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Evaluation of the 96-well format for screening of chromatographic buffer conditions

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Abstract

An important part in the development of monoclonal antibody (mAb) purification processes is the optimization of the capture step with Protein A. In this step the mAb is captured and host cell proteins (HCP) are removed in a washing procedure, giving a mAb purity of 98% in one single step. In this study different intermediate wash buffers for the Protein A based chromatography media MabSelect SuRe were tested. This was performed by screening of different wash buffers using the 96-well format. The results were then verified with chromatography. Different buffer additives such as detergents, solvents, amino acids, etc. in combination with 0.5M NaCl at pH 7.0 gave a significant decrease in HCP levels in the eluates without decreasing the recovery. The correlation between the 96-well format and the chromatography was good. Thus, the 96-well format can be used as a time saving and consistent method for screening of different buffer conditions.

Keywords

Monoclonal antibodies, Protein A, Host cell proteins, 96-well format

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Evaluation of the 96-well format for screening of chromatographic buffer conditions

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Sammanfattning

Monoklonala antikroppar (mAb) kan användas som läkemedel för behandling av t.ex. psoriasis och reumatism. För framställning av dessa läkemedel odlas celler, s.k. värdceller som uttrycker antikroppen. För att kunna använda antikroppen som läkemedel behöver den renas från värdcellsproteiner. Detta utförs med hjälp av Protein A, naturligt producerat av *Staphylococcus aureus*, som binder specifikt till antikroppen. Genom att fästa Protein A till en matris som packas i en kolonn kan antikroppen bindas upp medan värdcellsproteiner tvättas ur med specifika tvättbuffertar. En bra tvättbuffert ska avlägsna så mycket värdcellsproteiner som möjligt utan att stora mängder mAb går förlorade. Den rena antikroppen kan sedan frigöras från kolonnen. Denna metod kallas för affinitetskromatografi.

I det här examensarbetet har olika tvättbuffertar testats för MabSelect SuRe™ (GE Healthcare Bio-Sciences AB) ett Protein A baserat kromatografimedium. Detta har utförts i två olika format. Först användes en 96-håls platta där många olika tvättbuffertar testades parallellt. Sedan verifierades resultaten från 96-håls plattan kromatografiskt. Studien visade att resultaten från de två olika formaten stämmer överens, vilket innebär att 96-håls plattor kan användas för att effektivt screena kromatografiska buffertbetingelser. Denna metod kan spara mycket tid vid processutveckling inom biofarmaceutiska industrin.

Examensarbete 20p i Molekylär bioteknikprogrammet
Uppsala universitet oktober 2006

Table of contents

1 Abbreviations

aa = amino acid
AIEC = anion exchange chromatography
CHO = Chinese hamster ovary
CIEC = cation exchange chromatography
CIP = cleaning-in-place
CV = column volume
DBC = dynamic binding capacity
DNA = deoxyribonucleic acid
DoE = Design of Experiment
ELISA = enzyme linked immunosorbent assay
EtOH = ethanol
Fab = fragment antigen binding
Fc = fragment crystallisable
FCS = foetal calf serum
H = heavy chains
HCP = host cell proteins
HIC = hydrophobic interaction chromatography
HTS = high through put screening
IgG = Immunoglobulins
κ = kappa
L = light chains
λ = lambda
mAb = Monoclonal antibody
NS0 = murine myeloma
OD = optical density
pAb = polyclonal antibody
PBS = Phosphate buffered saline
pI = isoelectric point
rProtein A = recombinant Protein A
SDS-PAGE = Sodium Dodecyl Sulphate-Polyacrylamid gel electrophoresis

2 Introduction

Monoclonal antibodies (mAbs) have emerged as one of the fastest growing segments of the biopharmaceutical industry. The annual growth is 20% and it is calculated that the income from this section will reach $20 billion year 2010. Today there are 23 mAbs and mAb-related proteins accepted for medical treatment on the market. Diseases that can be treated using mAb-based pharmaceuticals are rheumatoid arthritis, inflammatory bowel disease and psoriasis. High doses of above 1mg/kg are required. (1)

The protein production process can be divided into two parts, the upstream cell culture and the downstream purification process. Monoclonal antibodies are often produced in recombinant mammalian cells, e.g. Chinese Hamster Ovary (CHO) or murine myeloma (NS0) cells, and secreted extra-cellularly. The produced mAbs are separated from the host cells by centrifugation and/or filtration. After separation the host cell clarified feed contains, apart from the target protein, process related impurities such as host cell proteins (HCP), DNA and viruses and also product related impurities like IgG aggregates and fragments. A well working downstream purification process is of high importance because very low levels of impurities are accepted in mAb based pharmaceuticals. (2)

Chromatography is the foundation of protein purification in bioprocesses and mAbs are typically purified using Protein A based chromatography media for capture and cation exchange (CIEC) and anion exchange (AIEC) chromatography for intermediate and polishing steps respectively. In these intermediate and polishing steps impurities such as HCP, DNA, viruses and IgG aggregates are removed. If the mAb contains large amounts of IgG aggregates, AIEC can be used in the second step and hydrophobic interaction chromatography (HIC) can be used for polishing.

GE Healthcare Bio-Sciences produces chromatography media and is a major supplier of Protein A media used in the first step in the mAb purification process. Furthermore, the company works with integrated processes and is building a platform for mAb purification. The first platform will be based on MabSelect SuRe™, which is a novel alkali-stabilized Protein A derived medium. The alkali tolerance makes it possible to clean the resin with 0.1-0.5M of NaOH. Other benefits with this resin are also more generic elution conditions (3) as well as enhanced protease stability.

It is desirable to optimize the mAb purification process regarding product purity and recovery. In the capture step with Protein A it might be possible to improve the product purity by introduction of intermediate wash steps after loading of feed onto the column. Therefore, it is desirable to study a number of different wash buffer additives, salt concentrations and various pHs that can reduce non-specific binding of impurities and increase the purity in the eluate.

Development of a chromatographic purification method is tedious with a large number of variables that need to be optimized. Earlier, trial and error approaches were often used and column chromatography was the method applied which was very time consuming. Instead it is desirable to systematically explore a number of different variables in a short period of time. For that purpose 96-well plates containing chromatography media can be used for parallel screening of different buffer conditions. A number of different buffer excipients, such as buffer additive, salt concentration and pH, could be evaluated and the procedure would take no longer than a couple of hours. (4)

The focus of this study was to evaluate the possibility of using a 96-well format for parallel screening of different intermediate wash buffers used in the Protein A chromatography step. In addition to the wash buffer study, the protease stability of the MabSelect SuRe ligand was compared with the protease stability of recombinant Protein A.

3 Background

3.1 Affinity chromatography

Affinity chromatography is an efficient way for protein purification. It is based on the fact that proteins can bind to other compounds, called ligands, reversibly and specifically. These ligands can be immobilized to different gel matrixes that make up a chromatography column. The result of this is that only the protein with specificity for the ligand binds to the matrix, and all other proteins can be washed away. Protein A is an example of an affinity ligand that can be used for purification of immunoglobulin (IgG) (5). Protein A is naturally produced in *Staphylococcus aureus*, and is a 42 kDa large protein consisting of five different domains: E, D, A, B and C (3). Protein A has high specificity and affinity for the Fc region of IgG, and if it is immobilized to a stationary phase and packed into a column it can be used for purification of IgG from a mixture of proteins in solution. Protein A affinity chromatography has become an important method for industrial purification of large amount of antibodies for therapeutic use (6).

3.2 MabSelect SuRe

GE Healthcare Bio-Sciences has developed a family of chromatography media called MabSelect^{TM} for purification of IgG. MabSelect SuRe, one of the members, is an alkali-stabilized Protein A derived chromatography medium and SuRe stands for Superior Resistance. The SuRe ligand consists of four modified B domains from Protein A. The B domains in the SuRe ligand have been modified by site directed mutagenesis where alkali sensitive amino acids (aa) were removed and replaced with alkali stable aa (Figure 1 and Figure 2). The alkali tolerance implies that the resin can be cleaned-in-place (CIP) with 0.1-0.5M NaOH (7). As an example of the alkali tolerance it has been shown that MabSelect SuRe can be cleaned with 0.1M NaOH for a contact time of 15 or 60 minutes for more than 100 cycles without loss of dynamic binding capacity (DBC) (Figure 3). Furthermore it is possible to use as harsh conditions as 0.5M NaOH for a contact time of 15 minutes for more than 50 cycles without significantly reducing the DBC (Figure 3). The DBC, $Q_{BX\%}$, was calculated from the volume applied to the column until a break through of 10%, X=10, was detected, meaning that the outlet concentration was 10% of the initial protein concentration, according to Equation 1.

Equation 1

$$Q_{BX\%} = C_0(V_{X\%}-V_0)/V_C \quad (1)$$

Where C_0 is the initial protein concentration in mg/ml, $V_{X\%}$ is the applied sample solution volume at X% break through. V_0 is the systems delay volume in ml and V_C is the bed volume in ml. (8)

The MabSelect SuRe resin is build up of cross-linked agarose that makes the gel matrix very rigid. The SuRe ligand has a similar specificity for the Fc region of IgG as Protein A and the DBC of the resin is 20-30 mg/ml gel (7).

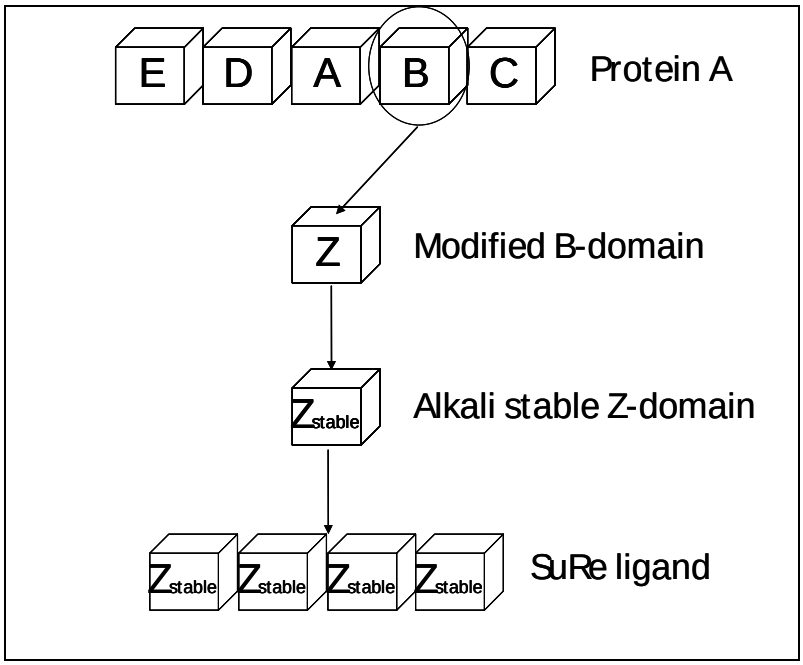


Figure 1 A schematic picture of the SuRe ligand. Protein A consists of five different domains, E, D, A, B and C. The SuRe ligand consists of four genetically modified B-domains. The B-domain was made alkali tolerant by site directed mutagenesis, where alkali sensitive amino acids were replaced by alkali stable amino acids.

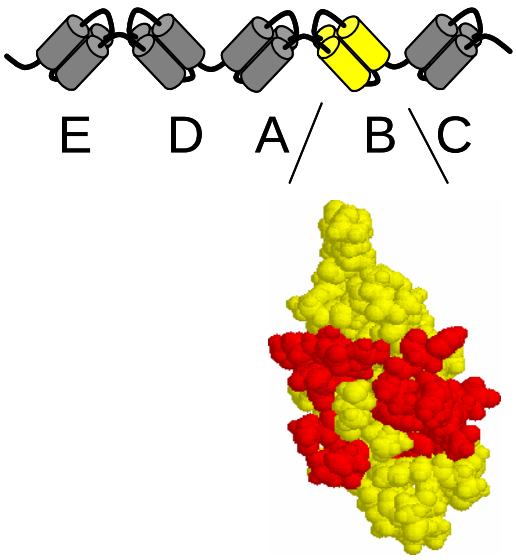


Figure 2 The structure of the SuRe ligand. Here the structure of one of the domains in the SuRe ligand is shown. The domain is made alkali tolerant by site-directed mutagenesis, where alkali sensitive amino acids are replaced by alkali stable amino acids (colored red). The SuRe ligand consists of four modified B-domains from Protein A. Illustration used with permission from Aman Mottaqui-Tabar, GE Healthcare Bio-Sciences.

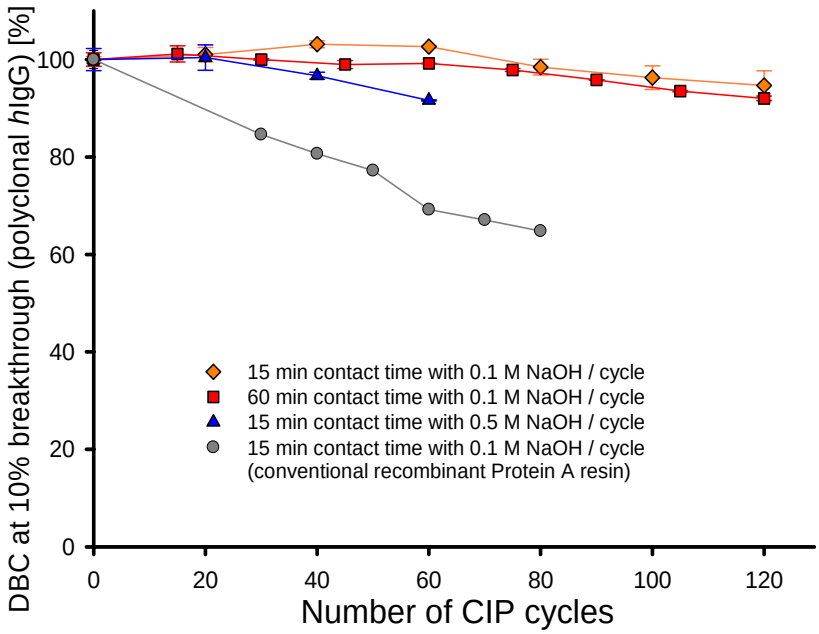


Figure 3 The number of cleaning in place (CIP) cycles possible for MabSelect SuRe. The picture shows the number of CIP cycles possible for MabSelect SuRe when using different concentrations of NaOH and different contact times. It can be seen that the dynamic binding capacity of the gel is not reduced to any larger extent after 120 cycles with 0.1M NaOH for contact times of 15 or 60 minutes. It is also possible to use as harsh conditions as 0.5M NaOH for a contact time of 15 minutes for 60 cycles with maintained binding capacity. Illustration used with permission from Aman Mottaqui-Tabar, GE Healthcare Bio-Sciences.

In Protein A, all five domains (E, D, A, B and C) can bind to the Fc region of IgG. Some immunoglobulins can bind to Protein A via the variable domain of the heavy chain. Such IgGs belongs to the V_H3 subfamily. IgGs containing heavy chains from this subfamily can bind to the D and E domains in Protein A via the Fab region (see 3.6). The interactions with the variable parts can affect the binding of V_H3 containing Ab during Protein A affinity chromatography. This theory was tested by Ghose *et al.* 2005 by investigating the elution pH for four different ab, V_H3-IgG2, V_H3-IgG1, non-V_H3-IgG2 and non-V_H3-IgG1. V_H3 containing Abs bind strongly to a conventional Protein A media and can only be eluted by decreasing the pH to below 3.5, whereas non-V_H3 containing ab elute at a pH higher than 3.5 from the same resin. The same V_H3 containing ab elute at a pH higher than 3.5 on the MabSelect SuRe resin, as does the non-V_H3 containing ab. This more generic elution from MabSelect SuRe is due to lack of variable interactions as the SuRe ligand consists of modified B domains only able to bind the Fc region of IgG. The fact that the elution takes place at a higher pH has positive effect on the mAb stability with less formation of aggregates. (3)

3.3 Development of a Protein A purification process

A typical Protein A cycle consists of different blocks: equilibration of the column, loading of feed, wash, elution of the target protein (mAb), regeneration, CIP and finally re-equilibration of the chromatographic column. A typical chromatogram from a Protein A capture step is presented in Figure 4. During loading, the feed containing mAb is added to the resin and the target protein binds specifically to it. Usually the load is approximately 80% of the DBC of the resin. The next step is to remove unbound proteins. This is done by washing with loading buffer and in some cases by using an intermediate wash buffer. The qualification for a good wash buffer is that it removes non-specific bound protein without eluting the target protein. The protein is eluted by decreasing the pH (9). As a last procedure before re-equilibration of

the column with loading buffer, a CIP is performed. This cleaning removes unwanted substances like precipitated, denatured or tightly bound proteins (7).

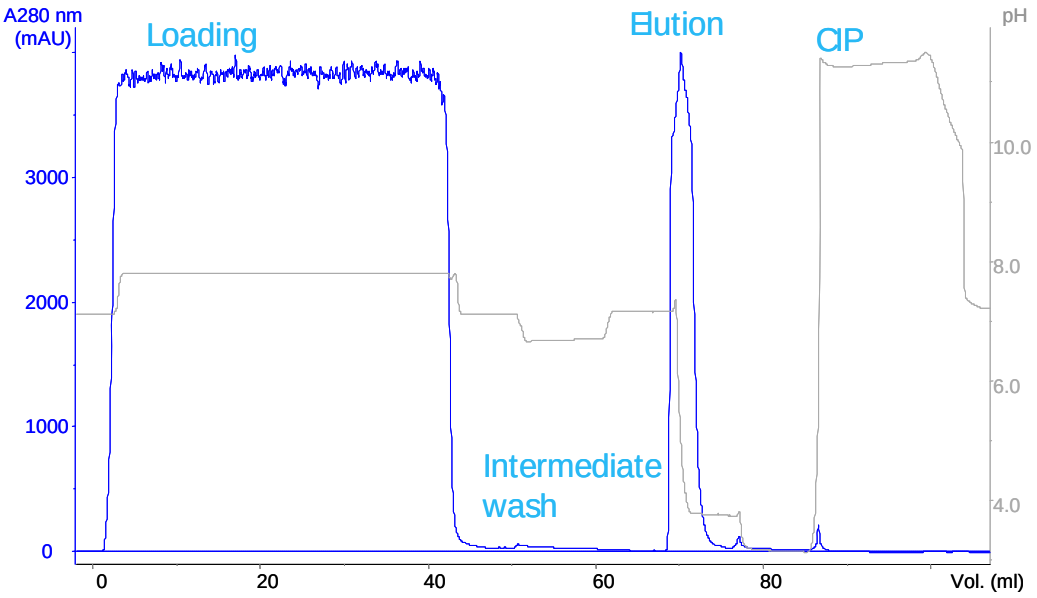


Figure 4 The different steps in the Protein A affinity chromatography. The different steps in Protein A chromatography include: loading of start material, intermediate wash, elution of protein, regeneration, CIP and re-equilibration of column. In the loading step the protein is added to the column and normally about 80% of the DBC of the resin is used. Unbound proteins are then removed by washing with loading buffer and sometimes with an intermediate wash buffer. To elute the protein the pH is decreased and the column is then regenerated. A CIP is performed on the column to remove unwanted substances like precipitated, denatured or tightly bound proteins. As a last procedure the column is re-equilibrated with loading buffer.

Time and resources can be saved by developing a platform process for mAb purification. It is desirable to develop a good generic process that works for different mAbs. This is not completely possible because molecules are different, and appear different in the purification process (3). Some steps in Protein A chromatography can be the same for all mAbs whereas some steps need to be optimized for each new mAb. The capacity of the resin differs between mAbs, and thus the loading needs to be optimized for each individual mAb. Furthermore, the intermediate wash and the elution also need to be modified due to differences in binding strengths between mAbs (Figure 5) (10). However, MabSelect SuRe shows a more generic elution compared to conventional Protein A chromatography media (3.2).

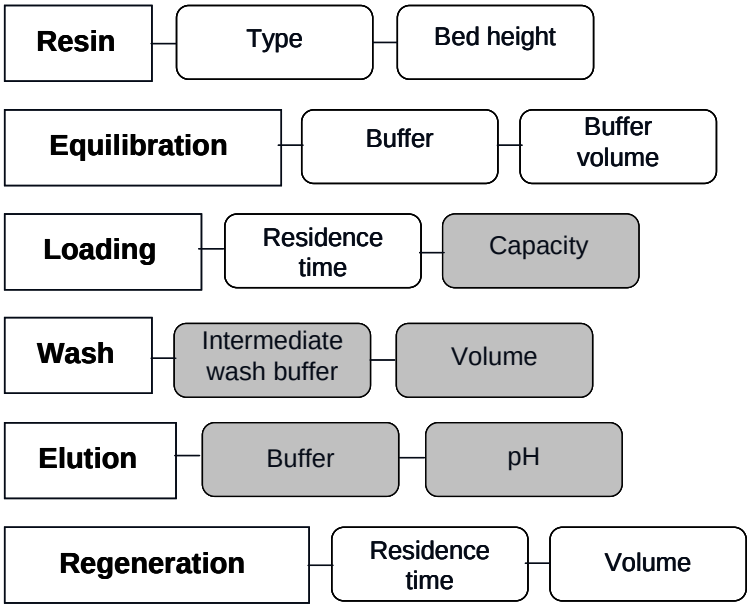


Figure 5 Development of the Protein A capture step The different steps in the Protein A purification step are shown. Those include resin type and bed height, equilibration buffer and buffer volume, residence time during loading and capacity, intermediate wash buffer and volume, elution buffer and pH and residence time for regeneration and volume of regeneration buffer. In the Protein A purification step the resin type and bed height can be predetermined. It is also determined which equilibration buffer should be used and the volume of that buffer. When loading the protein onto the column a residence time of 2.4 is normally used. The capacity of the resin differs between different mAbs and should be studied for each new mAb. In the wash it is optional to have an intermediate wash and wash buffer and buffer volume can be optimized for each protein. Because proteins are different they will elute at different pHs, so both elution buffer and elution pH needs to be optimized for each protein. However, MabSelect SuRe shows a more generic elution compared to conventional Protein A chromatography media (3.2). After the purification it is preferable to regenerate the column with regeneration buffer. The residence time of regeneration and the volume of regeneration buffer are predetermined. In the figure the white boxes represents predetermined steps, whereas other steps, here represented by grey boxes, need to be optimized for the specific protein that is purified. (10)

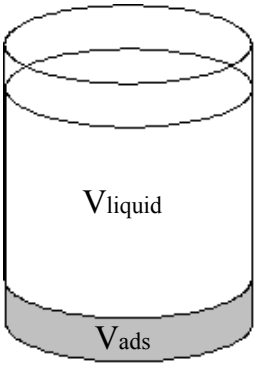
The use of Protein A in a purification process for mAbs is very effective because it can purify the product to more than 98% purity in one single step. After the capture step with Protein A, steps of viral inactivation, virus filtration, removal and polishing are needed. Virus inactivation is performed by decreasing the pH of the Protein A pool to between 3.3-3.8 and incubation of the solution for 45-60 minutes depending on stability of mAb. The pH is adjusted before the next step. A virus filtration step is also included where viruses are removed by size based separation. Commonly used techniques for removal and polishing are CIEC, AIEC and HIC. The first method mentioned is good at removing impurities such as HCP and DNA/RNA. AIEC is a useful method for virus clearance and other impurities such as DNA and endotoxins. Aggregates and dimers are effectively removed using HIC (2). For a mAb to be accepted as a pharmaceutical drug the levels of impurities in the final product should be: <5 ppm HCP, <10 ng rDNA /dose, <0.5% aggregates and <5 ppm Protein A (11).

3.4 The 96-well format

A lot of process development time can be saved by using a parallel purification method at small scale. For that purpose a 96-well format can be used for high through put screening (HTS) of chromatographic buffer conditions. The technique is based on parallel purification in 96-well plates. Different chromatography media depending on the purification method used can be added to the plate. The media will form small gel plugs, like micro-columns in the bottom of the plate and feed containing the target protein can be applied to the wells. After wash with loading buffer and, e.g. intermediate wash buffers, the target protein can be eluted

by an elution buffer. Between each step (loading, wash and elution) the supernatant can be collected for further analyses (4). This means that 96 purifications can be performed in parallel within a couple of hours. Using regular chromatography the same procedure could take weeks to perform. The method can be made totally automated by using robots for buffer preparation, gel and sample application, wash and elution. Making the method totally automated will save even more time and resources (9).

In the 96-well plate the added protein in solution is adsorbed by batch adsorption. The easiest way to perform a batch adsorption is to add adsorbent to the protein solution and stir for a couple of minutes, let the solution settle before filtering with suction (12).



The volume of adsorbent V_{ads} and liquid V_{liquid} are defined in Figure 6. The amount of protein adsorbed are described by Equation 2

Equation 2

$$m_{ads}=c_{ads}*V_{ads} \quad (2)$$

with c_{ads} as the concentration of adsorbed protein. The amount of protein not adsorbed are described by Equation 3

Equation 3

$$m_{free}=c_{free}*(V_{ads}+V_{liquid}) \quad (3)$$

with c_{free} as the concentration of free protein.

Figure 6 The definition of V_{ads} and V_{liquid} in batch adsorption

The partion coefficient α describes how successful the batch adsorption is. A value close to one for the proteins adsorbed is required for a good uptake. α is defined in Equation 4 (12).

Equation 4

$$\alpha = c_{ads}/(c_{free}+c_{ads}) \quad (4)$$

Then c_{free} can be written as $c_{ads}(1- \alpha)/ \alpha$.

Fraction of adsorbed protein f can be described by Equation 5.

Equation 5

$$f = c_{ads}*V_{ads}/(c_{free}(V_{ads}+V_{liquid})+c_{ads}*V_{ads}) \quad (5)$$

Another way of writing it is presented in Equation 6.

Equation 6

$$f = V_{ads}* \alpha /(V_{ads}+(1- \alpha)*V_{liquid}) \quad (6)$$

(12)
The adsorption can also be described by an equilibrium using following equations:

Initially the adsorption can be described by Equation 3, because all protein is still free in solution. At equilibrium,

Equation **7** can be used.

Equation 7

$$m_{final}=c_{final}*V_{liquid}+q*V_{ads} \quad (7)$$

where m_{final} is the protein amount in mg. c_{final} is the protein concentration in the liquid in the well at equilibrium. q is the capacity of the gel resin, i.e. the amount of protein in mg that is bound to the resin. V_{ads} is the volume of the gel as defined in Figure 6. The first term ($c_{final}*V_{liquid}$) describes the amount of protein in mg still present in the liquid at equilibrium. The second term ($q*V_{ads}$) describes how much protein that has bound to the gel resin. Since no protein is lost during the reaction the two formulas before equilibrium (Equation 3) and at equilibrium (

Equation 7) should be equal (Equation 8).

Equation 8

$$c_{free}*(V_{liquid}+V_{ads})= c_{final}*V_{liquid}+q*V_{ads} \quad (8)$$

and if q is solved out, the formula looks this way:

Equation 9

$$q= (c_{free}- c_{final})* V_{liquid}/ V_{ads}+c_{free} \quad (9)$$

From Equation 9 it can be seen that the capacity (q) of the gel is dependent on the volume and the protein concentration of the protein solution added to the well. q is also dependent on the gel volume in the well. The amount of protein that binds to the resin (q) can be increased by increasing the concentration or volume of the protein solution, or by decreasing the gel volume. (13)

3.5 Different buffer additives

By adding different additives to either the elution buffer or the wash buffer different effects have been seen. Such effects are for example reduced amount of protein aggregates and less electrostatic and hydrophobic interactions with the resin.

Both soluble and insoluble high molecular weight aggregates can form during elution from a Protein A column. The eluted product can be stabilized by adding arginine to the elution buffer (14). Effective elution of mAbs at pH 4.0 or higher was seen in affinity chromatography by adding arginine to the elution buffer. Some arginine derivatives were also tested as eluents and were shown to be almost as effective as arginine. Other aa such as lysine, proline, glycine and histidine were also added to the elution buffer and the mAb was eluted under identical pH conditions. However, the elution was not as effective for these aa as for arginine (15).

It has been shown that by adding 25 mM caprylic acid to the wash buffer during purification of polyclonal IgG from ovine serum using chromatography based on Protein A mimetics the level of non-specifically bound albumin could be reduced. The final product contained very low levels of albumin. The purity of IgG could be increased from about 80% to 95% by introducing this intermediate wash after loading of serum to the column. This was done without significantly decreasing the capacity of the resin. (16)

It has been examined whether or not variable interaction between Protein A and the variable domain of IgG can be reduced by adding mobile phase modifiers such as ethylene glycol and NaCl to the elution buffer. This was tested on MabSelect, a conventional Protein A media, and MabSelect SuRe. It has been shown that the elution pH for different Ab from the MabSelect SuRe resin has increased depending on the reduced amount of variable interactions (discussed in paragraph 3.2). If the variable interactions were eliminated by the mobile phase

modifiers the elution pH from the MabSelect media would also increase. However no such effect was seen because these Ab variable region interactions are too strong (3). Previous studies have shown that such modifiers can reduce the amount of electrostatic and hydrophobic interaction between HCP and the column (17).

It is known that HCP bind non-specifically to silica based chromatography media. Different wash buffer additives were therefore tested to find out whether the amount of non-specific interactions could be decreased. It was shown that Tween^{TM} 20 in combination with 0.5M NaCl at pH 5.0 was an effective wash buffer for silica based Protein A chromatography media (18).

3.6 Antibodies

Antibodies (Ab) belong to the family of immunoglobulins (IgGs). They all share the same core structure, with two identical heavy (H) chains and two identical light (L) chains. Between the L chain and H chain, and between the H chains there are disulfide bonds that hold them together (Figure 7). The chains fold into different globular domains, so called Ig domains. Every domain, both in the H chain and L chain, is either constant, C, or variable, V. V_H and V_L are the antigen binding site and are part of the fragment antigen binding (Fab) region. The constant heavy chains build up the fragment crystallisable (Fc) region (19). The Fab region of an Ab can recognize and bind to an antigen via the epitope. The fact that an antibody only binds one specific antigen results in high specificity and affinity. Antibodies can be divided into two types, polyclonal (pAb) and monoclonal (mAb). MAbs are produced by one cell type and are therefore identical; pAbs on the other hand are produced by different cell lines and have variable regions that are structurally different. This means that mAbs have specificity for only one epitope of an antigen whereas pAbs have specificity for different epitopes (20).

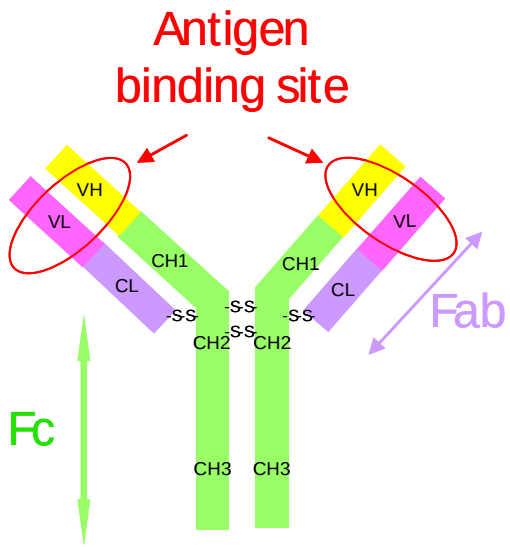


Figure 7 The structure of antibodies. Antibodies share the same core structure with two identical heavy (H) chains and two identical light (L) chains. V_H and V_L is the antigen binding site that recognize one specific antigen, and are part of the fragment antigen binding (Fab) region. The constant heavy chains build up the fragment crystallisable (Fc) region.

The immunoglobulins can be divided into different classes depending on their C_H chains. The reason for this is that different classes have different effector functions. In humans and mice there are five different classes of immunoglobulins: IgG, IgA, IgM, IgD and IgE. There is a subdivision of the IgG class, in humans they are called IgG_1 IgG_2 IgG_3 IgG_4 and in mice IgG_1,

IgG_{2a}, IgG_{2b}, IgG_3. The light chains can be divided into two classes, kappa (κ) and lambda (λ) (20). Humans have a ratio of 60:40 between κ and λ, whereas mice have a 95:5 ratio (19).

In the immune system a lot of different mechanisms and cells are cooperating, and their responses to foreign substances are called the immune response. The immune system functions as a defense against infective agents and antibodies belong to this defense. Abs can recognize infective antigens and activate different effector mechanisms, leading to elimination of the antigen. Different antibodies are specialized to activate different effector mechanisms (20).

3.7 Chymotrypsin

Chymotrypsin belongs to the class serine proteases that catalyze peptide bond hydrolysis in proteins. They are called serine proteases because a serine residue has an important role in the catalytic process. All serine proteases have a similar active site structure, meaning that the same residues build up the active site. Chymotrypsin cuts the polypeptide chain next to the C-terminal side of hydrophobic residues like phenylalanine. (21)

3.8 Analytical methods

3.8.1 Sodium Dodecyl Sulphate-PolyAcrylamid Gel Electrophoresis (SDS-PAGE)

SDS-PAGE can be used to separate proteins according to their size. A current is applied to the gel and the proteins start to migrate down the gel. Small proteins can pass through the gel pores easily whereas large proteins are retarded. The purity after each step in a protein purification process can be determined by collecting samples after each step and run them on a SDS gel.
There are different types of gels, homogenous gels and gradient gels. In the homogenous gel the acrylamid concentration is constant and in the gradient gel the acrylamid concentration varies from a smaller percentage at the top to a larger percentage in the bottom. As the acrylamid concentration increases the pore size decreases (5).
GE Healthcare Bio-Sciences has developed the Phast system™ Separation unit for rapid protein separation, with prepared gels, PhastGels™, and buffer strips and the Development unit for automatic gel dyeing.

3.8.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA can be used to determine the concentration of an antigen present in a solution. The sandwich ELISA is commonly used where a certain Ab is attached to the surface of a 96-well plate. The sample containing the unknown concentration of antigen is then added to the wells of the plate, and the antigen binds to the attached Ab. Any unbound antigen is removed by washing before an enzyme linked or radiolabeled second Ab is added. This labeled Ab binds to another epitope of the antigen, and unbound second Ab is washed away. The amount second Ab bound to the antigen can be measured in a spectrophotometer and thereby also the concentration of antigen in the solution. (20)

3.8.3 Analytical gel filtration

Gel filtration or size exclusion chromatography (SEC) separates the proteins according to size. Smaller proteins have entrance to a larger internal pore volume than larger proteins, and will therefore be more retarded in the column. Proteins are eluted from the column in decreasing molecular size meaning that very large proteins and aggregates pass through the column without penetrating any pores and are eluted first and the smallest proteins are eluted last (6). SEC can be used for determination of the aggregate concentration in an IgG sample

and it can also be used for determination of the IgG concentration. This is done by injecting a sample with unknown concentration to the gel filtration column and measuring the UV absorbance at 215 nm, 280 nm and at 410 nm. The mAb peak in the chromatogram for 215 nm is integrated for IgG concentration determination. The elution peak area is used for calculation of the IgG concentration from a standard curve previously generated from injection and elution of pure IgG of known concentrations.

3.8.4 IgG concentration determination using the MabSelect SuRe method

Protein A chromatography can be used for determination of IgG concentrations in samples with unknown IgG concentration. This method can be used both for IgG concentration determination in feed, i.e. a complex mixture of proteins and other components, or further downstream, i.e. in post-Protein A samples. However, since the samples after the Protein A step consist of highly purified IgG, the measurement of UV absorbance at 280 nm is sufficient for determination of the IgG concentration after capture, intermediate and polishing steps.

The MabSelect SuRe analytical method is based on the highly selective binding of IgG to a Protein A (MabSelect SuRe) column. A small amount of sample diluted to an approximate IgG concentration of 1 mg/ml is injected onto the MabSelect SuRe column and the bound IgG is eluted with an acidic buffer. The chromatogram is integrated and the elution peak area is used for calculation of the IgG concentration from a standard curve previously generated from injection and elution of pure IgG of known concentrations.

3.9 Aim of the study

The aim of this project was to evaluate the possibility of using a 96-well format for screening of potential intermediate wash buffers for Protein A (MabSelect SuRe) chromatography. It was also desirable to find effective intermediate wash buffer candidates for the above chromatography media. This was performed by first doing a parallel screening of different wash buffers using 96-well plates and then a verification of the results using chromatography. The idea was to see how well the results correlated between the 96-well plates and the column chromatography. The protease stability of the SuRe ligand was also examined by incubation of the ligand with proteases. The protease stability of the SuRe ligand was compared with the protease stability of recombinant Protein A (rProtein A).

4 Material and methods

4.1 The 96-well format

4.1.1 General description of 96-well plate method

4.1.1.1 Preparation of chromatography media

The Protein A chromatography media MabSelect SuRe (GE Healthcare Bio-Sciences) was used. Before the gel was loaded into the wells of a 96-well plate it was washed four times with one gel volume of MilliQ water followed by two times with one gel volume of 0.1M NaOH. The gel was then incubated for 10 minutes in one gel volume of 0.1M NaOH. After the incubation the gel was washed with three gel volumes of MilliQ water followed by ten gel volumes of 20 mM phosphate, 0.15M NaCl, pH 7.4 (Coricon AB, Uppsala). 10% gel slurry in 20 mM phosphate, 0.15M NaCl, pH 7.4 was prepared. The work-flow is presented in Figure 8.

4.1.1.2 Preparation of plate

The same 96-well filter plates as in the purification of histidine-tagged proteins were used, except for the gel media (26). The filter plates were made of polypropylene and polyethylene (26) and 500 µl of 10% gel slurry in 20 mM phosphate, 0.15M NaCl, pH 7.4 was distributed to each well by using a Thermo Electron Corporation Multidrop DW. The buffer was removed by centrifugation of the plate at 100*g for 2 minutes, allowing the gel to form plugs in the bottom of the wells. The protein solution, either feed or pure mAb, was added to the gel in the plate by using an Eppendorf Multi pipette Research Pro, 50-1200 µl. The gel was then washed in different steps with loading buffer and intermediate wash buffers before the target protein was eluted with elution buffer (20-100 mM citrate, pH 3.5-3.7). All buffers were added to the plate using the multi pipette. Between each step the supernatant was collected into either a 96-well UV-plate (Göteborg Thermometer factory, product no 11-003635) or a 96-well V-bottom plate (Göteborg Thermometer factory, product no 11-003957) by centrifugation at 100*g for 2 minutes at 18ºC in a Beckman Coulter Avanti J-20XP centrifuge (serial no ZXP02G19, IP no 23766) using a JS 5.3 rotor. The UV absorbance at 280, 320 and 410 was measured in a Spectramax plus Microplate Spectrophotometer. The results were evaluated using the data program Softmax pro 471. The amount eluted protein and amount proteins removed in the wash was calculated in an excel template designed for the 96-well format (22). The absorbance at 280 nm was measured in flow through, wash and elution fractions and also in the elution buffer and the different wash buffers. The absorbance values for the different wash buffers and the elution buffer were subtracted from the absorbance values for the wash and elution fractions respectively. In this way the background from the different wash buffers and from the elution buffer was subtracted before the recovery was calculated in the template. The work-flow is outlined in Figure 8.

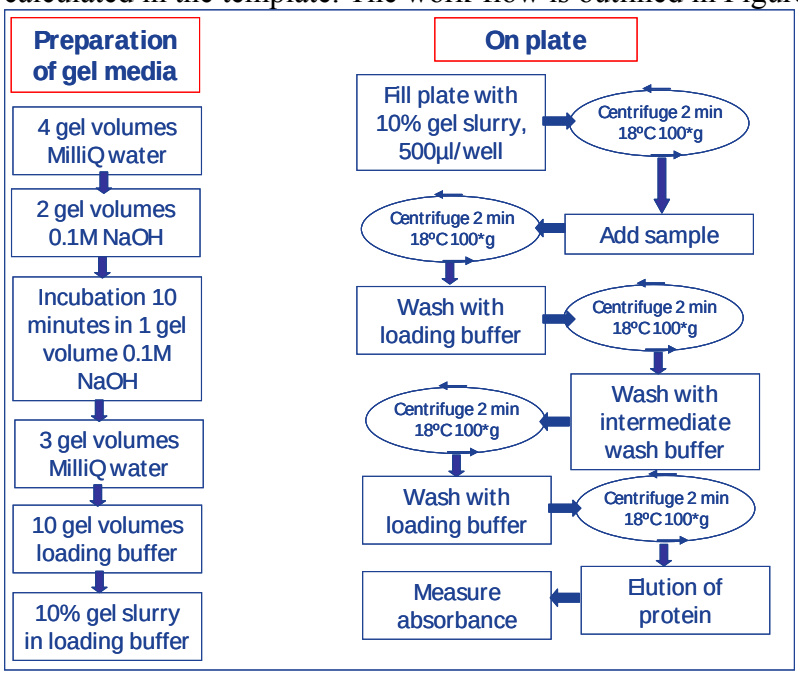


Figure 8 Outline of the multiwell experiment. Before the gel media was filled into the plate it was washed with four gel volumes of MilliQ water followed by two gel volumes of 0.1M NaOH. The gel was then incubated in 0.1M NaOH for 10 minutes before washed with 3 gel volumes of MilliQ water and equilibrated with ten gel volumes of 20 mM phosphate, 0.15M NaCl, pH 7.4. 10% gel slurry in 20 mM phosphate, 0.15M NaCl, pH 7.4 was prepared and 500 µl slurry i.e. 50 µl of gel was added to each well. The buffer was removed by centrifugation before the sample i.e. feed containing mAb or pure mAb was added to the plate. Several steps of washing and elution were done and between each step the plate was centrifuged for 2 minutes at 18ºC at 100*g for collection of flow through, wash and elution fractions. The absorbance of elution fraction and wash fractions were measured.

4.1.2 Development of the 96-well method using pure IgG

The method for loading of protein onto the plate, wash and elution was first tested using pure IgG. Different concentrations of IgG in different volumes were added to the 96-well plate, and different incubation times with or without shaking of the plate during incubation were evaluated. Both MabSelect SuRe with and without NaOH washes were evaluated. 20 mM phosphate, 0.15M NaCl, pH 7.4 was used as loading buffer and 0.1M Na-citrate, pH 3.0 was used as elution buffer.

4.1.2.1 Pure IgG

A purified CHO-cell derived IgG (POLYMUN SCIENTIFIC) with a concentration of 13.7 mg/ml was used for development of the 96-well method. The extinction coefficient (ε) of the mAb was 1.172.

4.1.2.2 Varying the volume of protein solution

The pure mAb was diluted to a concentration of 2 mg/ml in 20 mM phosphate, 0.15M NaCl, pH 7.4 before filled into the plate as described in paragraph 4.1.1.2. Different volumes of the IgG solution corresponding to different amounts of mAb, 300 µg, 400 µg and 500 µg were added to the wells. The protein was incubated for 0.5-3 minutes. The wash was performed with 500 µl loading buffer in two steps and the mAb was then eluted in two steps using 200 µl of elution buffer per step, after incubation for one minute in elution buffer. Between loading, wash and elution the plate was centrifuged and the flow through, wash and elution fractions were collected in UV plates and the UV absorbance was measured in a spectrophotometer. The background from the loading buffer and elution buffer was subtracted from the absorbance values in the wash and elution fractions as described in 4.1.1.2.

4.1.2.3 Varying the protein concentration

The experiment in paragraph 4.1.2.2 was repeated but now the protein concentration was varied instead of the volumes loaded to the matrix. The gel was washed as in paragraph 4.1.1.1 before filled into the plate. Three different concentrations of sample were tested: 1.0, 2.0 and 3.0 mg/ml, and volumes of 200 µl were added to the wells of a 96-well plate. The protein solutions were incubated for 3-7 minutes. The gel was washed with 200 µl loading buffer in three steps before elution. 200 µl elution buffer were added to each well and incubation was performed for one minute before centrifugation. The procedure was repeated one more time, giving a total of two elution steps. The background from the loading buffer and elution buffer was subtracted from the absorbance values in the wash and elution fractions as described in 4.1.1.2.

4.1.2.4 Evaluation of shaking incubation

The experiment described in paragraph 4.1.2.3 was repeated but now with shaking incubation (100 rotations per minute).

4.1.2.5 Evaluation of cleaning MabSelect SuRe with 0.1M NaOH before usage

The effect of cleaning the gel with 0.1M NaOH before usage was tested and compared to a gel not treated with 0.1M NaOH. The recovery was determined to see if it was different for the two gels. Three different mAb concentrations: 1.0, 1.25 and 1.5 mg/ml were tested, and 200 µl of the solutions was added to the wells of a 96-well plate. The gel was incubated for 3 minutes for all the concentrations. The background from the loading buffer and elution buffer was subtracted from the absorbance values in the wash and elution fractions as described in 4.1.1.2.

4.1.3 Screening of wash buffers using the 96-well plate method

4.1.3.1 Screening of wash buffers using a CHO-cell lysate

4.1.3.1.1 Preparation of CHO-cell lysate

CHO cells cultured in a standard medium (DMEM, Sigma) in presence of 10% Fetal Bovine Serum (HyClone) were obtained from the centre of Surface Biotechnology at Uppsala Biomedical centre (BMC) (batches: 2005-09-27, 2005-09-28, 2005-09-29 and 2005-10-04). The CHO cells were disrupted using the following procedure: The frozen cells were thawed and stored in 10 mM phosphate buffer pH 7.4 at 4ºC over night. The next day they were centrifuged at 15 100*g (11500 rpm) for 20 min at 4ºC, and the supernatant was collected. The supernatant was filtrated using a Sterivex-HV 0.45 µm Filter Unit. The filtrate was diluted two times in 30 mM phosphate, 0.3M NaCl to a final buffer concentration of 20 mM phosphate, 0.15M NaCl, pH 6.1. The HCP concentration in the lysate was determined to 135 µg/ml with CHO HCP-ELISA (Cygnus Technologies, Inc, product no F015).

4.1.3.1.2 Screening of 96 buffer conditions

96 different buffers containing different concentrations of buffer additives such as arginine (0.5-2M), glycine (0.5-2M), tryptophane (25-50 mM), Tween™ 20 (0.1-0.5%), isopropanol (1-5%), propylene glycol (20%) urea (1-2M) caprylic acid (25 mM), at two different pHs (5.0 and 7.0) different NaCl concentrations (0-1.0M) (Table 7 in appendix 10.1) were evaluated for intermediate wash. 200 µl of CHO-cell lysate was loaded into each well and incubated for 4 minutes. The washing procedure looked this way: two times 200 µl loading buffer followed by two times 200 µl intermediate wash buffer and finally 200µl loading buffer. The protein was eluted in three steps with 200 µl 0.1M citrate, pH 3.0 in each step. The background from the different wash buffers and the elution buffer was subtracted from the absorbance values in the wash and elution fractions as described in 4.1.1.2.

Some of the buffers (Table 1) were tested with pure IgG. 200 µl of 2 mg/ml IgG was loaded to each well of a 96-well plate. The wells were washed two times 200 µl loading buffer followed by two times 200 µl intermediate wash buffer and finally two times 200 µl loading buffer. The protein was eluted in three steps with 200 µl 0.1M citrate, pH 3.0 in each step.

Table 1 The different wash buffers tested with pure IgG

Additive	Buffer	NaCl (M)	pH
No additive (control)	20 mM phosphate	0.15	7.4
2M arginine	25 mM phosphate	0	7.0
0.5M arginine	25 mM phosphate	0.5	7.0
0.5M arginine	20 mM citrate	0.5	5.0
2M glycine	20 mM citrate	0	5.0
0.5M glycine	25 mM phosphate	0.5	7.0
0.5M glycine	20 mM citrate	0.5	5.0
1M urea	25 mM phosphate	0.5	7.0
1M urea	20 mM citrate	0.5	5.0
1% isopropanol	25 mM phosphate	0.5	7.0
1% isopropanol	20 mM citrate	0.5	5.0
0.1% Tween	25 mM phosphate	0.5	7.0
0.1% Tween	20 mM citrate	0.5	5.0
No additive	25 mM phosphate	1	7.0
No additive	20 mM citrate	1	5.0
No additive	20 mM citrate	0.5	5.0
25mM caprylic acid	25 mM phosphate	1	7.0
20% propylene glycol	25 mM phosphate	0.5	7.0

20% propylene glycol	20 mM citrate	0.5	5.0
No additive	0.3M citrate	0	5.0
No additive	1.2M acetate	0	5.0

4.1.3.2 Screening of wash buffers using NS0 clarified feed

4.1.3.2.1 NS0 clarified feed

A filtrated unpurified NS0 cell culture supernatant containing 1.4 mg/ml human IgG1 was obtained from BioInvent International. The NS0 cells had been cultured in a standard medium (DMEM, Gibco) in presence of 10% (v/v) foetal calf serum (FCS, Gibco). The extinction coefficient of the mAb was 1.4. The HCP concentration in the feed was determined to 1.25 mg/ml by NS0-ELISA (Cygnus Technologies, Inc, product no F220).

4.1.3.2.2 Loading to column followed by wash and elution in 96-well plate

A Tricorn™ 10/100 column packed with MabSelect SuRe to a column volume (CV) of 8.0 ml was used. An ÄKTAexplorer™ 10-system (GE Healthcare Bio-Sciences) was used for loading of feed onto the column. The column was cleaned-in-place with 0.5M NaOH for a contact time of 15 min. before usage. After the CIP the gel was equilibrated with 10 gel volumes of 20 mM phosphate, 0.15M NaCl, pH 7.4. Filtrated, non-purified NS0 cell culture supernatant from Bioinvent International was loaded onto the column to a load of 26.3 mg mAb/ml gel. After loading a wash was performed with loading buffer until the UV absorbance at 280 nm reached the baseline. The column was unpacked and 10% slurry of the loaded resin was prepared in 20 mM phosphate, 0.15M NaCl, pH 7.4. 500 µl slurry i.e. 50 µl of gel loaded with mAb was filled into each well in a 96-well plate. Each well was washed with 200 µl loading buffer followed by wash with two times 200 µl of intermediate wash buffer. 32 different intermediate wash buffers listed in Table 2 were evaluated in triplicates on one plate. After the intermediate wash the gel was washed with two times 200 µl of loading buffer before the protein was eluted in three steps with 200 µl 20 mM Na-citrate, pH 3.7 in each step. The eluate fractions were collected by centrifugation into UV-plates and the UV absorbance at 280, 320 and 410 was measured. The background from the different wash buffers and the elution buffer was subtracted from the absorbance values in the wash and elution fractions as described in 4.1.1.2. The protein recovery was calculated by using the excel template designed for the 96-well format (22).

Table 2 The different intermediate wash buffers tested with NS0 clarified feed

Additive	**Buffer**	**NaCl (M)**	**pH**
2M arginine	25 mM phosphate	0	7.0
0.5M arginine	20 mM citrate	0.5	5.0
0.5M arginine	25 mM phosphate	0.5	7.0
2M arginine	20 mM citrate	0.5	5.0
2M glycine	20 mM citrate	0	5.0
0.5M glycine	20 mM citrate	0.5	5.0
0.5M glycine	25 mM phosphate	0.5	7.0
0.5M glycine	20 mM citrate	1	5.0
2M glycine	20 mM citrate	1	5.0
No additive	0.3M citrate	0	5.0
No additive	1.2M acetate	0	5.0
0.1% Tween 20	20 mM citrate	0	5.0
0.% Tween 20	20 mM citrate	0.5	5.0
0.1% Tween 20	25 mM phosphate	0.5	7.0
0.5% Tween 20	20 mM citrate	0.5	5.0
0.1% Tween 20	20 mM citrate	1	5.0
1M urea	20 mM citrate	0.5	5.0

1M urea	25 mM phosphate	0.5	7.0
2M urea	20 mM citrate	0.5	5.0
2M urea	25 mM phosphate	0.5	7.0
1% isopropanol	20 mM citrate	0.5	5.0
1% isopropanol	25 mM phosphate	0.5	7.0
5% isopropanol	20 mM citrate	0.5	5.0
5% isopropanol	25 mM phosphate	0.5	7.0
No additive	20 mM citrate	0.5	5.0
No additive	25 mM phosphate	0.5	7.0
No additive	20 mM citrate	1	5.0
No additive	25 mM phosphate	1	7.0
20% propylene glycol	20 mM citrate	0.15	5.0
20% propylene glycol	20 mM citrate	0.5	5.0
20% propylene glycol	25 mM phosphate	0.5	7.0
No additive (control)	20 mM phosphate	0.15	7.4

4.1.3.2.3 Loading to a 96-well plate followed by wash and elution

The gel was washed and filled into the plate as described in paragraph 4.1.1.1 and 4.1.1.2. 200 µl NS0 clarified feed was loaded to each well. After removal of the flow through by centrifugation, wash and elution steps were performed as described in paragraph 4.1.3.2.2. The 32 different intermediate wash buffers listed in Table 2 were evaluated in triplicates on one plate. The eluate fractions were collected by centrifugation into UV-plates and the UV absorbance at 280, 320 and 410 was measured. The background from the different wash buffers and the elution buffer was subtracted from the absorbance values in the wash and elution fractions as described in 4.1.1.2. The protein recovery was calculated in the excel template designed for the 96-well format (22).

4.2 Column chromatography

All the chromatographic experiments were performed on an ÄKTAexplorer 10-system with a UV detector, a conductivity and a pH meter and a fraction collector Frac-900 (GE Healthcare Bio-Sciences, Uppsala). The UNICORN^{TM} 5.01 software was used for control of the ÄKTA system and for evaluation of the chromatograms. Tricorn 5/100 and 5/50 were packed with MabSelect SuRe. CIP with 0.5M NaOH for a contact time of 15 minutes was done on the columns before usage.
20 mM phosphate, 0.15M NaCl, pH 7.4 (Coricon AB, Uppsala) was used for equilibration of the columns (5 CV) and also used as loading buffer in all experiments. Different elution buffers (0.1M Na-citrate, pH 3.0 (Coricon AB, Uppsala) or 20 mM citrate, pH 3.7) were used depending on starting material.
In all the following experiments 20 mM phosphate, 0.15 M NaCl, pH 7.4 (Coricon AB, Uppsala) was used as a wash buffer control, to which the different intermediate wash buffers were compared.

4.2.1 Column packing and evaluation of column efficiency

Two Tricorn 5/50 columns (GE Healthcare Bio-Sciences, 18-1163-09) and one Tricorn 5/100 column (GE Healthcare Bio-Sciences, 18-1163-10) were packed with MabSelect SuRe (GE Healthcare Bio-Sciences, 17-5438-03). 50% gel slurry was packed in 20% EtOH and 0.2M NaCl, using a pack flow of 0.5 ml/min for 3 minutes. A compression flow of 3.0 ml/min was then applied for 5 minutes. After removal of the packing tube and positioning of the filter and the top adaptor the column was packed for additional 3 minutes using 3.0 ml/min. The packing efficiency was tested by injecting 50 µl of 1% acetone in pack buffer onto the column using a linear flow velocity of 25 cm/h. The area for the eluted acetone peak was integrated

and the asymmetry and number of plates were determined. An asymmetry between 0.8-1.5 and a number of plates >2800 should be accepted.

4.2.2 Chromatography using mAb spiked with CHO-cell lysate

Nine of the buffers from the screening (4.1.3.1.2) and the control (loading buffer) were evaluated using chromatography. The buffers are presented in Table 3. The start material was CHO-cell lysate (4.1.3.1.1.) pH adjusted to 7.0 and filtrated through a Sterivex-HV 0.45 µm filter. MAb was spiked into the lysate to a concentration of 2 mg/ml. 10 ml of the spiked lysate was loaded onto a Tricorn 5/50 column packed with MabSelect SuRe (CV: 1 ml) to a load of 20 mg mAb/ml gel. The CHO-cell lysate spiked with mAb was applied to the column using a 50 ml super-loop and a flow velocity of 0.25 ml/min was used in the method. After loading of sample a wash with loading buffer was done until the UV absorbance at 280 nm reached the baseline. The gel was then washed with additional 2 CV of loading buffer followed by 5 CV of the intermediate wash buffer. To avoid co-elution of excipients from the intermediate wash buffer the column was washed with additional 2 CV of loading buffer before the mAb was eluted with 0.1M Na-citrate, pH 3.0 (Coricon AB Uppsala). The pH in the eluate was adjusted to 5.0 by addition of 1M Tris-HCl, pH 8. Flow through, wash and elution fractions were collected. The eluates were analysed for HCP-concentration by CHO HCP-ELISA. The IgG concentration in the eluates was determined by analytical gel filtration and by measuring the UV absorbance at 280 nm on an Ultrospec 6300pro Spectrophotometer (Amersham Biosciences serial no 93942). The Beer-Lambert law (Equation 10) was used for determination of the mAb concentration in the eluates and the recoveries from the runs with different intermediate wash buffers were calculated by using Equation 11.

Table 3 The different intermediate wash buffers tested on column with CHO-cell lysate

Additive	Buffer	NaCl (M)	pH
No additive (control)	20 mM phosphate	0.15	7.4
0.5M arginine	20 mM citrate	0.5	5.0
0.5M arginine	25 mM phosphate	0.5	7.0
0.5M glycine	20 mM citrate	0.5	5.0
0.5M glycine	25 mM phosphate	0.5	7.0
2M glycine	20 mM citrate	1	5.0
0.1% Tween 20	20 mM citrate	0.5	5.0
0.1% Tween 20	25 mM phosphate	0.5	7.0
0.1% Tween 20	20 mM citrate	0	5.0
No additive	20 mM citrate	0.5	5.0

Equation 10 The Beer-Lambert law

$$A * \text{dilution factor} = \varepsilon * c * l \quad (10)$$

c = mAb concentration (mg/ml)
A = absorbance at 280 nm
l = path length (cm) = 1
ε = molar extinction coefficient (mg ml^{-1})

The amount of mAb in the eluates was determined by multiplying the mAb concentration in the eluate with the eluted volume. The recovery in percent was determined by dividing the eluted mAb amount by the amount of mAb loaded onto the column according to Equation 11.

Equation 11

$$\text{Recovery (\%)} = (c_E * V_E)/ (c_L * V_L) * 100 \quad (11)$$

c_E = mAb concentration in eluate (mg/ml)
V_E = Eluted volume (ml)
c_L = mAb concentration in loaded sample (mg/ml)
V_L = Volume of loaded sample (ml)

4.2.3 Chromatography using NS0 cell clarified feed

Bioinvent feed containing 1.4 mg/ml IgG was used as start material. 40 ml feed corresponding to a load of 28 mg/ml gel was applied to a Tricorn 5/100 column packed with MabSelect SuRe to a column volume of 2 ml. The feed was loaded using a 50 ml super-loop. A flow velocity of 250 cm/h was used throughout the method. After sample-loading the gel was washed with 3 CV of loading buffer followed by 5 CV of the specific wash buffer listed in Table 4. The column was washed with 4 CV of loading buffer before eluting the protein with 20 mM citrate, pH 3.7. After the elution the column was regenerated with 0.1M citrate, pH 3.0 before a CIP with 0.5M NaOH was done. The column was then re-equilibrated with loading buffer. The flow through, wash and elution were collected. The pH in the eluate was adjusted to 5.0 by addition of 1M Tris-HCl, pH 8.0. The elution fractions were analysed for HCP-concentration by NS0-ELISA. Start material, wash fractions and elution fractions were analysed for IgG concentration by the MabSelect SuRe concentration determination method. The IgG concentration was also determined by measuring the UV absorbance at 280 nm in the elution fractions by an Ultrospec 6300pro Spectrophotometer (Amersham Biosciences serial no 93942). The Beer-Lambert law (Equation 10) was used for determination of the mAb concentration in the eluates and the recoveries from the runs with different intermediate wash buffers were calculated by using Equation 11.

Table 4 The different intermediate wash buffers tested on column with NS0 clarified feed

Additive	Buffer	NaCl (M)	pH
No additive (control)	20 mM phosphate	0.15	7.4
0.5M arginine	20 mM citrate	0.5	5.0
0.5M arginine	25 mM phosphate	0.5	7.0
0.1% Tween 20	20 mM citrate	0.5	5.0
0.1% Tween 20	25 mM phosphate	0.5	7.0
5% isopropanol	20 mM citrate	0.5	5.0
5% isopropanol	25 mM phosphate	0.5	7.0
No additive	25 mM phosphate	1	7.0
20% propylene glycol	20 mM citrate	0.5	5.0
20% propylene glycol	25 mM phosphate	0.5	7.0

4.3 Protease stability

The protease stability for the SuRe ligand was compared to the protease stability for recombinant Protein A after labeling with different fluorophores. After pooling of the labeled proteins they were incubated with different concentrations of chymotrypsin for different times. The SuRe ligand and rProtein A were also incubated in CHO-cell lysate at both neutral and acidic pH.

4.3.1 Labeling of rProtein A and SuRe ligand

SuRe ligand (GE Healthcare Bio-Sciences produced by Repligen Corporation) and rProtein A (GE Healthcare Bio-Sciences produced by Repligen Corporation) of concentrations 1.5 mg/ml were desalted on NAP™ 10 gel filtration columns (Amersham Biosciences prod no 17-0854-01) and eluted in concentrations of 1 mg/ml. 20 mM $NaHCO_3$ was used for equilibration of, and elution from, the NAP columns. 125 µl of each protein, SuRe ligand and rProtein A, were then incubated with 1 µl Dye stock solution, CyDye™ DIGE Fluor Cy3 (Amersham Biosciences prod no RPK0273) and CyDye™ DIGE Fluor Cy5 (Amersham Biosciences prod

no RPK0275) in four combinations: Cy3 labeled SuRe ligand, Cy5 labeled SuRe ligand, Cy3 labeled rProtein A and Cy5 labeled rProtein A, for 30 minutes. During the incubation the protein vials were stored in the refrigerator wrapped up in aluminium foil to avoid light exposure which would lead to degeneration of the CyDye.

4.3.2 Incubation of rProtein A and SuRe ligand in different concentrations of chymotrypsin

The Cy5 and Cy3 labeled SuRe ligand and rProtein A were pooled, Cy3 labeled SuRe ligand with Cy5 labeled rProtein A or Cy5 labeled SuRe ligand with Cy3 labeled rProtein A, and incubated in chymotrypsin of concentrations: 0 (control), 1 and 5 µg/ml for 0-68.5 hours. The labeled proteins were also incubated in 0 (control), 0.1, 1 and 10 µg/ml chymotrypsin for 60 minutes. After incubation the proteins were analyzed by SDS-PAGE.

4.3.3 Incubation of rProtein A and SuRe ligand in CHO-cell lysate

The above experiment was repeated but this time the labeled proteins were incubated in CHO-cell lysate. Flow through fractions from loading of CHO-cell lysate spiked with IgG to a MabSelect SuRe column was used (4.2.2). The flow through was first filtered through a Sterivex-HV 0.45 µm Filter followed by a Sterivex-HV 0.22 µm Filter to remove any unwanted substances. The proteins were incubated in the flow through at both neutral and acidic pH. To obtain the acidic pH, one volume of 1M acetate buffer pH 3.5 was added to eight volumes of flow through (1:9). Cy3 labeled SuRe ligand/Cy5 labeled rProtein A and Cy5 labeled SuRe ligand/Cy3 labeled rProtein A were stored in flow through both at pH 7 and pH 3.5 for 0-168 hours, two controls: Cy3 labeled SuRe ligand/Cy5 labeled rProtein A + Cy5 labeled SuRe ligand/Cy3 labeled rProtein A were stored in PBS pH 7 and in acetate buffer pH 3.5 for 0-168 hours. The pooled proteins were also incubated in the same flow through material at pH 5 and for a shorter incubation time, 0-18 hours (23). After incubation the pooled proteins were analyzed by SDS-PAGE.

4.4 Analytical methods

4.4.1 SDS-PAGE

4.4.1.1 96-well screening and column chromatography

The samples were reduced by mixing them with the reducing agent DTT in sample buffer containing 10 mM Tris, 1.0 mM EDTA, 2.5% SDS, pH 8 either 1:1 or 1:3 and put in a 95ºC heating block for 5 minutes before application onto PhastGels gradient 8-25 (GE Healthcare Bio-Sciences product no 17-0542-01). After separation the gels were automatically Coomassie stained in the development unit of the Phast system by running a program that first washed the gels with fixing solution (containing 30% EtOH, 10% acetic acid), then with Coomassie stain solution (containing 0.05% PhastGel Blue R in 10%MeOH, 9% acetic acid, 2% ammonium sulphate), followed by destain solution (containing 10% acetic acid). The gels were finally washed with 20% glycerol and MilliQ water.

4.4.1.2 Protease stability study

The samples taken out after different incubation times were mixed 1:1 with the reducing agent DTT in sample buffer containing 10 mM Tris, 1.0 mM EDTA, 2.5% SDS, pH 8. The samples were then put in a 95ºC heating block for 5 minutes before the proteins were separated on homogenous 20 SDS PhastGels (GE Healthcare Bio-Sciences, product no 17-0624-01). To the samples incubated in acetate buffer, 2 µl of 2M Tris buffer was added to increase the pH before analyzed on SDS PhastGels. After the separation the gels were scanned using a

Typhoon™ 9410 scanner (Amersham Biosciences) to be able to see the fluorescence colours. The scanned images were analysed in the software ImageQuant™ and ImageQuant Tools.

4.4.2 ELISA

NS/0 HCP-ELISA and CHO HCP-ELISA (Cygnus Technologies, Inc, product no F220 and F015 respectively) was used for measuring of the HCP concentration. The High Sensitivity Protocol (according to manufacturer's protocol) was used for both ELISAs.
To the samples analyzed with the CHO HCP-ELISA a storage solution consisting of 2M Tris, pH 8.0, 0.5% Tween, 1% BSA, 0.2% acid was added to a final concentration of 10%. For the samples to be analysed by the NS/0 HCP-ELISA the same storage solution but without acid was used.

4.4.3 Analytical gel filtration

A Superdex™ 200 10/300 GL column (GE Healthcare Bio-Sciences, product no 17-5175-01) was used. The column was equilibrated with loading buffer 10 mM phosphate buffered saline (PBS) (Sigma, P4417-100TAB) at a flow of 0.5 ml/min. 50 µl of sample was injected onto the column and the protein was eluted using isocratic elution, meaning that the same buffer was used throughout the whole method, for both loading and elution. The UV absorbance at 280, 215 and 410 nm was measured.

4.4.4 IgG concentration determination using the MabSelect SuRe method

The IgG concentration in NS0-feed, flow through, wash- and elution fractions from the runs with different intermediate wash buffers was determined. A 1.0 ml MabSelect SuRe HiTrap™ column was used. Before the runs a standard curve was made by injecting IgG standards in the concentration interval 0-2.0 mg/ml. This made the method reproducible in the range 0.1-2 mg/ml. 50 µl of sample was injected onto the column using a flow of 1 ml/min. 10 mM PBS was used as loading buffer. The protein was eluted with 50 mM phosphoric acid, pH 3.0 using step-wise elution. In a control run a sample with 1.0 mg IgG/ml was injected onto the column. A blank sample was also added, containing PBS to further detect unwanted matrix effects. The absorbance at 280, 215 and 400 nm was measured and the peak at 280 nm integrated. The results were evaluated using the Quantitate function in UNICORN, where the protein concentration was determined.

4.5 Chemicals

Chemicals used are listed in Table 5.

Table 5 The chemicals used in the experiments

Chemical	Supplier	Product no
Arginine	Fluka Biochemical	11010
Glycine	Merck	1.04201.0250
Tryptophan	Fluka Biochemical	93660
Urea	Merck	1.08487.1000
Isopropanol	Merck	1.09634.1011
Tween 20	Aldrich	P7949
NaCl	Merck	1.06404.1000
Phosphoric acid	Merck	1.00573.2500
NaH_2PO_4	Merck	28013.264
Caprylic acid	Sigma	C2875
Propylene glycol	Aldrich	13,436-8
Tris	Merck	1.083820500
Citric acid	Merck	1.00244.0500
Acetic acid	Merck	1.00063.2511 or 1.00063.1011

NaOH	Merck	1.06469.1000
Chymotrypsin	Sigma-Aldrich	C-3142
EtOH	Kemetyl AB	3062T
Ammonium sulphate	Merck	1.01217.1000
Glycerol	Merck	1.04094.1000
NaAc x $3H_2O$	Merck	1.06267.1000
$Na_2S_2O_3$ x $5H_2O$	Riedel-de-Haen	13479
Silver nitrate	Merck	1.01512.0100
Formaldehyde	Merck	1.04003.1000
Glutaraldehyde	Fluka Biochemical	348514/1
Na_2-EDTA x $2H_2O$	Merck	1.08418.0250
Na_2CO_3	Merck	1.06392.1000
PBS	Sigma	P4417-100TAB
$NaHCO_3$	Merck	1.06329.1000

5 Results and discussion

5.1 Screening of 96 intermediate wash buffers using the 96-well format

Purification of IgG using Protein A chromatography for capture results in a highly purified product after one single chromatography step. It is possible to even further increase the mAb purity during capture on MabSelect SuRe by introduction of an intermediate wash step after loading of feed, and before elution of the bound mAb. In this study, 96 different intermediate wash buffers (Table 7 in appendix 10.1) with different additives, salt concentrations and pHs were screened using the 96-well format. A CHO-cell lysate without mAb was added to a 96-well plate filled with MabSelect SuRe. The wells were washed in different steps with loading and intermediate wash buffer. The intermediate wash fractions were collected and analysed using Coomassie stained SDS-PAGE to find out which wash buffers were efficient for clearance of CHO-cell proteins from the MabSelect SuRe resin.

5.1.1 Ranking of wash buffers

The wash fractions that contained most HCP and thus showed most bands on the SDS-PAGE, were considered to be efficient for removal of HCP. The wash buffers were ranked from 1 to 4 or from (–) to (++) where 1 (-) is the buffer the least efficient, and 4 (++) is the buffer the most efficient for HCP removal. The rating of the buffers was performed in the following way: The buffers where no bands were seen were ranked as 1 or (-) buffers. Buffers where weak bands were seen were ranked as 2 or (0) buffers. Buffers where strong bands were seen and also an additional band was darker were ranked as 3 or (+) buffers. For the most efficient buffers, 4 or (++), strong bands were seen and the additional band was darker than for the (+) buffers (Figure 9).

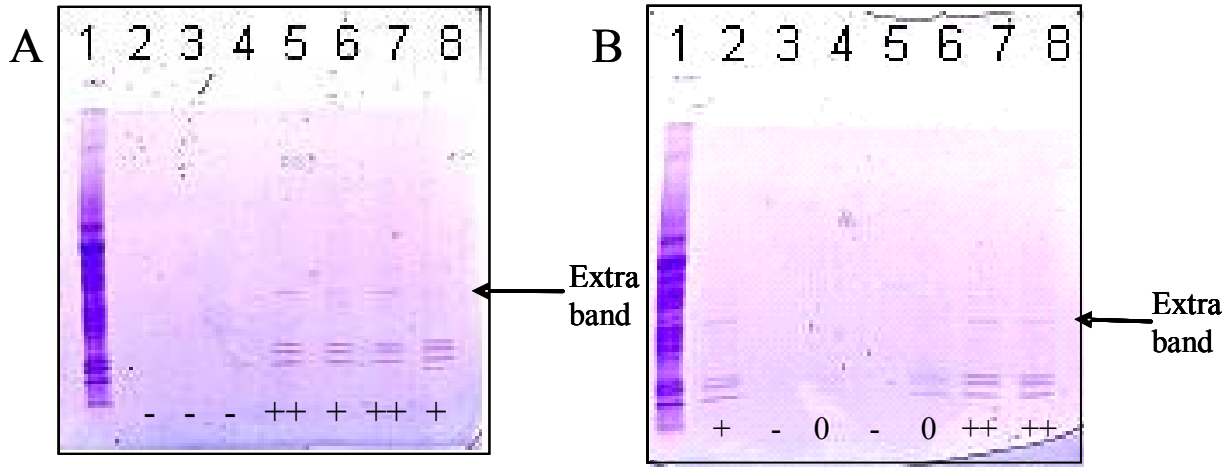


Figure 9 SDS-PAGE on wash fractions from different intermediate wash buffers. A screening of 96 different wash buffers was done on 96-well plates and the fractions from the wash with different intermediate wash buffers were analysed with SDS-PAGE. The different wash buffers were ranked from 1 (-) to 4 (++) where ++ is the buffer most efficient for HCP removal and – the buffer least efficient. The fractions from the different wash buffers shown on these gels are: **A 1)** CHO-cell lysate (start material) **2)** 1% isopropanol pH 7.0 (-) **3)** 5% isopropanol pH 5.0 (-) **4)** 5% isopropanol pH 7.0 (-) **5)** 1% isopropanol, 0.5 M NaCl, pH 5.0 (++) **6)** 1% isopropanol, 0.5 M NaCl, pH 7.0 (+) **7)** 5% isopropanol, 0.5M NaCl, pH 5.0 (++) **8)** 5% isopropanol, 0.5M NaCl, pH 7.0 (+) **B 1)** CHO-cell lysate (start material) **2)** 50mM tryptophan, 1M NaCl, pH 5.0 (+) **3)** 1M urea pH 5.0 (-) **4)** 1M urea pH 7.0 (0) **5)** 2M urea pH 5.0 (-) **6)** 2M urea pH 7.0 (0) **7)** 1M urea 0.5 M NaCl pH 5.0 (++) **8)** 1M urea, 0.5 M NaCl, pH 7.0 (++)

5.1.2 Characteristics of efficient wash buffers

From the 96 buffer screening it was apparent that some additives in combination with NaCl at pH 5.0 were most efficient for HCP removal from MabSelect SuRe. Additives that were most effective were 0.5-2M arginine, 0.5-2M glycine, 1M urea, 0.1-0.5% Tween 20, 1-5% isopropanol and 20% propylene glycol in combination with 0.5M NaCl. The pH 5.0 buffers with this combination of additive and salt concentration were classified as (++) buffers. The same combinations of additive and salt concentration at pH 7.0 were a little less effective and generally classified as (+) buffers. The control, 20 mM phosphate, 0.15M NaCl, pH 7.4, the buffer also used for equilibration of the Protein A gel and loading of feed, was classified as a (–) buffer. No bands were seen on the SDS-PAGE gel for that wash fraction which showed that no HCP was removed in the wash. This was expected because the loading buffer should not remove HCP to any larger extent.

All 96 wash buffers tested as well as the ranking from (-) to (++) according to the description above (5.1.1) can be seen in Table 7 in Appendix 10.1. The SDS-PAGE gels for all buffers are presented in Appendix 10.2.

5.1.3 Alternative analytical methods for evaluation of the wash buffer screening

Other analytical methods that were more quantitative than SDS-PAGE were also evaluated in the 96 buffer screening.

5.1.3.1 UV absorbance at 280 nm in wash and eluate fractions

The UV absorbance at 280 nm was measured on the wash and elution fractions collected in the screening. High absorbance in a fraction would correspond to a high protein (HCP) concentration. For the buffers with highest UV absorbance at 280 nm in the wash fractions one would have expected a low absorbance in the elution fractions and vice versa. Some differences were observed between different wash buffers. However the expected inverted

correlation between the UV absorbance in wash and elution was not seen. Despite subtraction of the background from the wash buffers it appeared as some wash buffer additives disturbed the absorbance measurements and these values could not be trusted. Such buffer additives were especially arginine, where the UV absorbance was very high and the values increased with increasing arginine concentration. Tryptophan seemed to bind to the resin or surface/filter of the 96-well plate leading to high absorbance values in these eluates. Measuring of the UV absorbance in wash and elution fractions would have been a fast and easy way to classify the wash buffers but since the buffer additives themselves absorb UV differently at 280 nm and the protein (HCP) concentration is very low this was not feasible.

5.1.3.2 CHO HCP-ELISA on intermediate wash and elution fractions

CHO HCP-ELISA was also used for measurement of HCP concentrations in some wash and eluate fractions from the 96-well plate. However, the eluates contained very low HCP concentrations of a few ng/ml and no differences could be seen between the different wash buffers. For the wash fractions, differences were seen between the different wash buffers but it was suspected that the buffer additives affected the ELISA in different ways. For example, arginine seemed to generate false low values, as arginine added to one of the standards, i.e. a sample of known HCP concentration, resulted in almost no HCP concentration in the ELISA. Therefore, it was decided only to analyze eluates by ELISA.

Consequently, using this CHO-cell lysate as start material, Coomassie stained SDS-PAGE seemed to be the best option for analysis. Further silver-staining of the Coomassie stained SDS-PAGE gels was also performed but did not result in any additional information.

5.1.4 Recovery

Twenty promising intermediate wash buffers ranked as (+) or (++) and the control with loading buffer rated as (-) in the 96-buffer screening were tested in the 96-well format with pure IgG to study whether the wash negatively affected the mAb recovery or not. 400 µg of mAb was loaded to each well and the amount of protein in flow through, and elution was determined by measuring of the UV absorbance at 280 nm. The recovery and the relative amount of protein in the flow through and wash were determined. The relative amount of IgG that was lost in the wash was determined by assuming that all mAb that was neither eluted nor found in the flow through was lost during the wash. Most of the wash buffers had no negative effect on the recovery as compared to the control (20 mM phosphate, 0.15M NaCl, pH 7.4). However two of the buffers: 0.5M arginine, 0.5M NaCl, pH 5.0 and 20% propylene glycol, 0.5M NaCl at pH 5.0 showed a decrease in recovery (Figure 10). It was later shown chromatographically that the recovery was decreased when different additives in combination with 0.5M NaCl at pH 5.0 was used (5.2.2).

Figure 10 also show that 15.9 (±1.4) % of the protein ended up in the flow through. This was due to the low binding capacity of the media in the 96-well plate. Less than 200 µg of mAb could be loaded to each well (50 µl gel) to obtain 100% recovery, and thus no loss of protein in the flow through. The low capacity in the 96-well format is further discussed in paragraph 5.5.3.

Measuring of the UV absorbance at 320 and 410 nm is useful for detection of aggregates and precipitate. Looking at this data, no differences were seen between the different elution fractions, which indicated that neither of the wash buffers affected the mAb in such a way that they formed aggregates or precipitated.

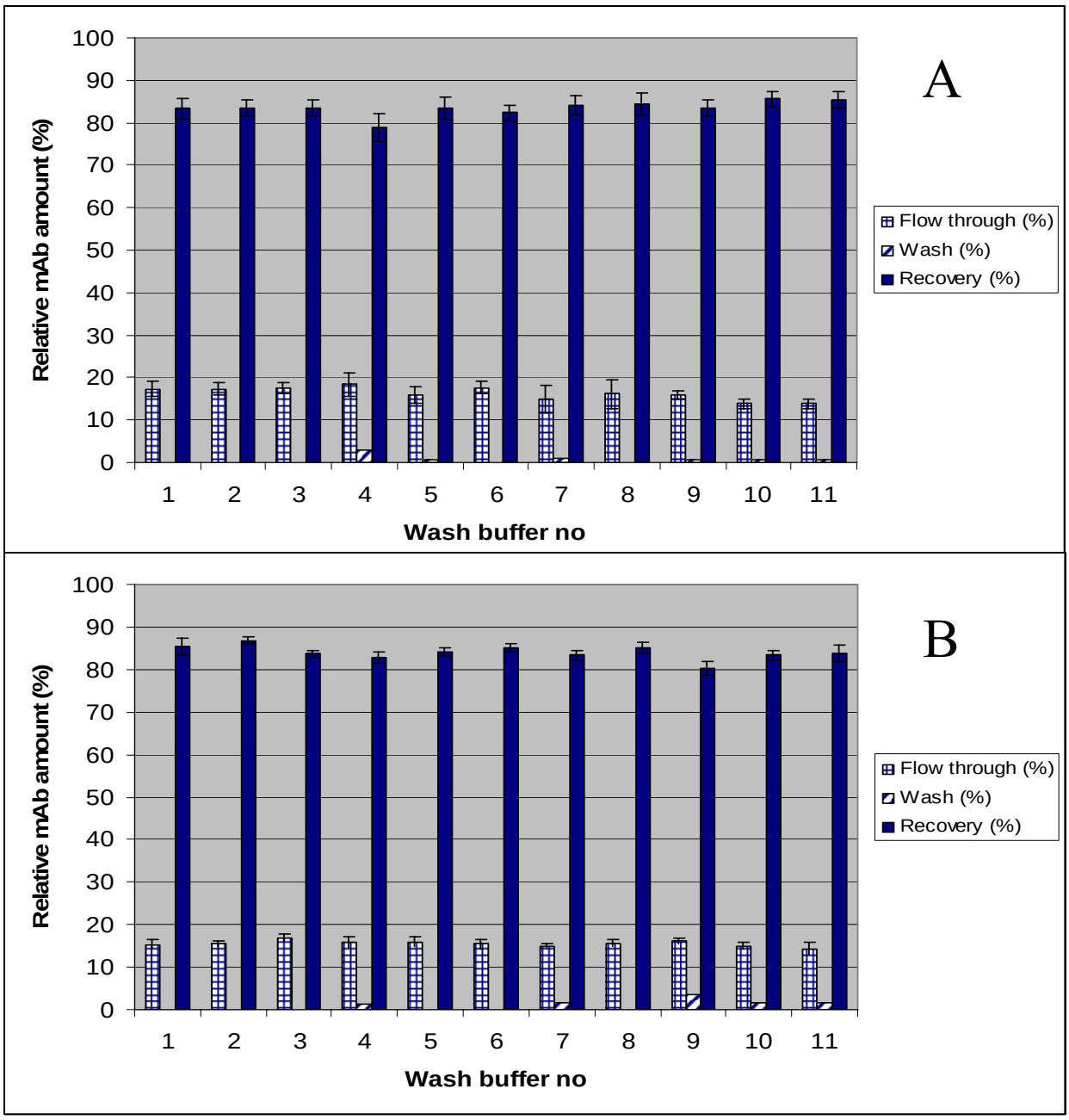


Figure 10 Recoveries after loading pure IgG to the 96-well plate and washing with different intermediate wash buffers. 400 µg IgG per well was loaded onto a 96-well plate. The gel was washed with loading buffer, intermediate wash buffer and then again with loading buffer before elution of IgG. The different intermediate wash buffers tested here were: **A 1)** 20 mM phosphate, 0.15M NaCl, pH 7.4 **2)** 2M arginine, pH 7.0 **3)** 0.5M arginine, 0.5M NaCl, pH 7.0 **4)** 0.5M arginine, 0.5M NaCl, pH 5.0 **5)** 2M glycine, pH 5.0 **6)** 0.5M glycine, 0.5M NaCl, pH 7.0 **7)** 0.5M glycine, 0.5M NaCl, pH 5.0 **8)** 1M urea, 0.5M NaCl, pH 7.0 **9)** 1M urea, 0.5M NaCl, pH 5.0 **10)** 1% isopropanol, 0.5M NaCl, pH 7.0 **11)** 1% isopropanol, 0.5M NaCl, pH 5.0. **B 1)** 20 mM phosphate, 0.15M NaCl, pH 7.4 **2)** 0.1% Tween 20, 0.5M NaCl, pH 7.0 **3)** 0.1% Tween 20, 0.5M NaCl, pH 5.0 **4)** 1M NaCl, pH 7.0 **5)** 1M NaCl, pH 5.0 **6)** 0.5M NaCl, pH 5.0 **7)** 25mM caprylic acid, 1M NaCl, pH 7.0 **8)** 20% propylene glycol, 0.5M NaCl, pH 7.0 **9)** 20% propylene glycol, 0.5M NaCl, pH 5.0 **10)** 0.3M citrate, pH 5.0 **11)** 1.2M acetate, pH 5.0. The recoveries for the runs with different intermediate wash buffers were calculated (filled bars). The relative amount IgG lost in the wash was determined by assuming that all mAb that was neither eluted nor found in the flow through (checked bars) was lost during the wash (striped bars). The recoveries and relative amount in the flow through were plotted with the standard deviation. As can be seen in A, some mAb was lost in the wash with buffer number 4, 0.5M arginine, 0.5M NaCl, pH 5.0, and in B with wash buffer number 9, 20% propylene glycol, 0.5M NaCl, pH 5.0, otherwise the losses were small.

5.2 Chromatography using NS0-feed compared with 96-well screening with CHO-cell lysate

The main purpose of this project was to determine whether the 96-well format could be used for screening of chromatographic buffer conditions or not. To be able to answer this question the result from the 96 buffer screening using the 96-well format was verified chromatographically. Nine wash buffers from the 96-well screening were selected together with the control. Apart from the control buffer which was a (-) buffer only promising buffers i.e. (+) and (++) buffers were selected. It would have been preferable to use the same start material for the chromatographic verification that had been used in the 96-well screening. Therefore, CHO-cell lysate spiked with pure mAb was first tested. However, it was suspected that the column and/or the mAb were degenerated by the CHO-cell lysate since very low recoveries were obtained even without wash with an intermediate wash buffer (paragraph 5.3). An NS0-feed containing mAb was therefore used for the chromatographic verification instead of the CHO-cell lysate. The feed was loaded onto a Tricorn 5/100 column packed with MabSelect SuRe with an asymmetry of 1.06 and 4825 N/m number of plates. The column was then washed with loading buffer and intermediate wash buffer and finally with loading buffer again before elution of the mAb. The eluates were analyzed for IgG and HCP concentrations. The purity in ppm (ng HCP/mg mAb) and the mAb recovery was calculated for each chromatographic run.

5.2.1 Purity

All nine wash buffers tested chromatographically removed more HCP and thus resulted in a purer product than, wash with loading buffer. The pH 5.0 buffers were more effective for removal of HCP than the pH 7.0 buffers containing the same buffer additive and NaCl concentration (Figure 11). This was in accordance with the 96-well screening. In the 96-well screening the intermediate wash buffers were classified as (-), (0), (+) and (++) buffers where (++) were the most effective wash buffers. The wash buffers classified as (++) in the 96-well screening removed more HCP than a (+) buffer when tested in column chromatography. Looking at the same buffer additives at the different pHs they were rated (++) buffers at pH 5.0 and (+) buffers at pH 7.0, except for 0.5M arginine, 0.5M NaCl where both pH 5.0 and pH 7.0 were rated as (++) buffers (Figure 11). 0.5M arginine, 0.5M NaCl, pH 7.0 was considered a false positive but both the pH 5.0 and pH 7.0 buffers with arginine and NaCl was well working wash buffers. Also 1M NaCl at pH 7.0 was classified as a (++) buffer in the 96-well screening, but during chromatography this wash buffer showed the same level of HCP in the eluate as a typical (+) buffer. A buffer that is a false positive in the 96-well screening such as 0.5M arginine, 0.5M NaCl, pH 7.0 or 1M NaCl, pH 7.0 can be excluded after column chromatography.

The results between the two formats are in great accordance which leads to the conclusion that the 96-well format can be used for screening of chromatographic buffer conditions. By using this format a lot of process development time can be saved. Materials such as protein, feed and buffers will also be saved because of the small volumes consumed.

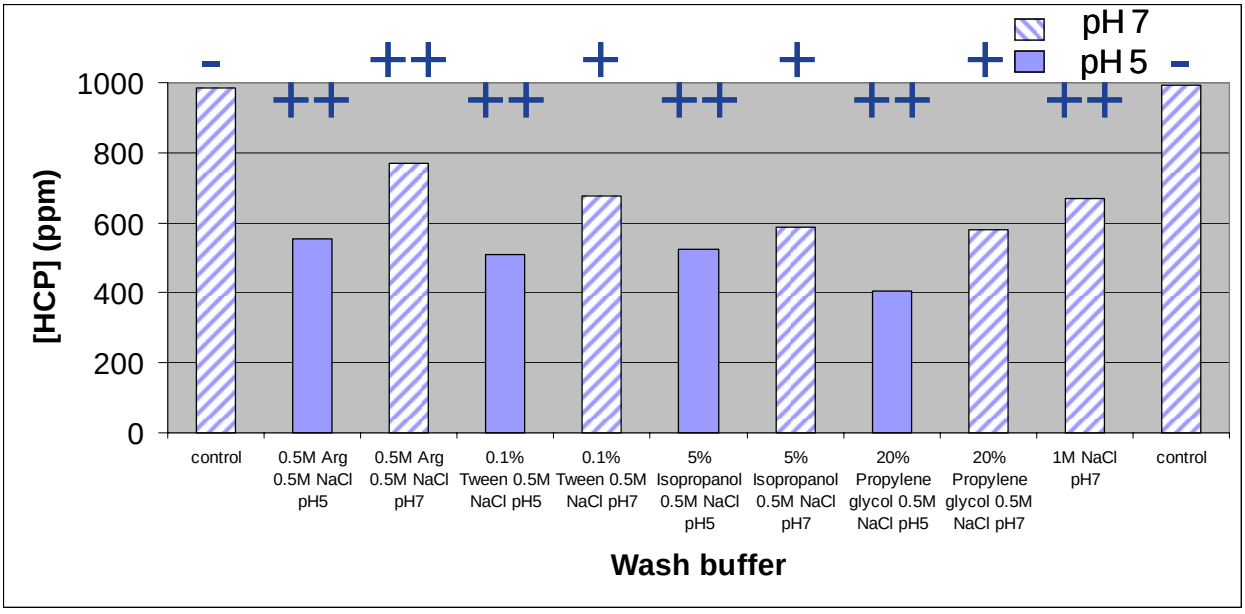


Figure 11 Comparison of chromatography with 96-well screening. The bars in the diagram show the HCP concentrations in the elution fractions from the chromatographic runs after wash with different intermediate wash buffers. The bars showing the HCP concentrations after wash with pH 7.0 buffers are striped in the figure whereas the bars for the HCP concentrations in eluates after wash with pH 5.0 buffers are filled. The pH 5.0 buffers removed more HCP in the wash than pH 7.0 buffers, because the HCP concentrations in the eluates after wash with pH 5.0 buffers are lower than the HCP concentrations in the eluates after wash with pH 7.0 buffers for the same additive. In the 96-well screening the intermediate wash buffers were classified as (-), (0), (+) and (++) buffers where (++) are the most effective wash buffers. The ranking of the buffers from the 96-well plate screening is presented above the bars in the figure. When comparing the results from the screening with the column chromatography it was observed that buffers classified as (++) buffers in the screening also removed more HCP in the wash during chromatography than a (+) buffer.

5.2.2 Recovery

In protein purification it is important to obtain a product with high purity but it is also necessary the get a high recovery. A recovery of more than 95% is desirable in a chromatographic step.

The mAb concentration in NS0-feed, wash fractions and elution fractions was determined and the relative amount of mAb in the wash fractions and eluates was calculated. It was observed that intermediate wash with pH 5.0 buffers removed not only HCP but also mAb during the wash, whereas intermediate wash with pH 7.0 buffers did not result in any loss of mAb as compared to the control buffer. The wash buffers that resulted in the highest loss of mAb and consequently the lowest recovery were 0.5M arginine, 0.5M NaCl, pH 5.0 and 20% propylene glycol, 0.5M NaCl, pH 5.0 (Figure 12). These buffers were earlier identified during the 96-well plate screening as buffers that resulted in low recoveries (5.1.4, Figure 10).

It is remarkable that the control run, wash with loading buffer (20 mM phosphate, 0.15M NaCl, pH 7.4), only results in a mAb recovery of approximately 90%. This was probably due to the fact that the delay volume was not correctly adjusted on the ÄKTAexplorer 10 system resulting in partial loss of the elution peaks. Similar runs using the same starting material had earlier resulted in recoveries of approximately 94% (9). However, relating the recoveries in the different chromatographic runs to the recoveries for the controls it was possible to identify wash buffers that did not decrease the recovery. Recoveries equal to the control even though they were approximately 90% were considered to be acceptable.

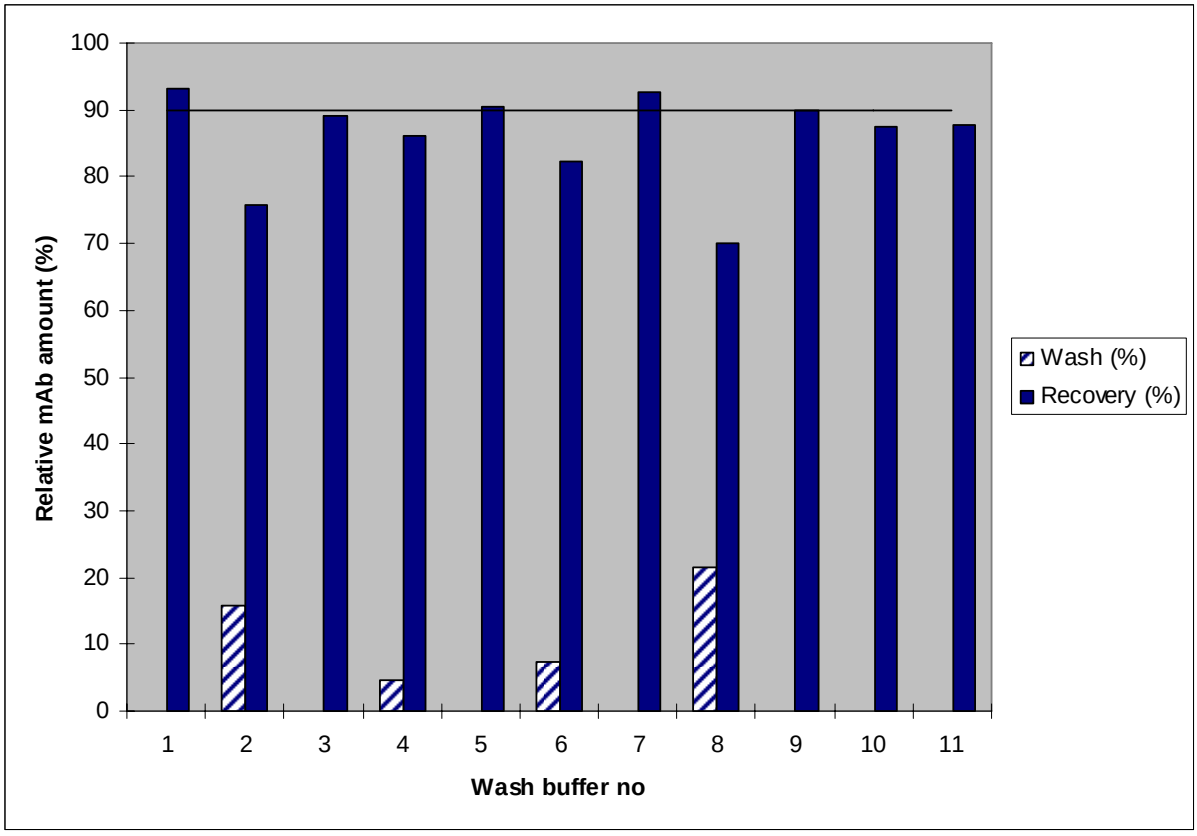


Figure 12 The recovery and the relative amount of mAb that was lost during the intermediate wash in the chromatographic runs NS0-feed was loaded to a column and the gel washed with different intermediate wash buffers before elution. The numbers on the x-axes corresponds to following buffers: **1)** 20mM phosphate, 0.15M NaCl, pH 7.4 **2)** 0.5M arginine, 0.5M NaCl, pH .05 **3)** 0.5M arginine, 0.5M NaCl, pH 7.0 **4)** 0.1% Tween 20, 0.5M NaCl, pH 5.0 **5)** 0.1% Tween 20, 0.5M NaCl, pH 7.0 **6)** 5% isopropanol, 0.5M NaCl, pH 5.0 **7)** 5% isopropanol, 0.5M NaCl, pH 7.0 **8)** 20% propylene glycol, 0.5M NaCl, pH 5.0 **9)** 20% propylene glycol, 0.5M NaCl, pH 7.0 **10)** 1M NaCl pH 7.0 **11)** 20mM phosphate, 0.15M NaCl, pH 7.4. The filled bars show the relative amount of eluted mAb i.e. the recovery for each chromatographic run and the striped bars show the relative amount of mAb that was lost during the intermediate wash. When wash buffers with different additives and 0.5M NaCl at pH 5.0 was used mAb was lost during wash. For the pH 7.0 buffers with the same additives and salt concentration no mAb was lost during wash and the recovery was equal to the recovery obtained in the control run with wash with 20 mM phosphate, 0.15M NaCl at pH 7.4. The relatively low recovery of approximately 90% obtained for the control run was due to an incorrect adjustment of the delay volume on the ÄKTA system.

Comparison of the chromatograms for each run with different intermediate wash buffers revealed differences between different wash buffers. The pH 5.0 buffers showed significant peaks during the intermediate wash. Examples of chromatograms from the runs where 20 mM phosphate, 0.15M NaCl, pH 7.4 (control), 5% isopropanol, 0.5M NaCl at pH 7.0 and 5% isopropanol, 0.5M NaCl at pH 5.0 were used as wash buffer are presented in Figure 13.

For the chromatographic run where loading buffer was used for intermediate wash, i.e. the control run (Figure 13 A) and the run where 5% isopropanol, 0.5M NaCl, pH 7.0 was used for intermediate wash (Figure 13 B) no large peaks were seen during wash. For the run where 5% isopropanol, 0.5M NaCl, pH 5.0 was used (Figure 13 C) a peak was detected when washing with the intermediate wash buffer. This peak represented mainly the mAb that was lost during wash. This pattern was seen for all the pH 5.0 buffers but not for the pH 7.0 buffers.

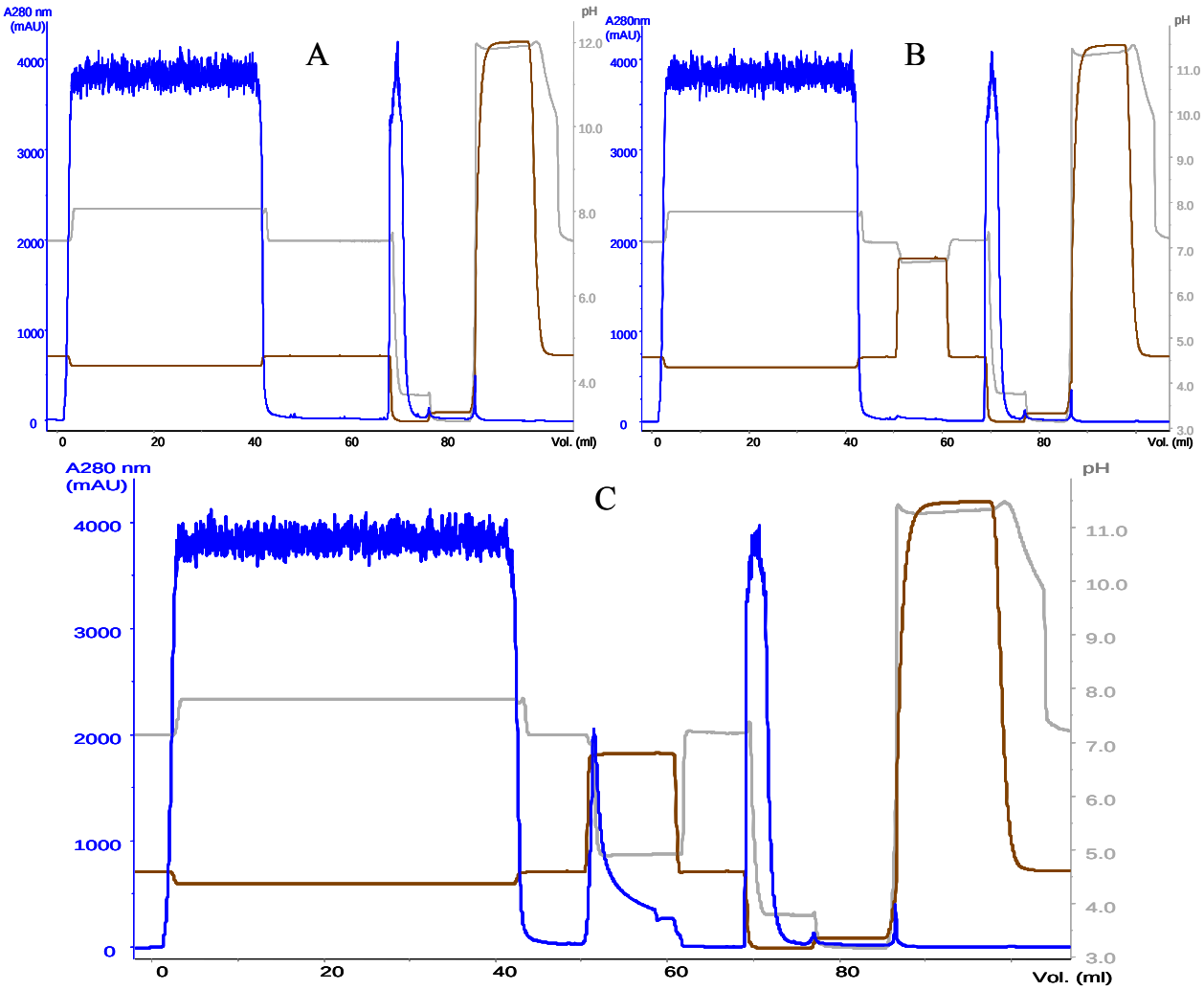


Figure 13 Chromatographic runs with different intermediate wash buffers 40 ml of NS0-feed was loaded onto a Tricorn 5/100 column packed with MabSelect SuRe to a column volume (CV) of 2 ml. This corresponded to a load of 28 mg mAb/ml gel. The feed was loaded onto the column using a 50 ml super-loop. A linear flow velocity of 250 cm/h was used throughout the method. After sample-loading the gel was washed with 3 CV of 20 mM phosphate, 0.15M NaCl, pH 7.4 (loading buffer) followed by 5 CV of the specific wash buffer. The column was washed with additional 4 CV of loading buffer before eluting the mAb with 20 mM citrate, pH 3.7. After elution, the column was regenerated with 0.1M citrate pH 3.0. A CIP was performed with 0.5M NaOH. The blue curve was the UV absorbance measured at 280 nm, the grey and brown curves were the pH and conductivity of the buffer respectively. Wash buffers tested: **A** 20 mM phosphate, 0.15M NaCl, pH 7.4, **B** 5% isopropanol, 0.5M NaCl, pH 7.0, **C** 5% isopropanol, 0.5M NaCl, pH 5.0.

For easier comparison of the chromatograms the wash and elution were zoomed and the curves were overlaid with baselines offset vertically for presentational clarity. In general the pH 5.0 wash buffers had a larger wash peak than the pH 7.0 wash buffers where almost no peak was detected during wash. The zoomed chromatograms for all wash buffers are presented in Figure 14. Size exclusion chromatography (SEC) on the wash fractions revealed that the main content in the wash peak was mAb that was lost during wash. This further verified the decrease in recovery that was seen for the pH 5.0 wash buffers. 20% propylene glycol, 0.5M NaCl, pH 5.0 seemed to elute most mAb in the wash followed by 0.5M arginine, 0.5M NaCl, pH 5.0, 5% isopropanol, 0.5M NaCl, pH 5.0 and 0.1% Tween 20, 0.5M NaCl, pH 5.0 (Figure 15).

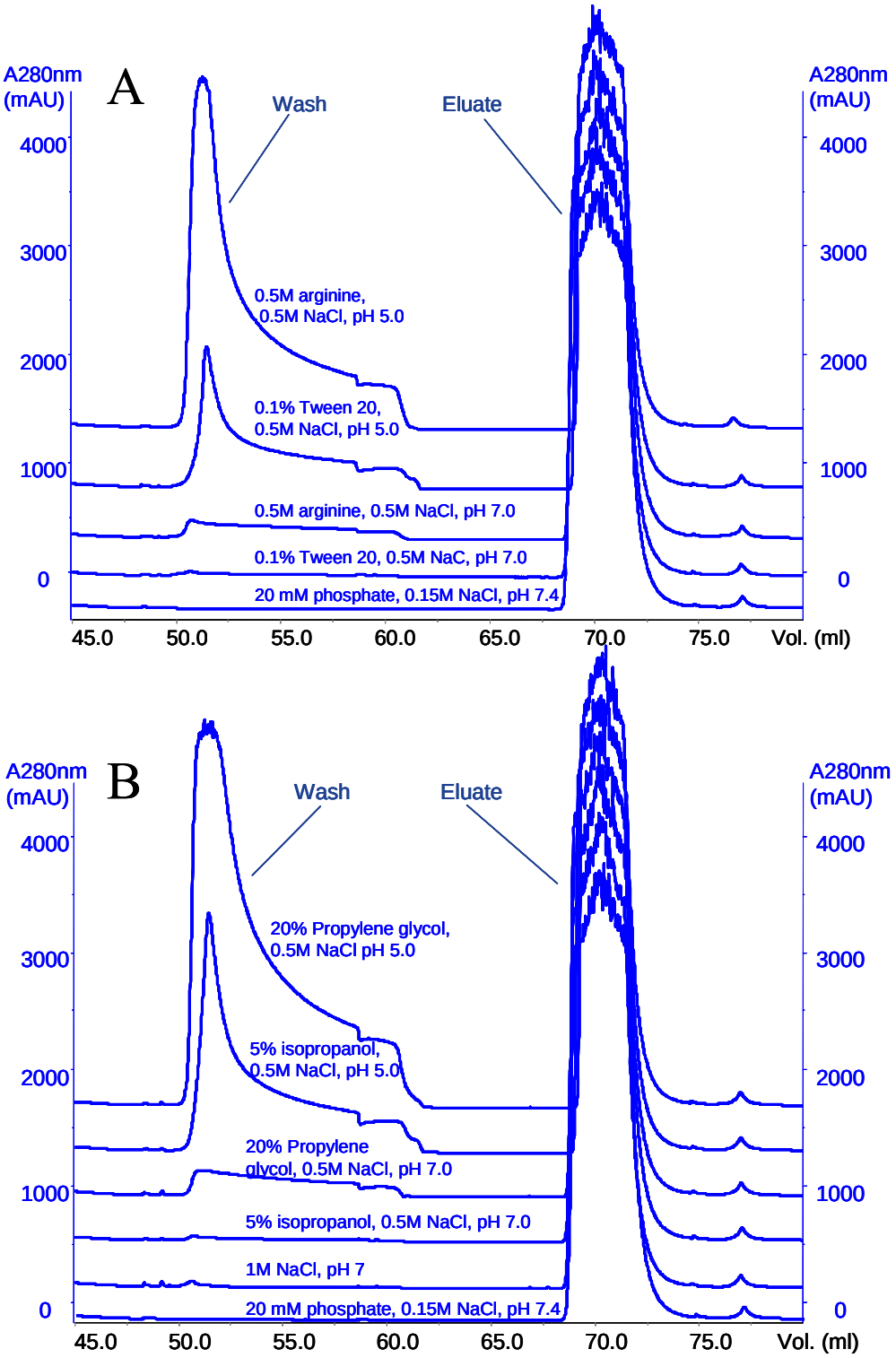


Figure 14 Comparison of the wash and elution peaks for the chromatographic runs with different intermediate wash buffers. Baselines of the chromatograms were offset vertically for presentational clarity. A peak was observed during intermediate wash between 50 and 60 ml for some of the wash buffers. The wash peaks were compared for the different buffers. **A:** 0.5M arginine, 0.5M NaCl, pH 5.0, 0.1% Tween 20, 0.5M NaCl, pH 5.0, 0.5M arginine, 0.5M NaCl, pH 7.0, 0.1% Tween 20, 0.5M NaCl, pH 7.0 and the control 20 mM phosphate, 0.15M NaCl, pH 7.4 **B:** 20% propylene glycol, 0.5M NaCl, pH 5.0, 5% isopropanol, 0.5M NaCl, pH 5.0, 20% propylene glycol, 0.5M NaCl, pH 7.0, 5% isopropanol, 0.5M NaCl, pH 7.0, 1M NaCl pH 7.0 and the control 20 mM phosphate, 0.15M NaCl, pH 7.4**.** As can be seen the peak was larger for the pH 5.0 buffers than for the pH 7.0 buffers. SEC on the wash fractions revealed that the main content in the wash peak was mAb that was washed off (Figure 15).

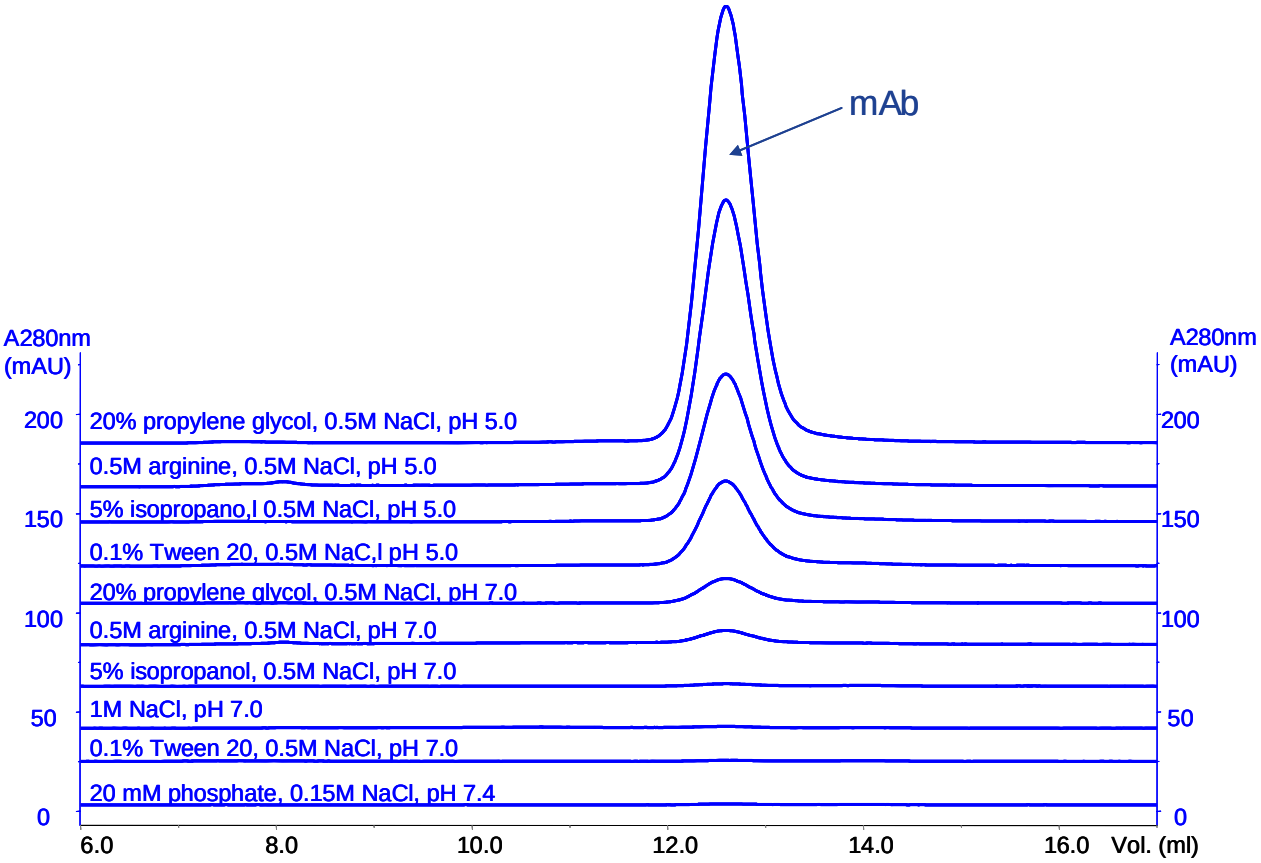


Figure 15 SEC/Gel filtration analysis on wash fractions. The wash fractions from the chromatic runs with different intermediate wash buffers were run on a Superdex 200 10/300 GL gel filtration column. The sample volume was 50 µl and the eluent was PBS buffer, pH 7.4 at a flow rate of 0.5 ml/min. Baselines of the chromatograms were offset vertically for presentational clarity. As can be seen the pH 5.0 buffers washed off more mAb compared to the pH 7.0 buffers. Most mAb was lost when washing with 20% propylene glycol, 0.5M NaCl, pH 5.0 and 0.5M arginine, 0.5M NaCl, pH 5.0.

5.2.3 The optimal wash buffer

An optimal wash buffer should remove as much HCP as possible without washing off the target protein. The NS0-feed, wash and elution fractions from the runs with different wash buffers were analysed with gel filtration. The result for 5% isopropanol, 0.5M NaCl, pH 7.0 is shown in Figure 16. No significant amount of mAb was detected in the intermediate wash and the eluate was very pure. With all these facts, no loss of mAb during the wash, good recovery and pure eluate (<600 ppm according to ELISA), 5% isopropanol, 0.5M NaCl, pH 7.0 seemed to be a good wash buffer. Other additives such as Tween 20, propylene glycol and arginine could also be used.

Different buffer additives such as detergents, solvents, amino acids, etc. in combination with 0.5M NaCl at pH 7.0 gave a significant decrease in HCP levels in the eluates without decreasing the recovery. However, it might be possible to even further improve the wash step by exploring a wider pH and conductivity range in combination with a wider range of buffer additive concentration using Design of Experiment (DoE).

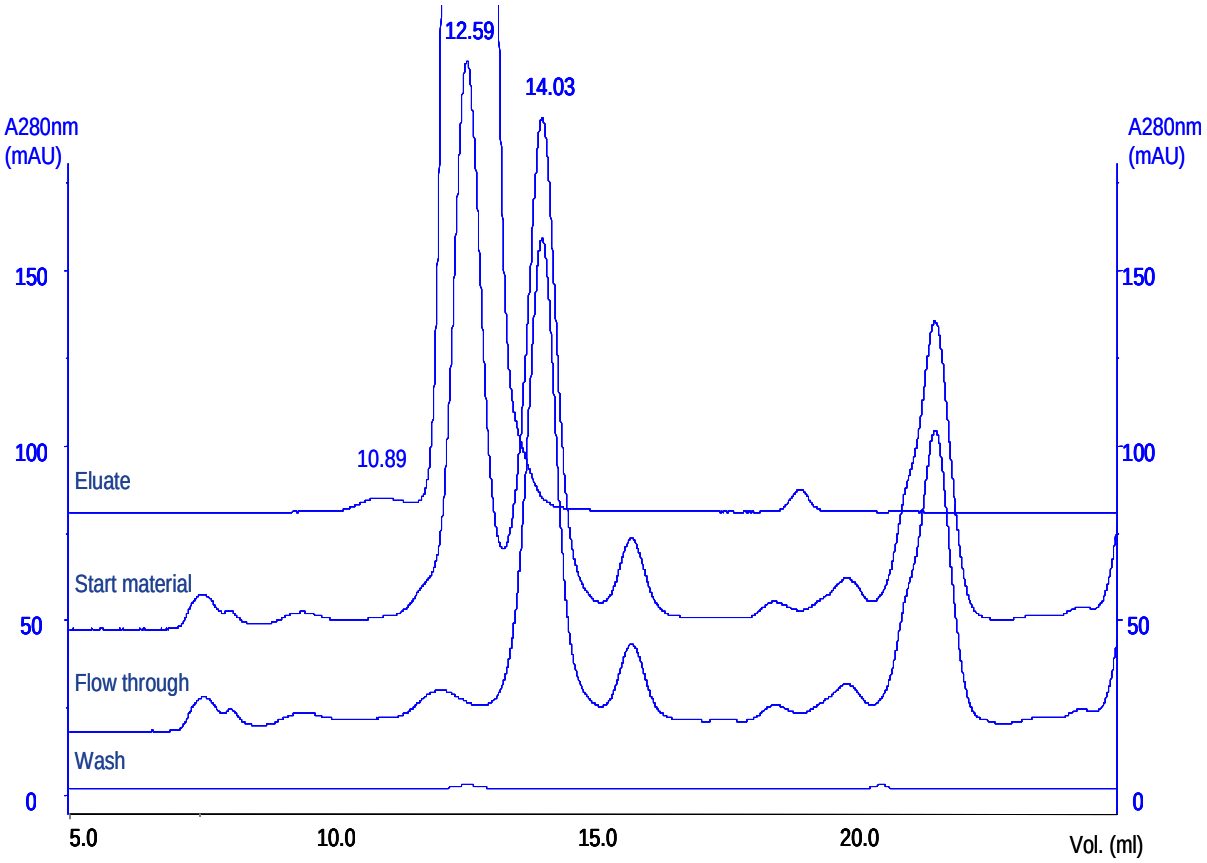


Figure 16 SEC on start material, flow through, wash with 5% isopropanol, 0.5M NaCl, pH 7.0 and elution fractions. The samples were run on a Superdex 200 10/300 GL gel filtration column. The sample volume was 50 µl and the eluent was PBS buffer, pH 7.4 at a flow rate of 0.5 ml/min. Baselines of the chromatograms were offset vertically for presentational clarity. The peak at 12.59 ml represents IgG and the peak at 14.03 ml represents albumin from the FCS in cell culture medium. The start material, second curve from top, contains both mAb and Albumin together with NS0-HCP. The content in the flow through was mainly albumin and HCP (second curve from the bottom). The intermediate wash fraction (bottom curve) contained very little mAb. The eluate (top curve) contains mAb monomer (12.59 ml), and some dimer eluting at 10.89 ml.

5.3 Chromatography using CHO-cell lysate spiked with IgG

The intent was to use the same start material for the chromatographic verification that had been used in the 96-well screening. Therefore, CHO-cell lysate spiked with pure mAb to a concentration of 2 mg/ml was used. The lysate was loaded onto a Tricorn 5/50 packed with MabSelect SuRe (asymmetry 0.99 and 1.01 respectively with 6468 N/m and 7111 N/m) followed by wash with loading buffer and intermediate wash buffer and finally wash with loading buffer again before elution of the mAb. The wash and elution fractions were analysed for HCP concentration using CHO HCP-ELISA. The eluates were also analyzed for IgG concentration. In the 96-well screening the intermediate wash buffers were classified as (-), (0), (+) and (++) buffers, where (++) were the most effective wash buffers. For the chromatographic verification with CHO-cell lysate 4 wash buffers classified as (++) buffers in the screening as well as one (+), one (0) buffer and one (–) buffer was used. The control, 20 mM phosphate, 0.15M NaCl, pH 7.4 classified as a (-) buffer was also included in the study.

Very low recoveries of approximately 85% were obtained in these chromatographic runs even without wash with an intermediate wash buffer. It was later shown (5.6.3.1) that a lot of proteases were present in the CHO-cell lysate and it was possible that these proteases degraded the chromatography resin and/or the mAb and that could explain the very low recovery. The HCP levels in the eluates were very low (7-24 ng/ml) resulting in HCP

concentrations in ppm between 0.5 and 2.0 for all chromatographic runs. The low HCP levels together with the uncertain recovery made it difficult to make use of these data. It was also impossible to evaluate the HCP data in the wash fractions since it seemed like some of the buffer additives affected the ELISA. A control analysis was performed where a known amount of HCP was diluted in buffer containing arginine. This dilution resulted in very low and incorrect HCP concentration in the ELISA. Thus, the conclusion was that only elution fractions and not wash fractions should be analysed by ELISA.

Looking at the chromatograms from these runs, peaks were seen during wash for all intermediate wash buffers except for 0.1% Tween 20, pH 5.0 and the control run with wash with loading buffer where no peaks were detected. Both 0.1% Tween 20, pH 5.0 and loading buffer were classified as (-) buffers in the 96-well plate screening. A lot of proteins seemed to bind very strongly to the column and did not elute with low pH but were released in the CIP with 0.5M NaOH. In the cycles where some protein were removed in the intermediate wash the CIP peaks were smaller than in the runs where (-) buffers were used for wash. Examples of chromatograms from the control run and washing with 0.1% Tween 20, 0.5M NaCl, pH 7.0 as intermediate wash buffer are presented in Figure 17.

The only conclusion that could be drawn from these experiments was that the buffers that were classified as (-) buffers in the screening did not remove large amount of protein during wash. The CHO-cell lysate was not an optimal start material to load to the column though it seemed like the gel matrix and/or the mAb was degraded. That was why further chromatographic verification was performed using a NS0-feed instead (paragraph 5.2).

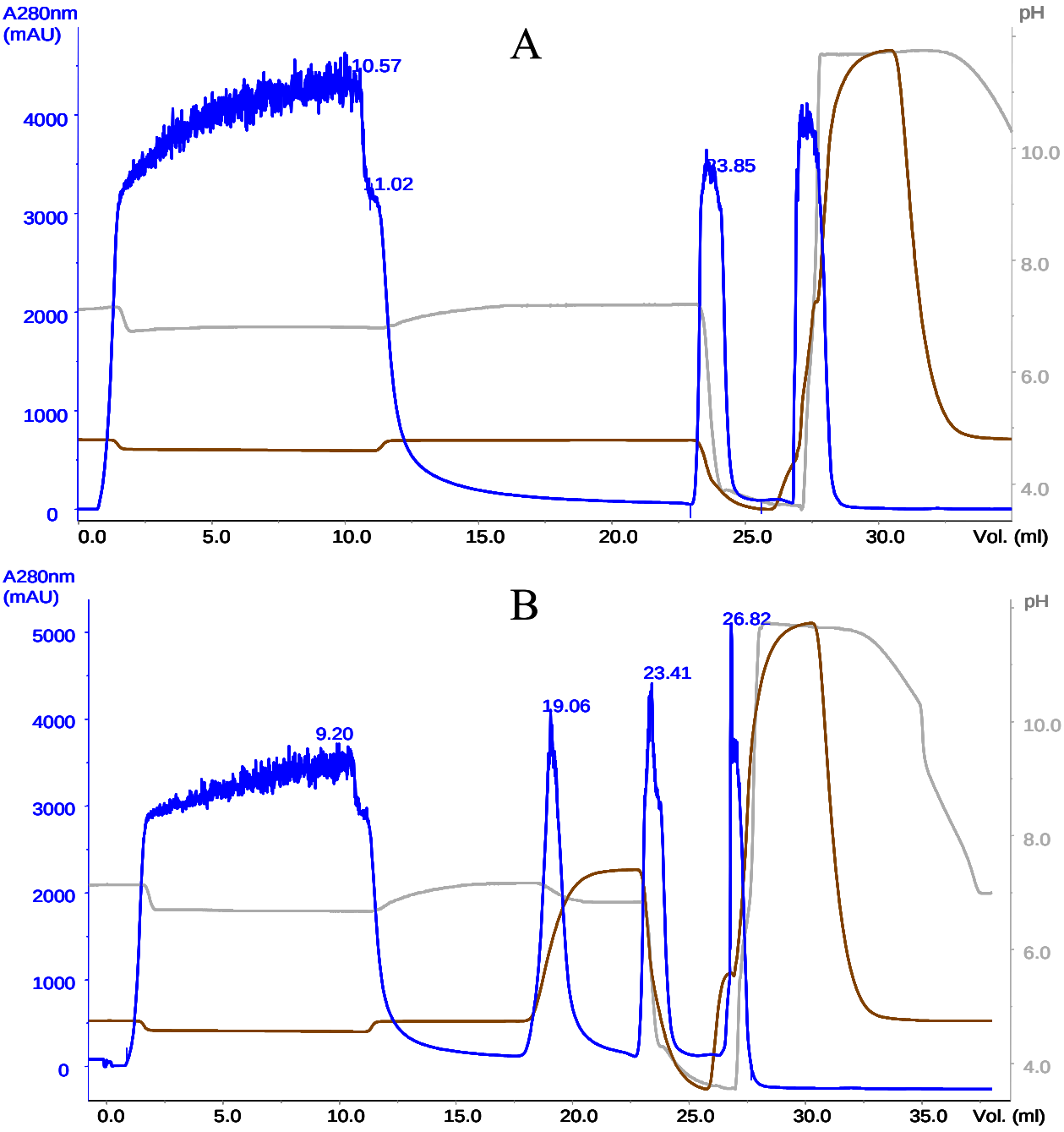


Figure 17 Loading of CHO-cell lysate spiked with 2 mg/ml mAb and washing with different intermediate wash buffers A Tricorn 5/50 column packed with MabSelect SuRe to a CV of 1.0 ml was used and spiked CHO-cell lysate was applied onto the column to a load of 20 mg mAb/ml gel. A 50 ml super-loop was used for loading and a flow velocity of 0.25 ml/min was used throughout the method. After loading of sample a wash with loading buffer was done until the UV absorbance at 280 nm reached the baseline. The gel was then washed with 2 CV of loading buffer followed by 5 CV of the specific wash buffer. The gel was washed with additional 2 CV loading buffer before the protein was eluted with 0.1M Na-citrate pH 3.0 (23 ml). A CIP with 0.5M NaOH was then done on the column and proteins that had bound strongly to the column were removed (27 ml). The blue curve was the absorbance measured at 280 nm, the grey and brown curves were the pH and conductivity of the buffer respectively. Wash buffers tested: **A** 20 mM phosphate, 0.15M NaCl, pH 7.4 (loading buffer) **B** 0.1% Tween 20, 0.5M NaCl, pH 7.0

5.4 NS0-feed loaded to column and wash and elution in 96-well plate

It was known that the capacity in the 96-well plate was low (5.5.3). Only about 3.8 mg mAb/ml gel could be bound in the plate without loss of mAb in the flow through, compared to a dynamic binding capacity of 20-30 mg mAb/ml gel in the column. To be able to see differences in HCP concentrations (ppm level) in the eluates from the plate it was preferable to load and elute as much protein as possible. In order to obtain a higher capacity of the gel the NS0-feed was loaded onto a column packed with MabSelect SuRe. The load was 26.3 mg mAb/ml gel. The pre-loaded gel was then distributed into the wells of a 96-well plate and wash and elution was performed in the plate.

5.4.1 Purity

After wash with loading buffer and intermediate wash buffer, the protein was eluted from the plate in three steps. Since the protein (mAb) concentration was highest in elution fraction 2, this fraction was collected and analyzed for HCP concentration by ELISA. The mAb concentration was also determined in this fraction by using the UV absorbance at 280 nm. The HCP concentration in ppm (ng HCP/mg mAb) was calculated as well as the mAb recovery. Low HCP concentration left in the elution fraction means that a lot of HCP was removed in the wash, which was desirable. The HCP concentration in the eluates differed between different wash buffers. In general the eluates after wash with a certain buffer additive at pH 7.0 had a lower HCP concentration than wash with the same buffer additive at pH 5.0 (Figure 18). Thus, it seemed like the pH 7.0 buffers worked better than pH 5.0 buffers when loading to column and performing wash and elution in the 96-well plate. This was neither in accordance with earlier 96-well screening where the CHO-cell lysate was used nor the column chromatography with NS0-feed. In those studies the pH 5.0 buffers were proven more efficient than pH 7.0 buffers.

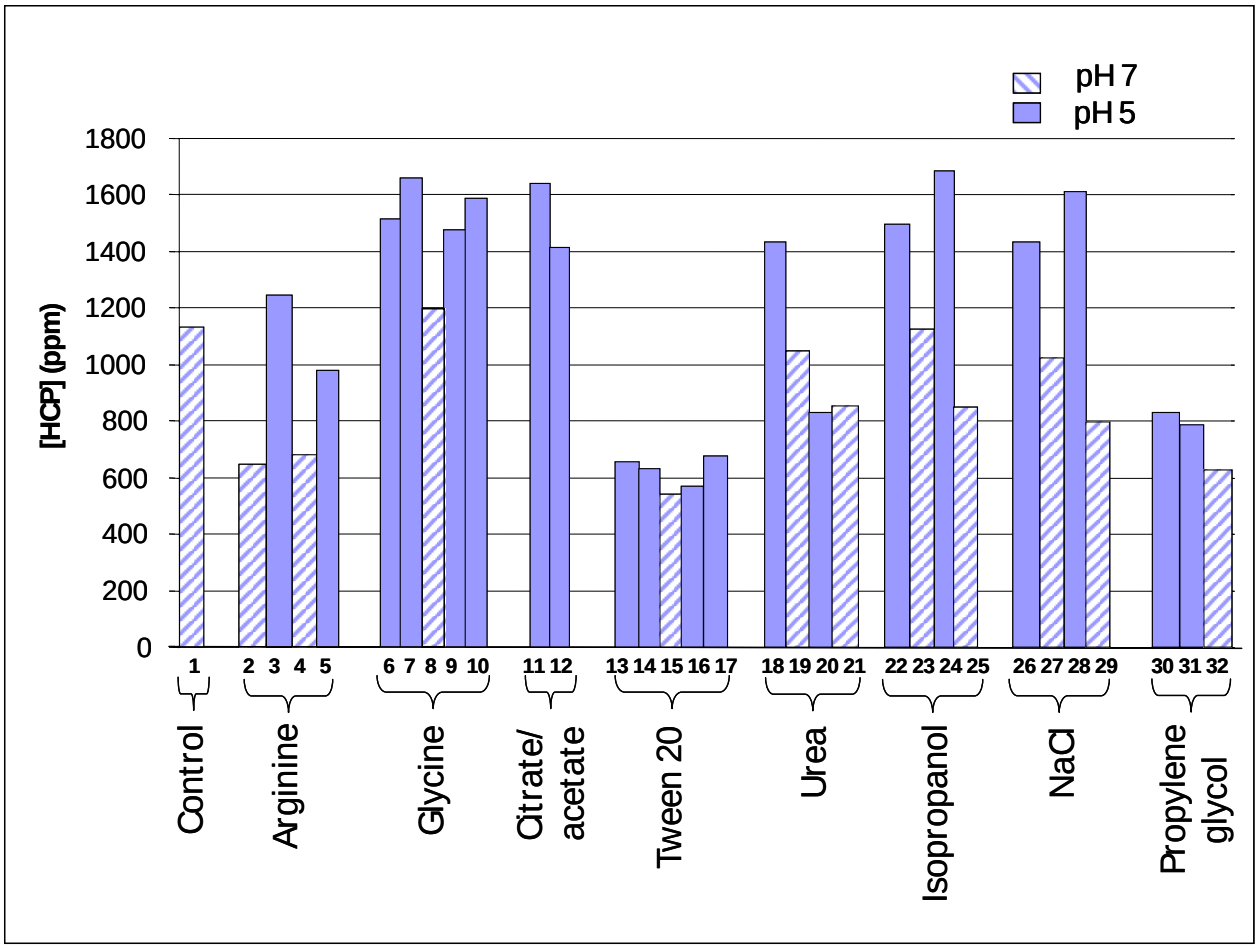


Figure 18 HCP concentration in elution step 2 from loading of NS0-feed onto a column followed by wash and elution in the 96-well plate. The amount of HCP in ppm (ng HCP/mg mAb) in the eluate was calculated after washing with different intermediate wash buffers. The striped bars are elution after wash with pH 7.0 buffers and the filled bars are elution after wash with pH 5.0 buffers. The different wash buffers tested are listed in Table 6 with their numbers 1-32 from the figure. It is desirable for a wash buffer to remove a lot of HCP in the wash. Consequently, the buffers with the lowest HCP concentrations in the eluates should be the most efficient. In this study wash with different buffer additives at pH 7.0 seemed to be more efficient than wash with pH 5.0 buffers. This was neither in accordance with earlier 96-well screening where the CHO-cell lysate was used nor the column chromatography with NS0-feed. In those studies the pH 5.0 buffers were proven more efficient than pH 7.0 buffers. The conflicting result of this study could be explained by non-specific interactions between HCP and the 96-well plate at pH 5.0.

Table 6 Wash buffers from Figure 18, Figure 20 and Figure 21

No	Wash buffer	HCP conc (ppm)
1	20 mM phosphate, 0.15M NaCl, pH 7.4	1141
2	2M arginine, pH 7.0	648
3	0.5M arginine, 0.5M NaCl, pH 5.0	1246
4	0.5M arginine, 0.5M NaCl, pH 7.0	679
5	2M arginine, 0.5M NaCl, pH 5.0	979
6	2M glycine, pH 5.0	1513
7	0.5M glycine, 0.5M NaCl, pH 5.0	1662
8	0.5M glycine, 0.5M NaCl, pH 7.0	1199
9	0.5M glycine, 1M NaCl, pH 5.0	1478
10	2M glycine, 1M NaCl, pH 5.0	1590
11	0.3M citrate, pH 5.0	1639
12	1.2M acetate, pH 5.0	1413
13	0.1% Tween 20, pH 5.0	654
14	0.1% Tween 20, 0.5M NaCl, pH 5.0	630
15	0.1% Tween 20, 0.5M NaCl, pH 7.0	542

16	0.5% Tween 20, 0.5M NaCl, pH 5.0	568
17	0.1% Tween 20, 1M NaCl, pH 5.0	676
18	1M urea, 0.5M NaCl, pH 5.0	1432
19	1M urea, 0.5M NaCl, pH 7.0	1049
20	2M urea, 0.5M NaCl, pH 5.0	831
21	2M urea, 0.5M NaCl, pH 7.0	856
22	1% isopropanol, 0.5M NaCl, pH 5.0	1495
23	1% isopropanol, 0.5M NaCl, pH 7.0	1126
24	5% isopropanol, 0.5M NaCl, pH 5.0	1684
25	5% isopropanol, 0.5M NaCl, pH 7.0	849
26	0.5M NaCl, pH 5.0	1432
27	0.5M NaCl, pH 7.0	1022
28	1M NaCl, pH 5.0	1612
29	1M NaCl, pH 7.0	796
30	20% propylene glycol, 0.15M NaCl, pH 5.0	828
31	20% propylene glycol, 0.5M NaCl, pH 5.0	788
32	20% propylene glycol, 0.5M NaCl, pH 7.0	628

The contradictory result obtained in this study could be due to non-specific interactions between HCP and the 96-well plate. Most HCPs are acidic i.e. have their isoelectric point (pI) around 5 which means that they are uncharged at that pH. This means that they can interact non-specifically with the surfaces and the filters of the 96-well plate. HCP that is removed from the resin during wash with a pH 5.0 buffer binds to the surface and filters of the plate, and co-elutes with the mAb. This can explain the higher levels of HCP present in the eluates for the pH 5.0 buffers (Figure 18). This theory is further confirmed by the fact that Tween 20 and propylene glycol, both known to break non-specific interactions, worked well at both pH 5.0 and 7.0 meaning that this problem was not seen for those buffers. An important lesson learned from this study was that in order to get a consistent result between the 96-well plate and the column chromatography it is necessary to load the protein solution directly to the plate and not pre-load it into a column. Loading directly into the plate will lead to saturation of surfaces and filters during loading. However, it is desirable to use the pre-loading method because the capacity of the gel is better utilized and the possibility to detect differences in HCP levels increases. A solution to the problem could be to use coated 96-well plates where non-specific interactions between HCP and the plate will be reduced.

Comparison of the result from the above experiment with the chromatographic result where NS0-feed had been used (5.2.1), but only looking at the pH 7.0 wash buffers, showed very similar HCP concentrations in the eluates (Figure 19). This further verified the theory of non-specific interactions between HCP and plate at pH 5.0. At pH 7.0 this was not a problem and the result at this pH is reliable. The conditions in the plate were very similar to the conditions in the column, meaning that the 96-well format could be used for screening of chromatographic conditions.

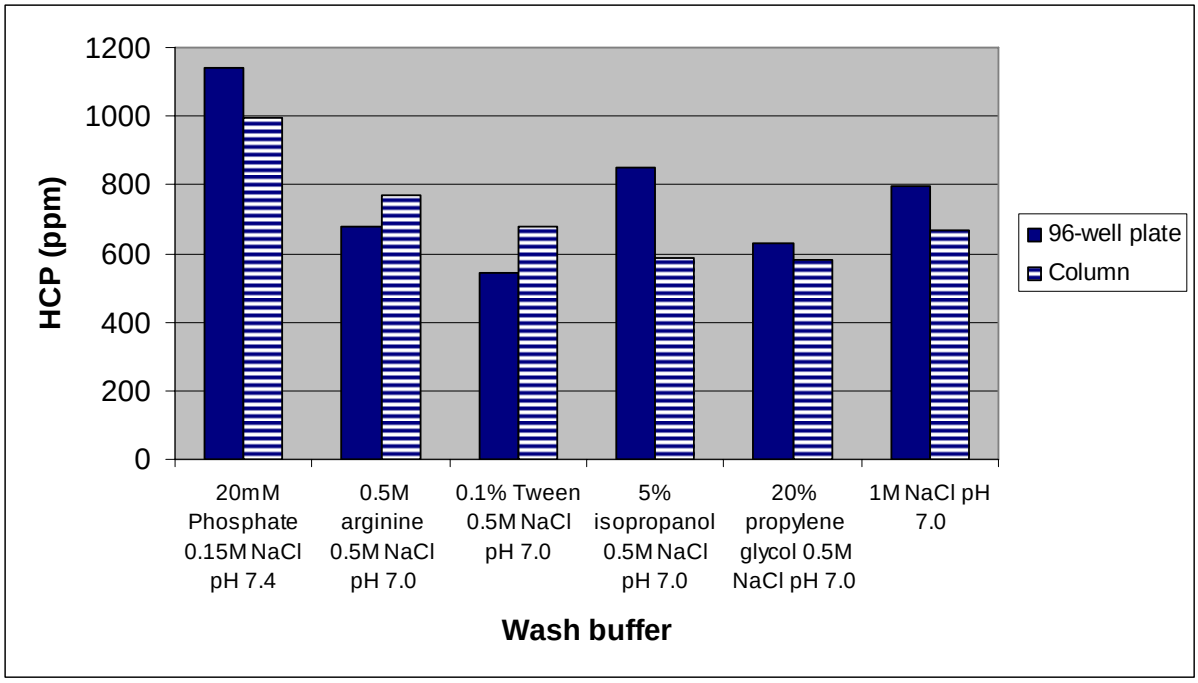


Figure 19 Comparison of HCP concentrations in eluates from 96-well format and column chromatography after wash with pH 7.0 buffers. The HCP levels in the eluates were compared between the 96-well format and column chromatography. The levels were very similar in the plate and on the column which indicates that the conditions in the plate were similar to the conditions on the column. A difference was seen for 5% isopropanol, 0.5M NaCl, pH 7.0.

5.4.2 Recovery

To determine the recoveries in the above study the UV absorbance at 280 nm was measured in the eluates from the 96-well plate. The recoveries for the different wash buffers were related to the control with phosphate buffer by dividing all values with that for the control, and multiplying them with 100. In general wash with pH 7.0 buffers showed higher recovery than pH 5.0 buffers (Figure 20). Wash buffers that resulted in low recovery were 0.5M arginine, 0.5M NaCl, pH 5.0, 2M arginine, 0.5M NaCl, pH 5.0, 2M urea, 0.5M NaCl, pH 5.0, 20% propylene glycol, 0.15M NaCl, pH 5.0 and 20% propylene glycol, 0.5M NaCl, pH 5.0.

Since high amounts of mAb had been loaded to the gel it was suspected that the measured UV absorbance should be out of the dynamic range of the spectrophotometer which was between 0-4 optical density (OD). This was true as many of the eluates had OD values above 4.0. Therefore a complementary study was performed where NS0-feed was loaded directly to a 96-well plate filled with MabSelect SuRe resulting in lower amount mAb adsorbed per well and lower amounts of mAb eluted. The same wash buffers were used as in the method where feed was loaded onto a column and distributed into a 96-well plate.
The same pattern as seen when feed was loaded onto a column and washed and eluted on the plate was seen for the experiment where feed was loaded to the 96-well plate; pH 7.0 buffers showed higher recovery than pH 5.0 buffers (Figure 21). However, the differences between the buffers were smaller when loading to plate compared to when loading to column.
It would have been interesting to analyze the eluates from the experiment where feed had been loaded to the 96-well plate for HCP concentration to see if the result was in accordance with the column chromatography. However, since time and resources were running out this was not done.

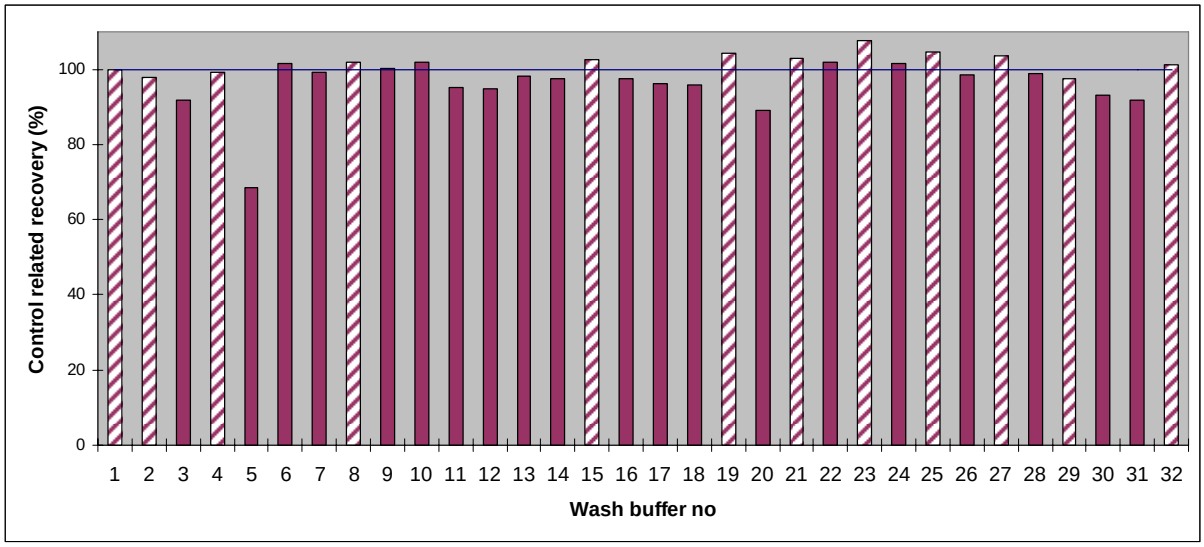


Figure 20 Control related recovery for loading of NS0-feed to column followed by wash and elution in 96-well plate. The recoveries from the runs with different intermediate wash buffers were calculated and related to the control. The line represents 100% recovery, and the striped bars are pH 7 buffers (including the control). It seemed like the pH 7.0 buffers had a better recovery than the pH 5.0 buffers for the same additive. Wash buffers with a bad recovery were especially no 3 0.5M arginine, 0.5M NaCl, pH 5.0, no 5 2M arginine, 0.5M NaCl, pH 5.0, no 20 2M urea, 0.5M NaCl, pH 5.0, no 30 20% propylene glycol, 0.15M NaCl, pH 5.0 and no 31 20% propylene glycol, 0.5M NaCl, pH 5.0. The different wash buffers with their numbers are listed in Table 6 in 5.4.1.

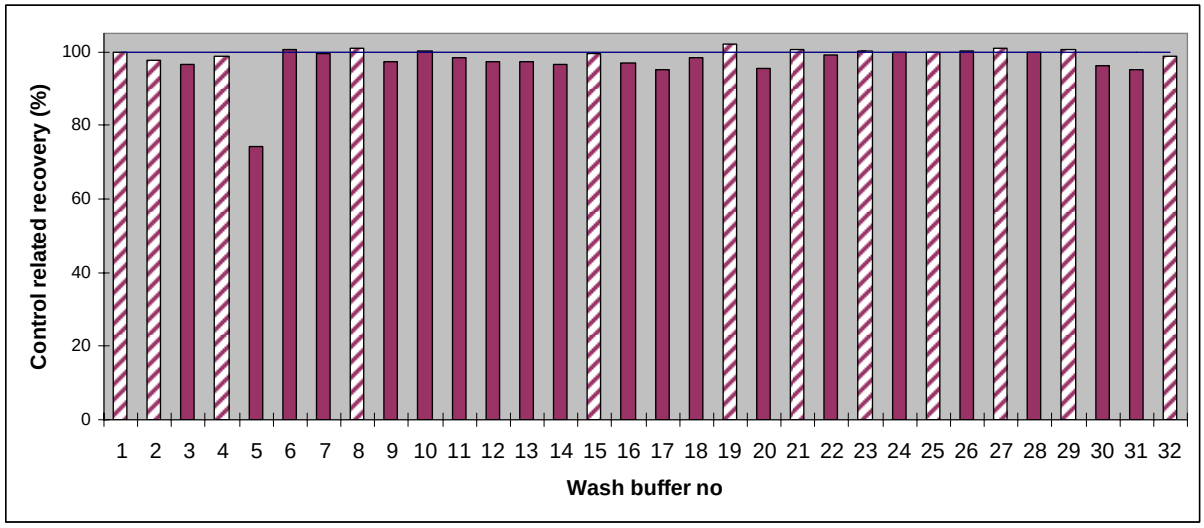


Figure 21 Control related recovery for loading of NS0-feed to 96-well plate followed by wash and elution in 96-well plate. The recoveries from the runs with different intermediate wash buffers were calculated and related to the control. The line represents 100% recovery and the striped bars are pH 7 buffers (including the control). The differences between the recovery for the pH 5.0 and pH 7.0 buffers seemed smaller than the values for loading to column. However, the same pattern as in Figure 20 was seen: pH 7.0 buffers had a higher recovery than the pH 5.0 buffers. Wash buffers with a bad recovery were especially no 3 0.5M arginine, 0.5M NaCl, pH 5.0, no 5 2M arginine, 0.5M NaCl, pH 5.0, no 20 2M urea, 0.5M NaCl, pH 5.0, no 30 20% propylene glycol, 0.15M NaCl, pH 5.0 and no 31 20% propylene glycol, 0.5M NaCl, pH 5.0. The different wash buffers with their numbers are listed in Table 6 in 5.4.1.

All these results (5.2, 5.3, 5.4) confirm that the 96-well format can be used as an effective time saving method for screening of buffer conditions. Good wash buffer candidates can be found using this screening method and verification can be performed by using column chromatography. A lot of process development time can be saved by finding good candidates in the plate and then not as many conditions needs to be tested on column chromatography.

An important lesson learned from this study is that in order to get a consistent result between the two formats the protein solution should be added directly to the plate alternatively coated plates should be used. Then the correlation between the 96-well format and column chromatography is good and the results will be comparable.

5.5 Development of the 96-well plate method

To be able to develop a well working 96-well plate method for screening of different wash buffer conditions the effect of variations in some parameters were evaluated using pure IgG. Parameters that were varied were volume and concentration of protein solution added, incubation time and incubation with and without shaking. The effect of cleaning the gel with 0.1M NaOH before usage was also examined. This was done to be able to understand the 96-well plate method and establish a method as effective as possible before the screening experiment started. The method was developed from an existing method for parallel screening of histidine-tagged proteins using the 96-well format (26).

5.5.1 Varying the volume of protein solution

Pure IgG was added to the wells of a 96-well plate in amounts between 300-500 µg to each well (50 µl gel). The incubation time was varied from 0.5-3 minutes. Flow through, wash and elution fractions were collected and the UV absorbance was measured in a spectrophotometer. The recoveries were then calculated and compared (Figure 22). The recoveries for the different amount of IgG loaded were for 300 µg approximately 100%, for 400 µg approximately 85% and for 500 µg approximately 80%. This showed that a smaller added amount gave higher recovery. The IgG that was not eluted ended up in the flow through. However, by adding a larger volume and consequently a larger amount of mAb the capacity of the gel increased. The capacity of the gel in the plate was 6 mg/ml, 6.8 mg/ml and 8 mg/ml when 300, 400 and 500 µg were added respectively. The calculated recoveries did not differ more than ±10% between the 8 replicates.

The plate used in this particular experiment had been used before, and perhaps some old proteins were still present in the plate despite cleaning before usage. This can explain the high yields of over 100% that was seen. This means that when calculating the mass balance (adding together the amount protein in flow through, wash and elution) the sum was larger than the amount protein added to the plate, which is impossible. Therefore the recoveries and capacities were a bit uncertain, but the trend is obvious.

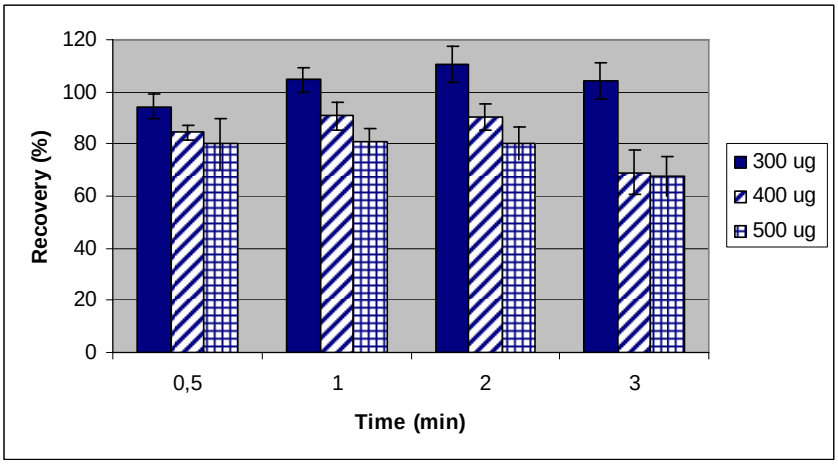


Figure 22 Recoveries from loading of different amount of IgG to the 96-well plate. Different amount of IgG was added to the 96-well plate by varying the volumes of the IgG solution. 300 µg, 400 µg and 500 µg of mAb was loaded and incubated for 0.5 to 3 minutes. The recoveries for 300 µg IgG (filled bars), 400 µg IgG (striped bars) and 500 µg IgG (checked bars) are shown. The recoveries did not differ more than ±10% between the 8 replicates. The recoveries increased with decreasing amount of protein added. The recoveries over 100% can be explained by the fact that the plate was reused and perhaps some old proteins were still present despite washing procedures.

5.5.2 Varying the protein concentration

Pure IgG was added to the wells of a 96-well plate in different amounts by varying the protein concentration from 1-3 mg/ml. 200-600 µg of mAb was added per well (50 µl gel). The incubation time was varied between 3-7 minutes. Flow through, wash and elution fractions were collected and the UV absorbance measured in a spectrophotometer. The recoveries were calculated and compared (Figure 23) and were for 200 µg approximately 95%, for 400 µg approximately 85% and for 600 µg approximately 80%. The calculated recoveries did not differ more than ±2% between the 8 replicates. The recoveries decreased with increasing amount IgG added, as was also seen in 5.5.1. Only small differences were seen between the recoveries for the same amount IgG added at the different incubation times. About 5-20% of the IgG ended up in the flow through. The capacity of the gel in the plate was 3.8 mg/ml, 6.8 mg/ml and 9.6 mg/ml when 200, 400 and 600 µg were added respectively.

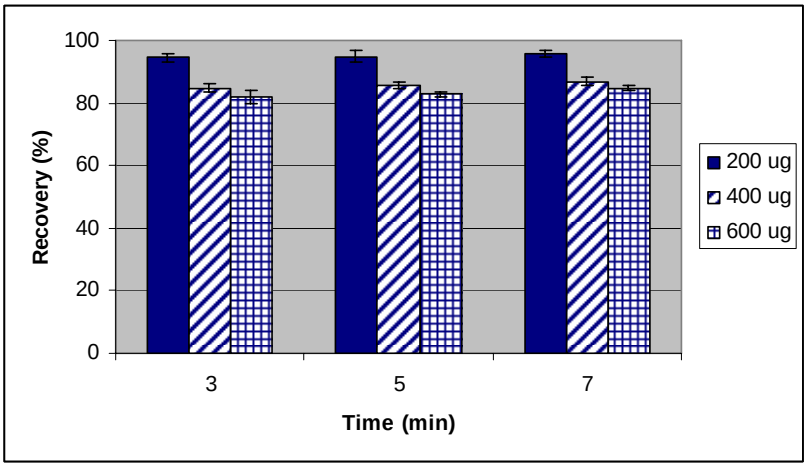


Figure 23 Recoveries for loading different amount of IgG to the 96 well plate. Different amount of IgG was added to the 96-well plate by varying the concentration of the IgG solution. 200 µg, 400 µg and 600 µg was loaded and incubated for different times, between 3 and 7 minutes. The recoveries for 200 µg IgG (filled bars), 400 µg IgG (striped bars) and 600 µg IgG (checked bars) are shown. The recoveries did not differ more than ±2% between the 8 replicates. The largest recoveries were seen for the smaller amount IgG added. Only small differences in recovery were seen between the different incubation times for the same amount IgG added.

5.5.3 Evaluation of shaking incubation

The experiment with results in 5.5.2 was repeated but this time with a shaking incubation. The recoveries for the different incubation times were calculated (Figure 24) and compared with the recoveries in Figure 23. It seemed like incubating the plate with shaking incubation did not increase the recovery in any larger extent compared to non shaking incubation. However only short incubation times were tested and perhaps it is necessary to incubate the plate for longer times to see any differences. The largest recoveries were seen for the smaller amount IgG added, as was also seen in both 5.5.1 and 5.5.2.

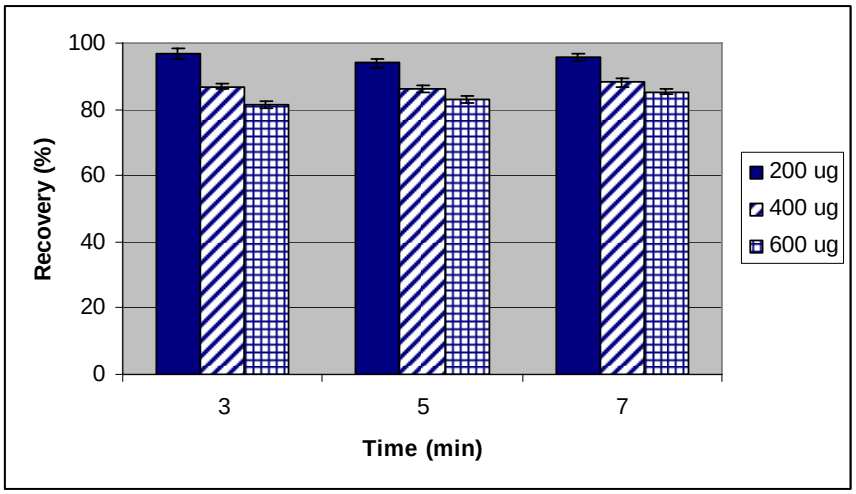


Figure 24 Recoveries for loading different amount of IgG to the 96 well plate and using shaking incubation. Different amount of IgG was added to the 96-well plate by varying the concentration of the IgG solution. 200 µg, 400 µg and 600 µg was loaded and incubated for different times, between 3 and 7 minutes using shaking incubation. The recoveries for 200 µg IgG (filled bars), 400 µg IgG (striped bars) and 600 µg IgG (checked bars) are shown. The recoveries did not differ more than ±2% between the 8 replicates. The largest recoveries were seen for the smaller amount IgG added. Small differences in recovery were seen for the same amount IgG added at the different incubation times.

The capacity of the gel in the 96-well plate is low, about 3.8 mg mAb/ml gel to get a recovery of approximately 95%, as was seen in both 5.5.2 and 5.5.3. This can be a problem because perhaps it is necessary to load more protein to be able to detect anything in the analytical methods used. The capacity in the plate can be increased either by adding a larger volume of protein solution of a higher concentration or by using a smaller gel volume, as discussed in paragraph 3.4 Equation 9. The time for developing the method for 96-well plate screening was very limited. It was not possible to further investigate the factors larger volume of protein solution or smaller gel volume even though it would have been preferable. Perhaps the incubation times tested in 5.5.1, 5.5.2 and 5.5.3 was not long enough to adsorb all the protein. It is possible that a longer incubation time and also an end over end rotation are needed for optimal protein uptake. Interesting would have been to increase the gel volume to 100 µl and adding the protein solution in multiple steps, to increase the capacity of the gel.

5.5.4 Evaluation of cleaning MabSelect SuRe with 0.1M NaOH before usage

Two differently treated MabSelect SuRe gels were used in this study, one pre-treated with 0.1M NaOH and one without this treatment. Different amount of IgG was added to the two gels, 210, 270 and 320 µg per well (50 µl gel), during incubation for 3 minutes. Small differences in capacities were seen between the gels (Figure 25). The highest recovery was seen for the lower IgG concentration for the pre-treated gel. The percentage IgG not eluted was found in the flow through. The calculated recoveries did not differ more than ±2% between the 8 replicates. It was decided to treat the gel with 0.1M NaOH in all experiments before usage.

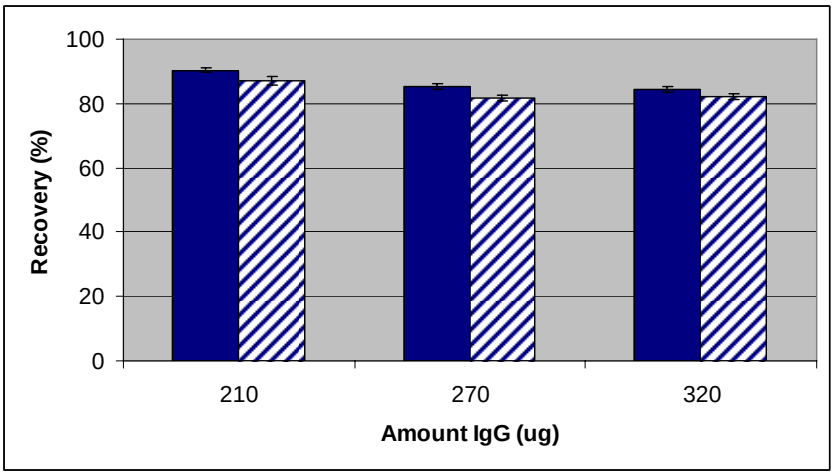


Figure 25 Comparison of recoveries for MabSelect SuRe with and without 0.1M NaOH treatment before usage. Different amount of IgG, 210 µg, 270 µg and 320 µg, was added to the 96-well plate during incubation for 3 minutes. The filled bars represent the recoveries from MabSelect SuRe that was treated with 0.1M NaOH before usage, and the striped bars represent the recoveries from gel not treated with 0.1M NaOH. A slightly higher recovery was seen when using a gel pre-treated with 0.1M NaOH. The recoveries did not differ more than ±2% between the 8 replicates.

5.6 Protease stability

When producing monoclonal antibodies for pharmaceutical use an affinity chromatography capture step such as immobilized Protein A is often preferred. One issue is then the ligand leaching and as the culture media after several weeks of culture of host cells might contain significant levels of proteases that might promote ligand leaching. As a result, a more protease resistant ligand will most likely reduce the level of leached ligand in the antibody pool after the capture step and a more robust purification can be achieved. The protease stability for the SuRe ligand was compared to rProtein A by incubating the two proteins in different concentrations of chymotrypsin and varying the incubation time. The proteins were also incubated in a CHO-cell lysate at different pHs and incubation times to investigate their protease stability.

5.6.1 Labeling of rProtein A and SuRe ligand

rProtein A and SuRe ligand were labeled with Cy3 and Cy5 and analysed on SDS-PAGE (Figure 26).

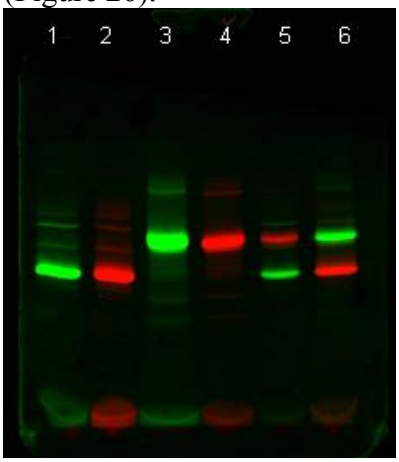


Figure 26 SDS-PAGE on Cy3 (green) and Cy5 (red) labeled SuRe ligand and rProtein A: 1) Cy3 labeled SuRe ligand **2)** Cy5 labeled SuRe ligand **3)** Cy3 labeled rProtein A **4)** Cy3 labeled rProtein A **5)** Pooled Cy3 labeled SuRe ligand and Cy5 labeled rProtein A **6)** Pooled Cy5 labeled SuRe ligand and Cy3 labeled rProtein A

5.6.2 Incubating rProtein A and SuRe ligand in different concentrations of chymotrypsin

5.6.2.1 Varying incubation time

The pooled proteins were incubated with 0, 1 and 5 µg/ml chymotrypsin for 0-60 minutes and samples were taken out after different incubation times and analysed on SDS-PAGE. The SuRe ligand was not degraded when adding 1 µg/ml or 5 µg/ml chymotrypsin and incubating 0 to 15 minutes (Figure 27 Gel A). rProtein A was partly degraded after incubation with 5 µg/ml chymotrypsin for 15 minutes (Figure 27 Gel B). rProtein A was even more degraded after incubation with 5 µg/ml chymotrypsin for 30 minutes and with 1 µg/ml after 60 minutes. rProtein A was almost totally degraded when incubated in 5 µg/ml chymotrypsin for 60 minutes, whereas only small parts of the SuRe ligand was degraded (Figure 27 Gel C). All these results confirmed that the SuRe ligand was more stable to this protease than rProtein A.

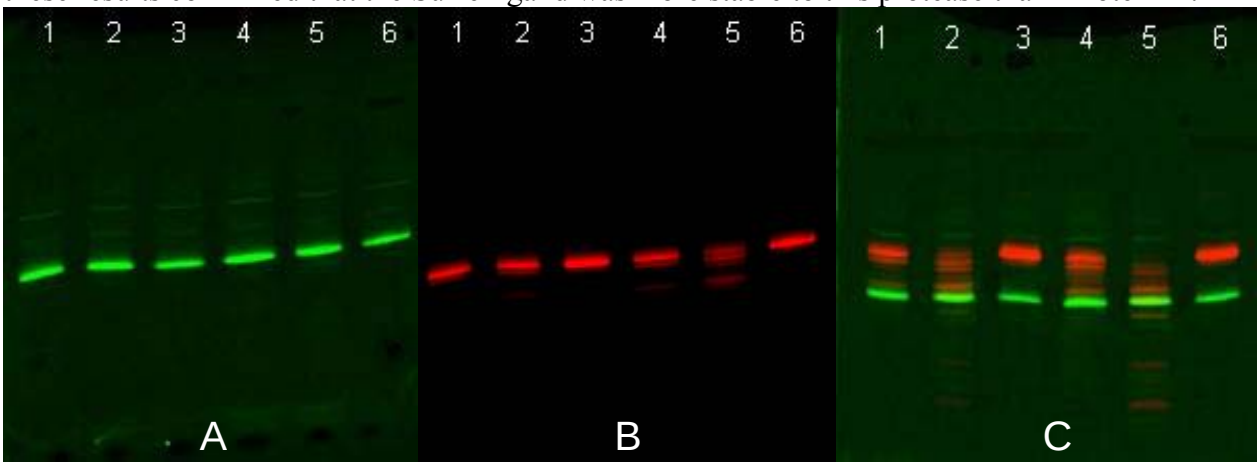


Figure 27 SDS PAGE on Cy3 (green) and Cy5 (red) labeled SuRe ligand and rProtein A incubated with 0-5 µg/ml chymotrypsin for 0-60 minutes: Gel A: Cy 3 labeled SuRe ligand incubated with different concentrations of chymotrypsin: **1)** 1 µg/ml 0 minutes **2)** 5 µg/ml 0 minutes **3)** control without chymotrypsin added 0 minutes **4)** 1 µg/ml 15 minutes **5)** 5 µg/ml 15 minutes **6)** control without chymotrypsin added 15 minutes. The SuRe ligand was intact after incubation with 0-5 µg/ml chymotrypsin for 15 minutes. **Gel B:** Cy 5 labeled rProtein A incubated with different concentrations of chymotrypsin: **1)** 1 µg/ml 0 minutes **2)** 5 µg/ml 0 minutes **3)** control without chymotrypsin added 0 minutes **4)** 1 µg/ml 15 minutes **5)** 5 µg/ml 15 minutes **6)** control without chymotrypsin added 15 minutes. rProtein A started to degrade after incubation with 5 µg/ml chymotrypsin for 15 minutes. **Gel C:** Pooled Cy3 labeled SuRe ligand and Cy5 labeled rProtein A incubated with different concentrations of chymotrypsin: **1)** 1 µg/ml 30 minutes **2)** 5 µg/ml 30 minutes **3)** control without chymotrypsin added 30 minutes **4)** 1 µg/ml 60 minutes **5)** 5 µg/ml 60 minutes **6)** control without chymotrypsin added 60 minutes. rProtein A was partly degraded after incubation with 1 µg/ml chymotrypsin for 30 and 60 minutes and totally degraded after incubation with 5 µg/ml chymotrypsin for 60 minutes. These results confirm that the SuRe ligand is more stable to chymotrypsin than rPrA. Note that Gel A and Gel B are the same gel and that the two channels Cy3 and Cy5 are separated for clarity, meaning that the two proteins are shown separately. In Gel C the two channels are shown simultaneously.

5.6.2.2 Varying chymotrypsin concentration

The pooled proteins were incubated with 0-10 µg/ml chymotrypsin for 60 minutes. The samples were analysed on SDS-PAGE and scanned. The SuRe ligand seemed to be more stable to chymotrypsin than rProtein A (Figure 28), because when 10 µg/ml chymotrypsin was added almost all rProtein A was degraded while just a small amount of the SuRe ligand was degraded. The same pattern was also seen with the other labeling combination. Then rProtein A was partly degraded when 1 µg/ml chymotrypsin was added whereas the SuRe ligand seemed to be intact. rProtein A was totally degraded when 10 µg/ml chymotrypsin was added but only small parts of the SuRe ligand was degraded. This showed that there was no difference between the two combinations of labeling; the same result was received for both

combinations. From the two studies in 5.6.2.1 and 5.6.2.2 where rProtein A and SuRe ligand were incubated with different concentrations of chymotrypsin for different incubation times the conclusion was drawn that the SuRe ligand was more stable to this protease than rProtein A.

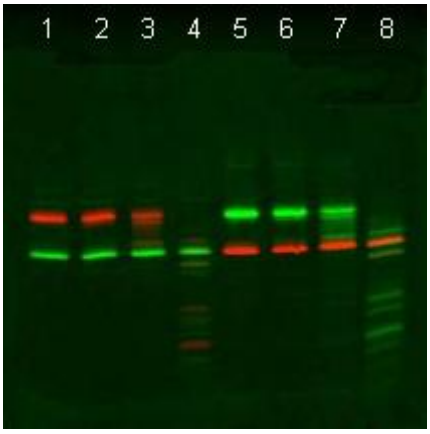


Figure 28 SDS-PAGE on Cy3 (green) and Cy5 (red) labeled SuRe ligand and rProtein A incubated with 0-10 µg/ml chymotrypsin for 60 minutes: Pooled Cy3 labeled SuRe ligand and Cy5 labeled rProtein A incubated with 0-10 µg/ml chymotrypsin: **1)** 0 µg/ml **2)** 0.1 µg/ml **3)** 1 µg/ml **4)** 10 µg/ml. Poled Cy5 labeled SuRe ligand and Cy3 labeled rProtein A incubated with 0-10 µg/ml chymotrypsin: **5)** 0 µg/ml **6)** 0.1 µg/ml **7)** 1 µg/ml **8)** 10 µg/ml. rProtein A was totally degraded after incubation with 10 µg/ml chymotrypsin for 60 minutes, lane 4 and 8, and the SuRe ligand seemed to be more stable to this protease. These results further confirm that the SuRe ligand is more protease stable than rProtein A.

5.6.3 Incubating rProtein A and SuRe ligand in CHO-cell lysate

5.6.3.1 Varying incubation time

It was suspected that a lot of proteases were present in the CHO-cell lysate used in the chromatographic runs in paragraph 5.3, because of the low recoveries. The pooled rProtein A and SuRe ligand were incubated in CHO-cell lysate at pH 3.5 and pH 7.0 for 0-168 hours to see if it contained any proteases. Samples were taken out after different incubation times and analysed on SDS-PAGE and scanned.

The two proteins were totally degraded after incubated 168 hours in CHO-cell lysate at pH 3.5, and also in the control, acetate buffer pH 3.5. In CHO-cell lysate at neutral pH rProtein A was partly degraded after 168 hours but the SuRe ligand did not seem to be affected. Interesting was the fact that rProtein A seemed to be degraded also in the acetate buffer control after 168 hours whereas the SuRe ligand seemed to be intact (Figure 29 gel A). It was here shown that the proteases present in this CHO-cell lysate were active at acidic pH, but it also seemed like the proteases started to degrade rProtein A after a long incubation time at neutral pH. This fact needs to be considered when the proteins are stored for a long time. Then perhaps the proteins are degraded even though the proteases present in the solution are inactive at the storing pH.
The two proteins were incubated in the same CHO-cell lysate at pH 5.0 for a shorter incubation time, 0-18 hours. rProtein A was degraded after incubation in CHO-cell lysate for 18 hours at pH 5.0 but the SuRe ligand seemed to be intact (Figure 29 gel B) (23). From this study where rProtein A and the SuRe ligand were incubated in CHO-cell lysate at different incubation times and pHs the following conclusions were drawn. The CHO-cell lysate contained a lot of proteases which explained the bad recoveries received in the chromatographic runs with this material and the SuRe ligand was more stable to proteases present in the CHO-cell lysate than rProtein A.

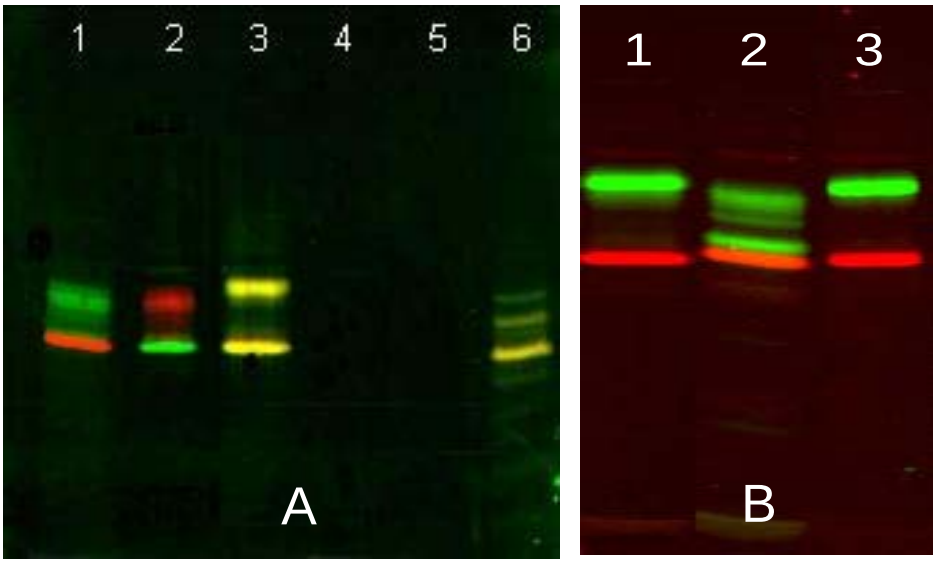


Figure 29 SDS-PAGE on Cy3 (green) and Cy5 (red) labeled SuRe ligand and rProtein A incubated in CHO-cell lysate for different times: Gel A: **1)** Pooled Cy5 labeled SuRe ligand and Cy3 labeled rProtein A at pH 7.0 incubated 168 hours **2)** Pooled Cy3 labeled SuRe ligand and Cy5 labeled rProtein A incubated 168 hours at pH 7.0 **3)** control (PBS) Cy3 labeled SuRe ligand and Cy5 labeled rProtein A + Cy5 labeled SuRe ligand and Cy3 labeled rProtein A incubated 168 hours at pH 7.0 **4)** Pooled Cy5 labeled SuRe ligand and Cy3 labeled rProtein A incubated 168 hours at pH 3.5 **5)** Pooled Cy3 labeled SuRe ligand and Cy5 labeled rProtein A incubated 168 hours at pH 3.5 **6)** control (acetate buffer) Cy3 labeled SuRe ligand and Cy5 labeled rProtein A + Cy5 labeled SuRe ligand and Cy3 labeled rProtein A incubated 168 hours at pH 3.5. rProtein A was partly degraded when incubated for 168 hours at pH 7.0 and both rProtein A and the SuRe ligand was totally degraded when incubated 168 hours at pH 3.5. **Gel B:** Pooled Cy5 labeled SuRe ligand and Cy3 labeled rProtein A incubated at pH 5 for different times: **1)** 0 hours **2)** 18 hours **3)** control 18 hours. rProtein A was degraded after 18 hours incubation in CHO-cell lysate at pH 5.0. This confirms that the SuRe ligand is more protease stable than rProtein A.

6 Conclusions

The aim of this project was to determine whether the 96-well format could be used for screening of chromatographic conditions. A goal was also to find good wash buffer candidates for the chromatography media MabSelect SuRe.

In the screening of 96 different wash buffers using the 96-well format it was found that some buffer additives worked better than others. Those were 0.5-2M arginine, 0.5-2M glycine, 1M urea, 0.1-0.5% Tween 20, 1-5% isopropanol and 20% propylene glycol in combination with 0.5M NaCl at pH 5.0 but also at pH 7.0. Since the recoveries when using pH 5.0 buffers were decreased it should be preferable to use wash buffers at neutral pH.

Consistency in the result was seen between column chromatography and the 96-well plate. Thus, it can be concluded that the 96-well plate can be used for screening of chromatographic conditions. By using this format a lot of process development time can be saved as well as amounts of protein and buffer additives due to the low volumes consumed.

There are some challenges with the 96-well format. First of all the capacity in the 96-well plate is low, 3.8 mg mAb/ml gel when adding 200 µl of a mAb solution of approximately 1 mg/ml per 50 µl gel. This can be compared with the DBC which is 20-30 mg mAb/ml gel using chromatography. The capacity can be more efficiently utilized by loading feed onto a chromatographic column and wash and elute in the 96-well plate. However this procedure seemed to lead to non-specific interactions between HCP and plate. The theory of non-

specific interactions between HCP and the surface of the plate needs to be investigated and the method for using the 96-well plate needs further optimization.

An investigation of the protease stability of the SuRe ligand and rProtein A was also done. The SuRe ligand was shown to be more protease stable than rProtein A both when incubating the protein with the protease chymotrypsin and in a CHO-cell lysate containing proteases. By immobilizing the SuRe ligand to a gel, as done in MabSelect SuRe, a more robust purification can be achieved because of lower levels of ligand leaching.

7 Future experiments

7.1 96-well screening

It would be interesting to investigate the theory of non-specific interactions between HCP and the surface of the 96-well plate. To examine this, coated 96-well plates could be used. The coating procedure can be performed by incubating the plate in a coating solution containing a compound that binds to the surface of the plate. When adding the feed sample, the compound bound to the surface will prevent non-specific interactions between surface and filters of the plate and proteins in the feed (24). An experiment designed to investigate the theory of non-specific interactions could be to use the two sample loading methods, pre-loading to column and loading to plate. Half of the 96-well plate could be coated and the sample from both loading methods distributed to both the coated and non-coated wells. The gel could be washed with buffers at pH 5.0 and pH 7.0. If the amount HCP left in the eluates after washing with for example a pH 5.0 buffer differs between the two sides of the plate, and between the two loading procedures, the answer to the question will be found.

In the screening of 96 different wash buffers, several wash buffer candidates were found. Different buffer additives such as detergents, solvents, amino acids, etc. in combination with 0.5M NaCl at pH 7.0 gave a significant decrease in HCP levels in the eluates. It might be possible to even further optimize the wash step by exploring a wider pH and conductivity range in combination with a wider range of buffer additive concentration for some specific additive.

7.2 Protease stability

An interesting experiment to perform would be to repeat the experiment outlined here but with another material, maybe the NS0-feed. If the same pattern is shown for that material then some further conclusions might be drawn. It would also be interesting to measure the protease clearance in elution fractions from a MabSelect SuRe column and compare with a regular Protein A column. This can be done by comparing the protease activity in the eluates from the two columns.

8 Acknowledgements

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10 Appendix

10.1 96 different wash buffers

96 different wash buffers were tested and are listed in Table 7.

Table 7 96 different wash buffers The wash buffers used in the screening of 96 different intermediate wash buffers are ranked from (-) to (++), where (++) is the most effective wash buffer for HCP removal and (-) is the least efficient.

Additive	Buffer	NaCl (M)	pH	Ranking
2M arginine	25 mM phosphate	0	7.0	++
0.5M arginine	20 mM citrate	0.5	5.0	++
0.5M arginine	25 mM phosphate	0.5	7.0	++
2M arginine	20 mM citrate	0.5	5.0	++
2M glycine	20 mM citrate	0	5.0	++
0.5M glycine	20 mM citrate	0.5	5.0	++
0.5M glycine	25 mM phosphate	0.5	7.0	++
0.5M glycine	20 mM citrate	1	5.0	++
1M urea	20 mM citrate	0.5	5.0	++
1M urea	25 mM phosphate	0.5	7.0	++
1% isopropanol	20 mM citrate	0.5	5.0	++
5% isopropanol	20 mM citrate	0.5	5.0	++

0.1% Tween 20	20 mM citrate	0.5	5.0	++
0.5% Tween 20	20 mM citrate	0.5	5.0	++
0.1% Tween 20	20 mM citrate	1	5.0	++
No additive	20 mM citrate	0.5	5.0	++
No additive	20 mM citrate	1	5.0	++
No additive	25 mM phosphate	1	7.0	++
20% propylene glycol	20 mM citrate	0.5	5.0	++
No additive	0.3M citrate	0	5.0	++
No additive	1.2M acetate	0	5.0	++
0.5M arginine	20 mM citrate	0	5.0	+
0.5M arginine	25 mM phosphate	0	7.0	+
2M arginine	20 mM citrate	0	5.0	+
0.5M arginine	20 mM citrate	1	5.0	+
0.5M arginine	25 mM phosphate	1	7.0	+
2M glycine	25 mM phosphate	0	7.0	+
2M glycine	20 mM citrate	0.5	5.0	+
2M glycine	25 mM phosphate	0.5	7.0	+
0.5M glycine	25 mM phosphate	1	7.0	+
25 mM tryptophan	20 mM citrate	0.5	5.0	+
25 mM tryptophan	25 mM phosphate	0.5	7.0	+
50 mM tryptophan	20 mM citrate	0.5	5.0	+
50 mM tryptophan	25 mM phosphate	0.5	7.0	+
25 mM tryptophan	20 mM citrate	1	5.0	+
25 mM tryptophan	25 mM phosphate	1	7.0	+
50 mM tryptophan	20 mM citrate	1	5.0	+
50 mM tryptophan	25 mM phosphate	1	7.0	+
2M urea	20 mM citrate	0.5	5.0	+
2M urea	25 mM phosphate	0.5	7.0	+
1M urea	20 mM citrate	1	5.0	+
1M urea	25 mM phosphate	1	7.0	+
2M urea	20 mM citrate	1	5.0	+
2M urea	25 mM phosphate	1	7.0	+
1% isopropanol	25 mM phosphate	0.5	7.0	+
5% isopropanol	25 mM phosphate	0.5	7.0	+
1% isopropanol	20 mM citrate	1	5.0	+
1% isopropanol	25 mM phosphate	1	7.0	+
5% isopropanol	20 mM citrate	1	5.0	+
5% isopropanol	25 mM phosphate	1	7.0	+
0.1% Tween 20	25 mM phosphate	0.5	7.0	+
0.5% Tween 20	25 mM phosphate	0.5	7.0	+
0.1% Tween 20	25 mM phosphate	1	7.0	+
0.5% Tween 20	20 mM citrate	1	5.0	+
0.5% Tween 20	25 mM phosphate	1	7.0	+
No additive	25 mM phosphate	0.5	7.0	+
25 mM caprylic acid	25 mM phosphate	0.5	7.0	+
25 mM caprylic acid	25 mM phosphate	1	7.0	+
20% propylene glycol	25 mM phosphate	0.5	7.0	+
No additive	1M tris	0	7.5	+
No additive	0.15M citrate	0	7.0	+
No additive	0.35M phosphate	0	7.0	+
2M arginine	25 mM phosphate	0.5	7.0	0
2M arginine	20 mM citrate	1	5.0	0
2M arginine	25 mM phosphate	1	7.0	0
2M glycine	20 mM citrate	1	5.0	0
2M glycine	25 mM phosphate	1	7.0	0
1M urea	25 mM phosphate	0	7.0	0
2M urea	25 mM phosphate	0	7.0	0
No additive	Milli Q water	0	6.16	0

0.5M glycine	20 mM citrate	0	5.0	-
0.5M glycine	25 mM phosphate	0	7.0	-
25 mM tryptophan	20 mM citrate	0	5.0	-
25 mM tryptophan	25 mM phosphate	0	7.0	-
50 mM tryptophan	20 mM citrate	0	5.0	-
50 mM tryptophan	25 mM phosphate	0	7.0	-
1M urea	20 mM citrate	0	5.0	-
2M urea	20 mM citrate	0	5.0	-
1% isopropanol	20 mM citrate	0	5.0	-
1% isopropanol	25 mM phosphate	0	7.0	-
5% isopropanol	20 mM citrate	0	5.0	-
5% isopropanol	25 mM phosphate	0	7.0	-
0.1% Tween 20	20 mM citrate	0	5.0	-
0.1% Tween 20	25 mM phosphate	0	7.0	-
0.5% Tween 20	20 mM citrate	0	5.0	-
0.5% Tween 20	25 mM phosphate	0	7.0	-
20 mM phosphate	NaH_2PO_4	0	5.0	-
20 mM phosphate	NaH_2PO_4	0.15	5.0	-
20 mM phosphate	NaH_2PO_4	0	7.4	-
No additive (control)	20 mM phosphate	0.15	7.4	-
No additive (control)	20 mM phosphate	0.15	7.4	-
25 mM caprylic acid	25 mM phosphate	0	7.0	-
20% propylene glycol	20 mM citrate	0	5.0	-
20% propylene glycol	25 mM phosphate	0	7.0	-
20% propylene glycol	20 mM citrate	0.15	5.0	-
20% propylene glycol	25 mM phosphate	0.15	7.0	-

10.2 Gels from screening of 96 different buffer conditions

In the screening of 96 different wash buffers a CHO-cell lysate was added to the 96-well plate followed by wash and elution. The wash fractions from the wash with different intermediate wash buffer were analysed on SDS-PAGE. The different wash buffers were ranked from 1 (-) to 4 (++) depending on the bands seen on the gels. The – buffer was the least efficient wash buffer for HCP removal and ++ was the most effective wash buffer. For the – buffer no bands were seen on the gel, and only bright bands were seen for the buffer ranked as a 0 buffer. A + buffer showed dark bands and an extra band, whereas a ++ buffer showed very dark bands and a dark extra band.

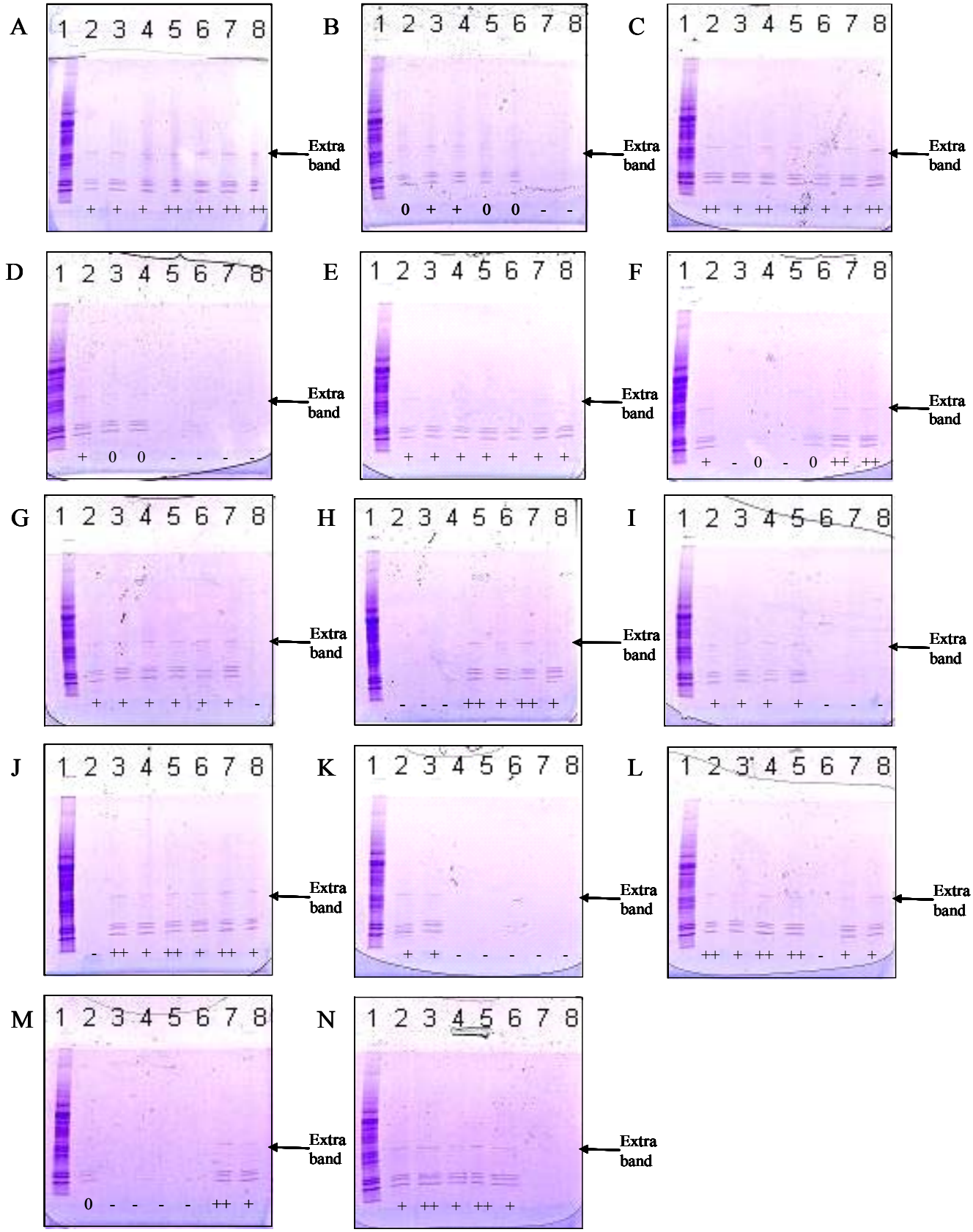

Figure 30 SDS-PAGE on wash fractions from different intermediate wash buffers. A screening of 96 different wash buffers was done on 96-well plates and the fractions from the wash with different intermediate wash buffers were analysed with SDS-PAGE. The different wash buffers were ranked from 1 (-) to 4 (++) where ++ is the buffer most efficient for HCP removal and – the buffer least efficient. The gels A to N show fractions from different wash buffers:

A 1) CHO-cell lysate **2)** 0.5M arginine, pH 5.0 (+) **3)** 0.5M arginine, pH 7.0 (+) **4)** 2Marginine, pH 5.0 (+) **5)** 2M arginine, pH 7.0 (++) **6)** 0.5M arginine, 0.5 M NaCl, pH 5.0 (++) **7)** 0.5M arginine, 0.5 M NaCl, pH 7.0 (++) **8)** 2M arginine, 0.5M NaCl, pH 5.0 (++)

B 1) CHO-cell lysate **2)** 2M arginine, 0.5M NaCl, pH 7.0 (0) **3)** 0.5M arginine, 1 M NaCl, pH 5.0 (+) **4)** 0.5M arginine, 1 M NaCl, pH 7.0 (+) **5)** 2M arginine, 1M NaCl, pH 5.0 (0) **6)** 2M arginine, 1M NaCl, pH 7.0 (0) **7)** 0.5M glycine, pH 5.0 (-) **8)** 0.5M glycine, pH 7.0 (-)

C 1) CHO-cell lysate **2)** 2M glycine, pH 5.0 (++) **3)** 2M glycine, pH 7.0 (+) **4)** 0.5M glycine, 0.5 M NaCl, pH 5.0 (++) **5)** 0.5M glycine, 0.5 M NaCl, pH 7.0 (++) **6)** 2M glycine, 0.5M NaCl, pH 5.0 (+) **7)** 2M glycine, 0.5M NaCl, pH 7.0 (+) **8)** 0.5M glycine, 1M NaCl, pH 5.0 (++)

D 1) CHO-cell lysate **2)** 0.5M glycine, 1M NaCl, pH 7.0 (+) **3)** 2M glycine, 1M NaCl, pH 5.0 (0) **4)** 2M glycine, 1M NaCl, pH 7.0 (0) **5)** 25 mM tryptophan, pH 5.0 (-) **6)** 25 mM tryptophan, pH 7.0 (-) **7)** 50 mM tryptophan, pH 5.0 (-) **8)** 50 mM tryptophan, pH 7.0 (-)

E 1) CHO-cell lysate **2)** 25 mM tryptophan, 0.5 M NaCl, pH 5.0 (+) **3)** 25 mM tryptophan, 0.5 M NaCl, pH 7.0 (+) **4)** 50 mM tryptophan, 0.5M NaCl, pH 5.0 (+) **5)** 50 mM tryptophan, 0.5M NaCl, pH 7.0 (+) **6)** 25 mM tryptophan, 1 M NaCl, pH 5.0 (+) **7)** 25 mM tryptophan, 1 M NaCl, pH 7.0 (+) **8)** 50 mM tryptophan, 1M NaCl, pH 5.0 (+)

F 1) CHO-cell lysate **2)** 50 mM tryptophan, 1M NaCl, pH 5.0 (+) **3)** 1M urea, pH 5.0 (-) **4)** 1M urea, pH 7.0 (0) **5)** 2M urea, pH 5.0 (-) **6)** 2M urea, pH 7.0 (0) **7)** 1M urea, 0.5 M NaCl, pH 5.0 (++) **8)** 1M urea, 0.5 M NaCl, pH 7.0 (++)

G 1) CHO-cell lysate **2)** 2M urea, 0.5 M NaCl, pH 5.0 (+) **3)** 2M urea, 0.5 M NaCl, pH 7.0 (+) **4)** 1M urea, 1 M NaCl, pH 5.0 (+) **5)** 1M urea, 0 1 M NaCl, pH 7.0 (+) **6)** 2M urea, 1 M NaCl, pH 5.0 (+) **7)** 2M urea, 1 M NaCl, pH 7.0 (+) **8)** 1% Isopropanol, pH 5.0 (-)

H 1) CHO-cell lysate **2)** 1% isopropanol, pH 7.0 (-) **3)** 5% isopropanol, pH 5.0 (-) **4)** 5% isopropanol, pH 7.0 (-) **5)** 1% isopropanol, 0.5 M NaCl, pH 5.0 (++) **6)** 1% isopropanol, 0.5 M NaCl, pH 7.0 (+) **7)** 5% isopropanol, 0.5M NaCl, pH 5.0 (++) **8)** 5% isopropanol, 0.5M NaCl, pH 7.0 (+)

I 1) CHO-cell lysate **2)** 1% isopropanol, 1M NaCl, pH 5.0 (+) **3)** 1% isopropanol, 1M NaCl, pH 7.0 (+) **4)** 5% isopropanol, 1M NaCl, pH 5.0 (+) **5)** 5% isopropanol, 1M NaCl, pH 7.0 (+) **6)** 0.1% Tween 20, pH 5.0 (-) **7)** 0.1% Tween 20, pH 7.0 (-) **8)** 0.5% Tween 20, pH 5.0 (-)

J 1) CHO-cell lysate **2)** 0.5% Tween 20, pH 7.0 (-) **3)** 0.1% Tween 20, 0.5M NaCl, pH 5.0 (++) **4)** 0.1% Tween 20, 0.5M NaCl, pH 7.0 (+) **5)** 0.5% Tween 20, 0.5M NaCl, pH 5.0 (++) **6)** 0.5% Tween 20, 0.5M NaCl, pH 7.0 (+) **7)** 0.1% Tween 20, 1M NaCl, pH 5.0 (++) **8)** 0.1% Tween 20, 1M NaCl, pH 7.0 (+)

K 1) CHO-cell lysate **2)** 0.5% Tween 20, 1M NaCl, pH 5.0 (+) **3)** 0.5% Tween 20, 1M NaCl, pH 7.0 (+) **4)** 20 mM phosphate, pH 5.0 (-) **5)** 20 mM phosphate, 0.15M NaCl, pH 5.0 (-) **6)** 20 mM phosphate, pH 7.4 (-) **7)** 20 mM phosphate, 0.15M NaCl, pH 7.4 (-) **8)** 20 mM phosphate, pH 7.4 (-)

L 1) CHO-cell lysate **2)** 0.5M NaCl, pH 5.0 (++) **3)** 0.5M NaCl, pH 7.0 (+) **4)** 1M NaCl, pH 5.0 (++) **5)** 1M NaCl, pH 7.0 (++) **6)** 25 mM caprylic acid, pH 7.0 (-) **7)** 25 mM caprylic acid, 0.5M NaCl, pH 7.0 (+) **8)** 25 mM caprylic acid, 1M NaCl, pH 7.0 (+)

M 1) CHO-cell lysate **2)** MQ pH 6.16 (0) **3)** 20% propylene glycol, pH 5.0 (-) **4)** 20% propylene glycol, pH 7.0 (-) **5)** 20% propylene glycol, 0.15M NaCl, pH 5.0 (-) **6)** 20% propylene glycol, 0.15M NaCl, pH 7.0 (-) **7)** 20% propylene glycol, 0.5M NaCl, pH 5.0 (++) **8)** 20% propylene glycol, 0.5M NaCl, pH 7.0 (+)

N 1) CHO-cell lysate **2)** 1M Tris, pH 7.5 (+) **3)** 0.3M citrate, pH 5.0 (++) **4)** 0.15M citrate, pH 7.0 (+) **5)** 1.2M acetate, pH 5.0 (++) **6)** 0.35M phosphate, pH 7.0 (+) **7)** blank **8)** blank