OSLO METROPOLITAN UNIVERSITY
STORBYUNIVERSITETET

Bachelor's thesis

Stability of Dynabeads Streptavidin coupled with biotinylated antibody

Biotechnology and applied chemistry
May, 2024

Filip Gierczak & Emma Nagel

KJTS3900 – Bachelor Thesis
20 ECTS

Faculty of Technology, Art and Design
Department of Mechanical, Electrical and Chemical engineering

Carried out at: Thermo Fisher Scientific
External supervisor: Ingrid Manger
Internal supervisor: Hanne Thomassen

Availability: ☐ Open ☐ Closed

Preface

This report is written as part of the bachelor thesis carried out at Thermo Fisher Scientific for the Department of Mechanical, Electrical and Chemical engineering at Oslo Metropolitan University.

We want to extend a huge thanks to our external supervisor Ingrid Manger, who has been great at giving us a proper introduction to the lab work of the project, as well as being a huge resource when it comes to the theory behind the chemistry.

We would also like to thank all the other amazing people at Thermo Fisher who has helped us out with everything under the moon.

Voin Petrović	Marc McGowan
Kamilla Jacobsen	Marie Holter-Sørensen
Christopher Stensrud	Ruth Martin Martin
Silje Bøen Torsetnes	Vegard Dammen
Padmapriya Kumar	Axl Neurauter
Terje Hekkelstrand	Erling S. Finne
Meley Johansen	

At last we would like to thank our internal supervisor Hanne Thomassen for being the fastest person alive when it comes to answering emails and giving feedback. She has been really helpful to us.

Contents

Abstract

Dynabeads™ MyOne™ Streptavidin T1 is a versatile platform that can be used for various applications due to its flexibility. By conjugating the beads with antibodies or antigen the customers themselves have biotinylated, this product can be used to produce assays tailored to each of their specific needs. Due to its flexibility and modifiability, the conjugated beads will sometimes be tailored in larger quantities, and then stored for extended periods of time under diverse conditions.

The streptavidin is covalent bound to the beads, while the biotin bound to either antibodies or antigen creates affinity bonds with amino acids on the streptavidin. Both of these bonds are susceptible to break apart from each other, rendering the complex less suitable for further use in the more sensitive immunoassays.

It is also important to avoid any free antibodies in the supernatant as they will compete with the antibodies on the beads, and reduce the signal of the assay.

Some of Thermo Fishers customers have the conjugated beads as a part of their assay kits. This makes for a situation where they want to keep the functionality of the beads even when storing them for longer periods of time.

This project was carried out to see if there was anything the customer could change in their routine that would reduce the leaching without losing the functionality.

Dynabeads™ MyOne™ Streptavidin T1 beads were conjugated with antibodies at varying concentrations, at different temperatures, and for different lengths of time. They were also stored in different buffer solutions, and had their excess binding sites blocked with biotin. This was all to assess how different treatments of the beads affect the shelf-life stability and accuracy of the assays.

The stability of the complex was assessed after each alteration of the treatment using immunoassay methods targeting different components of the complex that could potentially dissociate from it. Changes in the beads' functionality were also evaluated by using the beads in an assay model which sometimes included recovery tests. MODDE, a statistical software by Sartorius, was used for analysing the impact each change implemented on the treatment of the beads had, and how those correlated with the leaching of the components and the functionality of the immunoassay based on the beads.

By using higher concentrations of biotinylated antibodies under the conjugation,

and storing the beads at pH lower than physiological, with higher concentrations of BSA added to the buffer will reduce the leaching and increase the functionality of the immunoassays by reducing the noise. Blocking unoccupied binding seats on the streptavidin with pure biotin will also reduce the noise and the leaching, more so when higher quantities of pure biotin are added to beads.

All those treatments lead to mostly exaggerated and somewhat physically impossible results in the recovery tests. Yet. those values were quite evenly elevated, so further calibration of the assays employing modified beads would be required.

1 List of abbreviations and symbols

biotinylated AP	biotinylated alkaline phosphatase
BSA	bovine serum albumin
CLIA	chemiluminescent immunoassay
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
HRP	horseraddish peroxidase
IL-6	interleukin 6
MES	2-(N-morpholino)ethanesulfonic acid
RO	reversed osmosis
RSD	residual standard deviation
StA	streptavidin
S/N	mean MAX RLU to noise ratio
TBS	Tris buffered saline
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane

2 Introduction

In this project the stability of Dynabeads™ MyOne™ Streptavidin T1 (DB™ MyOne™ StA T1), a paramagnetic polymer-coated microbead conjugated with biotinylated antibody was investigated. Both the functionality in immunoassays and the leaching of various components from the complex was assessed after modifying various steps in the preparation process of the complex itself.

The leaching was assessed using different immunoassay methods targeting the various components of the complex. The functionality was evaluated using an immunoassay model based on interleukin 6 detection to simulate the real life use in clinical assays.

2.1 Thermo Fisher Scientific

Thermo Fisher Scientific is a biotechnology research company based in Waltham, Massachusetts, USA.

One group of products delivered by Thermo Fisherare Dynabeads^{TM}, which were originally developed and produced by the Norwegian company Dynal, based in Oslo. Dynal was an independent company until 2005, when it was acquired by Invitrogen, later known as Life Technologies. Thermo Fisher Scientific then acquired Life Technologies in 2013.

Today, Thermo Fisher Scientific still produces and develops Dynabeads^{TM} in Norway.

2.2 Dynabeads™

Dynabeads™ are monodisperse superparamagnetic polymer coated microspheres used as a mobile platform for a wide variety of molecules including proteins, DNA, and RNA.

The monodisperse polymer particles were first produced by professor John Uglestad in 1977.

Later, those particles were produced with a porous surface. Those pores could be filled with iron salts, giving the whole complex paramagnetic properties.

Their discovery also correlated in time with the production of the first monoclonal antibodies, and after a few years, in 1980, the first trials to couple Professor Uglestad's magnetic polymer beads with monoclonal antibodies were conducted. This led to the development of the bead based immunoassay techniques (Halvorsen, 2018). Today Dynabeads™ are produced in many various sizes, spanning from 1 μm to 4 μm.

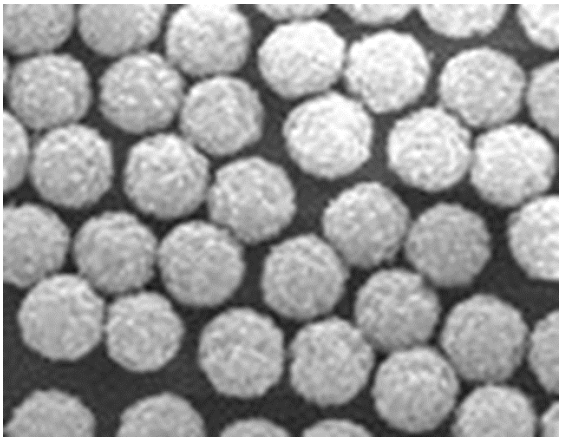

Figure 1: SEM micrograph of Dynabeads by courtesy of Thermo Fisher Scientific Norway.

Their surfaces are coated with different chemical groups, such as epoxy or tosyl, resulting in a diverse portfolio of products. Using different kinds of coating agents makes the different kinds of beads either more or less hydrophobic.

Using carboxylic groups as coating gives the beads hydrophilic properties, while utilising tosyl gives them everything from either slightly hydrophilic to moderately hydrophobic properties. Epoxy groups are hydrophilic and can also be used as coating. These choices will affect how the beads will behave in a given assay, and will make them better suited for given types of matrices they will be applied in later on.

2.3 Streptavidin

Streptavidin is a protein homotetramer (figure 2) which was first discovered in *Streptomyces Avidinii*. It is known for it's high affinity for biotin. This protein shows similarities to the more commonly occurring avidin, which can be found in egg white, and also shows a very high affinity for biotin (Taylor et al., 2006).

A single streptavidin tetramer weights about 60 kDa.

Due to it's high affinity for biotin and the low dissociation constant between those two compounds, about $4x10^{-15}$, streptavidin is widely used as a capture agent for biotin and by extension also biotinylated proteins or DNA.

Non-recombinant streptavidin has a pI close to neutral, which makes it easy to use for immunoassays, which are often carried out at slightly more basic conditions mimicking the physiological pH (Regnier and Cho, 2013).

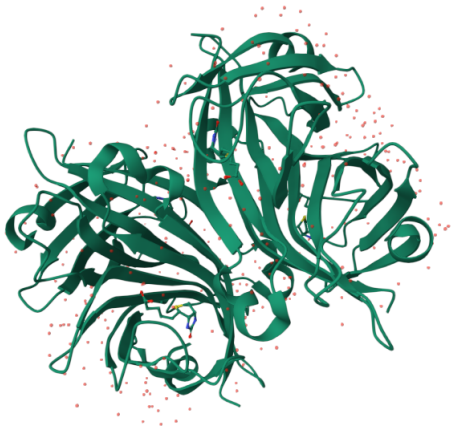

Figure 2: A streptavidine tetramer. (Weber et al., 1989, PBD ID: 1STA)

Streptavidin can therefore be used as a biotin capturing molecule. This requires the molecule to be either properly immobilised on a surface or easy to extract from the solution. Immobilising the streptavidin molecule on a Dynabead would allow for an easy and sure way to capture not only biotin but also the biotin-conjugated proteins or DNA from the solution and provide an easy way to extract or immobilise streptavidin together with captured compounds in for example a well on a well-plate. This makes streptavidin perfect candidate for use in combination with Dynabeads, and applying the complex in diagnostic methods such as immunoassays.

Utilising DB MyOne StA T1 as a platform for an immunoassay gives the advantages of easier automatiziation of the procedure, and greater surface areas to be covered in antibodies, compared to plate based assay formats such as ELISA.

2.4 Biotin

Biotin (figure 3), also known as vitamin B7 or vitamin H, is a small organic molecule with a molar mass of 244.31 g/mol, widely used by different organisms as coenzyme, signal molecule or for regulation of gene expression (Zempleni et al., 2009).

A part of this molecule, based on an imidazole ring, is effectively captured by streptavidin and held in place by affinity bond, in a 9 Å deep binding site. The other part of this

molecule is a pentanoic acid moiety (later called spacer arm), which sticks out from the binding site and therefore can be used as a conjugation site with other molecules.

Biotin can be modified to exchange the usual carboxylic group at the end of the spacer arm with other reactive groups such as N-hydroxysulfosuccinimide esters (NHS-esters), making it better suited for conjugation with proteins. A protein coupled with biotin in such way will also be captured by streptavidin.

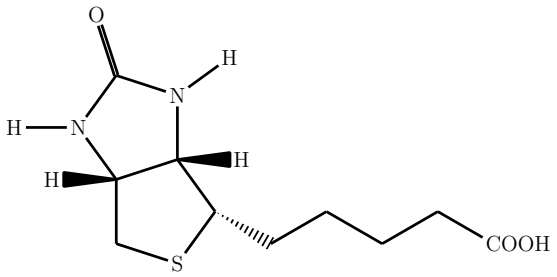

Figure 3: Structure of a biotin molecule.

Biotin molecules can also be modified to have a longer spacer arm. This can be beneficial when bigger, or sterically hindered proteins are to be captured by the streptavidin-biotin interaction.

This can be done through conjugating the pentanoic acid moiety of biotin with the amino acid lysine. Further modifications would include conjugation of lysine with a good leaving group such as previously mentioned NHS-esters.

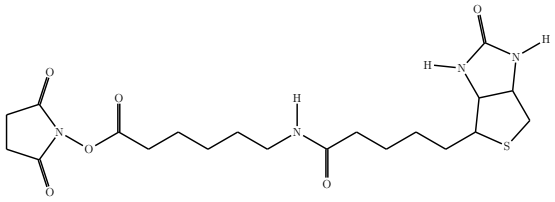

Figure 4: Structure of a NHS-LC-biotin molecule.

While a biotin molecule has a spacer arm about 13.5 Å long (figure 3), biotin molecules modified by coupling amino acid chains consisting of leucine such as LC-biotin or LC-LC-biotin can have spacer arms as long as 22.4 Å (figure 4) or 30.5 Å respectively (figure 5) (ThermoFisherScientific, 2023).

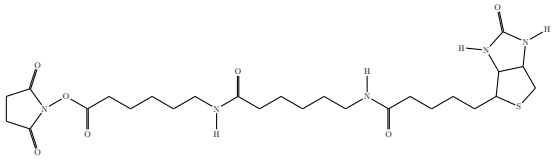

Figure 5: Structure of a NHS-LC-LC-biotin molecule.

Using biotin analogues such as desthiobiotin which has a spacer arm that's 9.7 Å long (figure 6) is also practiced.

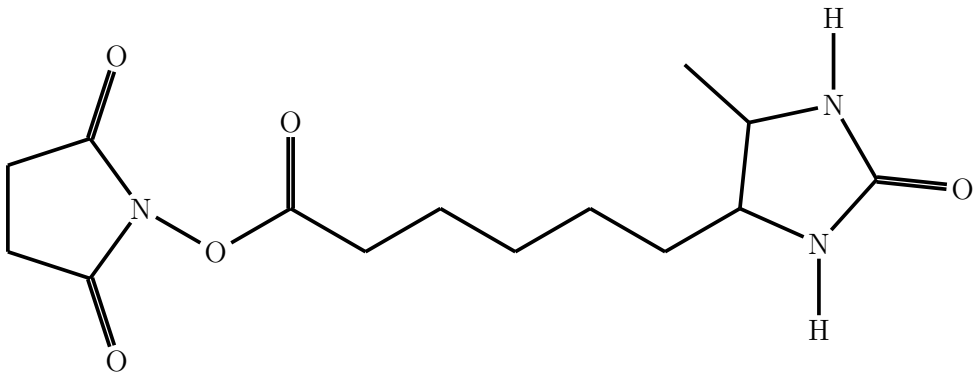

Figure 6: Structure of NHS-desthiobiotin molecule, a biotin analogue.

2.5 Antibodies and antigens

Antibodies, also called immunoglobulins (figure 7) are specialised proteins produced as a part of the immune response in the vertebrates. The purpose of the antibodies is the capture of antigens.Antigens are molecules or parts of molecules that are recognised as undesirable in an organism. In a healthy organism, antigens are in simple terms recognised as either related to illness or originating in other organisms.

Antibodies are made of two long and two short polypeptide chains called respectively heavy and light chains, held together by disulfide bridges, giving a typical antibody it's distinct Y-shape (Weaver and Murphy, 2022)

Heavy chains constitute the antibody's invariable region, and are used to classify antibodies into different classes. Therefore all IgG antibodies in a given class would have the same heavy chain structure. The heavy chain will serve as a scaffold for the light chain and also as a contact point with cells constituting the immune system.

The light chain is the most variable region of an antibody since antibodies of the same class will be made to target different antigens. For example antibodies from IgG class could be targeting toxins made by different bacteria, and those antibodies will vary only in their light chain structure. Even then, the site binding to the antigen will consist of amino acids from both the light and heavy chains (Walt et al., 2019, page 139.).

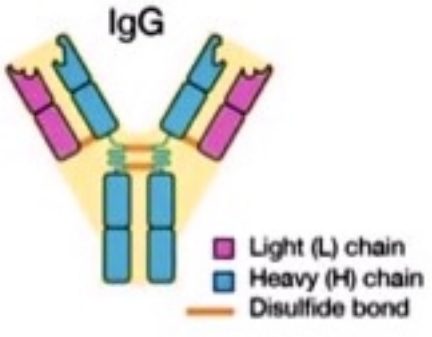

Figure 7: Schematic featuring the structure of a typical IgG molecule. It consists of two heavy and two light chains held together by disulfide bridges (EncyclopædiaBritannica, 2024).

This structure will capture specific parts of the antigens called epitopes, through an affinity binding between the amino acids from the light chain, the heavy chain and parts of the antigen itself.

2.6 Immunoassays

Immunoassays are bioanalytical methods utilising interactions between the antigens and antibodies to quantify the analytes (antigens) (Darwish, 2006).

The direct quantification of the antigens swimming freely in a solution at low concentrations might prove a challenging task. Therefore an indirect assay is used where an antibody towards the analyte is added. Other proteins or compounds are conjugated to the antibody to make the quantification easier. It assures that better sensitivity is obtained by enabling the quantification of the analytes with spectroscopic techniques.

Absorbance, fluorescence, and (flash and glow) luminescence are all widely used as methods for quantification.

For some of the most often used immunoassays, such as chemiluminescence immunoassay (CLIA) or enzyme linked immunosorbent assay (ELISA), sensitivity can be as low as $1x10^{-16}$M (Cinquanta et al., 2017).

Targeting specific molecules, such as bacterial metabolites, toxins, or disease markers using specific antibodies makes it possible to develop highly specific tests with low limits of detection (Darwish, 2006). Antibodies tailored for targeting specific epitopes will drastically improve the chances for detection by utilising the antibodies' high specificity for the analyte.

2.6.1 Immunoassay formats - sandwich vs. competitive immunoassays

Immunoassays can be constructed in many formats, each employing different arrangement for placement and the binding of antibodies, enzymes, and analytes. The most common formats for immunoassays used today are the sandwich assay, employing two antibodies, and competitive assays, which use only one antibody.

The sandwich immunoassays trap analytes between two antibodies. One of them, called the primary antibody will be immobilised on a surface and will capture analytes from a solution. The other antibody, called the secondary antibody will be added after a successful capture of analytes. The secondary antibodies will be conjugated with either the enzymes or the fluorophores. The quantification of analytes will be based on the signal produced by the sample. The stronger the signal, the more analyte was contained in the matrix.

The competitive assays will also employ an immobilised antibody to capture an analyte. In this method, the matrix containing the analytes will be supplied with an analog labelled with either an enzyme or a fluorophore. The unlabelled analytes will compete with the labelled analytes for binding to the immobilised antibodies. The quantification of analytes will be based on the signal produced by the sample. The weaker the signal, the more analyte was contained in the matrix, since the labelled ligands will have been outcompeted (Cox et al., 2012).

2.6.2 Models for immunoassay method development - interleukin 6 detection as an immunoassay model

When developing an assay or a product for use in an assay, for example Dynabeads, a model simulating how the consumers will utilize it is needed. The model does not need to be the exact same assay as the end user will be conducting, yet the principles and scale should be conserved to maintain the reliability and help develop a product which is better suited for the consumers needs. The assay model used in this project is based on the detection of interleukin 6.

Interleukins are intercellular signal molecules expressed by leukocytes. They are classified as cytokines. The overexpression of interleukin 6 (IL-6) is associated with a number of cancers such as myelomas, but also autoimmune diseases such as psioriasis and rheumatoid arthritis (Simpson et al., 1997).

Therefore, the detection and the optimisation of the assays detecting interleukin 6 are both valid areas for research. Using the IL-6 assay as a model, based on an easily available analyte, IL-6, makes it somewhat more cost effective.

2.6.3 Bead based immunoassays

Paramagnetic beads, such as Dynabeads™, serve as an effective platform for immunoassays. Specifically, they are utilized as a platform for primary antibodies, enabling the capture of analytes from the solution with precision and efficiency. Using paramagnetic beads as a carrier for the primary antibodies does not restrict the assay method, as both sandwich and competitive assays can be made using the beads.

In immunoassays utilising beads, antibodies are coupled to the surface of the beads and mixed with the solution containing analytes. Enhancing the contact between the antibodies and the ligands is achieved by suspending the beads in the matrix and agitating the resulting suspension. This allows for more effective capture than using antibodies attached to an immobile surface. Utilising the beads surface to anchor the primary antibodies, instead of the walls of a well in a microwell plate, provides much more surface

area for the analytes to bind to. Having more primary antibodies available the ligands can bind to can lead to an increase in the assay sensitivity. Agitating the beads in the suspension opens up for the antibodies and antigens to make more contact, and therefore a more effective capture of the analytes.

Because magnetic beads are either fished out from a solution with a magnet or retained in place when the liquid is discarded, using paramagnetic micro beads such as Dynabeads^{TM} would potentially allow for both more effective capture of the target molecules from the matrix, and concentrating given sample by allowing reconstitution of the beads in a smaller volume of buffer.

Utilising the paramagnetic properties of the beads also makes eventual automatization of the laboratory processes easier. The use of beads conjugated with antibodies in automatised laboratory tests rises the question of the beads stability in longtime storage. If big batches of beads are made and stored over a long time, leaching of the complex components can occur. Antibodies that are no longer properly immobilised on the beads' surface can be washed away between the incubation steps of an assay, potentially allowing for loss of analytes and unreliable results.

2.7 Factorial design of experiments

To optimise the amount of data collected and reduce the number of experiments, factorial design of experiments can be used. In factorial design of experiments a predetermined set of parameters to be investigated is specified.

Factorial design of experiments follows the formula n^m, where n is the number of parameters to be evaluated and m is the number points evaluated for each parameter. Therefore evaluating one parameter at two predetermined values requires 2^1 experiments, where 2 is a number of points evaluated for a given parameter and 1 is number of parameters. Therefore, a study evaluating 3 parameters at 2 points each will require 3^2, 8 experiments. This particular case can be visualised as a 3-dimensional box, as shown in figure 8.

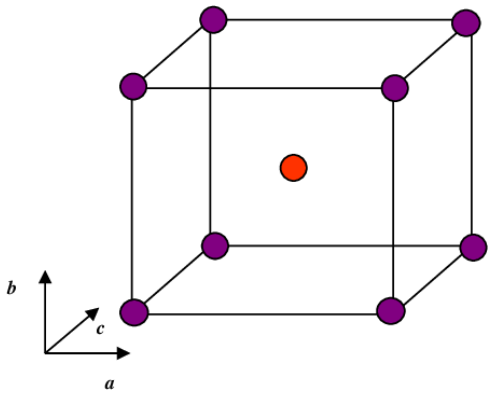

Figure 8: Model of a factorial DOE with a centre point. It has 3 factors with 2 levels. Rüttimann and Wegener, 2015

An analysis employing factorial design would have each parameter evaluated at determined edge points and at least one centre point. For example, pH can be used as one of the parameters and will be evaluated at pH 7.0 and 8.0 as edge points and using pH 7.5 as a centre point.

2.8 MODDE

MODDE is a design of experiments (DOE) software, previously developed by Umetrics and since 2017 owned and further developed by Sartorius®. This software is meant to help design and further analyse experiments by employing the principles of factorial design of experiments. It suggests a number of experiments for the user-defined set of parameters, and constructs analyses based on the provided results.

The MODDE software produce an analysis of the correlation between the parameters and determine the significance of any given parameter, for any given set of provided results. This is done through a variety of inbuilt statistical tools implementing various regression techniques such as partial least squares regression (PLS) or multiple linear regression (MLR). ANOVA analysis can be also be conducted, based on the selected regression mode.

For any model constructed by the software, a R^2 (square of goodness of fit) , Q^2 (model predictive power), model validity and reproducibility values are calculated (Sartorius, 2017). Q^2 itself is a measure of the model's robustness while working with incomplete sets of data. It provides an assumption of how good a statistical model is. Q^2 as a numerical value is based on a R^2 and tells the user how good the model would be to predict values between measured points that were used to calculate R^2.

If enough replicates are provided, the software will also calculate the model's validity and reproducibility scores. An example of summary of fit is presented in figure 9. This summary features R^2, Q^2, model validity, and reproducibility presented on the same plot and adjusted to a common Y-axis.

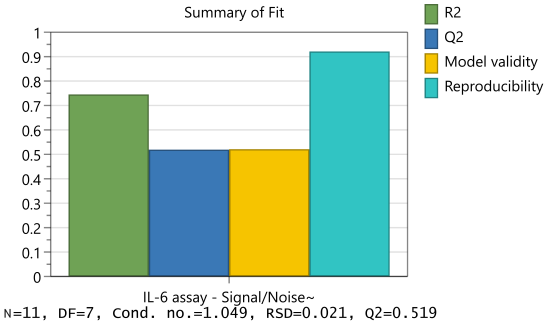

Figure 9: An example summary of fit plot generated by MODDE software. It features R^2, Q^2, model validity, and reproducibility scores generated for this particular model.

To construct a predictive model from the provided experimental data, the R^2 and Q^2 values are the most important. For a good model, those values shouldn't differ with more than 0.3 and R^2 should be as close to 1 as possible. Q^2 should have a numerical value greater than 0.1 to be considered a statistically valid model (Sartorius, 2017). Such a model would nevertheless be considered of having poor predictive strength due to the low correlation between the goodness of fit for the provided data and the correctness of the interpolation of the incomplete dataset.

2.9 ANOVA - Analysis of variance

Analysis of variance (ANOVA) is a statistical method for comparing three or more population means. It makes use of F-distribution to look at the mean values of n populations, and test if there are any significant differences between them (Bhattacharyya and Johnson, 2020

The ANOVA analysis can be done by the MODDE software. An ANOVA plot can be

constructed to graphically represent the analysis' results, as shown in figure 10. The plot shows SD regression, RSD, and RSD*$\sqrt{F(critical)}$.

The SD regression bar shows response variations in the constructed model as a square root of mean squares regression. It takes into account degrees of freedom of the model.

The RSD bar shows the residual standard deviation. Residuals are differences between the predicted and observed values. RSD is a measure of the variation of the responses that can not be explained by the constructed model. This measure is also adjusted for the degrees of freedom.

The RSD*$\sqrt{F(critical)}$ is calculated from the RSD value for a given model, multiplied by the square root of the critical F. The critical F is the value of the F-distribution over which SD regression is statistically significant for a given confidence level. If a confidence level of 5% is chosen, the F-value for this confidence level, F(0.05) for the given combination for degrees of freedom will be used further in calculations.

If the RSD*$\sqrt{F(critical)}$ value is smaller than the RD regression value (RSD*$\sqrt{F(critical)}$ bar is lower than RD regression bar), then the model will be considered significant at the chosen confidence level (Sartorius, 2017).

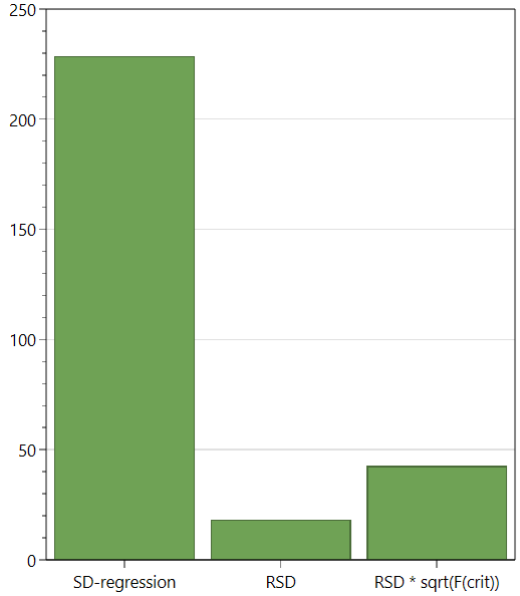

Figure 10: Example of an ANOVA plot generated by the MODDE software. The figure shows bars for SD regression, RSD, and RSD$\sqrt{F(critical)}$ values, all adjusted to a common scale. This example show a statistically significant model because the SD-regression value is much higher than the RSD*$\sqrt{F(critical)}$.*

3 Materials and methods

3.1 Materials

3.1.1 Equipment

analytical balance: Mettler Toledo XPR226DR^R

centrifuge: VWR Mini Star silverline

columns: GE Healthcare NAP TM-25 Lot:16836818

epoxy beadsd: Dynabeads MyOne Epoxy Anti-Streptavidin, internal coupled, 10 mg/mL

gravity flow chromatography columns: GE Healthcare NAP TM-25 Lot:16836818

heating cabinet: Termaks

incubator: Heidolph Inkubator 1000

magnet: Dynamag^{TM}-2

microplate for ELISA: ELISA Microplate 96-well C-Bottom High binding

microplate for StA and IL-6 assays: Greiner bio-one Microplate 96-well F-bottom (chimney well) white Lumitrac med. binding

microplate reader: AH diagnostics BioTek^R Synergy 2 microplate reader

microplate reader: Thermo Scientific Varioskan LUX

microplate washer: AH diagnostics BioTek^R 405 TS microplate washer

microvolume spectrophotometer: Thermo Scientific Nanodrop ONE^C

pH meter: Mettler Toledo SevenCompact^{TM} ph meter S220, ExpertProISM

precision balance: Mettler Toledo XP2002S

roller: Stuart^R roller mixer SRT6

sealing tape: Costar, Cat#6524, Clear Polyethylene non sterile

shaker: Heidolph Titramax 1000

thermal mixer: Eppendorf ThermoMixer C

3.1.2 Computer software

design of experients software: MODDE® by Sartorius

synergy software: BioTek Gen5

varioskan lux software: Thermo Scientific SkanIt

3.1.3 Reagents

Kuler:

Dynabeads™ MyOne™ Streptavidin T1, Invitrogen, Lot:252955630, Produktnummer:35602, 10 mg/mL

Dynabeads™ MyOne™ Streptavidin T1 (40 mg/mL)

Lot:2846074

Lot:2824565

Lot:2796130

Buffers:

1M $NaHCO_3$

PBS - 10 mM Na_2PO_4, 150 mM NaCl, pH 7.4

DMSO

TBST with NaN_3 - 50 mM Tris, 140 mM NaCl, 0.1% Tween, 0.02% NaN_3, pH 7.4

TBST with BSA and NaN_3 - 50 mM Tris, 140 mM NaCl, 0.1% Tween, 0.02% NaN_3, 0.1% BSA, pH 7.4

PBST 0.1% - 10 mM Na_2PO_4, 150 mM NaCl, 0.1% Tween 20, pH 7.4

Storing buffers:

50 mM MES with 0.05% Tween 20, pH 6.5

50 mM TBS with 70 mM NaCl and 0.05% Tween 20, pH 7.4

50 mM MES with 0.1% Tween 20, pH 6.5

50 mM TBS with 70 mM NaCl and 0.1% Tween 20, pH 7.4

50 mM MES with 0.05% Tween 20 and 0.1% BSA, pH 6.5

50 mM TBS with 70 mM NaCl and 0.05% Tween 20 and 0.1% BSA, pH 7.4

50 mM MES with 0.1% Tween 20 and 0.1% BSA, pH 6.5

50 mM TBS with 70 mM NaCl and 0.1% Tween 20 and 0.1% BSA, pH 7.4

50 mM TBS with 70 mM NaCl and 0.75% Tween 20 and 0.05% BSA, pH 7.0

biotin:

EZ-Link™ Sulfo-NHS-LC-LC-Biotin, No-Weigh™ Format, Lot:XC337449

D-biotin, succiniimidyl ester, B1513, Lot: 905708

EZ-Link™ Sulfo-NHS-LC-Biotin, B6353, Lot: 1004320

antibody for biotinylation: mAb Hu IL-6 Capture, (100 mg, 2.33 mg/mL), Lot:B21E13P1/01

detection antibody for IL-6 assay: anti-IL6 Det AB SACR-conj. 0.15 mg/mL, L004

standard antibody for IL-6 assay: anti-IL-6 0.5 μg/mL EXP 21-653

Trigger 1 - 0.43 M H_2O_2, 0.16 M HNO_3

Trigger 2 - 0.25 M NaOH, 26 mM CDAC

Pooled human serum, plasma derived,

Pooled human serum, plasma derived, spiked with 100 ng/mL IL-6.

Pooled human serum, plasma derived, spiked with 1000 ng/mL IL-6.

coating antibody: Gt x-Mouse Kappa Light Chain (1 mg/mL) A90-119A Lot:221209

blocking buffer: SuperBlock™ Blocking Buffer in TBS Lot:YD366735, Thermo Fisher Scientific

detection antibody for ELISA: Goat anti-Mouse IgG-h+I HRP conjugated, 0.5mg/mL, Lot: A90-216P-21

antibody for ELISA: IL6 antistoff, Lot: B21E13P1/01

substrate for ELISA: BioFX® TMB One Component HRP Microwell Substrate, Lot: TMBWW06

stop solution: 1% Sulfuric acid

Streptavidin, Roche, Lot:56267157 (1 mg/mL)

substrat for streptavidin assay: Dynalight Substrate with RapidGlow Enhancer Lot:2204044

biotinylert alkali phosphatase: biotinylert AP (1.2 mg/mL), Pierce 29339, Lot:RH2355259

StabilZym AP Conjugate, Surmodic SA01-0050 Lot:SA01X16

3.2 Methods

3.2.1 Biotinylation of anti-IL-6

The following procedure shows how anti-IL-6 were conjugated to biotin. This was to make the antibody ready to conjugate to the streptavidin coated Dynabeads.

Procedure:

- 1M $NaHCO_3$ was made by weighing in ca. 4.2 g $NaHCO_3$ and dissolving it in 50 mL RO water.

- 2.5 mM biotin solution was made by dissolving 1.000 mg sulfo-NHS-LC-LC-biotin in 600 μL RO water.
  2.5 mM biotin solution was made by dissolving 1.955 mg desthiobiotin in 2300 μL DMSO.
  2.5 mM biotin solution was made by dissolving 3.435 mg sulfo-NHS-LC-Biotin in 2500 μL RO water.
- In a tube 214 μL of 2.33 mg/mL anti-IL-6 antibody, 229 μL PBS buffer, 50 μL 1M $NaHCO_3$ and 7 μL of a 2.5 mM biotin solution was added.
- The tube was incubated on a roller at room temperature for 30 min.
- A NAP-5 column was equilibrated with 10 mL PBS buffer.
- 500 μL of the solution was added to the column.
- 1 mL PBS buffer was used to elute the biotinylated antibody.
- The concentration of biotinylated antibody was determined with nanodrop spectrophotometer.

3.2.2 Conjugation of biotinylated antibody with DB™ MyOne™ StA T1

The following procedure describes how previously biotinylated anti-IL-6 was conjugated to DB™ MyOne™ StA T1.

Conjugation of biotinylated antibodies with streptavidin coated Dynabeads is the last step in constructing the functional complex which will later be used in bead based immunoassays.

Procedure:

- 5 mg beads was washed three times in TBST-BSA buffer and resuspended in the buffer.

- 50 μg biotinylated antibodies was added.
- The beads were incubated on a roller in room temperature for a minimum of 30 min.
- The beads were washed three times and resuspended in TBST-BSA buffer.

One of the experiments used a modified version of this procedure but were either the temperature, incubation time or concentration of biotinylated antibodies varied.

3.2.3 IL-6 functionality assay

The following procedure describes the usage of Dynabeads conjugated with biotinylated anti-IL-6 in immunoassays under controlled conditions. To measure the functionality of a bead product, a comparative chemiluminescence immunoassay (CLIA) is performed (Cinquanta et al., 2017).

This method employs two types of antibodies, one for capture and one for detection of the target molecule, here IL-6 in an antibody-sandwich. The capture antibodies is conjugated to the bead and will capture IL-6 from a solution. The detection antibody labelled with acridinium ester will also recognise IL-6 as it's target. Acridinium ester will produce flash luminescence when added sodium hydroxide and hydrogen peroxide.

This assay was used to assess if the functionality of the assay was conserved after tweaking the methods. The standard curve was often ran without the serum samples.

Procedure for IL-6 assay in buffer and serum, with recovery test

- 5 mg/mL coupled beads where diluted to 0.2 mg/mL with TBST-BSA buffer.
- IL-6 antigen was diluted from 0.5 μg/mL to 10 ng/mL to 1000 pg/mL in buffer.
- The last dilution was further diluted to make a standard curve with the following concentrations; 500 pg/mL, 100 pg/mL, 10 pg/mL, and 0 pg/mL.

- Regular serum, serum spiked with a medium concentration of IL-6 antibodies and serum spiked with a high concentration of IL-6 antibodies were diluted 1:1 in the buffer.
- 50 μL of the different standard and serum dilutions were added to one row each on a microwell plate.
- 50 μL beads were added to each well.
- IL-6 SACR was diluted from 0.15 mg/mL to 0.1 μg/mL.
- 50 μL diluted IL-6 SACR was added to each well.
- The plate was sealed and incubated for 30 min in 37°C with 950 rpm.
- The plate was washed for three cycles with 300 μL on the plate washer.
- Flash luminescence was measured with Varioskan LUX plate reader.

3.2.4 ELISA anti-IL-6 leaching assay

Leakage of antibodies from the bead-streptavidin-antibody complex will negatively affect the sensitivity of the assays using the bead product.

The concentration of the antibodies leaching can be quantitatively measured using an immunoassay targeting the conjugated antibody.

This assay is made in an ELISA format, that is to say it's a plate based assay..

Procedure for coating the ELISA microwell plates:

- The coating antibody was diluted from 1 mg/mL to 2 μg/mL with PBS buffer.
- 100 μL of this solution was added to each well.
- The plate was sealed and incubated overnight in 4°C.
- The solution aspirated on the microplate washer.

- 300 μL blocking buffer was added to each well.
- The plate was sealed and incubated at room temperature for 1h.
- The solution aspirated on the microplate washer.
- The plate was stored in a bag with an absorbing pouch.

Procedure for ELISA immunoassay:

- IL-6 antibody was diluted to 100 μg/mL, 10 μg/mL, 1 μg/mL, and then 50 ng/mL using PBS buffer.
- The last dilution was used to make the standard curve with the following concentrations; 8 ng/mL, 5 ng/mL, 2.5 ng/mL, 2 ng/mL, 1.5 ng/mL, 1 ng/mL, 0.5 ng/mL, and 0 ng/mL.
- The supernatant of the coupled beads were diluted 1:80, 1:160, 1:320, and 1:640 in PBS buffer.
- 100 μL of the standard solutions and the diluted supernatant were added to each well on the microplate as triplicates.
- The plate was sealed and incubated at 37°C with 900 rpm for 1h.
- It was washed three times with 300 μL PBS with Tween 20 on the microplate washer.
- The HRP detection antibody was diluted from 0.5 mg/mL to 0.07 μg/mL.
- 100 μL diluted HRP was added to each well.
- The plate was sealed and incubated at 37°C with 900 rpm for 30 min.
- It was washed three times with 300 μL PBS with Tween 20 on the microplate washer.
- 100 μL TMB substrate was added to each well.
- The plate was sealed and protected aginst light with aluminium foil, before it was incubated at 37°C with 900 rpm for 15 min.

- 100 μL 1% sulfuric acid was added to each well to stop the reaction.
- Absorbance was measured at 450 nm with Varioskan LUX plate reader.

3.2.5 Streptavidin leaching assay

Some streptavidin molecules will dissociate from beads they were conjugated with. Leakage of streptavidin from the bead-streptavidin-antibody complex will also affect sensitivity of assays that use the bead product.

This assay employs affinity between streptavidin and biotin to quantify the streptavidin concentration in the supernatant. After capturing the free streptavidin from bead supernatant with anti-streptavidin coated Dynabeads, the captured streptavidin is then incubated with biotinylated alkali phosphatase. The alkali phosphatase will later be used to drive an enzymatic reaction which produces a luminescent compound used to measure concentrations of streptavidin in solution.

Procedure:

- Streptavidin was diluted to 100 ng/mL.
- This dilution was used further to make the standard curve with the following concentrations; 40 ng/mL, 20 ng/mL, 15 ng/mL, 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1 ng/mL, and 0 ng/mL.
- The supernatant of the coupled beads was diluted 1:16, 1:64, 1:256, and 1:512.
- 50 μL of the standard solutions and diluted supernatant were added to each well in triplicates on a microplate.
- 10 mg/mL Epoxy anti-StA beads were washed three times in TBST-BSA, and diluted to 0.2 mg/mL.
- 100 μL of the diluted epoxy beads were added to each well.
- The plate was sealed and incubated at 37°C with 900 rpm for 30 min.

- It was washed three times with 300 μL buffer on the microplate washer.
- Biotinylated AP was diluted from 1.2 mg/mL to 0.48 mg/mL.
- 150 μL diluted biotinylated AP was added to each well.
- The plate was sealed and incubated at 37°C with 900 rpm for ten minutes.
- It was washed three times with 300 μL TBST buffer and resuspended.
- Fluorescence was measured with Synergy 2.

4 Results

4.1 Testing different biotin types

The first series of experiments was conducted to compare how the different types of biotin would affect the leaching from the bead-streptavidin-biotin-antibody complex, and the functionality of the assays making use of those beads.

Anti-human-IL-6 monoclonal antibodies were conjugated with three different types of biotin. They were further conjugated with DB™ MyOne™ StA T1 and stored in TBST buffer at 37°C for one week before being used in IL-6 assay. The leaching of streptavidin and antibodies from each sample was evaluated after five and twelve days respectively.

The biotin types looked at for this series of experiments were Sulfo-NHS-LC-biotin, desthiobiotin and Sulfo-NHS-LC-LC-biotin with the molecular weights 556.59 g/mol, 311.33 g/mol, and 669.75 g/mol respectively. The spacer arm length was shortest for desthiobiotin, being only 9.7 Å. The spacer arm of LC-biotin was 22.4 Å, and 30.5 Å for LC-LC-biotin.

Sulfo-NHS-LC-biotin and desthiobiotin showed a similar degree of sensitivity in the IL-6 assays, based on the mean MAX RLU measurements (figure 13).

The measured mean MAX RLU for LC-biotin was 122133 RLU. For desthiobiotin the mean of highest values was 105200 RLU. The ratios of mean MAX RLU to noise (S/N) were calculated to be 22.9 and 22.1 respectively. The highest registered value for beads using LC-LC-biotin was 133300 RLU. S/N was calculated to be 34.7. The signal to noise ratios in the IL-6 assay for the beads using those three types of biotin are summarised in figure 13.

The leaching of IgG was calculated to be 335 ng/mg beads for the beads using sulfo-NHS-LC-biotin, 135 ng/mg beads for the beads using desthiobiotin and 219 ng/mg beads for the beads conjugated with antibodies using sulfo-NHS-LC-LC-biotin. The leaching of anti-IL-6 is summarised in figure 11.

The leaching of streptavidin was calculated to be 3.9 ng/mg beads for the beads using sulfo-NHS-LC-biotin, 2.3 ng/mg beads for the beads using desthiobiotin and 2.0 ng/mg beads for the beads using sulfo-NHS-LC-LC-biotin.

Figure 13 summarises the previously mentioned data for leaching of IgG (ng/mg beads), leaching of streptavidin (ng/mg beads), and S/N registered for the beads conjugated with antibodies employing different types of biotin.

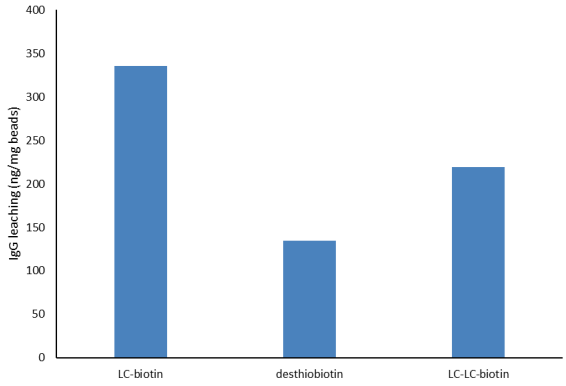

Figure 11: A comparison of IgG leaching (ng/mg beads) for beads conjugated with antibodies biotinylated with either LC-biotin, desthiobiotin, or LC-LC-biotin.

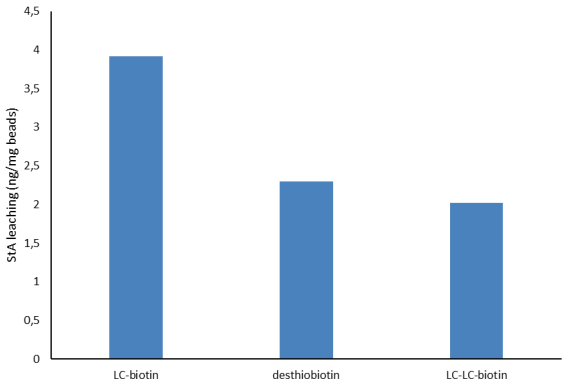

Figure 12: A comparison of StA leaching (ng/mg beads) for beads conjugated with antibodies biotinylated with LC-biotin, desthiobiotin, or LC-LC-biotin.

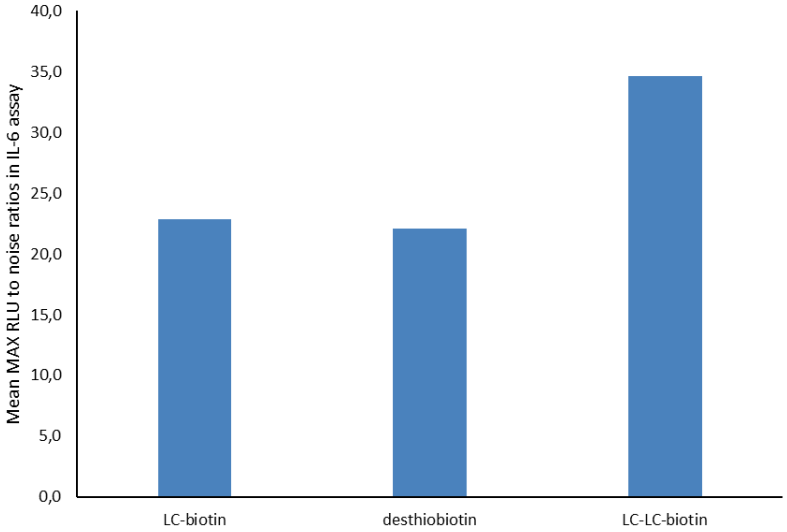

Figure 13: A comparison of mean MAX RLU to noise ratios registered in the IL-6 assay for beads conjugated with antibodies biotinylated with either LC-biotin, desthiobiotin, or LC-LC-biotin. The measurements of signal to noise ratios are based on RLU measured for samples with IL-6 concentration of 1000 ng/mL.

4.2 Testing different conditions under conjugation

The purpose of this experiment was to check how different conditions under the conjugation of the biotinylated antibodies to the beads coated with streptavidin would affect the leaching and functionality of the assays.

The parameters varied under this experiment were the incubation time, the temperature under incubation and the concentration of biotinylated IgG added to the reaction tubes. This experiment was designed with help of the MODDE software. The highest and lowest points were chosen somewhat arbitrarily, with the concern of which experimental conditions would be most easily attainable. The choice of centre points was proposed by the MODDE software.

The incubation time was evaluated between 10-60 minutes. The temperature under the conjugation was evaluated between 10-35°C. The concentration of biotinylated IgG added to the reaction tube was between 2-15 μg/mg beads.

All the nine combinations of temperature, concentration of IgG and incubation time are summarised in table 1.

Table 1: Different combinations of incubation time, temperature under incubation and concentration of biotinylated IgG used to make nine samples of beads to be later evaluated for leaching of IgG, leaching of streptavidin and the functionality in IL-6 immunoassays.

Sample number	Incubation time (min)	Temperature (°C)	Concentration of biotinylated IgG (μg/mg beads)
N1	10	10	2
N2	60	10	2
N3	10	35	2
N4	60	35	2
N5	10	10	15
N6	60	10	15
N7	10	35	15
N8	60	35	15
N9	35	23	8.5

After conjugation, all the samples were stored in TBST-BSA buffer at 37°C for five days before applying them in an IL-6 assay. The leaching of streptavidin was assessed after six days, and the leaching of biotinylated antibodies after seven days.

Figure 14 summarises the leaching of IgG in ng/mg beads. Figure 15 illustrates the changes in streptavidin leaching between the samples in terms of ng free streptavidin measured in supernatant per mg of beads. Figure 16 shows changes in S/N registered in IL-6 assays for beads from each sample.

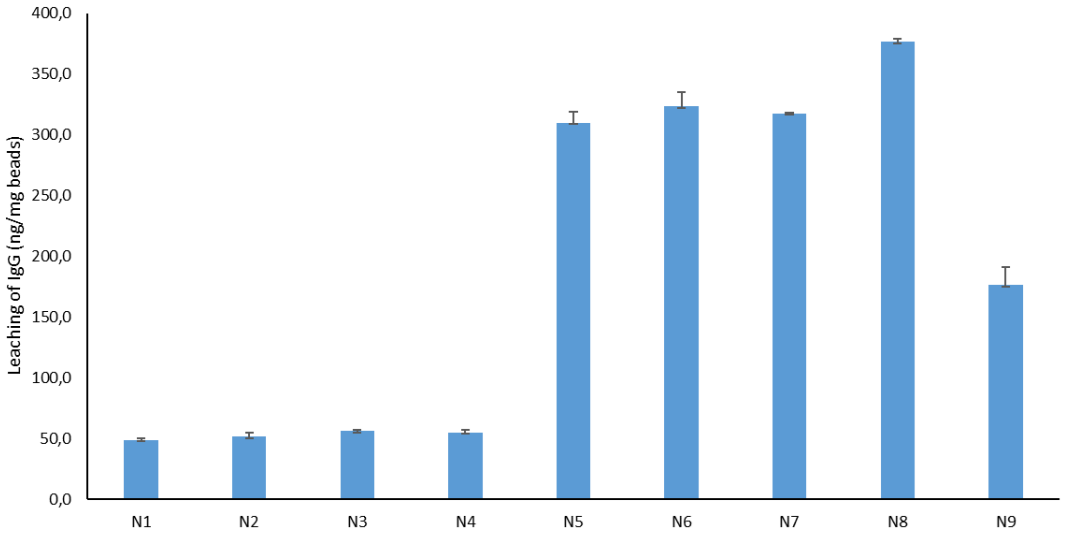

Figure 14: Leaching of IgG (ng/mg beads) measured for nine samples of beads conjugated with biotinylated antibody under varying conditions and stored at 37°C for seven days. Sample numbers in this graph correspond to the sample numbers from table 1.

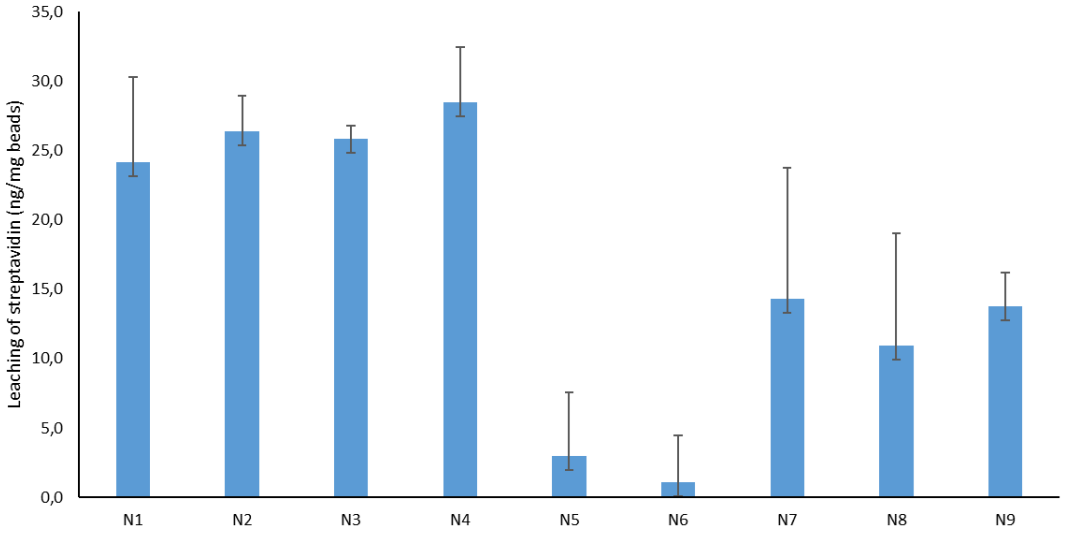

Figure 15: Leaching of streptavidin (ng/mg beads) measured for nine samples of beads conjugated with biotinylated antibody under varying conditions and stored at 37°C for six days. Sample numbers in this graph correspond to sample numbers from table 1.

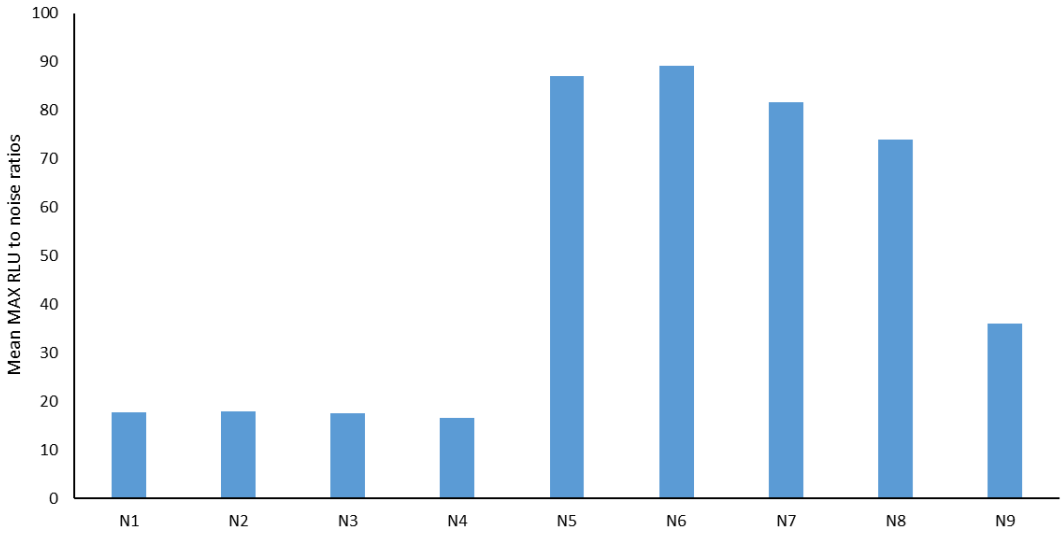

Figure 16: Mean MAX RLU to noise ratios in the IL-6 assay, measured for nine samples of beads conjugated with biotinylated antibody under varying conditions and stored at 37° C for five days. Sample numbers in this graph correspond to sample numbers from table 1.

After analysing the dataset with the MODDE software, an analysis of fit and the predictive strength of the model was constructed as shown in figure 17. Due to a calculation error in the scaling of the experiment, there was only one sample of beads to be used as a centre point. Because only one centre point was included (sample N9), the experimental data provided to the MODDE software were not enough to produce reproducibility, or validity score models for this dataset.

This can be seen in figure 17, where analysis of fit, R^2, and model predictive power, Q^2 are shown for the statistical models of biotinylated IgG leaching, streptavidin leaching and S/N of the IL-6 assay made using beads conjugated with antibodies under different conditions.

For the IgG leaching model, the analysis of fit was calculated to be $R^2 = 0.989$, while the model predictive power was calculated to be $Q^2 = 0.870$. For the streptavidin leaching model, the analysis of fit was calculated to be $R^2 = 0.930$, while model predictive power was calculated to be $Q^2 = 0.776$. For the mean MAX RLU to noise ratios model, the analysis of fit was calculated to be 0.981, while the model predictive power was calculated to be $Q^2 = 0.866$.

The p-values calculated for the IgG leaching model were 0.212, 0.225, and $4.08\text{x}10^{-6}$ for

the incubation time, temperature, and concentration of biotinylated antibody respectively. The p-values calculated for the StA leaching model were 0.969, 0.051, and 5.72x10^{-4} for incubation time, temperature, and concentration of biotinylated antibody respectively. The p-values calculated for the S/N in the IL-6 assay were 0.240, 0.110, and 1.84x10^{-5} for incubation time, temperature, and concentration of biotinylated antibody respectively.

The significance level chosen as an upper bar for a significant model in this analysis was 1% (0.01). Only the smallest p-values for each set were considered small enough to consider a factor significant with good enough certainty.

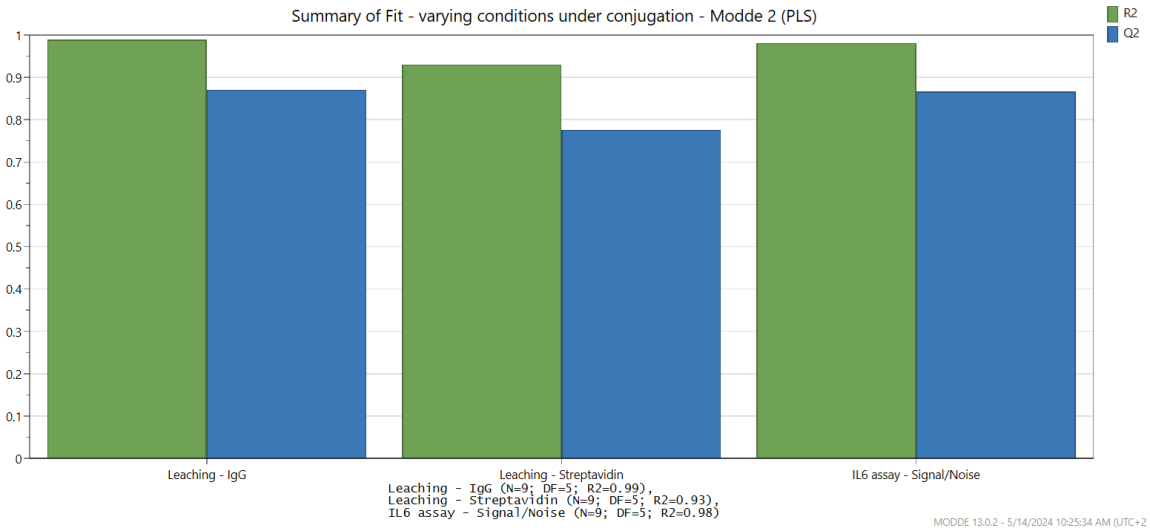

Figure 17: Analysis of fit, R^2, and model predictive power, Q^2 for the statistical models of biotinylated IgG leaching, leaching of streptavidin and mean MAX RLU to noise ratios in IL-6 assay made using beads conjugated with biotinylated antibodies under different conditions. The plot was generated by MODDE software.

An ANOVA analysis was conducted for the dataset presented in figure 18. The results of the ANOVA analysis including SD-regression, RSD, and RSD*$\sqrt{F(critical)}$ values are summarised in figure 18.

SD-regression was calculated for each model. The SD-regression values are 30.1, 16.2, and 228.7 for the signal to noise ratios in IL-6 assay, leaching of StA, and leaching of IgG respectively.

The RSD for each model was measured to be 3.26, 18.38, and 3.44 for RLU to noise ratios, IgG leaching, and StA leaching respectively.

The RSD*$\sqrt{F(critical)}$ is given to each model with the following values for signal to noise ratios in IL-6 assay, leaching of StA, and leaching of IgG respectively: 7.58, 42.76, and 8.00.

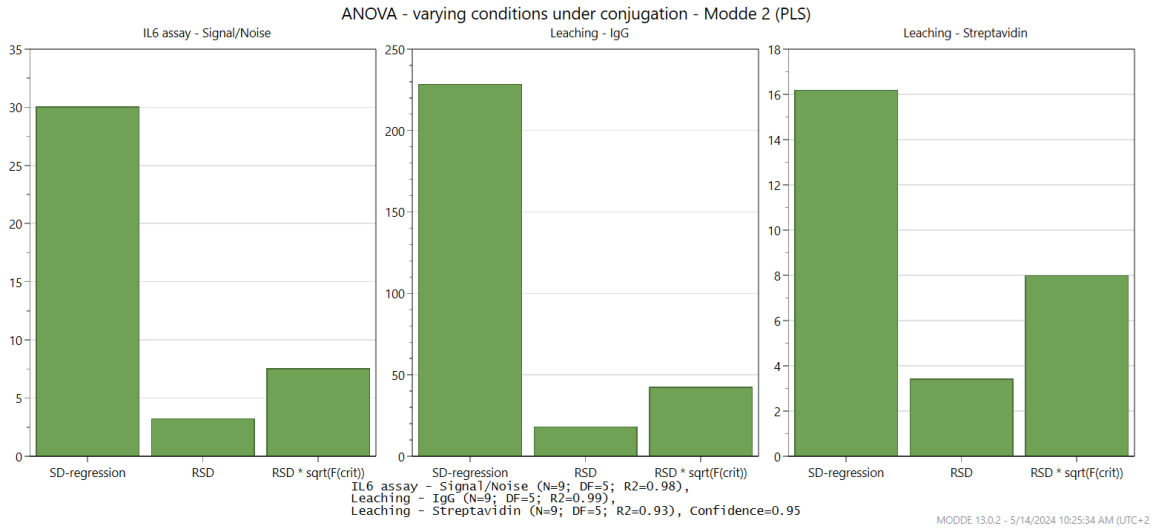

Figure 18: A summary of ANOVA analysis made for experiment evaluating various conditions under conjugation of biotinylated antibodies with Dynabeads™Streptavidin. SD-regression, RSD, and RSD$\sqrt{F(critical)}$ for each response evaluated: signal to noise ratios in IL-6 assay, leaching of IgG, and leaching of streptavidin.*

The significance each parameter had on the leaching of both streptavidin and biotinylated antibody from the bead-streptavidin-biotin-antibody complex, as well as the functionality of the assays was also evaluated. The significance of each parameter is summarised in figure 19. The concentration of biotinylated antibody used under conjugation is the only significant parameter for each model.

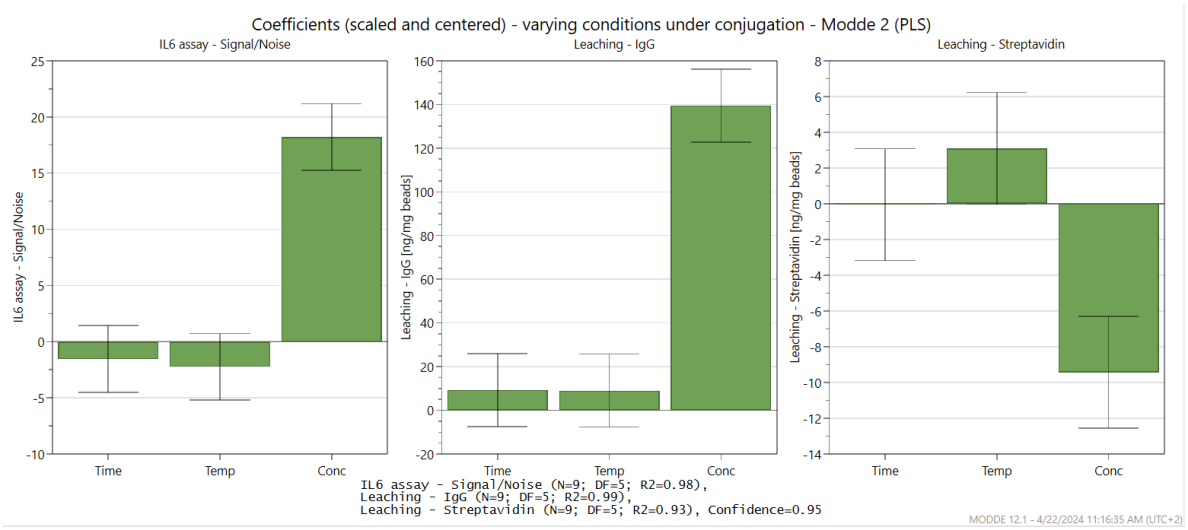

Figure 19: Coefficients plot for the statistical models constructed to evaluate the impact the varying conditions under conjugation had for the mean MAX RLU to noise ratios in IL-6 assay, leaching of biotinylated IgG, and leaching of streptavidin. The plot was generated by the MODDE software.

4D response contour plots were also constructed to visualise the effect the parameters have on the S/N (figure 20), IgG leaching (figure 21), and the StA leaching (figure 22).

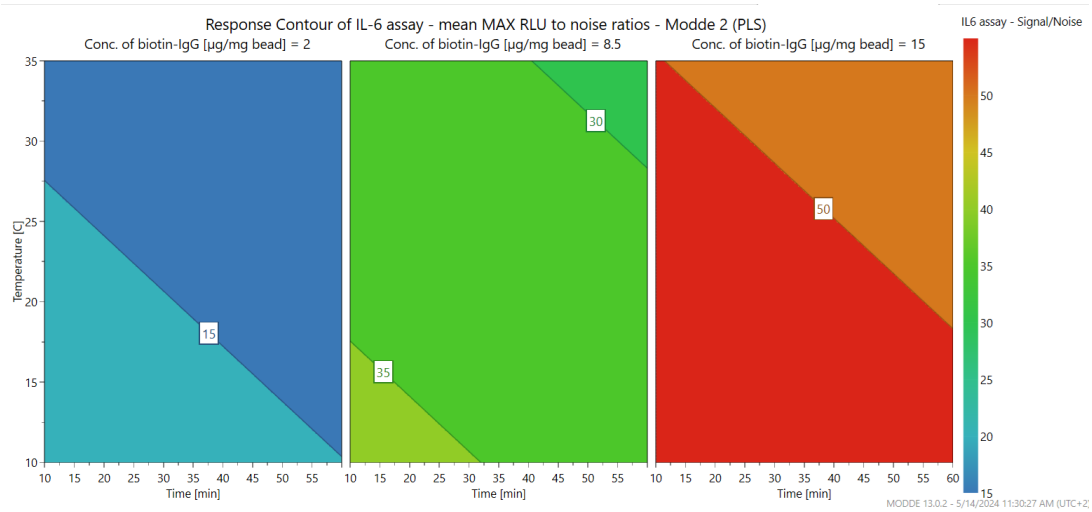

Figure 20: 4D response contour plot showing the impact changes in concentration of biotinylated antibody used under conjugation (µg/mg beads), temperature during reaction (° C) and incubation time (min) has for the sensitivity of IL-6 assay, expressed with measured mean MAX RLU to noise ratios. The plot was generated by MODDE software.

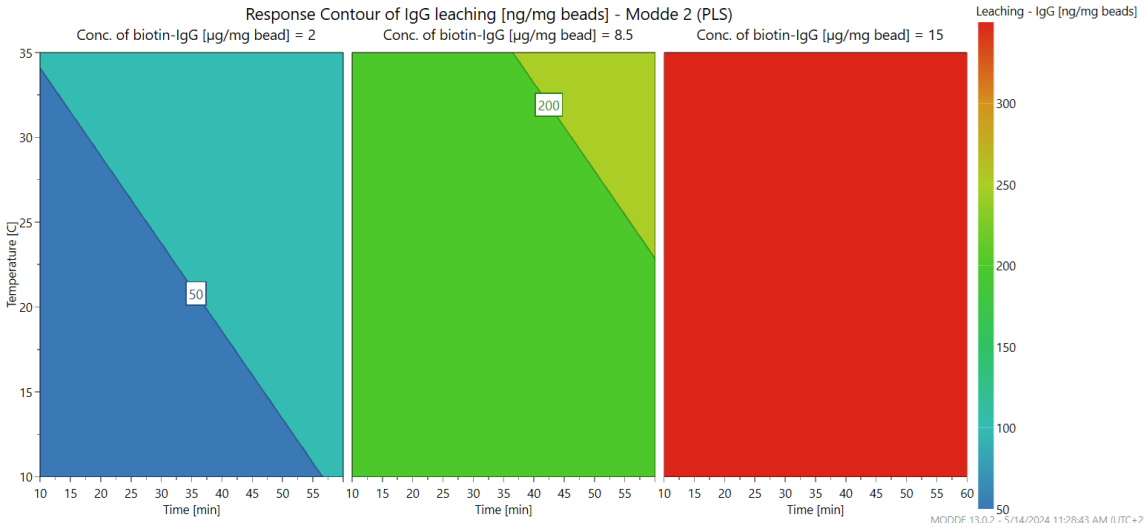

Figure 21: 4D response contour plot showing the impact changes in concentration of biotinylated antibody used under conjugation (µg/mg beads), temperature during reaction (°C) and incubation time (min) has for the leaching of biotinylated antibody from beads expressed in ng/mg beads. The plot was generated by MODDE software.

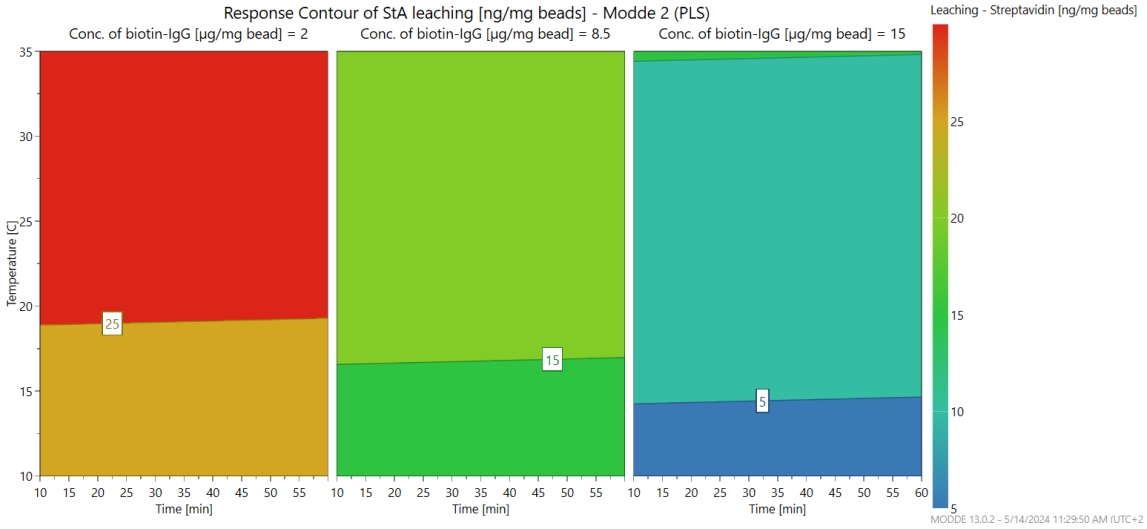

Figure 22: 4D response contour plot showing impact changes in concentration of biotinylated antibody used under conjugation (µg/mg beads), temperature during reaction (°C) and incubation time (min) has for leaching of streptavidin from beads expressed in terms of ng/mg beads. The plot was generated by MODDE software.

4.3 Varying conditions under storage

In this experiment, various storage conditions for the beads were evaluated.

DB™ MyOne™ StA T1 is delivered in the storage buffer PBS with 0.1% BSA and 0.02%

NaN_3, pH 7.4.

The buffers for storage after coupling with biotinylated IgG were based on both tris(hydroxymethyl)aminomethane (Tris) and 2-(N-morpholino)ethanesulfonic acid (MES) to cover a wider range of pH. The addition of different concentrations of polysorbate 20 (Tween 20), which acts as an anti-aggregation agent, and bovine serum albumin (BSA) which acts as a stabiliser was also evaluated.

The MODDE software was used to design this experiment, in total, 9 different buffers were made with variations of mentioned parameters. The constitutions of each buffer are summarised in table 2.

Table 2: Summary of the buffers made to evaluate how different storage conditions affect the leaching of IgG, streptavidin, and mean MAX RLU to noise ratios in the IL-6 assay. Buffer number for each buffer, pH, percentage concentration of Tween 20 and percentage concentration of BSA added to each buffer sample.

Buffer number	pH	Tween 20 (%)	BSA (%)
1	6.5	0.05	0
2	7.4	0.05	0
3	6.5	0.1	0
4	7.4	0.1	0
5	6.5	0.05	0.1
6	7.4	0.05	0.1
7	6.5	0.1	0.1
8	7.4	0.1	0.1
9	7	0.075	0.05

Each buffer was used to make one sample of beads. Excluding buffer number 9 which was designed as a centre point, and used to make three samples of beads which was used to estimate the pure error in the model.

4.3.1 The behaviour of DB™ MyOne™ StA T1 coupled with a biotinylated antibody and stored under various conditions

The purpose of this experiment was to assess how storage in different buffers affects the leaching of biotinylated antibody and streptavidin from DB™ MyOne™ StA T1 and how

the behaviour of the final product changes in the assays.

The leaching of streptavidin and anti-IL-6 was assessed after storing each sample of the bead-streptavidin-biotin-antibody complex in it's respective buffer at 37°C for five and six days respectively. The functionality of IL-6 assay based on those beads was measured after seven days of storage at 37°C.

Figure 23 summarises the leaching of IgG (ng/mg beads) measured for each sample. Figure 24 shows the leaching of streptavidin (ng/mg beads) for each sample. The ratios of mean MAX RLU to noise measured in the IL-6 assays based on the respective bead samples are presented in figure 25. The measurements of signal to noise ratios are based on the mean MAX RLU measured in triplicates for the samples with an IL-6 concentration of 1000 ng/mL.

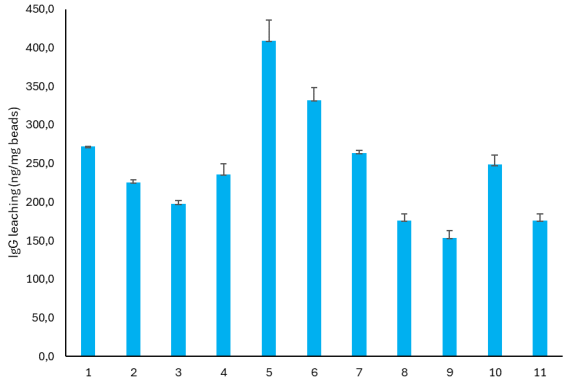

Figure 23: Measured concentration of IgG leaching (ng/mg beads) for the eleven samples of beads stored in different buffers at 37° C for six days. Bead sample number corresponds to buffer number used for storage, as stated in table 2. Buffer number 9 was used to prepare bead samples numbered 9, 10, and 11.

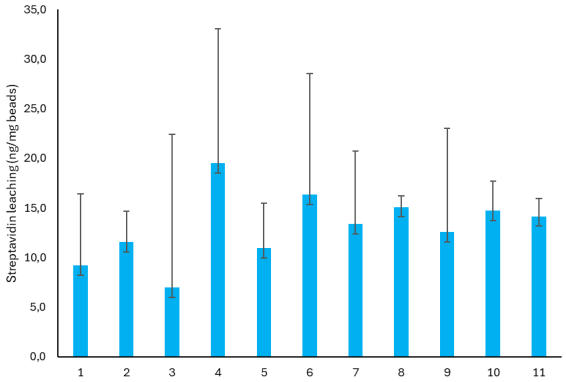

Figure 24: Measured concentration of streptavidin leaching (ng/mg beads) for the eleven samples of beads stored in different buffers at 37° C for five days. Bead sample number corresponds to buffer number used for storage, as stated in table 2. Buffer number 9 was used to prepare bead samples numbered 9, 10, and 11.

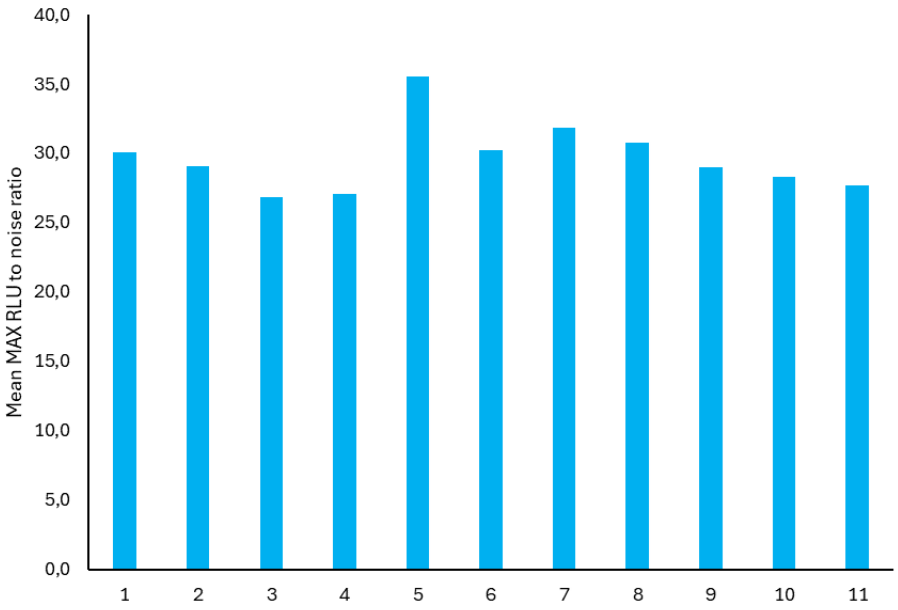

Figure 25: Measured mean MAX RLU to noise ratio for the eleven samples of beads stored in different buffers at 37° C for seven days. Bead sample number corresponds to buffer number used for storage, as stated in table 2. Buffer number 9 from table 2 was used to prepare bead samples numbered 9, 10, and 11.

After analysing the dataset presented in figures 23, 24, and 25 with MODDE, an analysis of fit and predictive strenght of the model was constructed by the software as shown in figure 26.

The figure shows the analysis of fit (R^2), model predictive power (Q^2), model validity score and reproducibility for the statistical models of biotinylated IgG leaching, leaching of streptavidin and S/N in IL-6 assay made using beads conjugated with biotinylated antibodies and stored under varying conditions.

For the mean MAX RLU to noise ratios, analysis of fit is calculated to be $R^2 = 0.745$, model predictive power to $Q^2 = 0.519$, model validity to 0.521, and reproducibility 0.921. For the streptavidin leaching, analysis of fit is calculated to be $R^2 = 0.518$, model predictive power at $Q^2 = 0.382$, model validity to 0.507 and reproducibility 0.899. For IgG leaching, analysis of fit is calculated to be $R^2 = 0.436$, model predictive power at $Q^2 = 0.192$, model validity to 0.768 and reproducibility 0.564.

The p-values calculated for S/N in IL-6 assay model were 0.133, 0.075, 0.008 for pH, concentration of Tween 20, and concentration of BSA in storage buffer respectively. The p-

values calculated for StA leaching model were 0.015, 0.347, and 0.256 for pH, concentration of Tween 20, and concentration of BSA in storage buffer respectively. The p-values calculated for IgG leaching model were 0.317, 0.077, and 0.199 for pH, concentration of Tween 20, and concentration of BSA in storage buffer respectively.

The significance level chosen as an upper bar for a significant model in this analysis was 1% (0.01). Only the smallest p-values for S/N model, and StA leaching model were considered small enough to consider a factor significant with good enough certainty. None of p-values calculated for the IgG leaching model was small enough to consider a significant factor.

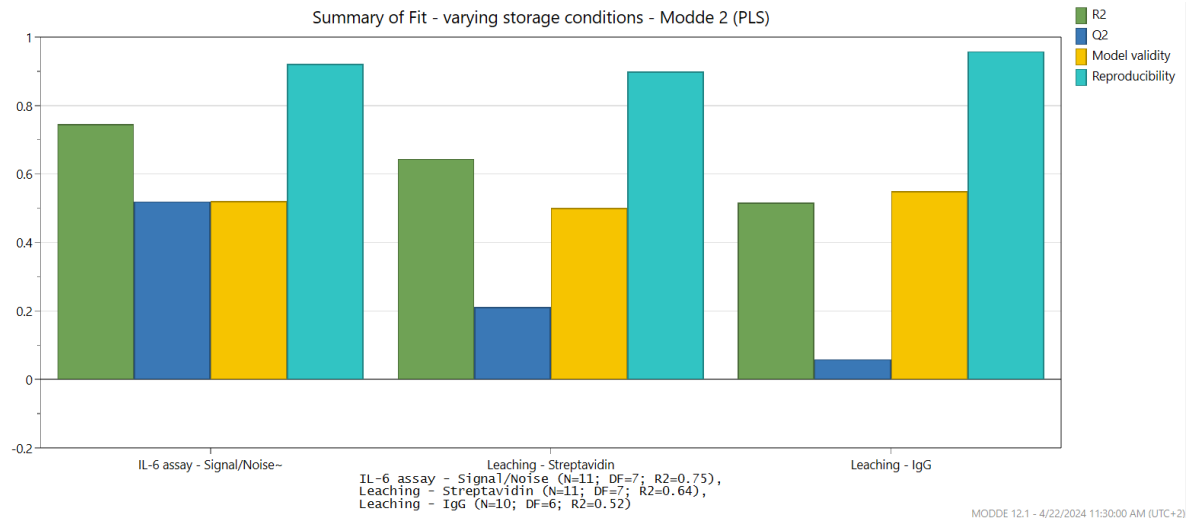

Figure 26: The analysis of fit, R^2, model predictive power, Q^2, model validity score and reproducibility for the statistical models of biotinylated IgG leaching, leaching of streptavidin and mean MAX RLU to noise ratios in the IL-6 assay made using beads conjugated with biotinylated antibodies and stored under varying conditions.

An ANOVA analysis was conducted for the datasets presented in figures 23,24, and 25. The results of the ANOVA analysis including SD-regression, RSD, and RSD*$\sqrt{F(critical)}$ values are summarised in figure 27.

SD-regression was calculated for each model. The SD-regression values are 0.06, 5.05, and 98.14 for signal to noise ratios in IL-6 assay, leaching of StA, and leaching of IgG respectively.

The RSD for each model was measured to be 0.02, 2.46, and 62.19 for RLU to noise ratios, IgG leaching, and StA leaching respectively.

The RSD*$\sqrt{F(critical)}$ is given for each model with the following values for signal to noise ratios in IL-6 assay, leaching of StA, and leaching of IgG respectively: 0.045, 5.12, and 129.67.

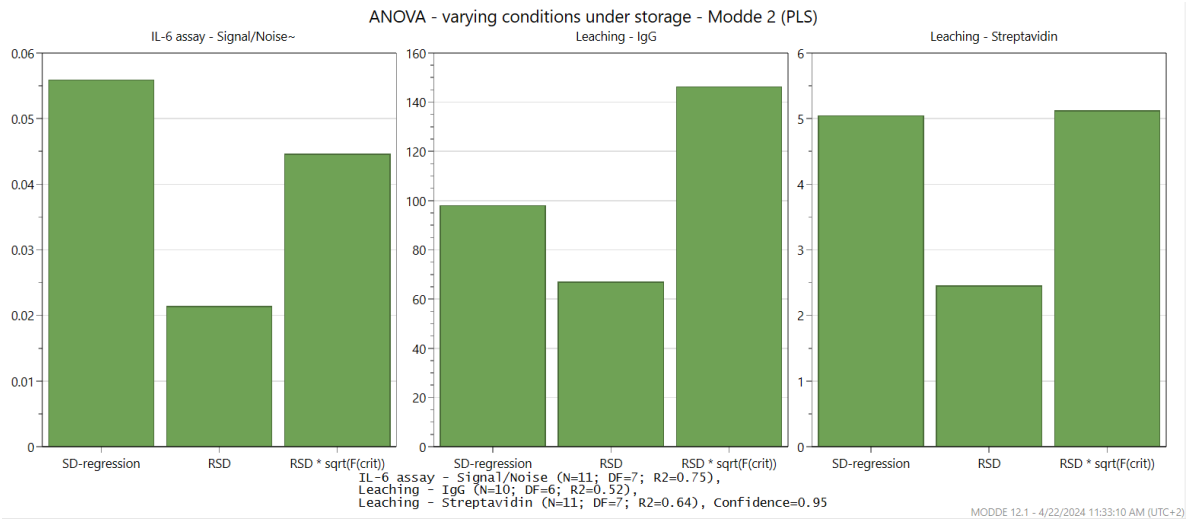

Figure 27: A summary of ANOVA analysis made for experiment evaluating various conditions under storage Dynabeads™MyOne Streptavidin T1 conjugated with biotinylated antibody. SD-regression, RSD, and RSD$\sqrt{F(critical)}$ for each response evaluated: signal to noise ratios in IL-6 assay, leaching of IgG, and leaching of streptavidin.*

The significance of each change in the buffer parameters for the leaching of streptavidin and biotinylated antibody from the bead-streptavidin-biotin-antibody complex as well as the sensitivity in assays was also evaluated. The significance of each factor is summarised in figure 28.

For the S/N in IL-6 assay, the BSA concentration in the storage buffer is the only significant factor. For the leaching of IgG, none of the evaluated factors is considered to be significant. The streptavidin leaching is only significantly affected by the pH of the storage solutions.

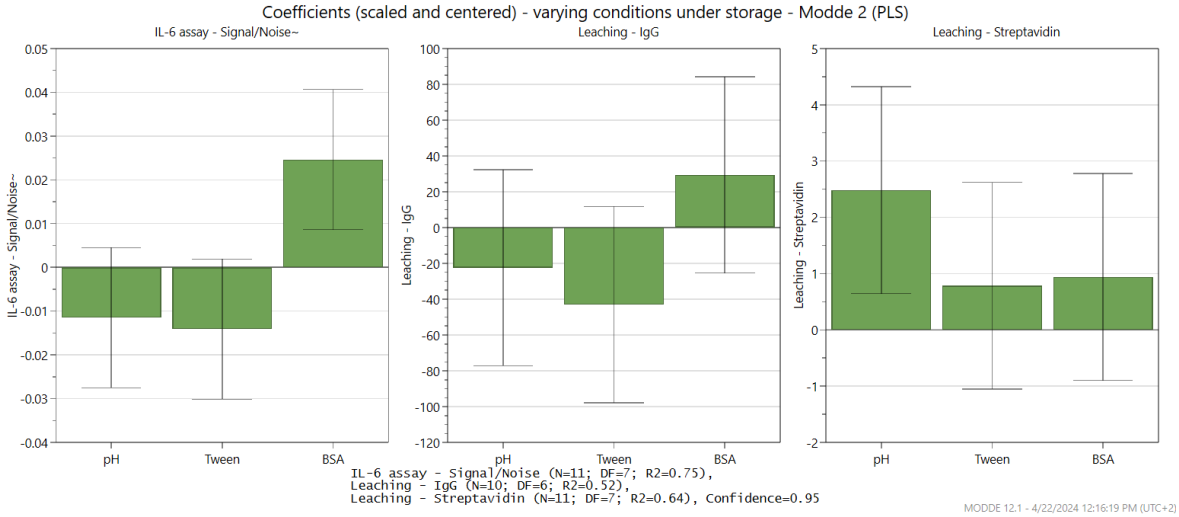

Figure 28: Coefficients plot for statistical models constructed to evaluate what impact storage buffers' composition has for the mean MAX RLU to noise ratios in IL-6 assay, leaching of biotinylated IgG, and leaching of streptavidin. The plot was generated by MODDE software.

4D response contour plots visualising effect of each parameter on S/N (figure 29), IgG leaching (figure 30), and StA leaching (figure 31) respectively.

Figure 29 shows that the signal to noise ratio is higher when there is more BSA in the buffer to stabilise the streptavidin-bead complex, and the pH of the buffer is lower. Figure 30 shows that leaching of IgG goes up at higher BSA concentrations, lower Tween 20 concentrations and lower pH. Figure 31 shows that leaching of streptavidin goes up at higher BSA, higher Tween 20 concentrations, and higher pH.

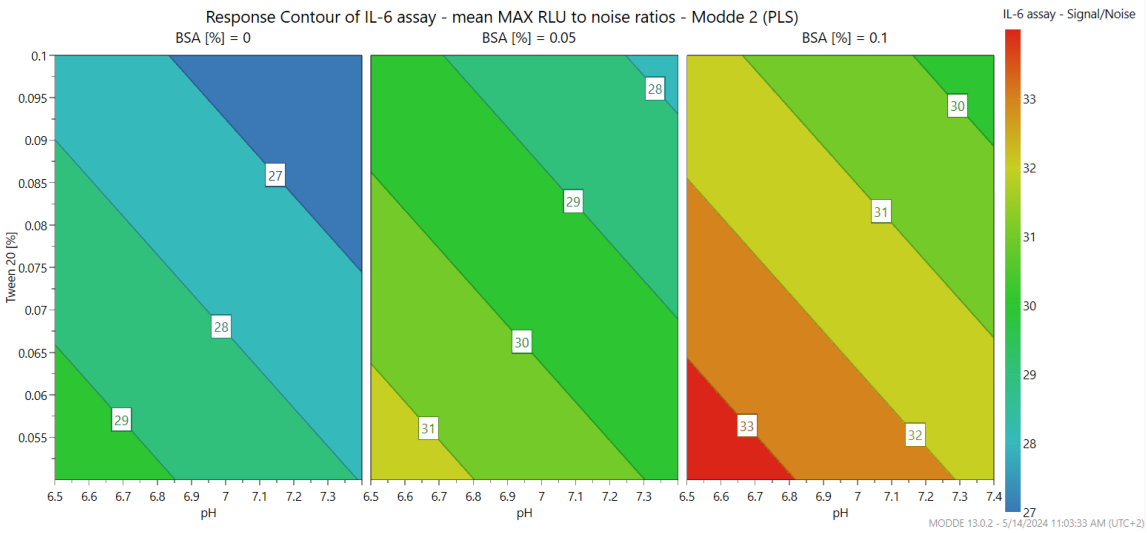

Figure 29: 4D response contour plot showing the impact the changes in concentration of BSA (%), concentration of Tween 20 (%) and pH of the buffer has for the sensitivity of the IL-6 assay, expressed in terms of measured mean MAX RLU to noise ratios.

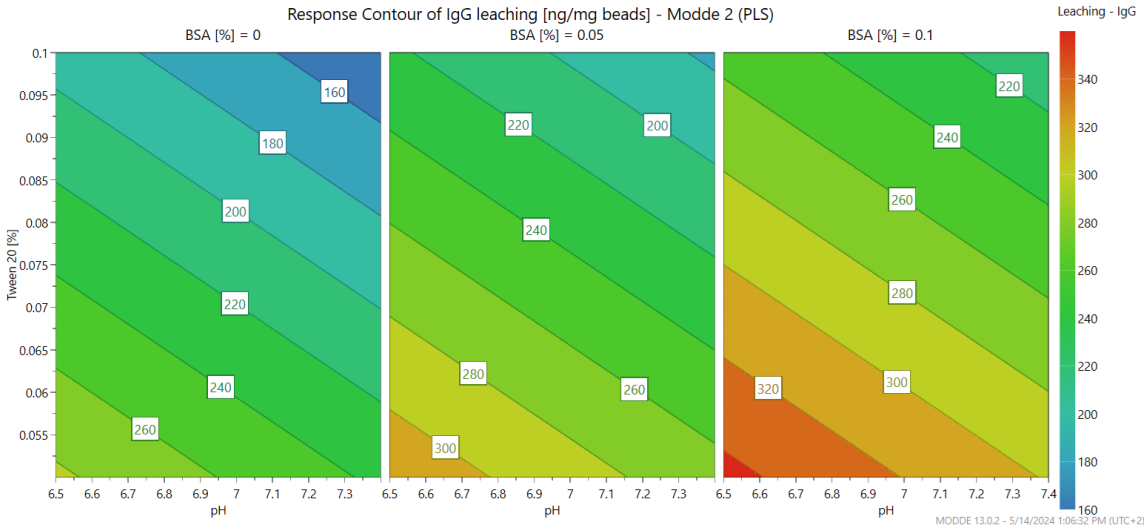

Figure 30: 4D response contour plot showing the impact the changes in concentration of BSA (%), concentration of Tween 20 (%) and pH of the buffer has for leaching of biotinylated antibody from the beads expressed in terms of ng/mg beads.

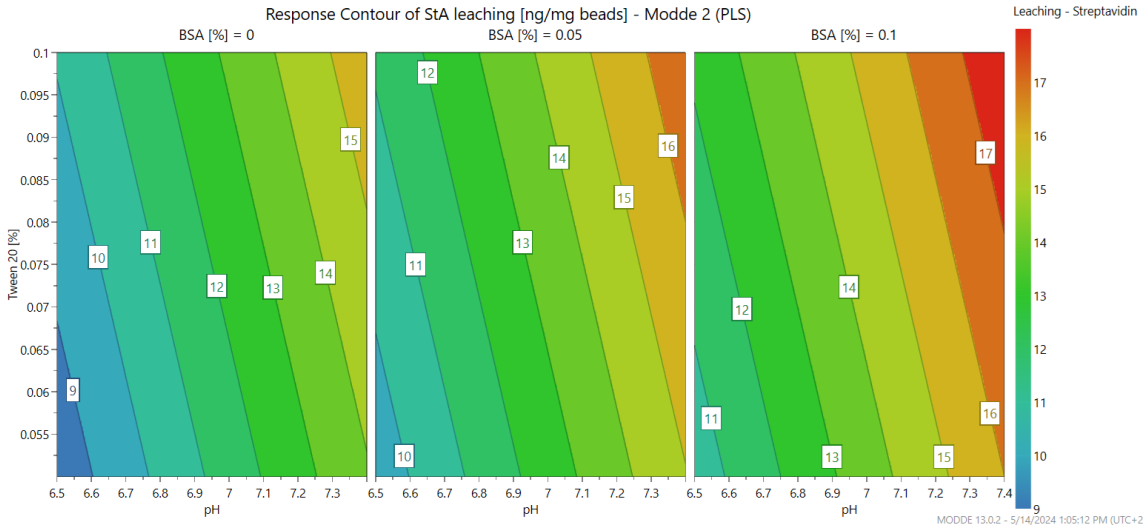

Figure 31: 4D response contour plot showing the impact the changes in concentration of BSA (%), concentration of Tween 20 (%) and pH of the buffer has for leaching of streptavidin from the beads expressed in terms of ng/mg beads.

4.3.2 The behaviour of DB™ MyOne™ StA T1 not coupled with biotinylated antibody - stability of streptavidin bond under different storage conditions

DB™ MyOne™ StA T1 not conjugated with biotinylated antibody were washed three times (to simulate how the conjugated beads were treated) and stored at 37°C in the buffers listed in table 2. The streptavidin leaching from those beads was measured after five days storage. The purpose of this experiment was to see how conjugation with biotinylated antibody affects the bead-streptavidin-biotin-antibody complex. The StA leaching from the negative test of beads is summarised in figure 32.

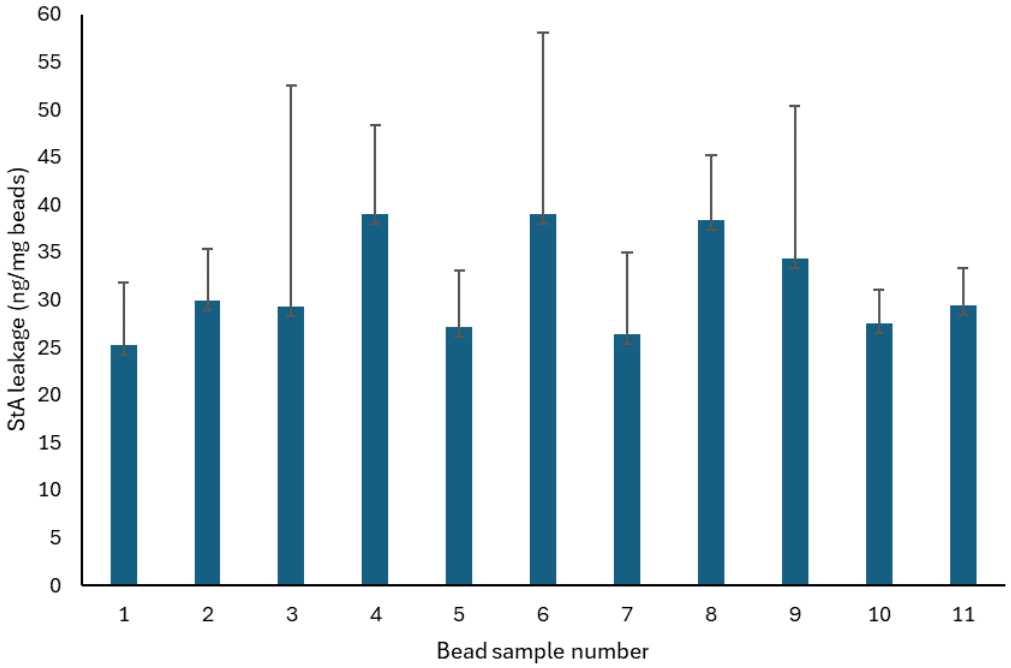

Figure 32: The leaching of streptavidin (ng/mg beads) measured for the beads not conjugated with antibody, washed and stored for five days at 37°C in buffers with varying concentrations of BSA and Tween 20, and with different pH levels.

Figure 33 shows comparison between measured streptavidin leaching for the beads not conjugated with antibodies and the ones conjugated with biotinylated antibody stored in the same buffers.

The graph shows the concentrations of streptavidin leaching for the beads not conjugated to antibody laying between 25 and 40 ng/mg beads. The beads conjugated to antibody show concentrations of streptavidin leaching laying between 5 and 20 ng/mg beads.

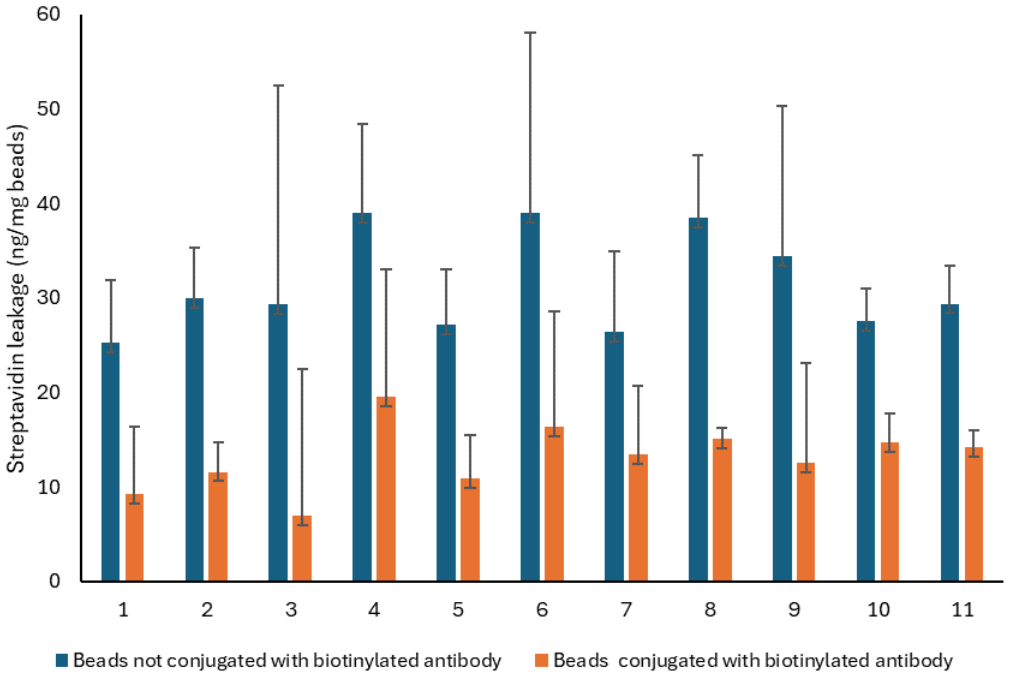

Figure 33: A comparison of the measured concentrations of streptavidin leaching for beads conjugated with biotinylated antibody and beads which weren't conjugated with antibody, stored with eleven different buffers with varying concentrations of BSA and Tween 20, and at different pH levels.

4.3.3 Evaluating the changes in assay functionality after excluding a washing step before using the beads in the assays

The purpose of this experiment was to assess how excluding the washing step before using DB™ MyOne™ StA T1 conjugated with a biotinylated antibody in the immunoassay would affect the sensitivity of the assay in means of mean MAX RLU to noise ratios.

Beads stored in buffers with the following compositions were chosen:

- 50 mM MES, 0.05% Tween 20, pH 6.5
- 50 mM TBS, 0.1% Tween 20, 70 mM NaCl, pH 7.4
- 50 mM MES, 0.1% Tween 20, pH 6.5
- 50 mM MES, 0.05% Tween 20, 0.1% BSA, pH 6.5

The beads were stored at 37°C for thirteen days before being used in the IL-6 assay.

The comparison of mean MAX RLU to noise ratios in IL-6 assays using beads washed in their respective buffers, or applied to the assay straight from storage is summarised in figure 34. The measurements of S/N are based on the mean MAX RLU measured for the samples with IL-6 concentration of 1000 ng/mL. Figure 34 shows on average a higher mean MAX RLU to noise ratio for the analysed beads which were washed in advance.

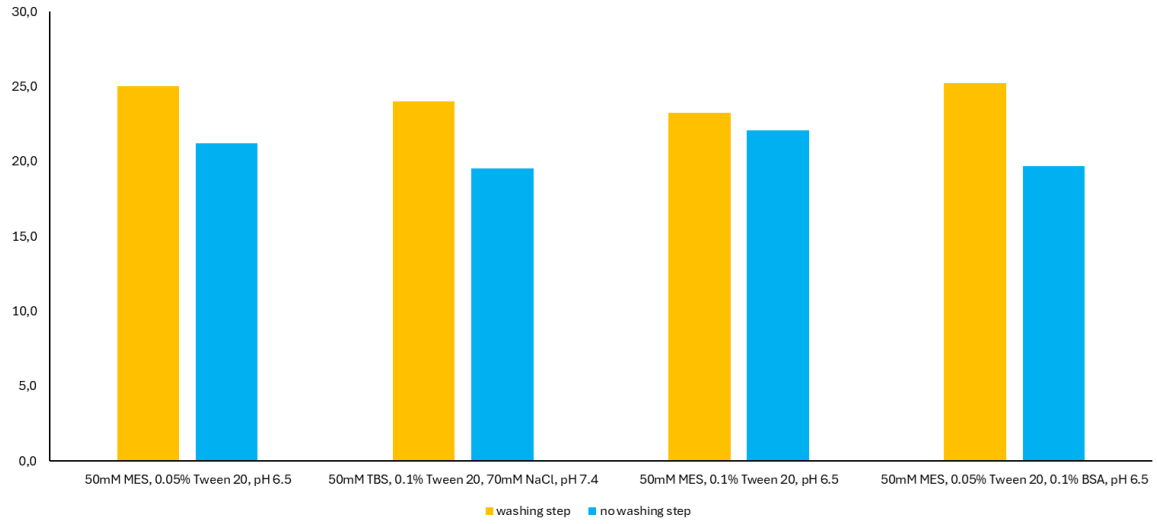

Figure 34: A comparison of measured mean MAX RLU to noise ratios in an IL-6 assay including or excluding the washing step before applying beads. Beads used for this experiment were stored in four different buffers for thirteen days at 37° C.

4.4 Evaluating variations between different lot of beads

The purpose of this experiment was to check if beads from different lot would be affected in a similar manner after being stored in the different buffers from the previous experiment. This was done to validate the results and predictions from the previously developed model.

DB™ MyOne™ StA T1 from three different lot were included in this experiment. Beads from lot 2846074 (lot 1), lot 2824565 (lot 2), and lot 2796130 (lot 3) were used in this experiment.

To minimise the workload, the beads from each lot were stored in four buffers chosen from the buffers used in the previous experiment.

The following buffers were chosen to validate the previously constructed model:

- 50 mM MES, 0.05% Tween 20, pH 6.5
- 50 mM MES, 0.05% Tween 20, 0.1% BSA, pH 6.5
- 50 mM TBS, 0.05% Tween 20, 70 mM NaCl, pH 7.4
- 50 mM TBS, 0.05% Tween 20, 70 mM NaCl, 0.1% BSA, pH 7.4

After conjugation, the bead samples were resuspended in their respective buffers and stored at 37°C for five to seven days (depending on the test used).

Leaching of biotinylated anti-IL-6 was measured for the different buffers with each sample after incubating the beads for seven days. The leaching of IgG (ng/mg beads) is summarised in figure 35. Leaching of streptavidin was measured after six days of incubation. The results of the streptavidin leaching is summarised in figure 36. The behaviour in the IL-6 assay is summarised in figure 37. The measurements of S/N are based on mean MAX RLU measured for samples with IL-6 concentration of 1000 ng/mL. The IL-6 assay was conducted after incubating the beads from each sample for five days.

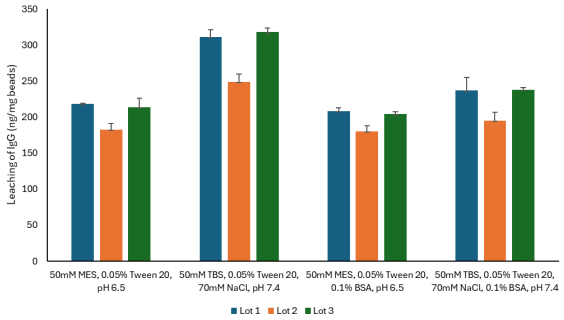

Figure 35: IgG leaching measured for beads from three different lot conjugated with biotinylated antibody and stored in four different buffers each for seven days at 37° C.

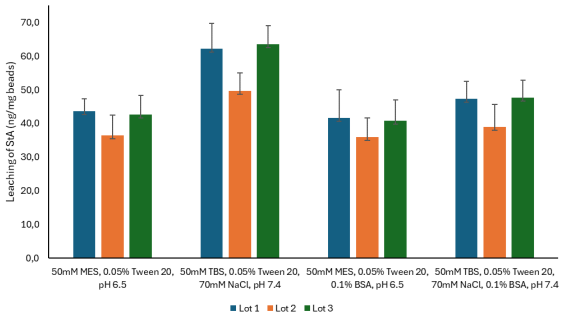

Figure 36: Streptavidin leaching measured for beads from three different lot conjugated with biotinylated antibody and stored in four different buffers each for six days at 37° C.

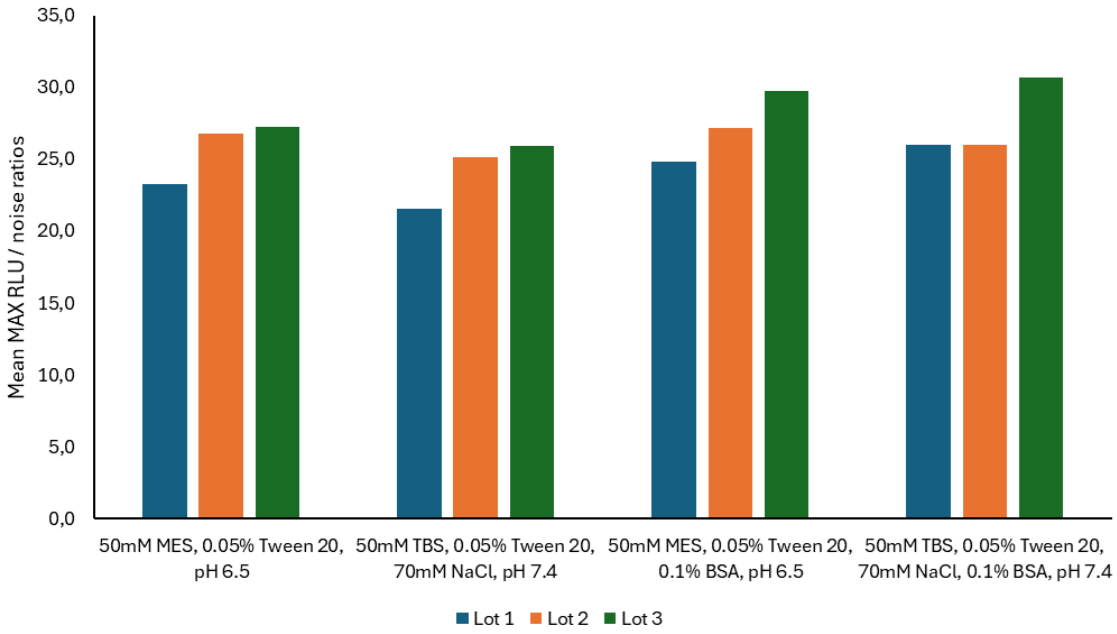

Figure 37: Measured mean MAX RLU to noise ratios for the IL-6 assay using beads from three different lot conjugated with biotinylated antibody and stored in four different buffers each for five days at 37° C.

The recovery tests using the IL-6 assay model were performed for the three lot of beads each stored in one of four different buffers. The recovery test was performed using two concentrations of IL-6 spiked human serum and a unspiked human serum as a zero control.

Serum spiked with 100 ng/mL IL-6 and serum spiked with 1000 ng/mL IL-6 was diluted 1:1 before use in recovery test.

The recovery % calculated for serum spiked with IL-6 and dilluted to 50 ng/mL, or 500 ng/mL are presented in figures 38 and 39 respectively.

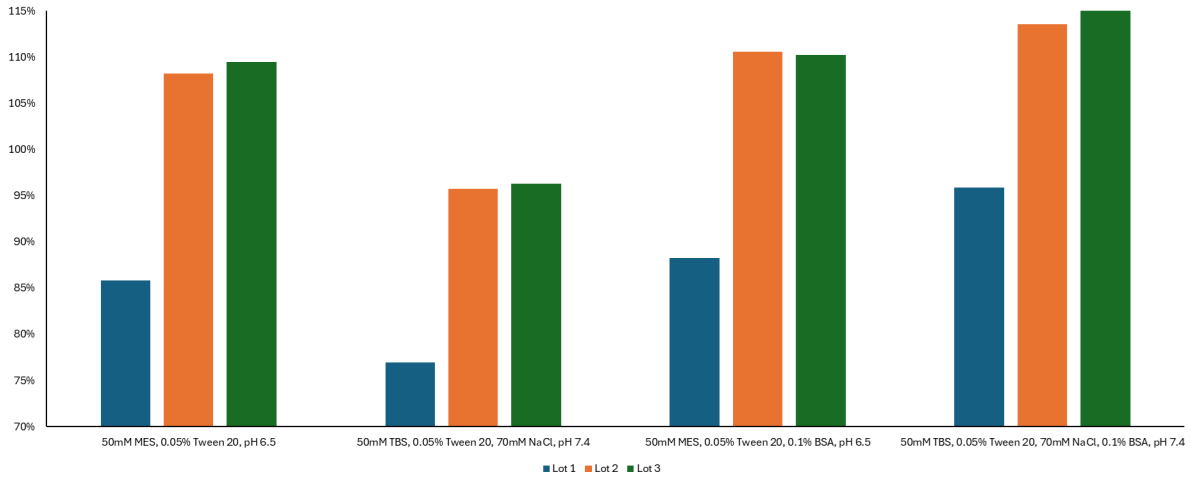

Figure 38: A comparison of recovery% in IL-6 assays for recovery test with human serum spiked with 50 ng/mL IL-6, using beads from three different lot conjugated with biotinylated antibody and stored in four different buffers for five days at 37° C.

Figure 38 shows recovery % at lower concentration of IL-6 added as spike. For lot 1, the recovery % varies from 77 to 96 %, depending on the buffer used. For lot 2, the recovery % varies from 96 to 114 %, depending on the buffer used. For lot 3, the recovery % varies from 96 to 115 %, depending on the buffer.

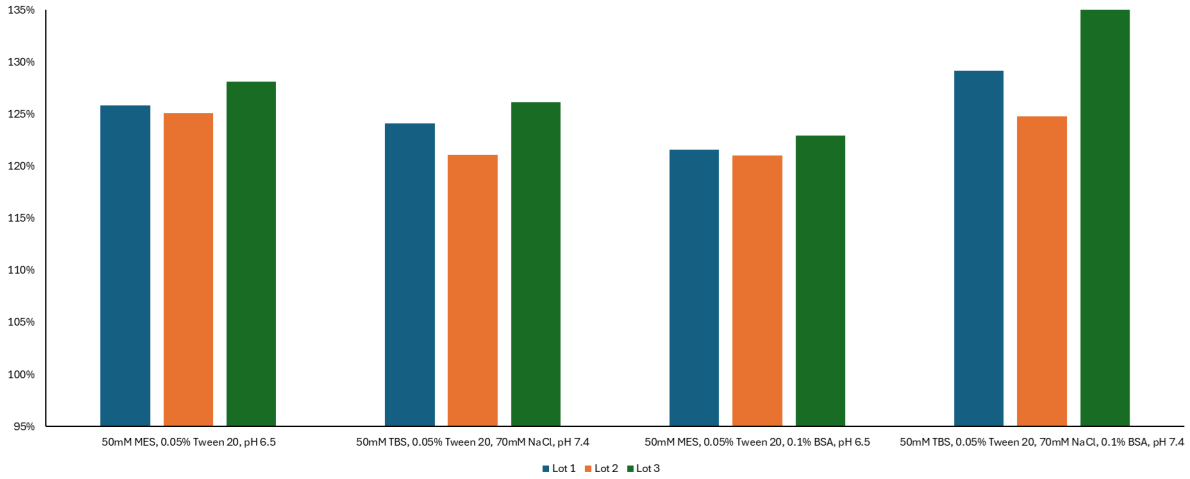

Figure 39: A comparison of recovery% in IL-6 assays for recovery test with human serum spiked with 500 ng/mL IL-6, using beads from three different lot conjugated with biotinylated antibody and stored in four different buffers for five days at 37° C.

Figure 39 shows recovery % at higher concentration of IL-6 added as spike. For lot 1, the recovery % varies from 122 to 129 %, depending on the buffer used. For lot 2, the recovery % varies from 121 to 125 %, depending on the buffer used. For lot 3, the recovery

% varies from 123 to 136 %, depending on the buffer.

4.5 Blocking binding-sites on streptavidin with pure biotin

The purpose of this experiment was to see how the blocking of unoccupied biotin binding sites on streptavidin would affect the leaching of streptavidin, the leaching of biotinylated antibody from the complex, and the sensitivity of the bead product in the IL-6 assay.

Samples form three lot productions of DB™ MyOne™ StA T1 was conjugated with biotinylated anti-IL-6. After conjugation of the beads and biotinylated antibodies, the beads were aliquoted into three sets. To each set pure biotin was added, either 30.5 ng/mg beads or 61 ng/mg beads. A control group consisting of beads from each lot without added pure biotin was also made. After washing the beads with TBST 0.05% with 0.1% BSA buffer, the products were stored at 37°C for five to seven days, depending on the test used.

The leaching of biotinylated IgG measured on beads from each lot blocked with different concentrations of biotin are summarised in figure 40. The leaching of streptavidin measured for beads from each lot blocked with different concentrations of biotin are summarised in figure 41. The S/N for beads from each lot blocked with different concentrations of biotin are summarised in figure 42.

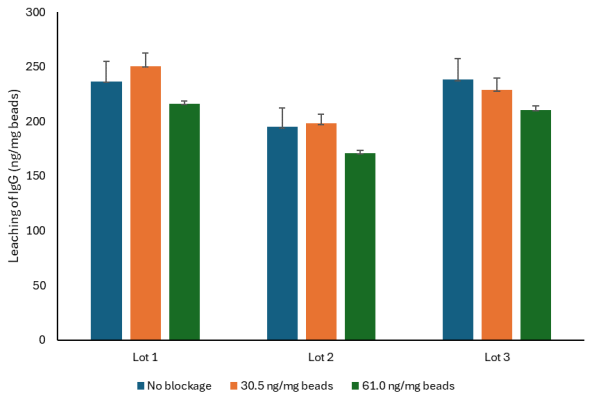

Figure 40: The concentrations of biotinylated IgG leaching measured on beads from three different lot treated with either no biotin, 30.5 ng/mg biotin, and 61.0 ng/mg.

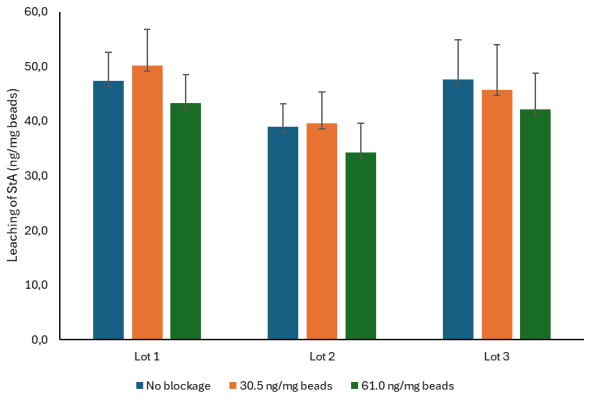

Figure 41: The concentrations of streptavidin leaching measured on beads from three different lot treated with either no biotin, 30.5 ng/mg biotin, and 61.0 ng/mg.

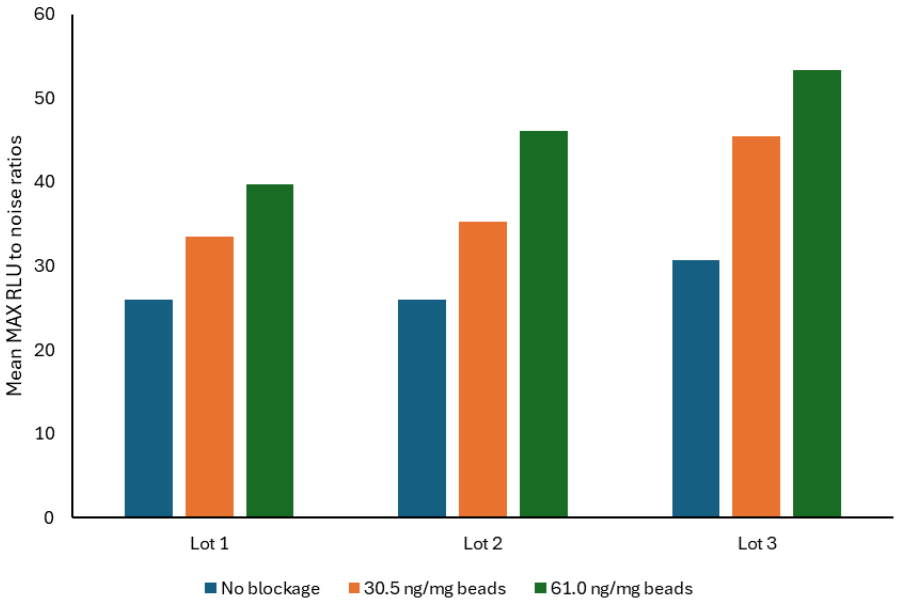

Figure 42: The mean MAX RLU to noise ratio calculated for beads from three different lot treated with either no biotin, 30.5 ng/mg biotin, and 61.0 ng/mg.

Recovery tests using the IL-6 assay model were performed for the three lot of beads treated with pure biotin right after conjugation with biotinylated antibody. The recovery test was performed using two concentrations of IL-6 spiked human serum and a unspiked human serum as a zero control. Recovery % calculated for serum spiked with 100 ng/mL IL-6 and serum spiked with 1000 ng/mL IL-6 are presented in figures 43 and 44 respectively.

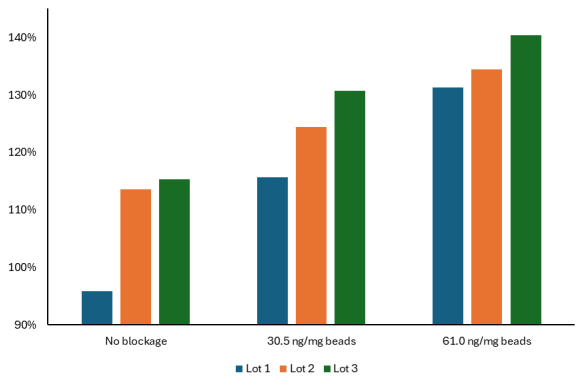

Figure 43: A comparison of recovery% in IL-6 assays for recovery test with human serum spiked with 100 ng/mL IL-6 and diluted to 50 ng/mL, using beads from three different lot conjugated with biotinylated antibody, treated with pure biotin after conjugation and stored for five days at 37° C.

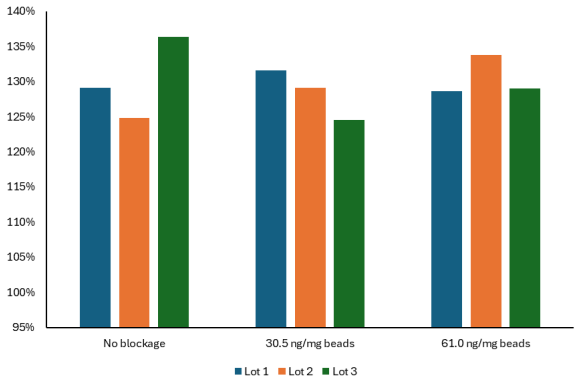

Figure 44: A comparison of recovery% in IL-6 assays for recovery test with human serum spiked with 1000 ng/mL IL-6 and diluted to 500 ng/mL, using beads from three different lot conjugated with biotinylated antibody, treated with pure biotin after conjugation and stored for five days at 37° C.

Figure 43 shows recovery % at lower concentration of IL-6 added as spike. For lot 1, the recovery % varies from 96 to 131 %, depending on the buffer used. For lot 2, the recovery % varies from 114 to 134 %, depending on the buffer used. For lot 3, the recovery % varies from 115 to 140 %, depending on the buffer.

Figure 44 shows recovery % at lower concentration of IL-6 added as spike. For lot 1, the recovery % varies from 129 to 132 %, depending on the buffer used. For lot 2, the recovery % varies from 125 to 134 %, depending on the buffer used. For lot 3, the recovery % varies from 125 to 136 %, depending on the buffer.

5 Discussion

The purpose of this project was to investigate the stability of DB™ MyOne™ StA T1 coupled with biotinylated antibody under simulated long-term storage. We looked at how different treatments would affect the leaching of different components from the bead-streptaindin-antibody complex, as well as the functionality of the beads in an immunoassay.

5.1 Testing different types of biotin

The first step in this project was to compare how the use of different types of biotin, featuring different lengths of spacer arms, would affect the leaching and the performance in immunoassays. This part of the study was done on a small sample pool. For each type of biotin used to biotinylate the antibodies, only one sample of beads was prepared.

Despite being prepared and treated in the exact same way, the conjugated bead batches showed considerable differences in leaching of both streptavidin and antibodies, as well as a difference in the functionality of the immunoassay.

NHS-LC-LC-biotin was chosen to be used in further experiments. This was because it had moderate measured leaching of biotinylated IgG, the lowest measured leaching of StA, as well as an above average S/N registered in IL-6 assay for measurements at 1000 ng/mL IL-6.

The other types of biotin used in this experiment gave almost identically, much lower S/N. The background noise registered as mean MAX RLU for all three samples featuring different types of biotin was smallest for desthiobiotin at about 3845 RLU, and highest for LC-LC-biotin at about 5100 RLU.

Even though desthiobiotin has shown less IgG leaching than what LC-LC-biotin had, the functionality of the assay was comparable to that of LC-biotin.

The leaching of StA from the sample utilising desthiobiotin was also somewhat higher than that of LC-LC-biotin, but lower than for the sample using LC-biotin.

This jump in sensitivity can be attributed to less steric hindrance when biotin with longer spacer arm is used.

5.2 Testing different conditions under conjugation

The next step in this project was to change the conditions under which the conjugation of the biotinylated antibodies with the streptavidin beads happened. Then to further evaluate how those changes would affect the leaching and the functionality of the assays.

This experiment was designed using the MODDE software. The goal here was to evaluate how the different lengths of incubation periods, the temperature, and the concentration of biotinylated antibody in the reaction solution under the conjugation process affected the stability of the bead complex.

The data from this experiment were evaluated using PLS regression. PLS regression was used as a basis for ANOVA.

Interactions between the parameters were attempted to be investigated by constructing interaction terms with the MODDE analysis. Those terms were left out from further analysis due to being insignificant altogether.

Because only one centre point was included, the statistical software used to analyse the experimental data couldn't calculate a proper model validity or reproducibility scores for neither model constructed as a part of this experiment.

The leaching of biotinylated antibodies was measured for the samples conjugated with lower concentrations of biotinylated IgG to be much lower than for other the bead samples. On the other side, the leaching of biotinylated IgG for the samples conjugated with concentrations of biotin that were 6.5 times greater was measured to be from about 5.5 to almost 7 times greater, when comparing the samples conjugated at the same temperatures and incubation times. This shows that the leaching of biotinylated IgG from the beads is relative to the quantity of the biotinylated IgG conjugated to begin with.

The leaching of streptavidin seems to be dependent on both the temperature under the

conjugation and the concentration of biotinylated antibody. As shown in figure 15, the samples where both a higher concentration of antibodies and a lower temperature were used under conjugation (sample 5 and 6) measure the least streptavidin leaching. Yet, with a closer look at the statistical analysis using MODDE, the temperature under conjugation is still considered an insignificant parameter. As can be seen in figures 19, and 22, the leaching of streptavidin are reduced when a higher concentration of biotinylated antibody are used under the conjugation.

When it comes to the S/N in the assays, the concentration of biotinylated antibody is again the only significant factor, with greater concentrations giving bigger ratios. The other factors can have some significance, yet their contribution is rather uncertain, as seen in figure 19.

5.3 Testing different conditions under storage

This experiment was also designed with help of the MODDE software. It was performed to evaluate how different conditions under storage, more precisely the composition of the storage buffers will affect the leaching and functionality of the assays. The parameters studied in this experiment were pH, concentration of polysorbate 20 (Tween 20), and concentration of BSA in the storage buffers.

In total, 9 storage buffers were prepared and used to make a total of 11 bead samples, using one of the buffers to make 3 identical samples used as centre points. The beads were conjugated with biotinylated antibodies and stored in their respective buffers at 37°C to simulate long term storage.

After evaluating the dataset with MODDE, the concentration of polysorbate 20 was not recognised as a significant factor in any evaluated response, as seen in figure 28. The pH was deemed a significant factor for streptavidin leaching. pH closer to physiological values showed more leaching. The pH was not recognised as a significant factor for neither the IgG leaching, or the sensitivity in assays.

The concentration of BSA was deemed to affect the sensitivity in the assays, giving

somewhat bigger S/N , while it bared no significance for leaching of either streptavidin or IgG.

The data from this experiment were evaluated using PLS regression. PLS regression was used as a basis for ANOVA. Despite considering pH a significant factor for the leaching of streptavidin, results of the ANOVA analysis for the whole StA leaching model make this conclusion somewhat optimistic. The whole model is characterised by a somewhat high RSD compared to the SD-regression value. The RSD combined with F(critical) chosen for this analysis gives a picture of a rather insignificant model, as seen in figure 27.

The concentration of BSA in the storage buffers is the only significant factor for S/N, as seen in figure 28. According to ANOVA, the statistical model describing responses in IL-6 assay is more reliable, having lower RSD compared to SD-regression, and having a lower RSD*$\sqrt{F(critical)}$.

The statistical model constructed for the leaching of IgG is rather untrustworthy. This model is characterised by high RSD, and RSD*$\sqrt{F(critical)}$ which is much higher than score for the SD-regression, as seen in figure 27.

5.3.1 Stability of Dynabead - streptavidin bond under different storage conditions

As a part of this experiment, the leaching of streptavidin was examined separately by making another series of bead samples to be stored under varying conditions. Those beads were not conjugated with biotinylated antibodies (negative samples) before being transferred into their respective buffers. This was done to check if conjugation with biotinylated antibody would stabilise the complex, or if the conjugation didn't have any effect on the streptavidin leaching.

The negative samples were compared to the samples which had been conjugated with antibodies before being stored in different buffers.

Each sample showed more StA leaching when not conjugated with biotinylated antibodies. The leaching of StA was at least two times greater than what was measured for the beads

conjugated with biotinylated antibodies. This shows that higher concentrations of proteins in the storage buffer leads to less leaching from the bead-streptavidin part of the complex. This is because of le chatelier's principle, which states that a system put under stress, will try to adjust itself back to equilibrium (Chang, 2005, page 206). So the proteins on the beads will drift out into the solution if it has a too low protein concentration, to equilibrate the overall protein concentration.

5.3.2 Evaluating the washing step's contribution to the assay's functionality

A small experiment was performed to check how excluding the washing step before IL-6 assay would affect the functionality. This experiment utilised four samples of beads stored under different conditions. The bead samples were picked somewhat arbitrarily, based on measured IgG, and StA leachings, as well as the functionality of the assays.

An IL-6 assay was performed with the chosen samples either after diluting them to the working concentrations straight from storage or after performing the washing step with TBST with NaN_3 buffer before dilution.

The S/N were then compared for the washed/unwashed samples showing about a 20 percentage points increase in S/N for the beads washed before use in IL-6 assay, as seen in figure 34.

The most discrepancy was registered for the beads stored in 50 mM MES, 0.05% Tween 20, 0.1% BSA, pH 6.5. This is the buffer that gave the most leaching of biotinylated IgG across all four buffers used in this experiment. Yet, beads stored in 50 mM MES, 0.1% Tween 20, pH 6.5 had shown least difference in S/N, despite not being the worst sample when it comes to leaching of IgG. This can be attributed to the leaching of StA, as this sample has shown almost twice as much leaching of StA compared with formerly mentioned sample from buffer 50 mM MES, 0.05% Tween 20, 0.1% BSA, pH 6.5.

The explanation for this phenomenon can be that IgG not conjugated with beads will be washed away under the later steps of the IL-6 assay, either alone or with unbound streptavidin that captures it. If those antibodies capture analytes only to be washed

away later, the quantity of binding sites available for secondary antibodies will decrease. As noticed previously, higher concentrations of antibodies on the beads surface give better sensitivity in assays, since more analytes can be captured. Higher concentrations of antibodies used for conjugation will also somewhat reduce the leaching of streptavidin from the beads. If the analytes are captured, but not retained in the well where the reaction takes place, the effect will be comparable to having less binding sites to begin with.

5.4 Evaluating variations between product lots stored under different conditions

This experiment was preformed to validate the previous findings and models constructed for leaching of biotinylated IgG and StA, as well as S/N in IL-6 assays. The point of this experiment was to see if those results would hold for beads from different lots.

To make the workload more manageable, a limited choice of buffers was used. The buffers were chosen somewhat arbitrarily, focusing on obtaining a spread in pH values and BSA concentrations, while Tween 20 concentrations were held constant at 0.05%.

Looking at the results, the leaching and functionality trends are indeed conserved between lots stored in different buffers. The most leaching of both StA and IgG was registered for samples stored in TBST 0.05%, without BSA. The least leaching was measured in MEST 0.05% with 0.1% BSA. Those results are conserved between all three evaluated lots.

The beads from all three lot were also used in IL-6 assay with recovery tests. The recovery tests were conducted with two concentrations of IL-6, 50 ng/mL and 500 ng/mL. As summarised in figure 38, one of the lots showed considerably lower recovery %, thought the values themselves were at least physically possible. The other two lots registered similar recovery %, yet those were over 100% each making them physically impossible to obtain.

Figure 35 compares the recovery tests conducted for higher concentrations of IL-6. Those were more evenly conserved between different lots, yet all of those were calculated to be

over 120% making the results unreliable.

Finding the exact reason for those discrepancies was beyond the scope of this experiment. Those can be attributed to either a scaling error in the design of the assay, or a calculation error under the dilution of spiked pooled human serum used for this test could be the simplest explanations for these unreliable results. Unspecific binding of antibodies used in the assay to other proteins present in the buffer can also play a role here.

5.5 Blocking the binding-sites on streptavidin with pure biotin

This experiment was performed to check how blocking unoccupied binding seats on streptavidin with pure biotin would affect the leaching and functionality of the beads. The storage buffer used in this experiment was 50 mM TBS, 0.05% Tween 20, 70 mM NaCl, 0.1% BSA, pH 7.4. DB MyOne™ StA T1 from the same three different lots were used to check if the results of this experiment would be conserved between the lots.

The leaching of both StA and IgG were measured, as well as S/N in IL-6 assay, including recovery tests. One set of samples featured beads conjugated without the blockage step, while the other two made use of either 30.5, or 61.0 ng/mg beads of pure biotin added to the reaction solution after conjugation and incubated for 30 min.

The leaching of IgG in lot 1 and 2 was somewhat higher in the samples with the lowest concentration of pure biotin added post conjugation, than for the samples where blocking was not performed (figure 40). The StA leaching in the samples treated with a higher concentration of biotin after conjugation was lower than the samples with no blocking. This was conserved in all three lots.

The leaching of StA shows the exact same trends as the leaching of IgG (figure 41).

The S/N in IL-6 assay was increasing with every increase in the concentration of biotin used for the blocking of binding seats for all three lots (figure 42). This is due to the noise being reduced rather than the mean MAX RLU increasing with higher concentrations.

The samples from this experiment were also used in recovery tests. The recovery % was

growing with every increase in the concentration of pure biotin used for blocking the binding sites on streptavidin. While the S/N ratios went up here as well, the recovery % in those assays remained physically impossible at well over 110% for all the tests except one.

These high recovery rates could be an effect of unspecific binding of antibodies used in the assay to other proteins present in the buffer, present there as either a stabiliser or pollution.

6 Conclusion

In this project, we looked at different bonds present in the bead-streptavidin-biotin-antibody complex used in diagnostic laboratory assays. We checked how different conditions under preparation of the complex, and under storage affects the functionality and leaching of StA and IgG.

This project confirmed that using biotin or it's analogues featuring longer spacer arm will positively affect the beads' functionality in assays, due to less steric hindrance.

Using higher concentrations of biotinylated antibody under conjugation with the streptavidin beads will help reduce the leaching of streptavidin. This can be explained with le Chatelier's principle. The leaching of IgG will then increase, but so will the S/N in assays, viewing this increase as insignificant.

When the beads are stored in buffers with pH closer to physiological, the streptavidin leaching will again increase, yet the functionality and leaching of IgG will not be affected. Higher concentration of BSA in the storage buffer will on the other hand increase the S/N in the immunoassays, yet to no practical degree for the interval of concentrations explored so far.

We also checked if the acquired results would hold up for different lots of beads, or if it was a one time occurrence for the main lot of beads we were working with until then. All the previous findings were conserved between the different lots, validating our claims to some degree.

When preparing the beads to use in immunoassays, including a washing step before diluting them to the working concentrations positively affect the S/N by reducing the background noise. The ratios measured for the beads washed before the dilution had S/N about 25% higher than their unwashed counterparts.

Blocking the unoccupied binding seats on streptavidin with pure biotin reduces the leaching. It also increases the S/N by reducing the noise. This claim was also confirmed by using beads from different lots.

Possible areas for further research

There was a lot of focus on the storage buffers in this project, yet what components could be evaluated was limited due to the time constraints.

Polysorbate 20 has solely been used in these experiments. So, checking how other, longer polysorbates such as polysorbate 40 or 80 would affect the leaching and functionality of the immunoassays could also be looked further into.

The influence of pH was evaluated at a small interval, ranging from 6.5 to 7.4. Checking a broader pH interval, possibly including pH higher than physiological should be considered in further research.

The blocking of unoccupied binding seats on streptavidin with pure biotin was done using two different concentrations of pure biotin. Looking into if even higher concentrations of pure biotin would further reduce the noise in IL-6 assays as well as the leaching of StA and IgG could be done at a larger scale, with more variations in the concentrations.

References

Bhattacharyya, R. A., & Johnson, G. K. (2020). *Statistics: Principles and methods, emea edition*. John Wiley; Sons UK.

Chang, R. (2005). *Physical chemistry for the biosciences*. University Science Books.

Cinquanta, L., Fontana, D., & Bizzaro, N. (2017). Chemiluminescent immunoassay technology: What does it change in autoantibody detection? *Autoimmunity Highlights 8*, (9). https://doi.org/10.1007/s13317-017-0097-2

Cox, K. L., Devanarayan, V., Kriauciunas, A., Manetta, J., Montrose, C., & Sittampalam, S. (2012). Assay guidance manual [internet]. Eli Lilly; Company; the National Center for Advancing Translational Sciences.

Darwish, I. A. (2006). Immunoassay methods and their applications in pharmaceutical analysis: Basic methodology and recent advances. *International journal of biomedical science : IJBS*, 217–235.

EncyclopædiaBritannica. (2024). *Classes of antibodies*. https://www.britannica.com/science/antibody/Antibody-structure-and-classes#/media/1/27783/17661

Halvorsen, A. G. (2018). *En kule varmt : Historien om dynal*. Thermo Fisher Scientific.

Regnier, F. E., & Cho, W. (2013). Chapter 13 - affinity targeting schemes for biomarker research. In H. J. Issaq & T. D. Veenstra (Eds.), *Proteomic and metabolomic approaches to biomarker discovery* (pp. 197–224). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-394446-7.00013-3

Rüttimann, B., & Wegener, K. (2015). The power of doe: How to increase experimental design success and avoid pitfalls. *Journal of Service Science and Management*, *08*, 250–258. https://doi.org/10.4236/jssm.2015.82028

Sartorius. (2017). *Modde® 12 user guide* (1992-2017). https://www.sartorius.com/download/544636/modde-12-user-guide-en-b-00090-sartorius-data.pdf

Simpson, R. J., Hammacher, A., Smith, D. K., Matthews, J. M., & Ward, L. D. (1997). Interleukin-6: Structure-function relationships. *Protein Science*, *6*(5), 929–955. https://doi.org/https://doi.org/10.1002/pro.5560060501

Taylor, C. R., Shi, S.-R., Barr, N. J., & Wu, N. (2006). Chapter 1 - techniques of immunohistochemistry: Principles, pitfalls and standardization. In D. J. Dabbs (Ed.), *Diagnostic immunohistochemistry (second edition)* (Second Edition, pp. 1–

42). Churchill Livingstone. https://doi.org/https://doi.org/10.1016/B978-0-443-06652-8.50007-7

ThermoFisherScientific. (2023). *Biotinylation*. Retrieved May 23, 2024, from https://www.thermofisher.com/no/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/biotinylation.html

Walt, B. A., Hopkin, K., Johnson, A., Morgan, D., Raff, M., & Roberts, P. K. (2019). *Essential cell biology, 5th edition*. W. W. Norton Company.

Weaver, K., & Murphy, C. (2022). *Janeway's immunobiology, 10th edition*. W. W. Norton Company.

Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J., & Salemme, F. R. (1989). Structural origins of high-affinity biotin binding to streptavidin. *Science*, *243*(4887), 85–88. https://doi.org/10.1126/science.2911722

Zempleni, J., Wijeratne, S. S., & Hassan, Y. I. (2009). Biotin. *BioFactors*, *35*(1), 36–46. https://doi.org/https://doi.org/10.1002/biof.8