is expected that their concentration does not impact, as there is only a limited amount of bound HCPs to bind. The concentration of HRP-labeled Abs was seen to be proportional to the signal up to a saturated concentration (data not shown), but for the FITC-labeled antibody the concentration was not known and still did not impact the assay much. This means that a lower FITC Ab concentration probably could be found useful. To premix or remove one of these antibodies would be a step of simplifying the assay, and should be feasible after more investigation.

5.1.7 HRP Substrate

To remove the DAB would make the assay much more flexible as it does not have to be handled in a fumehood, does not necessarily require its own buffer and can be put directly in a liquid handler. That the AEC gave high signals can be of use for this and other assays in the future. The problems with the "broken" signals when using the AEC is probably due to lack of substrate, especially in the wells with sensors with high concentration of HCP. It could also be because the bound molecules fall off the sensors during rotation, or that the outer reflective surface is blocked so that no wavelength shift is detected. However, DAB showed the same patterns when being run

simultaneously with the AEC. As there is not much knowledge about using AEC in this kind of assays, it might be that side reactions are happening that for example inhibits the enzyme activity, which could be why the signals "break". Another theory is that the sensors are somehow impeded and can't detect signals over a certain value.

The impact of the detection time implies that a long detection time should give higher signals, but as the signals "break" this might not always be true. That the last readable signal was used for the signals that were "broken" at 600 seconds can also impact the results of the DOE in an incorrect way as they cannot be used in a real assay. At the same time, setting them to zero would skew the results in the other direction. The optimal reading time probably must be investigated and determined for each assay until an environment where AEC can be used in its full potential is found.

That no interactions between the parameters were found from the DOE can imply that there are one or two parameters that have a much greater impact than the others, just as is seen in figure 11. Shake speed impact can be neglected compared to the other three. As the possibility of changing the reading time is restricted the concentration and buffer should also be closely considered. That a PBS and a low concentration of AEC seem to be favourable is welcome, as NN buffer and small reagent volumes are advantageous for the customization of the assay.

As figure C.2 shows, the AEC gives higher signals especially at high concentrations, and slightly lower at the lower concentrations. This could possibly affect the sensitivity of the assay, but at the same time the analytical range is wider with AEC as it has both higher and lower signals than DAB. A signal enhancing substrate anyway increases the assay sensitivity.

Sartorius claimed that a DAB alternative had never been tested, which was surprising due to its toxicity. There are many other precipitating substrates available on the market, the AEC and StayYellow seemed promising, but other precipitating substrates could probably give similar results. Although the AEC worked well it would be very interesting to keep investigating it with a further DOE with more parameters, especially for gaining more stable signals with the AEC so that the high binding rates at 140 nm can be enjoyed.

In spite of the problems with the stability for the longer reading times, the kit and yeast assays with AEC show that they work and give concentration dependencies which fit well to the 4PL standard curves, as seen in figure 12.

5.1.8 Full Assays Buffers' Exchange

The buffers have shown to have a major impact, as seen in figure 14 the yeast signals increased with more than 10 times with the right buffer compared to with a less suitable buffer. This is probably one of the explanations to why the CHO assay does not work well - the correct buffer has not been found. The same buffers as used in the corresponding ELISAs have primarily been tested, which was why the TBS was chosen for yeast and PBS for CHO. The recipe of the kit sample buffer is not known, but as it works well for both of the organisms it must contain some crucial ingredient which enhances and facilitates the bindings in the assay. The used PBS does not have a great buffer capacity, but adding BSA or tween could possibly help inhibit unspecific binding. The TBS, on the other hand, contains both BSA, tween and TRIS which should inhibit most of the unspecific binding. Possibly there is also a TBS buffer used for CHO ELISAs in the department that could be more suitable for the CHO BLI analysis, and which is more enriched than the here used PBS.

TBS was not used for loading the sensors due to its BSA content, although it might have helped the assay further. Normally, when working with biotin-streptavidin bindings unspecific binding is not a problem as their affinity is so strong. However, the BSA could make it hard to distinguish the loading from the background, but maybe these drawbacks could be weighed up by using TBS for the loading at it showed such benefits for the rest of the assay.

Domnowski et al. [39] have investigated how different buffer compositions affected the binding rates in the BLI assay for self-interaction of a monoclonal antibody. The pH had a big impact, and salt composition and concentration affect in varying grade. Also Naik et al. [40] described the great impact of the salt in the buffer, here especially described the impact of NaCl. The pHs of PBS and TBS used in this study are quite similar, 7.4 respectively 8.0, why that should not be the main reason, although it has not been specifically investigated. It should rather be the salt content or ingredients impeeding unspecific binding that produces the notable difference.

As very small volumes and binding surface area is used in the BLI assay, a small change in buffer makes a big difference for the micro-environment, why the buffer composition probably should be thoroughly investigated for each assay developed in the BLI.

5.1.9 Other Improvements

The reuse and the loading in 384 wells gives a further flexibility to the assay which is needed for running many samples at a time, and is especially beneficial for the automated assay. The high throughput of the Octet® is an advantage for fast drug development. Premixing some steps would be interesting for reducing assay time even more, but needs to be further investigated.

5.1.10 Most Impactful Parameters

From the findings in this study, it seems like some parameters are of more impact for the full assay signal than others, see table 7. Overall, the loading seemed to be the most crucial step, but has many parameters woved in (sensor type, biotinylation, loading steps). Also buffers play a major part. If further optimizing this assay, these are the steps that primarily should be investigated.

Table 7: ***Parameters' impact on the full assay signal.*** *The loading of the biosensors together with buffers seem to have a big impact of the final assay signal, while other assay steps seem to have less impact.*

Big impact	**Small impact**
Biosensor type	FITC-labeled Ab
Dilution and storage buffers	HRP-labeled Ab
b-Ab loading time	b-Ab concentration and MCR

5.1.11 Customized Assays

It is curious that the yeast worked better than the CHO, as the initial kit was designed for CHO. As discussed, the CHO assay seems to be very affected by the buffer and the loading parameters, although the results obtained from the yeast assay give hope that a well-working customized CHO assay can also be developed if the right circumstances are found. The reversed pattern (figure C.3) is probably due to high background and unspecific binding, possibly affected by the buffer.

That the BLI CHO standard curve, see figure 17, is flat up to a concentration of around 100 ng/ml is not optimal as the ELISA's LLOQs are down

to a few ng/ml. To be able to compete with the ELISAs, the BLI's LLOQ would need to be similar or lower.

A yeast curve reaching 55 nm and that overall gives higher signals than the kit assay for corresponding concentrations, see figure 16, is a success. The explanation could be because of antibody differences, but this also shows that the yeast assay is well elaborated and will have a high sensitivity.

Why the AEC gives lower signals than DAB in the yeast assay is probably a question of finding the right circumstances for the use of AEC so that detection time can be extended.

5.1.12 Comparison of Yeast Assay to ELISA HCP Quantifications

There are many differences in the ELISA and BLI assays, yet the goal has been to obtain the same or comparable results. When considering the context of measuring concentrations in downstream processing, the exact number of the HCP concentration is not the most important value, but the magnitude of the value. Because of this, a variation of up to 20% is normally expected when working with bioassays [21]. The higher HCP concentrations show a better proximity to the ELISA measurements when using the customized yeast DAB assay than the lower concentrations. It could imply that the BLI assay has challenges when measuring the low concentrations and that the sensitivity is not as low as needed. At the same time, the LLOQ show great results. More measurements are needed to be able to draw any conclusions from this data.

The routine yeast ELISA does not show a full sigmoidal curve, which implies that the antibodies can be problematic to work with. The BLI results give a full standard curve which on the other hand implies that a good environment has been found for the antibodies. Compared to the kit CHO BLI assay, this customized assay shows results comparable to ELISA, which is reasonable due to the use of the same polyclonal antibodies, see Appendix A. The repeatability of the yeast assay has not been thoroughly investigated, but the F test for parallelism implies a good repeatability.

For the AEC assay, some more optimization would be needed before considering it for use in a real analysis environment. The variation in signals is probably because they don't have the time to stabilize before reading, due to lack of AEC substrate at the highest HCP concentration i.e. before all signals are saturated.

5.1.13 Analytical Parameters

As implied in earlier studies, the analytical parameters should be acceptable for customized BLI assays, also when working with polyclonal antibodies and complex media such as sera [20]. The estimated LLOQ is very low (down to a few ng/ml) no matter how the reasoning is made, and has approximately the same value as the yeast ELISA, which was the goal. The precision estimate is promising, although it is not under 20% as desired. Still it is not remarkably distinct from the wanted value, and because the measurements are only made twice the extra variation could be due to variation between just these two measurements. More samples are needed to be analyzed to get a more reliable result, however the estimated value implies that the variation probably can get within the required values after some further optimization.

That earlier studies have succeeded to measure polyclonal antibodies with streptavidin sensors and acceptable analytical parameters and wide analytical range further implies that this assay has the potential of working with good analytical parameters [4]. Some assays imply that the sensitivity of the Octet® assays can not compete with sensitivity to ELISA [21],[4], but none of these have used signal enhancing substrates, but have focused on kinetic assays. Another sandwich assay, using DAB as precipitating substrate and loading their own biotinylated antibodies, show the same sensibility as comparable ELISAs [22], which implies that the signal enhancing step increases the sensibility and the chances of competing with ELISAs. More studies to confirm those initial estimates of the parameters are needed.

5.1.14 Automation

Automating the assay with a liquid handler would make the assay even more attractive as it increases flexibility and reduces the number of manual handlings. Automation would increase the number of samples in each run from around 3 to around 10. The possibility of preparing the second plates for storage at 4°C and to analyze samples from different organisms in the same run because the plate is not pre-coated are big advantages which would save time and increase the flexibility greatly. If the automated assay in the future could be used as an *at line* analysis, a lot faster results and alterations of the purification process could be made.

Although the 384 well plate setup of the first plate would require a predilution of the standards and samples (in a liquid handler) and add one trans-

ferring step, it would remove the loading as an additional step. The assay would hence still be simplified with the 384 well format.

5.1.15 Regeneration

The regeneration was not deeply investigated, but it would be interesting to look into as it would save a lot of reagents and sensors and be very economically and sustainably smart. Knowing regeneration is possible, and proven in earlier works [38],[21], it should be possible also here. Although, the challenge for regenerating the sensors from this assay would require that the precipitating substrate is first washed off, before the loaded antibodies could be regenerated. AEC is for example soluble in alcohol and organic solvents, which would be very interesting to investigate further. If, as Kol et al. [38] describes, regeneration can be made while keeping the biotin-streptavidin bound antibodies, the loading could be performed only once and the sensors used up to ten times.

Even if sensors can not be regenerated, maybe the reagents can. As the reagents can be stored, the sensors are not sensitive to the surrounding environment and some impurities in the wells shouldn't affect the binding events as each binding step is specific, the reagent plates could probably be used several times. It is implied in the Tech Note 24 [24], that a loading plate with b-Abs can be used up to ten times, depending on the concentrations in the wells.

5.1.16 Other Thoughts and Reflections

This study has shown that it is possible to make fully customized assays for HCP quantification for different organisms by the use of BLI. Each step had to be investigated and optimized to get a reasonable full assay signal. The showings that it is possible are important as it can lead to more effective and automized analyses.

The hope was that the customized assays would reach the same full assay signals as the kit assay. Almost each step exchanged impacted the signals negatively, and when they all sum up the reduction of the full assay signal is obvious, which initially gave customized assays with low signals. On the other hand, the yeast assay gave higher signals for the lower standard concentrations why it could be claimed that the customized assay give, on average, similar signals as the kit assay, see figure 16.

To see the big difference between the CHO and yeast fully customized assay raises questions about if the assay is dependent on the organism, or more on the antibody pool. Why the yeast and CHO are so different is probably mostly due to antibody differences and how well those certain polyclonal antibodies work in the laboratory environment. Some antibody pools give more challenges than others. Also in the ELISAs different concentrations and buffers are used and the two organisms give different challenges.

5.2 Further Studies

Improved and partly optimized assays were found from the parameters varied in this project, but several other parameters can and needs to be investigated. Examples are buffer content, assay and biotinylation pH, time and temperature for biotinylation, type of biotin, purification level of the biotinylated Abs, and how all the parameters correlate. Most of the parameters examined in this study could be more deeply investigated. To continue investigating the AEC conditions would be a big subject of investigation, and for understanding better the "breaking" of the signals.

This study has shown that the BLI can be used for CHO assays, but worked even better for yeast. This implies that the customized BLI assay could also be used for other organisms such as *E. coli* or other species. As NN and many other companies need assays for several organisms, this study suggests that is should be possible to develop the assay for more organisms.

To further enhance the flexibility of the assay, it would be very interesting to minimize and simplify the assay and ask questions like: Are all the signal enhancing steps really needed? Could the FITC-labeled antibody not just be directly HRP-labeled? Are two α-HCP Abs really needed, or could one of them be removed? Could more reagents be premixed - or could the whole assay be premixed and then just loaded onto the sensors? Could another enzyme than HRP be used together with another substrate? Yeast showed a concentration dependency already at the HCP loading step, so is the sandwich assay even necessary? Could incubation times be reduced? And so forth.

Also investigating the incubation times of each step one by one, the assay total time could probably be reduced more. Additionally, it seems that the buffers have a great impact, and in what steps the different buffers are put could probably have an impact on final assay. Assay temperature has not at all been investigated, so it would also be interesting to investigate if

temperature has an impact on the assay or the HRP precipitating reaction.

To setup a full DOE over the loading and biotinylation step, as well as the signal enhancing step would probably render a lot of useful information. Together with buffers, these are the steps that primarily need to be further studied.

6 Sustainable Development and Ethical Considerations

The aim of this project is to minimize and automize routine analysis. With the automation of the assay, smaller amounts of reagents and less plastics can be used as more samples can be run on the same plates using plates with small wells. Reuse of sensors and reagents is hopefully possible in later stages of the project. In this way an implementation of BLI in the laboratory would contribute to more sustainable working routines.

To exchange the DAB to AEC improves the safety in the working environment, and contributes to a more sustainable way of working. As the reagents are not moving fluids and because the assay is not affected by the surrounding environment, the reagent plates (figure 20) could probably be used several times.

Furthermore, if the developed method can contribute to a faster development of future drug products, which will lead to a shorter timespan from idea until the product reaches the patient, it is a win both for the company and the patients.

7 Summary and Conclusion

This project has showed that

- the commercial BLI kit for HCP quantification gives results in the same order of magnitude as corresponding ELISAs
- fully customized HCP quantification assays can be developed
- AEC can be used instead of DAB as signal enhancing substrate, and give higher signals and a safer assay
- customized HCP assays can be optimized for different organisms such as yeast and CHO
- the analytical parameters for fully customized BLI assays show promising results for using as a functioning and reliable assay
- the BLI assay can be automated together with a liquid handler and run 10 samples in full dilution curves simultaneously

This means that BLI can be used for HCP quantification of different organisms and shows good possibilities to be comparable to ELISA. The assay setup is flexible and can be further elaborated, and the automation gives possibilities for running 10 samples at a time in the parallelly run 96 wells. There is a lot to investigate further, but setting up fully customized HCP quantification assays in the BLI is possible.

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A Introductory Experiments

As a pre-study to the thesis project, a development of a ready-to-use kit was carried out. The kit could have been used for quantification, but as it uses antibodies from Cygnus technologies and not NN in house-antibodies, a company specific, customized method was developed after the introductory experiments.

A.1 Introductory Material and Methods

A ready-to-use kit from Sartorius was used and a well working assay was developed. The material is listed and specified in section 3 and the General Protocol in section 3.2 has been followed for all experiments.

Standard Curve for BLI Kit Assay

It was established how the ready-to-use Anti-CHO HCP Detection Kit from Sartorius was best run to get a standard curve with an sigmoidal shape with a near linear interval in the middle of the concentration range. Those are the requirements for a good working standard curve. The NN CHO WSA HCP was mixed with sample buffer from the Anti-CHO HCP Detection Kit in several different dilution series to evaluate the optimal dilution series. A logged 4PL curve was fitted to the data.

Incubation Times

With the optimized standard curve protocol the incubation times for the standard antigen (NN CHO WSA), FITC-labeled α-CHO-HCP antibody and the HRP-α-FITC antibody were reduced in two assessments according to table 8. The assays were carried out following the BLI Kit General Protocol, except for changing the three incubation times.

Regeneration Ability

The possibility to reuse or regenerate the sensors was investigated with the CHO sensors from the kit. The aim was to wash away all molecules except for the first α-CHO HCP antibody, and to get the same results when running the same assay again.

Table 8: ***Incubation times of reagents in the BLI.*** *The three sets of times that were tested for investigating time reduction of incubation times for the HCP and antibodies in the BLI kit assay.*

Reagent	Original time (s)	New time 1 (s)	New time 2 (s)
Standard	3600	1800	600
α-HCP	3600	1800	600
α-FITC	1800	240	60
Total time	2 h 30 min	1 h 4 min	21 min

The regeneration was conducted with two solutions - with a low pH solution (10 mM glycine-HCl, pH 1.5) and with a salt solution (4M $MgCl_2$). After a full assay of standard curve (see section 3.2) the sensors were regenerated by dipping into the regeneration solution three times with a neutralizing solution (sample buffer) for 5 seconds in between. Regeneration times for pH was 5 and 10 seconds, and for salt 5 and 15 seconds.

Comparison between ELISA and BLI HCP quantification

Four HCP samples were quantified both by the standard ELISA method and the Anti-CHO HCP Detection Kit. It should be kept in mind that the two assays use different antibodies - ELISA uses NN Abs and BLI uses the kit Abs from Cygnus technologies. The standard material NN CHO WSA was the same in both assays, although the standard curve dilutions differed - for ELISA the routine curve was used, and for BLI the curve described in A.2 was used.

A.2 Introductory Results and Discussion

Standard Curve for BLI Kit Assay

For the NN CHO WSA HCP the optimal standard curve was established to a 3 times dilution with 8 points of dilution: 10 000, 3333, 1111, 370.4, 123.5, 41.2, 13.7, 4.5 ng/ml, see figure 7a. This curve showed a sigmoidal shape with a near linear interval in the middle, and platforms at high and low concentrations. It also gave high signals which makes it easier to distinguish between smaller concentration changes. The binding rates reached almost 80 nm for the highest concentration in successful assays.

Incubation Times

The *New time 1* in table 8 was chosen as it gave a very similar standard curve to the original times. The *New time 2* gave results that differed considerably and the standard curve got an offset shape. The *New time 1*, 1 hour and 4 minutes in total, was then used for the rest of the experiments.

Regeneration Ability

The regeneration for the CHO sensors only seemed to work for the lower concentrations. In figure A.1 the most successful regeneration results from the two approaches are shown. As the results were not similar to figure 7, regeneration was considered to not work for the CHO sensors. A probable reason is that the DAB precipitates and sticks to the tip, while the regeneration ability no longer is dependent on the antigen-antibody binding. As regeneration did not give promising results it was not further investigated in the prolongation of the project.

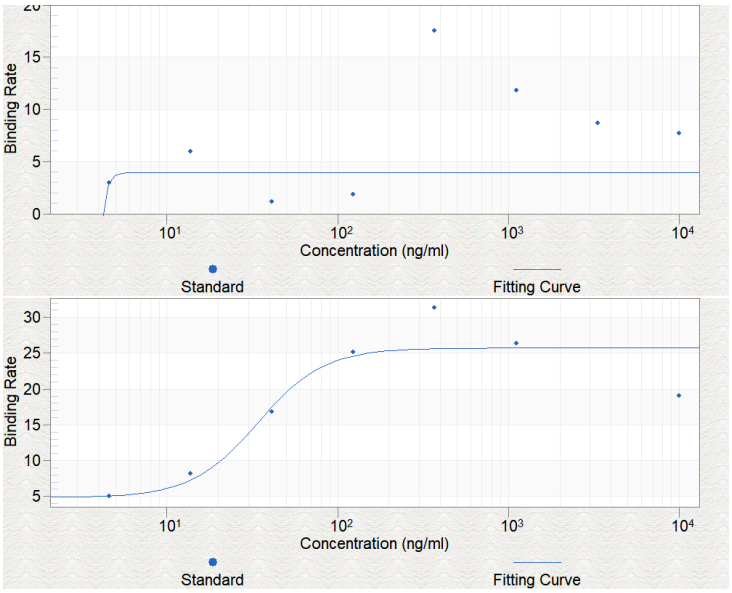

Figure A.1: ***BLI standard curves after regeneration.*** *The y-axis shows the signal measured in nanometer. Top figure: Salt as regeneration solution. Bottom figure: Low pH-solution as regeneration solution. The aim was to get the same shape of the curves as in figure 7, which was not obtained.*

Comparison between ELISA and BLI HCP quantification

The HCP quantification results between ELISA and BLI varied, especially for the highest concentration measurements. In the sample order seen in figure A.2 the relation BLI/ELISA was: 1.52, 0.20, 0.60, 0.27. The BLI generally gave lower results, which was hardly surprising as the kit antibodies were not generated for this exact HCP why all antigens were probably not detected. However, the BLI assay show the same trend as ELISA, even if it doesn't measure the exact same concentrations. This is promising for the continuation of the project.

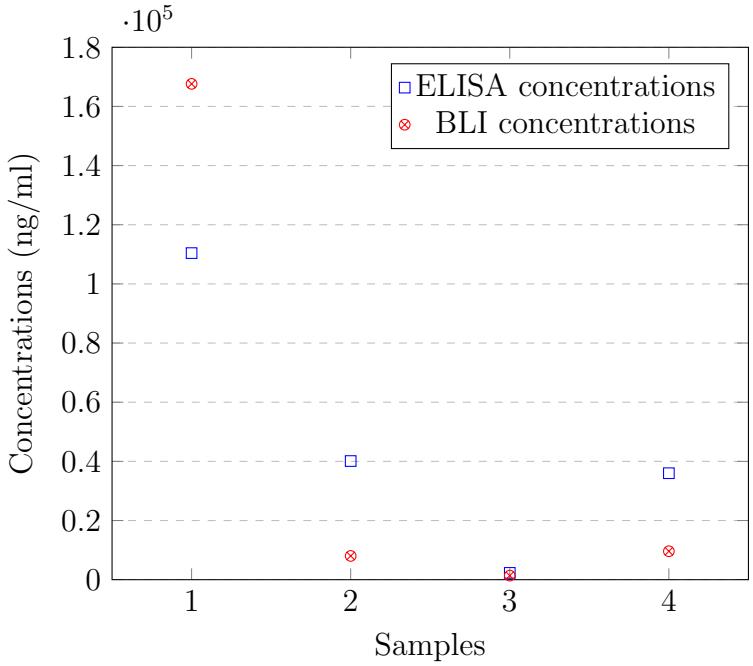

Figure A.2: ***HCP concentrations measured with BLI and ELISA.*** *A comparison between the measured HCP concentrations with the routine ELISA method and the BLI Anti-CHO HCP kit.*

B Article Draft for Scientific Paper

Customized Yeast Host Cell Protein Quantification with Bio-Layer Interferometry Using Safer HRP Substrate

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Novo Nordisk

Using BLI for measuring impurities in drug purification processes

Biopharmaceuticals are the biggest group of drugs in pharmaceutical development today [1], and development of new and safe drugs is on high demand. Biopharmaceuticals are drug substances derived from biological sources and are produced in living cells. Common production cell lines include bacterial or mammalian cells e.g. *Escherichia coli* (*E. coli*), Chinese Hamster Ovary (CHO) cells and yeast.

During the production process the cell line does not only produce the pharmaceutical product but also many proteins, including housekeeping proteins to uphold the basic function of the cell. These proteins are called host cell proteins (HCPs) and are considered impurities in the final drug product as they can affect the efficacy and safety of the drug. To ensure that the final drug product is pure, the HCP concentration needs to be minimized by removal during the drug production purification process. A suitable analysis method must be used for monitoring the HCP concentration in the purification steps.

Enzyme-linked immunosorbent assay (ELISA) has historically been the best-practise method, exceeding other methods with its high sensitivity. It is cheap, has high throughput and is easy to perform, but it requires long incubation times and several manual handling steps. Other immunoassay-platforms are available, one of them being the bio-layer interferometry assay (BLI), which is an optical method that can be performed almost completely automatically. This enables an HCP detection time from dilution to results of only 2 hours for BLI instead of the 1-2 days for a corresponding ELISA. In this study it has been investigated if currently used ELISAs can be setup on the BLI platform using process specific antibodies and reagents.

BLI is an easy-to-use optical platform

The BLI platform is based on small, optical biosensors which are dipped into the wells of a microtiter plate containing the reagents of the assay, such as the HCP. The molecular assay setup is similar to ELISA, but the molecular binding takes place on the tip of the biosensor [13]. Working with BLI is easy, as the only tasks needed are to setup the run in the software, choose the desired number of sensors and dilute the reagents in a microtiter plate. Then the assay takes place in the BLI machine in around an hour. Dilution steps can also be setup in a liquid handler, leaving only the step of moving the sensors and reagents into the BLI machine as a manual step.

When molecules bind the biosensor tip surface, white light is sent towards the tip of the sensor and is reflected on two surfaces. First, an internal reference layer, and secondly where the molecular assay meets the surrounding solution. This creates a signal measured as a wavelength shift, and which can be correlated to an HCP concentration from a standard curve, see figure B.1.

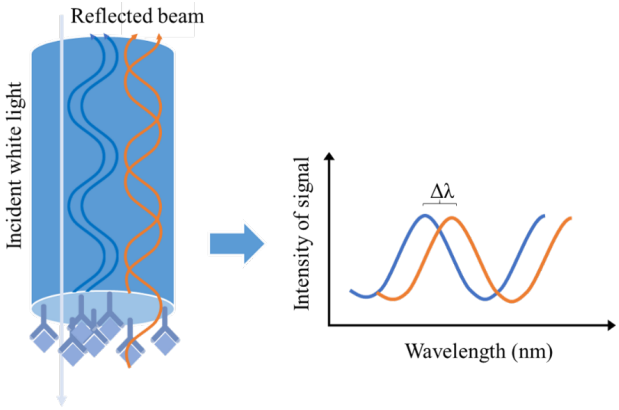


Figure B.1: ***A bio-layer interferometry (BLI) biosensor and its optical properties.*** *To the left the tip of the BLI biosensor, with a molecular binding event on the tip. White light is sent to the sensor tip where it is reflected on an internal reference layer, and on the molecular surface. Protein interaction causes a wavelength shift ($\delta\lambda$) between the reflected beams. The $\delta\lambda$ will be the output of the assay and can detect, quantify of measure kinetics of binding events.*

The signal is a measurement of the optical thickness of the assay, meaning that the thicker the molecular layer on the sensor tip is, the higher the signals obtained. This means that the more molecules and signal enhancing substrates in the molecular assay, the higher the signal. As the measurement

happens inside the sensor and is not a measurement of the refractive index, the signal is not affected by the surrounding media which makes it suitable for working also in very crude matrices.

A 21 CFR part 11 (GMP) compliant automated BLI platform, Octet® HTX, is produced by Sartorius, as well as kits for CHO HCP quantitation. Commonly, kits are used during early phases of drug development, but to gain optimal coverage and sensitivity HCP assays with custom made polyclonal antibody reagents are needed. Because of this, this study has focused on developing a fully customized BLI assay for quantification of yeast HCP. To investigate the performance of the assay, a direct comparison was performed between yeast HCP ELISA and yeast HCP BLI, see figure B.2.

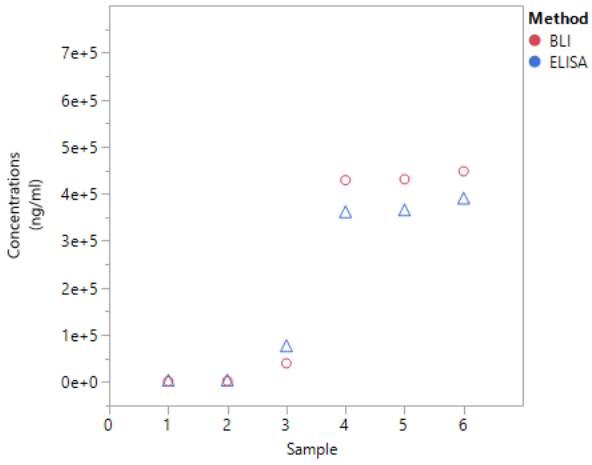


Figure B.2: ***Quantification of yeast HCP samples in ELISA and BLI.*** *Six samples were analysed for HCP with two assays, yeast HCP ELISA and yeast HCP quantification by BLI using the same interaction buffers. Measurements show results in same order of magnitude.*

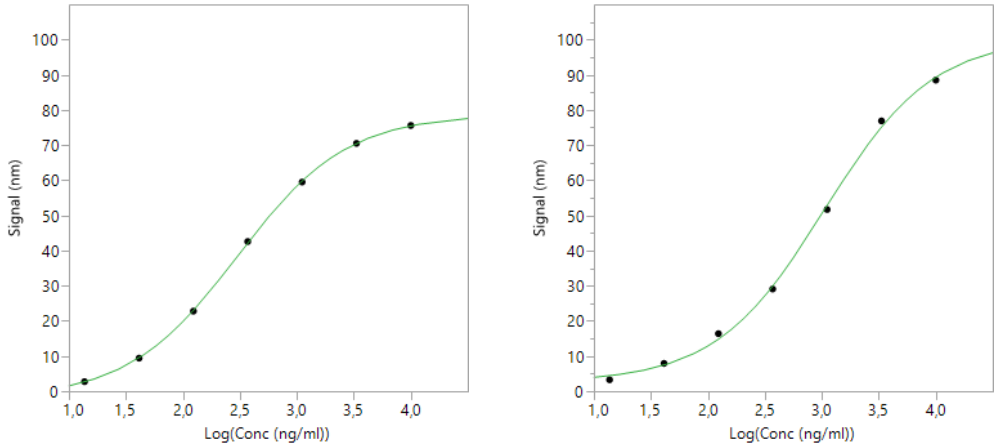
ELISA sandwich assay setup on the BLI platform

The kit for CHO HCP quantification is based on a molecular sandwich assay with polyclonal anti-HCP antibodies. There are several kinds of biosensors coated with streptavidin onto which a biotinylated polyclonal anti-CHO HCP antibody can be loaded. The sandwich assay has several layers of binding molecules, as more mass bound to the sensor tip gives higher signals. A horse radish peroxidase (HRP) labelled antibody is used in the last step and catalyses a precipitating reaction of the signal enhancing substrate 3,3'-

diaminobenzidine (DAB).

Proceeding from the BLI kit, each reagent was exchanged and optimized with the exact same reagents used in the corresponding ELISA. This included all reagents such as interaction buffers and labelling molecules, except the signal enhancing HRP substrate as the TMB used in ELISA is not applicable for the function of BLI as it does not precipitate. Sensor type and software settings were also examined, as well as alternative signal enhancing substrates. A BLI assay was developed where all steps were exchanged to process specific reagents, here called a customized assay.

Firstly, the standard curve was investigated according to its four parameter logistic regression (4PL) fit and dimension of signals. High signals are desirable as it gives a broad analytical range and higher signal to noise ratio and thereby higher sensitivity. Signals obtained with the kit reached around 80 nm for an HCP concentration of 10 000 ng/ml, as seen in figure B.3, which was considered a high signal. For comparison, six samples with different yeast HCP concentrations were then quantified with both the customized yeast BLI assay and yeast ELISA, see figure B.2.



(a) ***DAB*** *(b)* ***AEC***

Figure B.3: ***Standard curves from a BLI assay when using two different HRP substrates.*** *The enhanced sandwich assay used in the BLI assay uses a secondary antibody labelled with HRP. Two precipitating HRP substrates have been tested: the 3,3'-diaminobenzidine (DAB) used in the BLI kit and 3-amino-9-ethylcarbazole (AEC). Higher output signals are seen using AEC in the BLI assay, creating a higher sensitivity. A reading time of 100 seconds for the signal enhancing step has been used.*

Fully customized yeast HCP quantification assay shows promising analytical parameters

The standard curve produced with the yeast HCP quantification BLI method showed data with a good fit to a full 4PL plot and reproducible results. High raw data signals were obtained, up to 55 nm for an HCP concentration of 900 ng/ml, yielding a high sensitivity. The HCP detection in samples evaluated in both the BLI HCP quantification assay and in the traditional ELISA showed comparable levels, as can be seen in figure B.2. From this, a preliminary evaluation of LLOQ and precision was made, which showed values comparable between the BLI HCP quantification assay and the currently used ELISA. The LLOQ reached as far down as to only a few ng/ml for both yeast ELISA and BLI.

This assay is based on polyclonal antibodies on different platforms. High variation in bioanalytical methods is commonly observed and variations of 20% or less is expected within bioassays [21]. In this HCP quantification BLI assay, variation around 20% is expected which might be reduced with further optimizations. As the kit is directed at CHO and the customized assay was developed for yeast, this shows that fully customized assays can be developed for different production cell lines on the BLI platform with promising analytical parameters. This also implies that it should be possible to use for more cell lines, for example bacterial cells such as *E. coli*.

Safer HRP substrate also gives higher assay signals

HRP was used for signal amplification in the ELISA as well as in the BLI assay for HCP quantification. The BLI assay needs a precipitating, insoluble product from the HRP-substrate reaction, and DAB is most commonly used as amplification substrate. Nevertheless, it is hazardous, acute toxic and must be handled in a fume hood, why it challenges safety.

To get around this problem, other precipitating HRP substrates were tested such as 3-amino-9-ethylcarbazole (AEC), a less hazardous substrate. AEC is mostly used for precipitating HRP reactions in immunohistochemistry, but it was discovered that AEC could also increase BLI assay signals considerably. Not only did AEC improve the signal-to-noise ratio, but it also enhanced the assay safety due to its lower hazardousness. This also increases the flexibility of the assay as there is no need for using a fume hood, facilitating the automation of the assay. The 4PL fit to the standard curves of

the two substrates are similar, but the AEC shows a broader analytical range due to the higher signals, see figure B.3.

A small design of experiment was setup to improve the AEC conditions, investigating dilution buffer, AEC concentration, shake speed and reading time. A middle range HCP concentration was chosen, and the four parameters were varied in 12 experiments, using the commercial kit and varying only the signal enhancing step. Results gave clear implications that using a low concentration such as 10%v/v AEC in a PBS buffer or similar gave the highest assay signals, rather than using a peroxide buffer as is used for DAB. A reading time of 60 seconds gave consistent assay signals, while a reading time of 600 seconds could increase the signals significantly, but with the risk of saturating the sensors. Shake speed did not seem to have any impact.

BLI is fast and can be automized

Optical sensors in bioanalysis is an evolving field and is used more and more for characterization and analysis of samples, both with e.g. BLI and surface plasmon resonance (SPR). BLI has many applications and can also be used for other drug purity evaluations, for example by protein A bindings. Simpler assay setups can also be used with even more reduced time consumption than in the HCP quantification BLI assay.

BLI has several advantages over ELISA, for example the reduction in hands-on time and the overall time reduction from days to hours. The Octet® gives possibility for a fully automated assay together with laboratory robots, which saves time and resources. Several steps of ELISA can also be automized with liquid handlers, but this does not reduce the long incubation times. While ELISA needs scheduled watching times, BLI is a walk away assay that can also be run overnight. As BLI gives real time analysis data, incubation times can be narrowed down to a minimum without compromising with the data quality. Rather, a generous amount of data is generated for each sample point which can reduce the need of thorough safety measures with numerous control samples and replicates.

A considerable drawback of BLI would be the high costs both for introducing and maintaining the system, as the one-time cost and sensor price is high compared to ELISA equipment. However, the sensors have the ability of being regenerated and reused [21], [38], although it has not been shown in this study. Reagents can also be reused, as the sensors dip into the wells and does not consume the reagent completely. The machinery does not have

any tubing which reduces the need for maintenance compared to other automated platforms. Another advantage is that an automated and quick assay could be used as an at line analysis, which would create a faster feedback loop and increase the possibility for earlier adjustments of the purification process. Today, sending samples back and forth and setting up an ELISA can take days or weeks.

Fully customized BLI HCP quantification assays can be developed

In conclusion, BLI works well for HCP quantifications in a rapid and sensitive manner. Fully customized assays with host specific antibodies and interaction buffers may be developed, improving the hands-on and analysis time without compromising assay sensitivity, accuracy or precision.

C Additional Figures and Tables

Table 9: ***DOE experiments for AEC.*** *The 12 experiments carried out in the DOE for investigation of optimal conditions for AEC. The AEC concentration is described as a dilution where 1 means undiluted AEC (100%), 0.5 means 50% (v/v) AEC and 0.1 means 10% (v/v) AEC diluted in the different buffers. Rpm is a measure for shake speed.*

	Buffer	**Detection time (s)**	**rpm**	**Concentration**
1	Peroxide buffer	60	400	0.1
2	Peroxide buffer	60	400	0.1
3	PBS	60	400	1
4	PBS	60	400	1
5	Sample buffer	600	400	1
6	PBS	60	1000	0.5
7	Sample buffer	60	400	0.1
8	Sample buffer	600	1000	0.1
9	PBS	600	1000	0.1
10	Sample buffer	60	1000	1
11	Peroxide buffer	60	1000	1
12	PBS	600	400	0.5

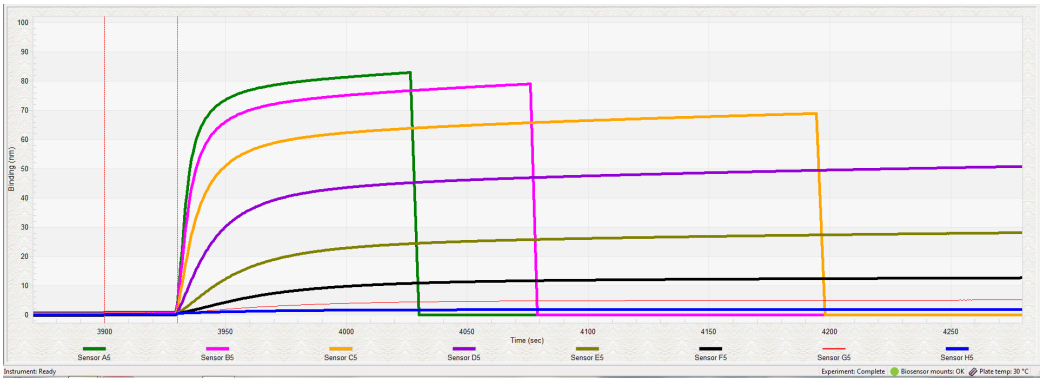


Figure C.1: ***Broken signals when using AEC in the BLI.*** *An example of how the broken signals looked like in the Octet® software, here the DAB signals from a standard dilution series when running them at the same time as the AEC. The top three concentrations have the typical pattern of what is here called "broken" signals.*

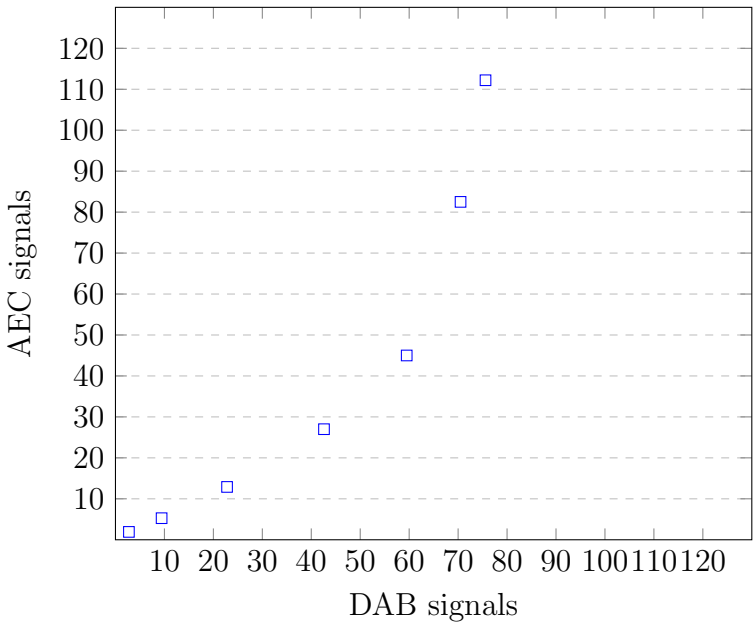


Figure C.2: ***Comparison of AEC and DAB signals obtained using equal standard concentrations.*** *The trend of AEC rendering higher signals is especially clear for higher concentrations. The data points are the same as in the two curves in figure 12, and using a CHO kit assay with 100 seconds reading time for AEC and 60 seconds for DAB. Signal unit is nanometer.*

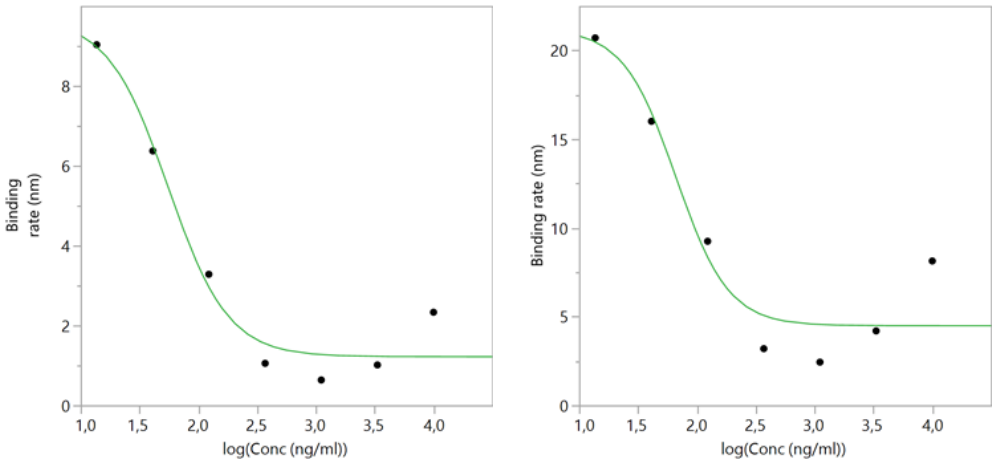


Figure C.3: ***Customized CHO assay standard curve with DAB and AEC.*** *Left, DAB assay and right AEC assay, both with the same standard curve (NN CHO HCP WSA starting at 10 000 ng/ml with 3 times dilution). The curves show a reversed pattern to the functioning standard curves. The background signal was 3.18 nm for DAB assay and 19 nm for AEC assay.*