2-D DIGE
to facilitate downstream process development for recombinant therapeutic antibodies

Dissertation

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Abstract (in English)

About 25% of biopharmaceutical drugs on the market are recombinant monoclonal antibodies. Deeper knowledge and understanding of the production processes are very important for manufacturing of safe pharmaceutical products. In this project, Two-Dimensional Fluorescence Difference Gel Electrophoresis (2-D DIGE) was applied for downstream process development. Tracking of impurities, detection of critical contaminants, visualization of protein isoforms during the process as well as quality control were supported with this technique. The characterization and comparison of purification processes and the impact of different feed stocks on the product quality and impurities pattern were investigated.

Abstract (in German)

20% von der Biopharmazeutika, die sich auf dem Markt befinden, sind rekombinante monoklonale Antikörper. Charakterisierung und Verständnis der Produktionsprozesse spielen eine wichtige Rolle bei der Herstellung von sicheren, hochqualitativen Arzneimitteln. In diesem Projekt wurde Zweidimensionale Fluoreszenz Differenz Gelelektrophorese (2-D DIGE) für die Optimierung und Entwicklung der Reinigungsprozesse angewendet. Diese Methode ermöglicht sowohl das Monitoring von Verunreinigungen, die Detektion von kritischen Kontaminationen, Visualisierung der Proteinisoformen während verschiedener Reinigungsschritte, als auch eine Qualitätskontrolle. Die Charakterisierung und der Vergleich von Reinigungsprozessen und der Einfluss der Kulturüberstände aus verschiedenen Fermentationen auf die Qualität des Produktes und der Verunreinigungsmuster wurden untersucht.

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1 Introduction

1.1 Recombinant therapeutic monoclonal antibodies on the market

Recombinant monoclonal antibodies (rMAb) account for approximately 25% of the annual sales for biopharmaceutical drugs, and this percentage is increasing rapidly due to their excellent pharmaceutical properties and success in clinical applications. The rMAb market is expected to have annual sales of $15 billion in 2010 [1].

The potential for therapeutic monoclonal antibodies [2] started in 1975 with the development of hybridoma technology by Kohler and Milstein [3, 4]. A major breakthrough in rMAb production occurred in the 1980's with the development of recombinant DNA techniques[5]. The first recombinant monoclonal antibody was expressed in *Escherichia coli* using phage display technology [6]. The use of mouse antibodies for human therapeutics results in the undesired production of anti-murine antibodies in the human with the immunogenic response mainly directed against the mouse Fc region. Using recombinant DNA technology, more recent rMAb have replaced the murine regions of the IgG with the human fragments providing chimeric, humanized and full human antibodies as shown in Figure 1.

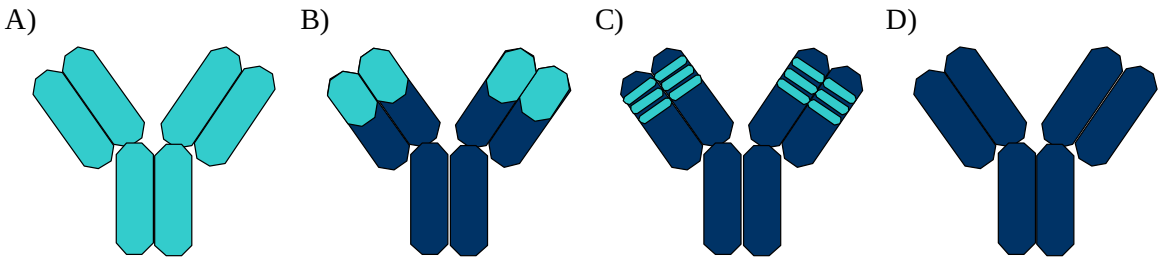

Figure 1. Recombinant monoclonal antibodies. A) mouse, 100 % murine; B) chimeric, 30 % murine; C) humanized, 5 % murine; D) full human, 100 % human.

In chimeric IgG only the variable regions are derived from the native mouse antibodies. In humanized antibodies, alternatively called CDR-gafted antibody, only the hyperactive, complementary determining region (CDR), responsible for antigen binding originates from mouse antibodies. Fully humanized rMAb have less immunological issues, but they may

have decreased avidity to the targeted antigen. In addition to full-length rMAb, antibodies can be expressed as fragments or as fusion proteins.

1.1.1 Antibody fragments and full size molecules

Antibody fragments are produced in *E.coli* as monovalent fragments [5, 7, 8] as shown in Figure 2.

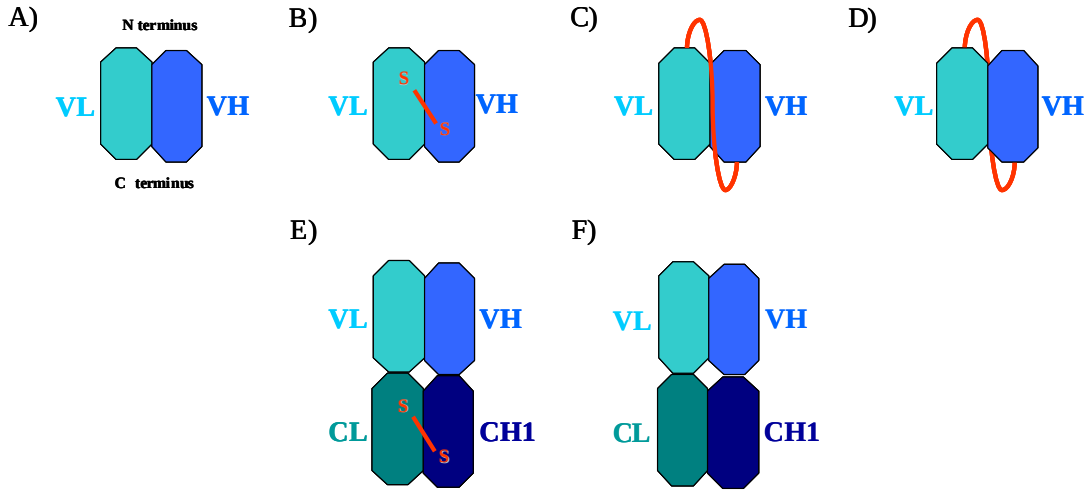

Figure 2. Monovalent antibody fragments expressed in E.coli. A) Fragment variable (Fv); B) disulfide stabilized Fv (dsFv); C) single-chain Fv (scFv), V_H-linkerV_L; D) scFv, V_L-linker-V_H; E) antibody binding fragment (Fab) covalently linked; F) Fab non-covalently linked. V_L – heavy region of light chain; V_H – variable region of heavy chain; C_L- constant region of light chain; C_{Hl} – heavy region of heavy chain [5].

The fragment variable (Fv) has a molecular weight of approximately 28 kDa and is the smallest segment produced in a single gene construct determining the binding size of the antibody to the antigen (A). Due to a non-covalent interaction between the variable region of the heavy (V_H) and light (V_L) chains, the stability of the Fv is very low. The use of a single covalent disulfide bound (Figure 2B) or flexible linkers between the C-terminal of the heavy chain and the N-terminal of the light chain (Figure 2C-D) composed of approximately 15 amino acids [9] (e.g., $(Gly)_4Ser(Gly)_4Ser(Gly)_4Ser$) can be used to stabilize the molecule. Additionally, an antibody binding fragment (Fab) with molecular weight of approximately 55 kDa consisting of four immunoglobulin domains, V_L and C_L of the light chain and V_H and C_{H1} are frequently used. Two chains of the Fab are encoded in separate gene constructs [10].

scFv and Fab have clinically demonstrated the ability to block infection of cells cultured in the presence of human rhinovirus [11]. Fab have the advantage of improved pharmacokinetics and low immunogenicity, due to absence of Fc region. *E.coli* does not glycosylate proteins, such as in the C_H2 domain at Asn-297 of IgG molecules, and thus is not viable for the production of intact, glycosylated IgG molecues. Whole IgG molecules can be produced in eukaryotic cell cultures, including mammalian cells, insect cells [12], transgenic animals [13], algae and higher-order plant cells [14]. The production of IgG in goat milk with titers ranging from 5 to 30 g/L was reported by Werner [15]. Tabacco, Nicotina [16, 17], corn and soybeans [18] are also used for large-scale production of rMAb, named plantibodies [19]. Unfortunately, even though production costs are very low, the rMAbs do not have the same glycosylation patterns compared to human posttranslational modifications [20]. Based on these facts, most rMAb for therapeutic indications are expressed in mammalian cells, such as fibroblast from ovary of Chinese hamster (Chinese hamster ovary cells, CHO cells) [21, 22] or cancer cells from kidney of Syrian hamster (Baby hamster kidney cells, BHK cells) [23, 24]. Table 1 shows a summary of the approved rMAb to date.

Table 1. Recombinant monoclonal antibodies approved to the marked [1]

Drug name	Indication	Company	Year of aproval
ReoPro	Prevention of blood clot	Centocor	1994
Rituxan	Non-Hodgkin's lymphoma	Genentech/Biogen-IDEC	1997
Panorex	Colorectal cancer	GalxoSmithKline	1995
Zenapax	Kidney transplantation rejection	Hoffman-LaRoche	1997
Simulect	Prophylaxis of organ rejection in alogenic kidney transplantation	Novartis	1998
Synagis	Respiratory synctial virus	Medimmune	1998
Remicade	Rheumatoid arthritis	Centocor	1998
Hereceptin	Metastatic breast cancer	Genetech	1998
Mylotrag	Acute myleogenous lymphoma	Wyeth-Ayerst	2000
Campath-1H	B-cell chronic lymphocytic leukemia	Millenium/ILEX	2001
Zevalin	Non-Hodgkin's lymphoma	Biogen IDEC	2002
Humira	Rheumatoid arthritis	Abbott	2002
Bexxar	Non-Hodgkin's lymphoma	Corxia/GSK	2003
Xolair	Allergy	Genentech/Novartis	2003
Erbitux	Colon cancer	Imclone/BMS/Merck	2004
Avastin	Metastatic colon cancer	Genentech	2004
Raptiva	Psoriasis	Genentech/Xoma	2004
Tysabri	Multiple sclerosis	Biogen-Idec	2006
Vectibix	Metastatic colorectal cancer	Amgen	2006

The approved rMAb are either chimeric, humanized or fully human as shown in Figure 1. ReoPro, Rituxan, Simulec, and Remicade are chimeric antibodies, while Zenapax, Synagis, Hereceptin, and Capath-1H are humanized proteins. Humira is an example of a fully human therapeutic rMAb [6]. The approved rMAb are used in cancer therapy [25], inflammatory

diseases, cardiovascular diseases, transplant rejection suppression, allergic diseases, arthritis and renal prophylaxis. Additionally, there are at least 400 molecules in various stages of development and clinical trials [6], including Anti-Rh(D) IgG1, a humanized antibody against Rhesus D antigen. This protein was used as a model antibody in this study. Anti-Rh(D) IgG_1 is expressed in CHO cells in a protein-free chemical defined medium using fed-batch cultivation system. Anti-Rh(D) IgG_1 is a basic antibody with a molecular weight of 150 kDa. The heavy chain has a molecular weight of 51.3 kDa and contains 468 amino acids, whereas the light chain has a molecular weight of 25.0 kDa and contains 231 amino acids. The isoelectric point (pI) was calculated from the amino acid sequence as 8.36 for the whole protein, 8.69 for the heavy chain and 6.07 for the light chain. The pI of the heavy chain was determined to be 7.5 to 9.5 and the pI of the light chain was determined to be 6.5 using 2-D electrophoresis under reducing conditions with verification by Western blot. Anti-Rh(D) IgG_1 has been used for alloimmunization of fetuses and new born children to prevent hemolytic disease [26-30].

1.1.2 Soluble Fc fusion proteins

Another antibody-type molecule is a soluble Fc-fusion protein where the Fc region (hinge-CH2-CH3) is merged to the protein of interest containing the biologically active site, frequently a receptor or cytokine. Advantages of this type of molecule are higher half-life *in vivo*, easier purification and large-scale production in kilogram quantities. There are three Fc-fusion proteins on the market today, as shown in Table 2, which exceed $9 billion in annual sales [1].

Table 2. Soluble Fc fusion proteins approved to the marked [1]

Drug name	Indication	Company	Year of aproval
Enbrel	Rheumatoid arthritis, psouriasis, ankylosing spondylitis	Amgen	1998
Amevive	Psouriasis	Biogen-Idec	2004
Orencia	Rheumatoid arthritis	Bristol Myers Squibb	2005

Recombinant Fc-fusion proteins are also expressed in mammalian cells due to the need for post-translation modifications, in particular glycosylation, and were used for immunotherapeutic treatments. In Figure 3 the structure of a soluble Fc-fusion protein is shown.

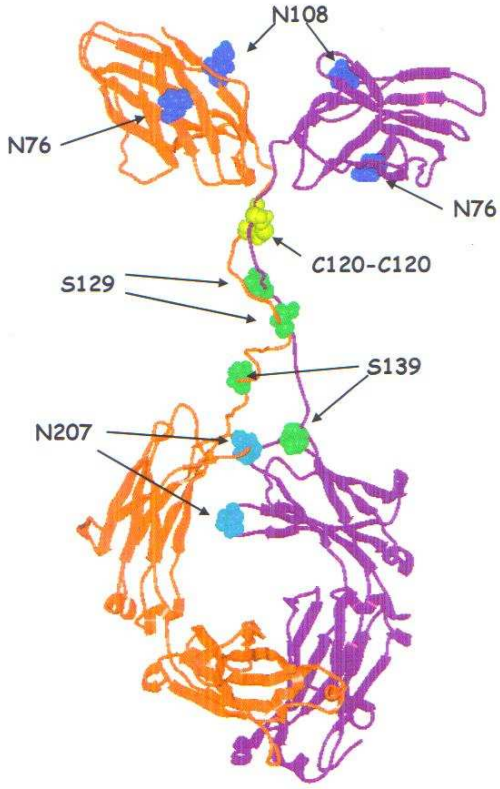

Figure 3. Structure of a soluble Fc fusion protein expressed in CHO cells.

The fusion protein shown in Fig. 3 has a molecular weight of 92.3 kDa with a pI ranging from 4.5 to 5.5. The Fc-fusion protein contains two 357 amino acid homologous polypeptide chains. Each CH_2 and CH_3 consist of about 110 amino acids residues. The protein is stabilized with one interchain disulfide bound between Cys120 residues. Additionally, the protein has five glycosylation sites, two O-linked glycosylation positions (S129 and S139) and three N-linked glycosylation positions (N76, N108 and N207). A minimum of 20 isoforms were detected using 2-D electrophoresis under reducing conditions.

1.2 Production

1.2.1 Upstream

Chinese hamster ovary (CHO) cells are the most common expression system for large scale rMAb and Fc-fusion protein production [31, 32]. The advantages of mammalian cells are secretion of desired antibodies in comparison to the *E.coli*, where the product is aggregated in inclusion bodies and protein refolding is required [9], and correct posttranslational modifications, such as glycosylation. Figure 4 shows typical glycoforms of human IgG with non-, mono- and digalctosylations and optimal sialylations.

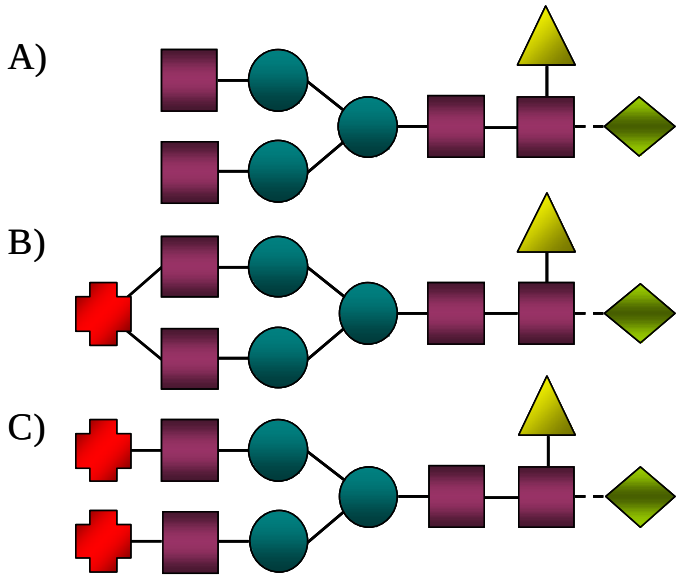

Figure 4. Glycosylation pattern of IgG. A) non-galactosylation; B) mono-galactosiyation; C) di-galgctosylation. – galactose; – N-acetyl-D-glucosamine; – mannose; – fucose; – sialic acid.

Antibodies have 2 to 3% (w/w) glycosylation. The glycosylation pattern is influenced by the culture conditions, like ammonia, dissolved, oxygen, glucose level, lipid compositions, pH and protein content in the medium [33]. CHO cells are sensitive to shear forces and require complex media with grow factors, such as plateled-derived-growth factor (PDGF), that regulate growth and differentiation, hormones, like insulin, hydrocortisone, estrogen, that regulate membrane transport and structure of cell surface, essential nutrients and metabolites [34]. Therefore calf (SC) and fetal bovine serum (FBS) at concentrations of 10% (v/v) with

high protein content are often supplemented to the growth medium. Serum (SC and FBS) is not chemical defined, has high batch-to-batch variation and has the risk of virus, endotoxin and bovine spongiform encephalopathy (BSE) contamination. Alternatively, serum-free media, such as Ham's F10 and F12, or protein-free media [35], where all animals constituents are replace with recombinant proteins are used [36]. The benefits of animal component free media are lower degree of variability, minimization of contamination risk and low cost.

Another advantage of using CHO cells in rMAb production is the ability to use a fed-batch cultivation system [37] with low cell density. Low cell densities often offer substantial benefit to the subsequent downstream process (DSP). However the cell density must be optimized to provide a sufficient titer, often by extending the cultivation time, which increases host cell proteins (HCP) and may increase the aggregate content. The primary and secondary cell metabolites have to be removed during the purification process. Often there will be a critical impurity, such as a monocyte chemoattractant protein-1 (MCP-1) that must be removed to a very low level (often to the sub ppm range). MCP-1 also named CCL2 [38, 39] is an acidic protein with molecular weight of ~ 30 kDa. It has a pro-inflammatory effect in humans [40] and is involved in nonalcoholic fatty liver disease [41], multiple sclerosis [42], human hearth [43], and prostate cancer [44, 45]. Additionally, a pathogenic effect in bowel diseases [46], atherosclerosis [47] and chronic lung diseases, like panbronchiolitis, asthma, cystic fibrosis and bronchitis [48] has been reported. In instances like this, the production of rMAb requires collaboration between upstream and downstream processing to provide sufficient quantities of pure, high quality material.

1.2.2 Downstream

The objective of DSP development is the establishment of sufficient, safe and fast processes for the purification of the rMAb from culture supernatants and broth. Common DSP goals are process yields over 50%, reduction of aggregates below 5%, HCP reduction to below 100 ppm, reduction of DNA to ppm levels and a minimum of 10-15 logs of viral clearance. By far, the DSP is the most cost intensive part of the manufacturing process. Numbers for the cost of DSP range from 60% to 80% of total production costs. The expenses are the cost of the chromatography media, buffers, water for injection, labor, hardware, utilities and

analytical tools. In large scale production processes the throughput and productivity are critical to the success of the product. Throughput and productivity are depended on size, type and backbone of chromatographic media, flow rates, separation efficiency, and column length [49].

For chromatography, there are three main factors to consider when designing a step: productivity, yield and purity. Unfortunately, it is not possible to maximize all three in one DSP step. At best, you can maximize two, but at a significant cost to the third factor. The productivity triangle is shown in Figure 5; to maximize productivity a compromise between a high yield and sufficient purity is required.

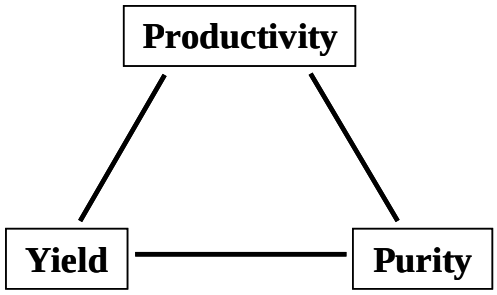

Figure 5. Productivity triangle. The productivity depends on the arrangement between yield and purity [49].

The optimization of each DSP step includes not only the individual parameters of that step, but also the interaction with the preceding and subsequent steps. The process specifications need to be addressed globally. Therefore, a minimal number of steps and simplicity of each step are highly desired during DSP development.

Downstream processing can be broken down to clarification, capture, intermediate purification and polishing steps. In between steps, buffer exchange and/or concentration/dilution may be required as well. Figure 6 shows the general overview of DSP.

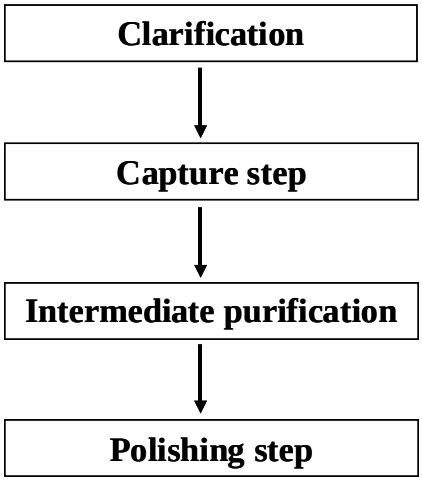

Figure 6. General scheme of downstream process of recombinant proteins.

1.2.2.1 Clarification

The first DSP step is the clarification of the desired protein from the cells, cell debris and other insolubles using filtration or centrifugation. The critical issue for this step is cell damage and the subsequent introduction to the product stream of additional host cell proteins, nucleic acids and other cell metabolites. Non-adsorptive and adsorptive membranes are available. Non-adsorptive membranes are chemically inert depth filters; adsorptive membranes contain functional groups that bind either the product or impurities. The membranes have the pore sizes between 0.1 to 10 μm and are utilized to eliminate microparticles, such as cells, cellular debris, aggregates, precipitates, bacteria and other non-dissolved moities. For large-scale processes, depth or microfiltration (MF) is often used. This technique provides easy-to-use disposable filter units with low initial costs, and relatively simple validation.

Following the cell removal step, ultrafiltration (UF) is often used to remove soluble impurities that are smaller than your target protein from the process stream, such as low molecular weight HCP, salts and other media components. The pore size of the UF membranes is given in molecular weight cut-off and is typically 10 – 100 kDa.

Dead-end filtration (e.g. depth filtration) and cross-flow filtration (e.g. UF) modes are often used in series during the clarification step.

1.2.2.2 Capture step

The two main objectives of the capture step are to reduce the volume and remove as many harmful contaminants, like proteases, DNA, HCP, as possible. Unit operations such as precipitation and extraction are economical, but less specific then the more specific and costly chromatography techniques. Chromatography is the most popular method for the capture step due to high efficiency, throughput and yield. For the capture step speed, throughput and recovery are more important than resolution. Time can be reduced by applying high flow rates and using resins with larger particle diameters (100-300 µm) and improve flow properties (e.g., convective flow resins or monoliths) to allow higher flow rates with the viscous culture supernatants. The capture step should have step yields of approximately 85% and increase the purity to around 70%.

1.2.2.3 Intermediate purification

Intermediate purification can include one or more steps. The steps should be orthogonal such as an ion exchanger followed by a hydrophobic interaction step. Typically resins used in intermediate purification steps have particle diameters between 30-100 µm. During this treatment clearance of remaining contaminants, such as HCP, nucleic acids, endotoxins and virus particles, occurs. Large capacity, high recovery and good resolution are requirements for this DSP stage.

1.2.2.4 Polishing step

The last purification step removes residual impurities that are closely related to the product, such aggregates, deamidated isoforms or glycocoforms. Very high resolution using high performance chromatographic media with particle diameters between 10 to 30 µm and extremely high recovery (>95%) are expected. Reduced flow rates and relatively low binding capacity are common for polishing steps. Size exclusion chromatography, ion exchange, hydrophobic interaction and affinity chromatography can be used.

1.2.2.5 Additional steps

In addition to the main purification stages, various intermediate activities such as concentration and buffer exchange are required. The conventional concentration and buffer

exchange method applied in large-scale DSP is ultrafiltration using tangential flow filtration (TFF). The concentration of the feed material is more important for non-adsorptive chromatography, such as size exclusion chromatography (SEC), where only 5% of the total column volume can be loaded, than for adsorptive chromatography. At small scale, other concentration and buffer exchange methods are also used, e.g. batch adsorption, dialysis, adsorption chromatography, and precipitation. The number of concentration and buffer exchange steps should be minimized to maximize throughput and productivity. An example of this would be a process using affinity chromatography as capture step, followed by ion exchange and finally hydrophobic interaction chromatography (HIC). For affinity chromatography, the culture supernatant only needs to be concentrated to reduce the loading time, without pH and conductivity adjustment. The affinity column eluate may either be directly loaded onto the ion exchange resin or may require a simple pH adjustment. The ion exchange eluate may only require a simple addition of high salt buffer to allow for efficient loading onto the polishing HIC column.

1.2.3 Impurities clearance

1.2.3.1 Virus clarification

Virus contamination can be caused by use of cell lines from infected animals, contaminated reagents or equipment and improper handling of the cell line [50]. The use of serum-free, protein-free and/or BSE-free media decreases the contamination risk. Although virus-free cell lines and raw materials are required today, the DSP must have the demonstrated capability to provide sufficient virus clearance. Virus clearance of 10 – 15 logs is common in the industry.

1.2.3.2 Endotoxin

Endotoxins, also called pyrogens, are highly negatively-charged lipopolysaccharides released by gram negative bacteria. They consist of a non-polar lipid region (lipid A), oligosaccharide core and a heteropolysaccharide region (O-antigen) (ref). Due to the fact, that bacteria are ubiquitous, the risk of endotoxin contamination is very high. For the sufficient clearance adsorptive chromatography, such as AEC, HIC or affinity chromatography should be used in

the DSP. However, AEC does not provide sufficient endotoxin clearance for acidic proteins. Endotoxin specific ligands have been developed and are commercially available (ref). CHO cell processes have lower endotoxin removal requirements than *E.coli* processes, for example. The limit of endotoxin in intravenous treatment is 5 endotoxin units (EU) per kilogram body weight per hour. Therefore, the size and duration of the dose is also important in determining the required endotoxin clearance.

1.2.3.3 DNA

Deoxyribonucleic acid (DNA) is an important issue especially for clarification and capture step in large scale. DNA increases the viscosity of the product stream and can impede the filtration and chromatographic steps by increasing the pressure drop across the unit operations. The DNA content in CHO cell culture supernatant is relatively low in comparison to other expression systems, such as *E.coli*. The advantage is the secretion of expressed product and minimization of cell damage during early processing. DNA is highly negatively charged, and therefore, high reduction can be achieved using AEC.

1.3 Purification platforms

1.3.1 Affinity based platform purification

Unfortunately, there is no general purification scheme available for all biopharmaceutical products. Due to the similar structure of rMAb however, there is a generally accepted platform process available [1, 51] that is based on Protein A affinity chromatography, see Figure 7.

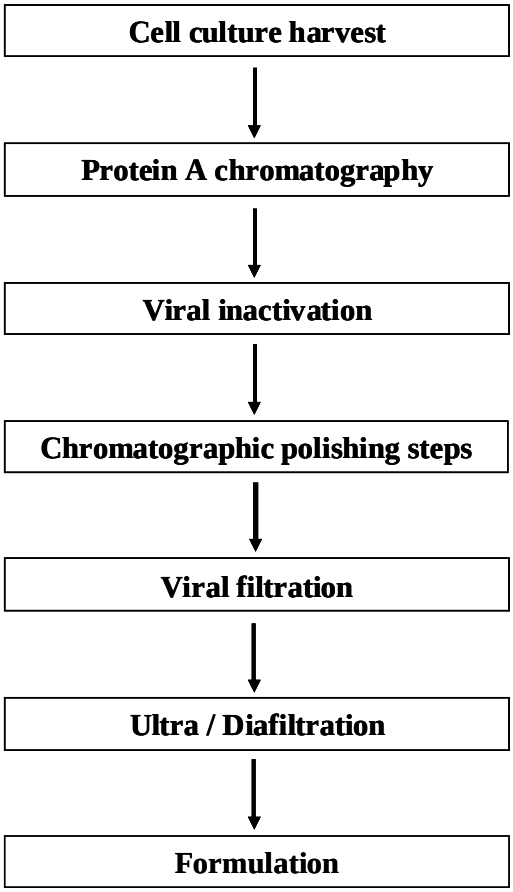

Figure 7. Platform downstream process for monoclonal antibodies [1, 51].

The platform process is based upon the use of *Staphylococcus* Protein A (SpA) or a modified SpA chromatography resin used as a capture step followed by two polishing steps using ion exchange chromatography. SpA binds selectively to the Fc-region of rMAbs and Fc-fusion proteins. Chromatography using SpA or modified SpA resins is very efficient, often resulting in 99.5% reduction in HCP [52] with relatively good dynamic binding capacities [53, 54] and step yields [55] > 99% [1]. Additionally, due to the specificity, clarified culture supernatant can be directly loaded onto the column without requiring pH conductivity adjustment. The stability of SpA and modified SpA resins have demonstrated effective use at 50 cycles [56] to 300 cycles [51]. Leaching of the SpA A ligand into the product stream requires demonstration of adequate removal. With the availability of modern SpA media and the subsequent purification steps, the leachable SpA levels remaining in the purified product stream are very low. Jungbauer et al. reported a value of < 4 ppm [57]. Additionally, by

using a short loading time and thus decreasing the contact time between culture proteases and the SpA media, the SpA leaching rate can be further reduced [57, 58]. Unfortunately, SpA media are very expensive, and the extra expense must be taken into consideration if the process is to be transferred to a large-scale manufacturing site. One solution is to use a smaller SpA column and cycle the column several times per batch which decreases the per batch cost of the SpA media.

Following the SpA capture step, two orthogonal chromatography steps are used, e.g. AEC for DNA and endotoxin removal and CEC or HIC for elimination of aggregates and/or degradation products [1]. Other options could be hydroxyapatite and immobilized metal affinity chromatography (IMAC) or CEC and AEC operated in flow-through mode [32]

Application of platform approaches speeds up the development of purification process, resulting in rapid progression to clinical trials and subsequent time-to-market [59].

1.3.2 Non-affinity based process

Currently, many companies attempt to replace the SpA capture step despite its excellent performance, mainly to reduce the cost of goods [60-62].Follman and Fahrner [52] used factorial screening to successfully replace SpA as the capture step. They described three non-affinity based column sequences with HCP reduction to < 2 ppm, which is comparable to the SpA-affinity based process. For the purification of rMAb, the high pI of IgG is advantageous. The rMAb binds to a CEC column at high pH allowing most of the HCP to be removed in the flow-through. If the rMAb pI is low, it is difficult to find an alternative method to SpA-based purification [63]. Nevertheless, for the acidic Fc-fusion protein used in this study, a non-affinity based process was investigated. The process development examined combinations of CEC, HIC and AEC. The tracking of impurities is more important in non-affinity based processes than for process with SpA as capture step.

1.4 Analytical methods

1.4.1 Traditional analytical tools

To ensure the safe and robust production of high quality rMAb products, established analytical approaches are required for qualitative and quantitative analysis of the product, product- and process-related impurities and contaminants. The International Conference on Harmonization (ICH), the European Medicines Evaluation Agency (EMEA), the Food and Drug Administration (FDA) provide a list of established analytical techniques to ensure the purity, identity, integrity, and activity of drugs [64].

1.4.1.1 Electrophoresis

1 dimensional electrophoresis

Electrophoresis is a common technique for the visualization and semi-quantification of proteins under native or reduced conditions. Commonly, 1-dimensional (1-D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used for process monitoring [65]. SDS binds to the protein in the ratio of 1.4 g_{SDS} per $g_{protein}$ and generates negatively charged micelles around the protein. Proteins migrate from the cathode to the anode in the presence of an electrical field at a rate proportional to the molecular weight of the protein. Proteins are visualized on the gel using a silver stain which results in a detection range of 0.5 - 1 ng of protein per band [66]. Up to 17 samples can be analyzed on one gel. The development of commercial available pre-cast gels and buffer systems have resulted in shorter separation times (approximately 50 min) and higher band resolution, thus making 1-D SDS-PAGE a suitable method for tracking of impurities and product-related materials in DSP development.

2 dimensional electrophoresis

2-D electrophoresis [67] provides a higher level of resolution compared [68] to 1-D gels and can be used to analyze protein patterns of complex samples. The first dimension separates proteins based upon the protein's isoelectric point (pI) using isoelectric focusing gels (IEF). The second dimension separates proteins based on molecular weight using SDS-PAGE. The

disadvantage of 2-D electrophoresis is high gel-to-gel variation due to long focusing time and drifting of gradients resulting in results that are not comparable. Additionally, the throughput is very low, only one sample per gel can be analyzed. This method requires an extensive sample preparation process and is generally not applicable for DSP development.

1.4.1.2 Immunoassays

Western blot

After electrophoresis proteins can be blotted to a nitrocellulose [69, 70] or polyvinylidene difluoride (PVDF) membrane and visualized using specific antibodies. It is a very helpful tool to distinguish between product-related bands and impurities. For the detection of IgG molecules, an anti-human IgG antibody is used. For the identification of HCP, antisera against all HCP proteins from a null cell line are used [71]. Detection antibodies are conjugated with horseradish peroxidase or alkaline phosphatase and are identified due to a color reaction with an appropriate subtract, 3,3' diamino benzidine tetrahydrochloride (DAB), H_2O_2 or 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 4- nitro blue tetrazolium chloride (NBT), respectively. Alternatively, the detection antibody can be identified by a secondary antibody for a two-step development method. Western blot is used as semi-quantitative analysis for process characterization. The limit of detection is usually between 20 - 200 ppm. However, Western blotting is time consuming, requiring approximately 3 hours for electrophoresis and blotting without including time required for detection and development.

Enzyme-linked immunosorbent assay (ELISA)

ELISA, first reported by Wide and Porath [72], is applied for direct quantification of products, HCP and critical impurities. The desired proteins are immobilized on a solid surface, usually a polystyrene microtiter plate due to adsorption or affinity binding to another antibody for “sandwich” ELISA. After immobilization the detection antibody is added and binds specifically to the desired protein based on affinity reaction. The detection antibody can be covalently linked to an enzyme, or it can be detected by a secondary antibody, like described above. ELISA results often not have a high level of precision dependent on the complexity of the samples and the binding strength of the antibodies. However, the detection limits for the ELISA are established using comparisons of samples between different process steps [64]. There are automated systems available with detection limits between 1 - 100 ppm.

1.4.1.3 High Performance Liquid Chromatography (HPLC)

Analytical size exclusion chromatography (SEC)

SEC is non-adsorptive chromatography technique that detects molecules based upon size in a large variety of conditions. For example, the detection of aggregates under native conditions which allows for the identification of covalent and non-covalent interactions can be accomplished [73]. The resolution of monomers, dimer, and higher-order aggregates as well as low molecular weight compounds can be observed using SEC. Additionally, SEC can be used to identify sample purity using ultraviolet detection at 214 nm or 280 nm. The linear detection range for antibodies is typically 0.1 - 500 µg, whereas the lower detection limit for aggregates is approximately 0.1%. Analysis can be completed in 30 - 60 minutes.

Analytical Protein A

For IgG quantification from crude samples a very specific, affinity chromatography method is required. Typically, rMAbs are quantified using a Protein A HPLC assay. The application of a high performance monolith Protein A chromatography assay with a linear range of 23 - 250 µg/ml, monitored by measurement of absorbance of UV light at 280 nm and a 5 minute analysis time has been reported [74].

1.4.2 A novel approach, 2-D DIGE

The two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) developed by Orange et al. [75] utilizes spectrally resolvable dyes, CyDye™ DIGE Fluor, to label protein samples prior to 2-D electrophoresis. Up to three different samples can be marked with CyDye DIGE Fluors, pooled together and co-separate simultaneously in the same isoelectric focusing and SDS-PAGE gel. The analyzing conditions are identical and the gel-to-gel variation is avoided. The principle of this method is shown in Figure 8.

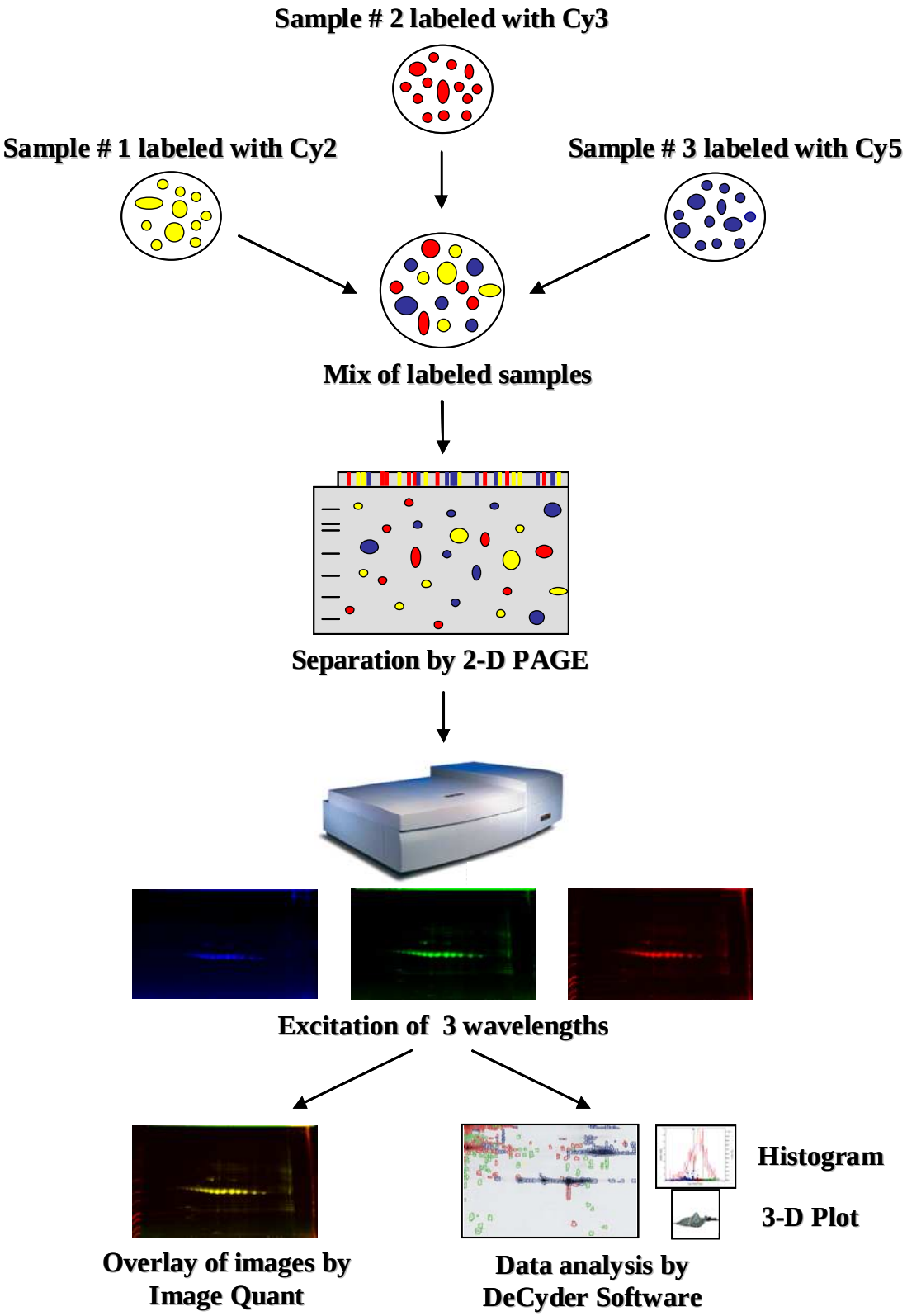

Figure 8. 2-D DIGE workflow in a CyDye DIGE Fluor minimal dye system

The three CyDye DIGE Fluor minimal dyes, Cy2, Cy3 and Cy5, include NHS ester active groups that covalently bind to the epsilon amino group on lysine of protein Figure 9.

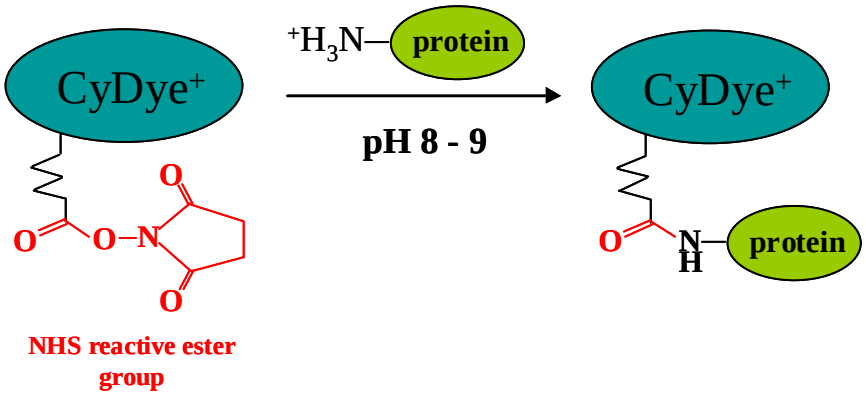

Figure 9. Binding of the CyDyes DIGE Flour minimal dyes to the back bound of lysine.

The labeling reaction requires 50 µg of protein per sample, and the reaction is limited by the ratio of dye to protein. This ensures the labeling of one lysine per protein and that ~3% of the total protein in sample will be tagged. CyDyes are size- and charge-matched, to allow migration of different proteins from different samples to the same position on a gel. The molecular weight of the CyDyes is ~500 Da which is comparable to unlabeled proteins after silver staining. CyDye DIGE Fluor minimal dyes carry a positive charge and replace the positive charge of lysine. Therefore, the pI of the protein does not change after labeling. The use of fluorescence yields high sensitivity, detection of up to 125 pg protein per spot, and acceptable for use to in applications with low abundance proteins.

After labeling, the protein samples are mixed together and loaded onto a single gel. The samples co-migrate on a single gel based upon their pI in the first dimension and based upon their molecular weight in the second dimension. Protein detection is accomplished by using the variable mode imager, Typhoon, with automated multicolor scanning. Individual gels can be scanned between glass plates without disturbing, drying or shrinking the gels. Excitation and emission spectra are presented in Figure 10.

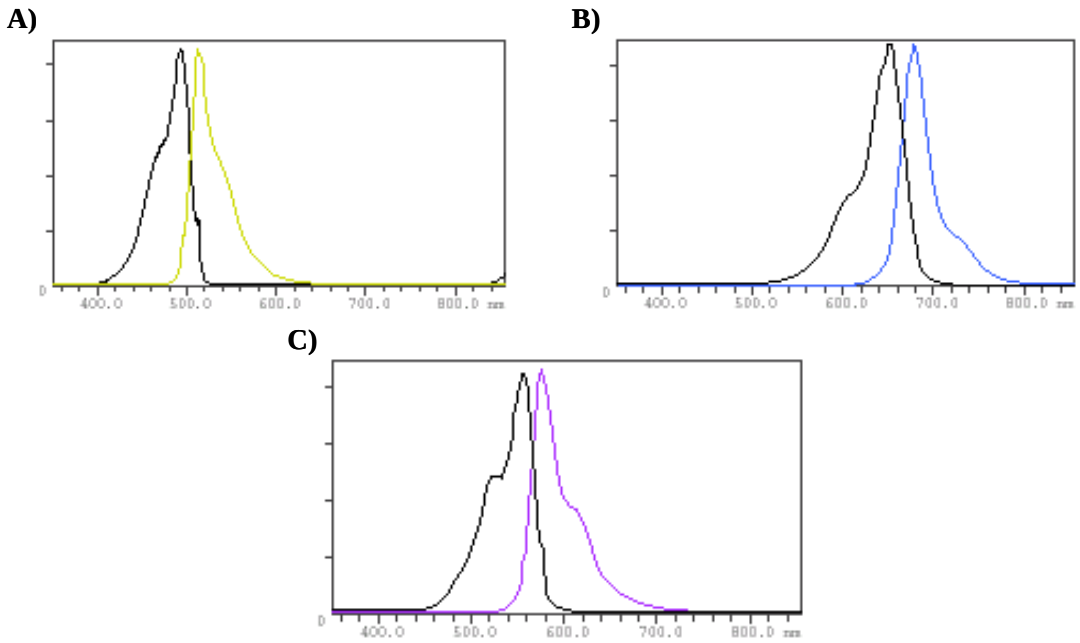

Figure 10. Excitation (black line) and emission spectra (color line) of CyDyes DIGE Flour minimal dyes. A) Cy2; B) Cy3; C) Cy5.

The data analysis is carried out using ImageQuant and DeCyder™ 2-D Differential Analysis Software. These tools automatically locate and analyze multiple spots.

Image Quant Software allows for the overlay of detected images from multiple samples. The spots that are identical in both samples generate a new color; e.g., if green spots represent proteins in sample labeled with Cy3 and red spots represent proteins in samples labeled with Cy5, than spots that are in the same concentration in both samples appears as a yellow spot.

DeCyder™ 2-D Differential Analysis Software includes two modules: Differential In-Gel Analysis (DIA) and Biological Variation Analysis (BVA), where multiple images from different gels are analyzed using statistical tests, such as ANOVA and student's t-test. For our study only DIA was used for protein spot detection from the same gel. The identical spot patterns are generated automatically using a novel, patented, co-detection algorithm. Background subtraction and gel artifact removal are further stages of the gel processing. The protein spot quantization is calculated as a volume ratio relating to the first loaded image. The spots are identified as decreased when the proteins in the first gel are in higher concentration; increased if the proteins in the first gel are in lower concentration; and similar if the spots are the same in both samples. The result can be summarized in a table or as a histogram. The number of detected, increased, decreased and similar spots is specified

allowing for the semi-quantitative analysis of removed proteins during the DSP. This analysis allows spot detection and minimizes user-to-user variation, while having high throughput, accuracy and reproducibility.

2 Objectives

The goal of this study was the application of two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) for downstream process (DSP) development. 2-D DIGE was used to characterize the purification steps during process development. This technique was used for the DSP development of a therapeutic monoclonal antibodies, a human monoclonal antibody IgG_1 against Rhesus factor D, called Anti-Rh(D) IgG1, and a Fc-fusion protein.

The first objective was to optimize the 2-D DIGE conditions for these two different molecules to achieve high spot resolution and identification of key isoforms and impurities. Anti-Rh(D) IgG1 is a basic, lowly glycosylated antibody, and the Fc fusion protein is an acidic protein with 10 glycosylation sites.

The second objective was to demonstrate, that 2-D DIGE is a reproducible and complementary method that can be used to aid DSP development. To demonstrate that this was possible, product fractions from various steps in the DSP process were analyzed with SDS-PAGE, Western blot, analytical SEC, analytical Protein A and ELISA in addition to 2-D DIGE.

Additionally, 2-D DIGE was tested as a tool to track and visualize impurities and isoform throughout the DSP process. Different DSP steps were compared, e.g. load sample, capture step and polishing step, to visualize the clearance of impurities during the processes and finally to apply this method for quality control.

The last objective was to utilize 2-D DIGE to compare different DSP. Two processes, one including SpA chromatography, the other without an affinity step were compared with respect to impurity patterns and process efficiency. 2-D DIGE is expected to provide easier process characterization and a better understanding of the unit operation steps.

3 Conclusions and Discussion

The development, control and validation of rMAb manufacturing processes are regulated by the International Conference on Harmonization (ICH), the European Medicines Evaluation Agency (EMEA), the Food and Drug Administration (FDA), and other worldwide government agencies. The regulation requires the use of established methods for qualitative and quantitative analysis of the product, product-related impurities and process-related impurities. Commonly used methods include 1-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, enzyme-linked immunosorbent assays (ELISA), quantitative polymerase chain reaction (qPCR), analytical HPLC including SEC, reversed phase and protein A, etc. These methods were selected to provide information for the control and monitoring of rMAb processes. Additionally, process analytical technology (PAT) has become more important not only during the manufacturing process to assure drug substance and drug product critical quality attributes are met [64] but also as a framework for innovative pharmaceutical development, manufacturing and quality assurance [76].

1-D SDS-PAGE is routinely used to visualize proteins of different molecular weight, whereas Western blots display only product-related bands. High and low molecular weight compounds are identified using analytical SEC. Analytical protein A HPLC analyses quantifies IgG concentration to allow for mass balance and yield calculations. ELISA tests quantify HCP, critical impurities, like MCP-1 and determine glycoforms giving an average of high and low glycosylated isoforms. .Two-dimensional (2-D) gel electrophoresis is not routinely used due to high labor requirements including sample preparation and high levels of gel-to-gel variation. 2-D gel electrophoresis does offer high resolution. A relatively new tool, 2-D fluorescence difference in-gel electrophoresis (2-D DIGE), reported by Orange et al. [75], overcomes the gel-to-gel variation issue. 2-D DIGE allows for the analyses of up to three different samples using fluorescence markers, called Cy2, Cy3, and Cy5. The dyes are mixed with a sample and up to three samples are analyzed on a single isoelectric focusing (IEF) gel in the first dimension and the using SDS-PAGE as the second dimension. Due to the identical conditions for all three samples, the gel-to-gel variation is minimized. Also, the use of fluorescent dyes allows for the quantification of very low differences in protein abundance. If one of the three samples is an internal standard, then the comparison of

multiple samples can be achieved using different gels with two samples and an internal standard per gel. The internal standard allows the software package, DeCyder, to detect the spots, compare them across gels due to the ability to normalized based on the internal standard. Decyder also allows the calculation of volume ratio, application of statistical tests, such as analysis of variance, ANOVA and student's t-test to detect minor differences in protein abundance between samples and spots.

2-D DIGE has high accuracy and reproducibility, relatively short analysis time, minimal user-to-user variation and can analyzing up to three samples on the same gel. 2-D DIGE is an established tool for protein analysis from complex biological samples [77]. The most common applications of 2-D DIGE are studies on protein expression levels in mammalian cells [78-80], microorganisms [81-83], and plant cells [84, 85]. 2-D DIGE is a helpful tool for understanding of the pathogenesis mechanism [86-88], and for the identification of biomarkers [89, 90] to develop novel drug targets or to monitor therapeutic processes. This approach has been used in the field of neuroscience [91], cancer proteomics [92-94], haematology [95], osteoporosis [96] , Parkinson´s-pathology, and Alzheimer´s-pathology.

This study was the first reported project using 2-D DIGE for DSP development. Two model proteins were used: a recombinant IgG_1 antibody against Rhesus D antigen, called Rh(D) IgG_1 and a Fc-fusion protein. Both proteins were expressed in CHO cells. Humanized Rh(D) IgG_1 was provided by Bioprocessing Technology Institute, A*STAR (Agency for Science, Technology and Research), Biopolis, Singapore and Fc-fusion protein was supplied by Bristol-Myers Squibb.

A two step affinity-based process using MabSelect Xtra as the capture step and CEC using Source30S as the polishing step were characterized using 2-D DIGE (publication 1). We elucidated the influence of cell viability and clone selection on the HCP pattern as well as the influence of different purification steps on the HCP and antibody pI characteristics. For this study, culture supernatants of two different MAb expressing clones, called M250-9 and M500-11, were harvested at high (~90%) or low (~40%) viabilities. The process was characterized using SDS-PAGE, Western blot, analytical SEC and 2-D DIGE. Up to 800 different spots were identified using 2-D DIGE, and it could be shown that the differences in cell viabilities had more influence on the spot pattern than the use of different clones. The 2-D DIGE spot pattern of MabSelect Xtra eluate pools from experiments using either the high or the low cell viability at harvest were analyzed Although SDS-PAGE analysis suggested

that the antibody had been a high degree of purity, 2-D DIGE analysis demonstrated that additional polishing step were required. Approximately 160 spots were detected by 2-D DIGE, and of the 160 spots, 60% were host cell proteins. The rest were derived from the antibody.

SDS-PAGE with silver staining has a limit of detection of 0.5 - 1 ng of protein per band [66], which is often not sufficient to detect of low abundance impurities. Additionally, 1-D separations do not have enough resolving power for adequate protein identification. 2-D DIGE overcomes both of these concerns and provides high resolution with a detection range down to 125 pg of protein per spot [75]. Furthermore, 2-D DIGE avoids the gel-to-gel variations seen with other gel electrophoresis techniques and provides a deeper understanding of the presence and quantities of impurities during the DSP. The robustness of the purification process can also be demonstrated by the identical spot patterns observed after purification of IgG from different culture supernatants. Additionally, product homogeneity can be monitored by 2-D DIGE. From this point of view, 2-D DIGE is a complementary tool for DSP development.

It is well known that Protein A resins are very expensive and thus many rMAb manufacturers seek alternatives to Protein A resins. Several commercial rMAbs already use non-affinity based processes, e.g., Humira. Non-affinity rMAb processes use combinations of ion exchange, HIC and/or mixed mode resins. In this work, a three-column DSP composed of CEC with SP Sepharose Fast Flow (SPFF), AEC with Q Sepharose FF (QFF) in flow-through mode and HIC with Phenyl Sepharose 6 FF (low sub) (PSFF) for clone M250-11 at low viability was explored (publication 2). Affinity and non-affinity processes were directly compared using standard analytical tools, such as SDS-PAGE, Western blot, analytical Protein A, analytical SEC and 2-D DIGE. Whereas the SDS-PAGE and analytical SEC show high impurity removal in both processes, the use of 2-D DIGE allowed for the detection of low abundance impurities. We concluded from the 2-D DIGE data that the affinity-based process was more efficient than the non-affinity process.

In addition, we also showed that the affinity-based process was very efficient without any additional optimization. To demonstrate this, we used a single post Protein A polishing step, instead of the two or more commonly used in platform processes. Even with the single post Protein A column, the product had high purity determined by 2-D DIGE. The endotoxin, DNA and viral clearance levels were not confirmed by orthogonal analysis. Jungbauer et al.

also reported the use of a two step affinity-based process for pilot scale purification of monoclonal antibody against human immunodeficiency virus HIV-1 [97]. With proper optimization, affinity-based rMAb platform processes may require only a single post Protein A chromatography step.

The non-affinity based rMAb process we used for our study was described by Follman and Fahrner [52]. They reported HCP removal comparable with an affinity-based process. In our experiments, the polishing step had very low step yields due to the formation of aggregates on the HIC column. The comparison of two polishing steps with 2-D DIGE showed a higher efficiency of the affinity-based process. Therefore, application of 2-D DIGE for optimization steps can decrease the development and optimization time for DSP.

The Fc-fusion protein example involved the development of a three column non-affinity based DSP (publication 3). Using factorial screening, four sequences containing different compositions of AEC, CEC, HIC in flow-through and bind-elute mode were tested. The critical issues for DSP, such as reduction of additional steps and volume reduction during the process, were considered. Comparison of column sequences, confirmed Follman and Fahrman's conclusion [52], that the order of chromatography steps in the sequence influences the purification result. A process consisting of AEC using Q Sepharose XL (QXL) as the capture step followed by HIC with Phenyl-650M (P650M) and AEC using Q Sepharose FF (QFF) as the polishing step met the established quality specifications but the overall yield (41%) was less than the goal (50%). A detailed investigation of the four sequences indicates that process optimization must be global. An example is a comparison of HIC steps using Phenyl-Sepharose FF (PSFF) in flow-through mode and P650M in bind-elute mode. The QXL and HIC step yields were comparable, but since the P650M step had a significantly higher MCP-1 clearance, the yield of the QFF step could be increased to 94%. Thus, the overall yield was 41% with the P650M column compared to 33% with the PSFF column.

2-D DIGE during process development was utilized to visualize the DSP, including the characterization of product isoforms and the ability to track critical impurities. The Fc-fusion protein had 20 glycoforms that were visible in the harvest sample, but only 17 were present in the purified product pool. The ELISA declares the protein glycosylation as SA ratio and gives information about the average of high and low glycisilated isoforms. Using 2-D DIGE, it is possible to detect each individual isoforms and to investigate the ratio of each species

using the spot size. The qualitative analysis of glycoforms and the monitoring of glycoform removal during the process are essential for process understanding. Additionally, 2-D DIGE allows for the identification and tracking of critical impurities using their molecular weight and isoelectric point.

A $1 billion drug loses about $3 million dollars every day it is not on the market. Thus, any acceleration of process development can result in faster time to market and a significant increase in profits.

In this study we demonstrated the following:

1) The use of 2-D DIGE allowed for improved and shortened DSP development for two proteins: Anti-Rh(D) IgG1 and a Fc-fusion protein;
2) 2-D DIGE is a reproducible and complementary method for DSP development;
3) 2-D DIGE is a very useful tool for impurity tracking and visualization of isoform removal during the DSP;
4) 2-D DIGE can be utilized for comparison of different downstream processes.

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5. Publications

2-D DIGE to expedite downstream process development of human monoclonal antibody
Julita K. Grzeskowiak, Anne Tscheliessnig, Poh Choo Toh, Janet Chusainow, Yih Yean Lee, Niki Wong, Alois Jungbauer
For submission to Protein Expression and Purification

2-D DIGE for comparison of affinity and non-affinity based downstream process of recombinant monoclonal antibody
Julita K. Grzeskowiak, Anne Tscheliessnig, Poh Choo Toh, Janet Chusainow, Yih Yean Lee, Niki Wong, Alois Jungbauer
For submission to Journal of Chromatography A

Use of 2-D differential gel electrophoresis to facilitate development in downstream process
Julita K. Grzeskowiak, Junfen Ma, Ronald C. Bates, Alois Jungbauer
For submission to Electrophoresis

For submission to Protein Expression and Purification

2-D DIGE to expedite downstream process development of human monoclonal antibody

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Abstract

Two dimensional fluorescence differential gel electrophoresis (2-D DIGE) is an established method for assessing protein expression strategies, understanding pathogenesis mechanisms, characterizing biomarkers, and controlling therapeutic processes. We applied 2-D DIGE to facilitate the development of the purification process of a recombinant IgG_1 antibody against Rhesus D antigen expressed in Chinese hamster ovary cells. The variability of two expression clones as well as the influence of cell viability on host cell protein pattern could be quantitatively assessed. Up to 800 different spots were identified, and it could be shown that the differences in cell viabilities had more influence on spot pattern than the expression clones. Furthermore, we could demonstrate that the purification scheme is robust, as identical spot patterns were observed after purification of IgG from different culture supernatants.

Keywords: 2-D DIGE; IgG; Recombinant antibody; *Staphylococcus* ProteinA chromatography; Affinity chromatography

1 Introduction

Recombinant antibodies are the largest class of biopharmaceuticals [1-4]. They are preferably produced in Chinese hamster ovary (CHO) cells [5-8] in fed-batch culture [9] with serum-free, defined culture medium [10-12]. In order to maximize titers, the cultivation time of fed batch cultures are often extended and culture broths are harvested at low cell viabilities. This may lead to increased host cell protein and aggregate content in the harvest. The culture conditions [13, 14] will also influence glycosylation [15, 16] or other post-translational modification of the antibody resulting in the secretion of different variants [17] that give the typically observed broad isoelectric point (pI) pattern of recombinant antibodies [18, 19]. Among other parameters this pI pattern defines the final product [20, 21]. Nevertheless, the variations in post-translational modification of the antibodies are limited and introduce less variability in comparison to other recombinant proteins, like recombinant human erythropoietin [22, 23]. Downstream processing (DSP) has the task to compensate for the variations resulting from the cultivation process and for the inability to produce exact batches with consistent protein patterns and host cell impurities.

The most frequent downstream processing of antibodies is composed of a *Staphylococcus* Protein A (sPA) affinity chromatography [24-27] as capture step followed by one or two additional chromatography steps such as anion or cation exchange chromatography (AEC, CEC) [28], respectively. Often, also hydrophobic interaction chromatography (HIC) or hydroxyapatite are included as orthogonal steps [29]. In addition to chromatography, dedicated virus inactivation steps have to be included [30, 31]. Combining the different steps processes are designed that are capable of reducing host cell proteins and DNA to the required levels.

A variety of different methods are applied to verify product characteristics or process performance. Common assays founds are among others sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, analytical Protein A chromatography, analytical size exclusion chromatography (SEC) and enzyme-linked immunosorbent assay (ELISA) [32]. 2-D electrophoresis is not included due high gel-to-gel variation. 2-D DIGE, as described by Orange et al. [33] and recently applied for identifying of biomarkers [34, 35], designing novel drug targets [36] and monitoring of therapeutic processes [37], overcomes this disadvantage of 2-D gel electrophoresis. Three different samples labeled with three different fluorescence markers, called Cy2, Cy3 and Cy5 (Table 1), can be pooled together and analyzed on one isoelectric focusing (IEF) and SDS-PAGE gel. Thereby the gel-to-gel variation is avoided [38-40]. Differences in protein pattern between cell lines and culture supernatants form different culture conditions, or changes during DSP can be reliably

visualized making 2-D DIGE. Thus, 2-D DIGE is a potentially complementary method for process development of biopharmaceuticals.

The objective of this work was to demonstrate 2-D DIGE as a valuable tool for DSP development, using a recombinant anti-Rh(D) IgG_1 [41, 42] as model system. We elucidated the influence of cell viability and clone selection on the host cell protein pattern as well as the influence of the different purification steps on the host cell protein and antibody pI characteristic. The selected purification process comprised of sPA chromatography using a new-generation resin, MabSelect Xtra, as capture step followed by a polishing step employing Source 30S. In this case, two clones from two fermentations with high and low viability at harvest were studied.

2 Material and methods

Culture supernatant

Parental dihydrofolate reductase (dhfr)- deficient CHO DG44 (Invitrogen, Carlsbad, CA, US) cells were transfected with two vectors, encoding the heavy chain and light chain of an IgG against RhD antigen (gift from Professor Robert Raison, University of Technology Sydney, Australia, with slight modification to the light chain expression vector). Stable cell pools obtained after selection were used to isolate single cell clones and subjected to gene amplification using step-wise increments of methothrexate (MTX). Clone M250-9 and M500-11 were high producer clones derived from an additional round of single cell cloning after amplification at 250nM and 500nM MTX respectively, and were adapted to in-house proprietary protein-free chemically defined media. Batch bioreactor cultures were conducted with each of the clones and harvested at two time points: one at high viability (>90%) (specific productivities of M250-9 and M500-11 were 18 and 33 pg/cell-day respectively) and the other at low viability (~ 40%) [43].

The culture broth was clarified by centrifugation at 4000 rpm (Beckman GS-6R, Palo Alto, CA, USA) for 10 min followed by filtration (0.45 + 0.2 μm, Sartobrand 150, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The supernatant was then frozen at -20ºC until further use. Prior purification, the samples were again filtrated using a 0.22 μm filter and concentrated using Tangential Flow Filtration (Millipore, Billerica, MA, US) with regenerated cellulose membranes, cut off 10 kDa (Millipore, Billerica, MA, US).

Preparative chromatography

Preparative chromatography was performed using ÄKTA*explorer 100* (GE Healthcare, Uppsala, Sweden). 4.3 ml MabSelect Xtra (GE Healthcare, Uppsala, Sweden) and 0.6 ml Source30S (GE Healthcare, Uppsala, Sweden), were packed into Tricorn™10/50 column and Tricorn™5/50 column (GE Healthcare, Uppsala, Sweden), at the bead high of 5.5 cm and 3 cm, respectively. If not given otherwise all chemicals were purchased from Merck, Vienna, Austria. All buffers were filtered using 0.22 μm regenerated cellulose filters (Millipore Corporation, Billerica, MA, USA) and degassed prior to use. All experiments were performed at room temperature.

The MabSelect Xtra column was first equilibrated with 5 column volumes (CVs) of Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.2 mM Ha_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4) followed by loading of the filtered (0.22μm) sample. Unbound sample was washed out with 7 CVs of PBS and adsorbed IgG was then eluted using a step gradient of 0.1 M glycine

(pH 3.5) and collected in fractions of 1 ml which were immediately neutralized using 1 M Na_2CO_3 to a final pH of 9. The column was reequilibrated with 5 CVs of PBS. The linear flow rate was 61 cm/h except for elution when a linear flow rate of 76 cm/h was applied. Detection was carried out by measurement of the absorbance at 280 nm and 214 nm.

For the second purification step, the elution pool of the sPA chromatography was diafiltrated into 10 mM CH_3COONa pH 5.0 using PD10 Column (GE Healthcare, Buckinghamshire, UK) containing Sephadex™ G-25 M resin. The procedure was carried out adhered to 'PD-10 desalting column' instruction manual: The column was equilibrated using 25 ml equilibration buffer. The sample, with the total volume of 2.5 ml, was loaded and proteins were eluted with 3.5 ml equilibration buffer.

Desalted and filtrated (0.22 µm) samples were loaded into Source 30S column. Again the column was first equilibrated using 10 mM CH_3COONa pH 5.0. The sample was loaded and unbound material was washed out with 7 CVs of equilibration buffer. IgG was eluted with a 20 CVs linear gradient using 10 mM Na_2HCOOH, 1 M NaCl, pH 5.0 buffer. The column was regenerated using 5 CVs of 1 M NaCl and finally reeqilibrated with 5 CVs of. All steps were performed at the linear flow rate of 76 cm/h.

Total protein concentration

Total protein concentration was determined by absorbance at 280 nm using U2001 UV/Vis Spectrophotometer (Hitachi, Pleasanton, CA, US). The protein concentration was calculated using Beer-Lambert equation with 1.2 as molar extinction coefficient.

Analytical SEC

Analytical SEC experiments were performed on a high pressure liquid chromatography (HPLC) system, Agilent 1100 Series (Waldbronn, Germany) in isocratic mode using PBS buffer at flow rate 0.5 ml/min**.** 50 µl samples were loaded into a Superdex™ 200 HR10/30 column (GE Healthcare, Uppsala, Sweden). Detection was carried out by measurement of the absorbance at 280 nm. The molecular mass calibration was performed using polyclonal IgG obtained from Octapharma, Vienna, Austria.

SDS-PAGE and Western blot

Electrophoretic separation was carried out using the NOVEX System (Invitrogen, Carlsbad, CA, US). Samples were combined with 4X NuPAGE® LDS Sample Buffer (106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM Phenol Red, pH 8.5), reduced with 0.1% DTT, heated at 100°C for 10 min

and loaded on the NuPAGE® Novex 4–12% Bis-Tris Gels (1.0 mm). Runs were performed using NuPAGE® MES SDS Running Buffer (50 mM MES, 50 mM Tris base, 35 mM SDS, 1 mM EDTA, pH 7.3) at 200 V, 400 mA, 40 W for 50 min. The gels were stained using silver staining as described by Heukeshoven [44] or blotted to a 0.2 μm nitrocellulose membrane at 400 V, 200 mA for 2 hours. The transfer was facilitated using blotting buffer composed of 50 mM $NaB_4O_7 \times 10\ H_2O$, 0.1% (v/v) SDS, 10% (v/v) methanol. Membranes were blocked with 3% bovine serum albumin, 0.1% Tween20 in PBS for 2 hours or over night. After three 5-minute wash steps with 0.1% Tween 20 in PBS, the membrane was incubated with a 1:1000 dilution of the anti-human IgG Fc specific conjugated to horseradish peroxidase in 0.1 % Tween20 in PBS. After further two 5-minute washing steps the IgG heavy chain was detected by color reaction using 1.7 mM 3,3'-diaminobenzidine tetrahydrochloride, 30% H_2O_2 in PBS buffer.

2-D DIGE

2-D DIGE was performed using Ettan DALT System (GE Healthcare, Uppsala, Sweden). If not given otherwise all chemicals were purchased from Merck, Vienna, Austria.

Sample preparation included desalting using the 2-D Clean Up Kit and labeling using CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3 and Cy5) obtained from GE Healthcare (Uppsala, Sweden). The desalting assay was prepared followed the '2-D Clean-Up Kit' procedure for sample volumes of 1-100 μl containing 100 μg protein per sample. 300 μl precipitant was mixed by vortexing with 1-100 μl protein sample and incubated on ice for 15 min. After adding of 300 μl co-precipitant and mixing, the tube was centrifuged at 12 000 × g, 4ºC for 5 min and the supernatant was removed. The pellet was washed with 40 μl of co-precipitant and incubated on ice for 5 min. The sample was centrifuged for 5 min and the wash solution was removed. The pellet was dispersed in 25 μl distilled water by vortexing (5-10 seconds). The next wash step was carried out using 1 ml of wash buffer, pre-chilled for at least 1 h at -20°C, and 5 μl wash additive. After mixing and fully dispersing the pellet, the protein solution was incubated on ice for at least 30 min and vortexed for 30 seconds once every 10 min. The tube was centrifuged at 12 000 × g for 5 min and the supernatant was removed and discarded. The pellet was air dried briefly for no more than 5 min and resuspended in an appropriate volume of labeling buffer (8 M urea, 4 % CHAPS (Sigma, St. Louis, MO, US), 30 mM Tris, pH 8.5).

The desalted samples, each containing 50 μg total protein, were labeled by a 30 min on ice incubation with 2 μl of 200 pmol/μl marker solutions in 99.8% anhydrous dimethylformamide (USB Corporation, Cleveland, OH, US) with. The reaction was stopped by adding 1 μl 10 mM L-Lysine. Three samples labeled with Cy2, Cy3 and Cy5, respectively were pooled together

and mixed with rehydration buffer (2 M thiourea, 6 M urea, 4% CHAPS, 20 mM DTT (GE Healthcare, Uppsala, Sweden) and 0.25% appropriate IPG Buffer (GE Healthcare, Uppsala, Sweden)). For complex culture supernatant samples, 50 µg of labeled protein, and for purified samples, 30 µg of labeled protein were loaded into the loading cup on the anode of the rehydrated Immobiline™ DryStrip precast IEF gels with pH range between pH 3-11 with none-linear gradient (NL) or 6-11 with linear gradient. The isoform separation was performed using the Ettan IPGphor II IEF System (GE Healthcare, Uppsala, Sweden) with 50 µA/strip and total kVh of 65 for pH 3-11NL or 50 kVh for pH 6-11 strips at 20°C. For the second dimension, the IPG strips were denatured and reduced in equilibration buffer I (5 mM Tris/HCl pH 6.8, 6 M urea, 2% SDS, 30 % glycerol, 2% DTT) and subsequently alkylated with equilibration buffer II (5 mM Tris/HCl pH 6.8, 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide (Sigma, St. Louis, MO, US)). The incubation time in each buffer was 15 min. Prepared Immobiline™ DryStrip gels were placed on the top of the 12.5 % tris-glycine gels and sealed using an agarose sealing buffer. The second dimension used an Ettan DALT*six* Large Vertical System (GE Healthcare, Uppsala, Sweden) at 2.5 W per gel, 600 V, 400 mA, for 45 min to allowed migration of proteins form the IPG strip to the gel. The run was continued at 100 W, 600 V, 400 mA for ~ 4 hours at room temperature. All gels were scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare, Uppsala, Sweden) with a resolution of 100 µm and photomultiplier tube set in the 400-530 V range. Spectral settings for CyDye™ DIGE Fluor minimal dyes are combined in Table 1. For comparison of protein abundances in different samples, ImageQuant and DeCyder Software (GE Healthcare, Uppsala, Sweden) were applied.

3 Results

To assess the complementary value of 2-D DIGE for downstream process (DSP) development we have analyzed the culture supernatants of two different mAB expressing CHO clones called M500-11 and M250-9, harvested at high (~ 90%) and low (~ 40%) viabilities and tracked the protein patterns of a purification process consisting of Protein A and CEC (Fig. 1). The culture supernatants harvested at high viability showed much lower total protein concentration in the range of 1.7-1.9 mg/ml, whereas supernatant with low viability with total protein concentration around 2.5 mg/ml. (Table 2). The lower protein content at high viability was confirmed by conventional SDS-PAGE (Fig. 2A) and analytical SEC (Fig. 3). Western blot (Fig. 2B) was used to identify IgG based on the heavy chain band.

The culture supernatant from clone M500-11 at high and low viability and clone M250-9 at low viability had been directly compared with 2-D DIGE and evaluated with the ImageQuant Software. This software identifies protein spots and compares the spots intensity of up to three samples run simultaneously in a single 2-D gel. Fig. 4 shows qualitative comparison of clone M500-11 at low viability labelled with Cy2 (Fig. 4A), clone M500-11 at high viability marked with Cy3 (Fig. 4B) and M250-9 at low viability tagged with Cy5 (Fig. 4C) analyzed on a single gel. Overlaying of images A and B (Fig. 4D) allows direct comparison of the same clone with different viabilities. Blue spots are proteins which are present in higher concentration in the culture supernatant at high viability, green spots are proteins present in higher concentration in the culture supernatant at low viability. Spots in similar concentration range in both samples appeared in turquoise colour. Comparison of different clones with the same viabilities is shown in Fig. 4E, where images B and C were superimposed. Red spots represent proteins that were more abundant in clone M250-9. Spots with similar concentration appeared in yellow colour. Fig. 4F presents the overlay of all three samples.

Semiquantitative analysis was performed using Difference In-Gel Analysis (DIA) (a tool of DeCyder Software) on the two samples at high and low viability from clone M500-11 (Fig. 5). Blue spots represent an abundance in proteins from high viability sample, while green spots are proteins enriched in low viability samples. Red spots represent proteins that are of the same concentration in both samples. In Fig. 5B the histogram shows the number of decreased, increased and similar spots. Spots found at the position of the IgG heavy (~50 kDa, pH 9-10) and light (~25 kDa, pH 6-7) chain are more frequently observed in the high viability sample. This is consistent with the result from SEC analysis (Fig. 3), but not with the results obtained by ELISA (high viability: 102 µg/ml; low viability: 226 µg/ml). The latter method also recognizes partly degraded IgG resulting in a falsely high IgG concentration of the supernatant

from low viability cultures. The quantitative analysis was based on 700 resolved spots. The differences between spot volumes were determined using the 1.5 fold threshold mode. Spots with a volume ratio between -1.5 and 1.5 were defined as identical. Based on this assumption 32% (228 blue spots) were defined as decreased, 14% (100 green spots) as increased and 53% (375 red spots) as similar.

Then we compared the two different clones at similar viabilities (Fig. 6). 611 spots could be resolved, where 60% of spots were similar (red spots), 13% of spots were decreased and 27% of spots were increased relative to clone M500-11. Spots representing IgG (green spots at light chain: ~25 kDa, pH 6-7; heavy chain: ~50 kDa, pH 9-10) were also increased in M500-11. When subtracting the spots for IgG, we found that the similarity between two different clones with the same viabilities was much higher than that of the same clone with different viabilities.

Also, the host cell impurities and spot patterns were tracked during the purification process of this antibody. The flow scheme is shown in Fig. 1. We compared the protein patterns of sPA-eluates from different feed stocks derived from cultures harvested at high and low cell viabilities. Fig. 7A shows the overlay of the chromatograms of all clones. Eluate from a feed stock with low viability and degraded antibody (M500-11, low viability) eluted in a broad tailing peak as compared to the others which eluted in a more symmetrical peak. This elution fraction of M500-11 at low viability also shows a different protein pattern by analytical SEC (Fig. 7B) than other eluates. In this sample lower IgG concentration and aggregates with molecular weight of ~1790 kDa were detected. All other samples include dimers and small aggregates with molecular weight of ~409 kDa. In all clones low molecular weight compounds (~3 kDa) were observed. SDS-PAGE of the eluates (Fig. 8A) suggested that the antibody had already been purified to a high degree of purity by sPA affinity chromatography. Western blot (Fig. 8B) verified the presence and integrity of the heavy and light chains of our IgG in all samples. Comparison of the eluates from different clones of low viability using 2-D DIGE (Fig. 9) showed that an additional polishing step was required. 157 spots were detected: 42% (66 spots) were decreased, 63% (40 spots) were similar and 18% (28 spots) were increased. Approximately 60% of the spots were host cell proteins, the rest was from IgG.

CEC was used to perform a final purification of IgG. The chromatogram of the separation of the M500-11 low viability is shown in Fig. 10A together with analytical SEC of the eluates (1^{st} peak) of all clones (Fig. 10B). All samples gave only one peak in analytical SEC without aggregates and degradation products. When we compared the CEC eluate derived from the M500-11 and M250-9 clone, both at low viability, using 2-D DIGE, only identical spot patterns were observed (Fig. 11). The 32 detected spots were confirmed to be similar. Although after

sPA the host cell protein patterns of the two clones were different, the CEC provides purification efficiency to purify the antibody to homogeneity.

4 Discussions

In this study we compared the two clones M500-11 and M250-9, each harvested at high and low viabilities, and investigated the influence of feed stocks on the two steps affinity based purification process. We used 2-D DIGE in comparison to conventional analytical methods, like SDS-PAGE, Western blot and SEC. We identified higher host cell protein content in supernatants from cultures harvested at low viability than in cultures harvested at high viability. Differences were detected using SDS-PAGE and SEC. Directly comparison of clones using 2-D DIGE indicated that the heterogenity in cell viabilities had more influence on spot patterns than the expression clones. 53% of detected spots were defined as similar when comparing clone M500-11 at high and low viabilities and 60% when comparing in comparison both clones at similar viabilities. Additionally a degradation of IgG in clone M500-11 at low viability was observed. It could be an explanation for different elution behaviour of this clone on the Protein A column and aggregation content in elution fraction detected by SEC. Due to the high sensitivity of 2-D DIGE, which can detect up to 125 pg protein/spot [45, 46], we are able to visualize the low abundance impurities in Protein A eluates. This gave us deeper knowledge and better understanding of capture step using affinity chromatography. CEC as polishing step removed critical contaminants and high molecular weight compounds from all samples, so that a homogenous protein pattern in all clones was achieved. All spots resolved by 2-D DIGE were described as similar.

Using 2-D DIGE we could demonstrate that the chosen DSP-scheme was able to compensate for the variations in the feed stock. Thus we belief, that this method is of interest when working with cell banks or when master cell banks have to be renewed. It helps to demonstrate that the products generated from cells belonging to the old and new cell banks are identical. It also helps to understand product differences due to process changes and helps to establish product biosimilarility or bioequivalency. 2-D DIGE is a useful complementary tool for understanding purification processes. It allows tracking of impurities in a qualitative and semiquantitative manner and controlling of product quality. Therefore, it was helpful for the targeted development of a platform process, which showed robustness despite variabilities in the feedstock. It can also be a valuable tool for the development of biosimilars, because the degree of product similarity can be tracked easily.

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Table 1
Laser wavelenght and emission filters used for scanning of CyDyes™ DIGE Fluor minimal dyes

Dye	Laser (nm)	Emission filter (nm)
Cy2	blue 488	520 BP 40
Cy3	green 532	580 BP 30
Cy5	red 633	670 BP 30

Table 2
Summary of clones

Clone	Cell viability [%]	Titer IgG [µg/ml]	Total protein concnetration [mg/ml]
M500-11	86	102,0	1,9
M500-11	34	225,6	2,1
M250-9	95	118,9	1,7
M250-9	40	160,0	2,4

Legend to figures

Fig. 1. Experimental design

Fig. 2. Comparison of clones using A) SDS-PAGE, B) Western blot. Lanes: (M) Molecular weight markers; (1) clone M500-11, high viability; (2) clone M500-11, low viability; (3) clone M250-9, high viability; (4) clone M250-9, low viability. kDa, molecular mass (M_r) x 10^{-3}. The analyses were normalized to 0.5 µg IgG.

Fig. 3. Comparison of clones using SEC. Runs was normalized to 5 µg IgG. kDa, molecular mass (M_r) x 10^{-3}

Fig. 4. Comparison of clones using 2-D DIGE. A) Clone M500-11 at high viability labeled with Cy2; B) clone M500-11 at low viability labeled with Cy 3; C) clone M250-9 at low viability labeled with Cy5; D) overlay of images A & B, comparison of the same clone at different viabilities; E) overlay of images B & C, comparison of different clones; F) overlay of images A & B & C. The analysis was prepared using ImageQuant Software. kDa, molecular mass (M_r) x 10^{-3}.

Fig. 5. Comparison of clone M500-11 at high viability labeled with Cy2 and low viability labeled with Cy3. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins that occur in higher concentration in clone at high viability, green spots (increased spots) represent proteins that occur in higher concentration in clone at low viability, red spots (similar spots) show proteins with the same concentration in both clones; B) Histogarm shows spot frequency plotted against log volume ratio.

Fig. 6. Comparison of clones M500-11 at low viability labeled with Cy2 and M250-9 at low viability labeled with Cy3. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins in higher concentration in clone M500-11, green spots (increased spots) represent proteins in higher concentration in clone M250-11, red spots (similar spots) show proteins in the same among in both clones; B) Histogarm shows spot frequency plotted against log volume ratio.

Fig. 7. Purification using Protein A chromatography as capture step. A) Chromatogram of Protein A runs of clone M500-11 and M250-9 in high and low viabilities; B) Comparison of elution peaks using SEC.

Fig. 8. SDS-PAGE (A) and Western blot (B) analysis of elution pools from affinity chromatography runs in comparison to load materials. (M) Molecular weight markers; (1) clone M500-11 high viability; (2) clone M500-11 low viability; (3) clone M250-9 high viability; (4) clone M250-9 low viability; a) culture supernatant; b) purified fraction. kDa, molecular mass (M_r) x 10^{-3}. The analyses were normalized to 0.5 µg IgG.

Fig. 9. Comparison of protein pattern in elution fractions from affinity chromatography run of clone M500-11 at low viability labeled with Cy3 and M250-9 at low viability labeled with Cy2. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins in higher concentration in clone M500-11, green spots (increased spots) represent proteins in higher concentration in clone M250-11, red spots (similar spots) show proteins in the same concentration in both clones; B) Histogarm shows spot frequency plotted against log volume ratio.

Fig. 10. Purification using CEC as polishing step. A) Chromatogram of CEC run of clone M500-11 at low viability as an example; B) Comparison of 1. peak of all clones using SEC

Fig. 11. Comparison of IgG_1 fraction after polishing step from clones M500-11 at low viability labeled with Cy5 and M250-9 at low viability labeled with Cy3. Both samples were separated one a single gel and analyzed using DeCyder Software. A) Red spots (similar spots) are proteins in the same concentration in both samples; B) Histogarm shows spot frequency plotted against log volume ratio.

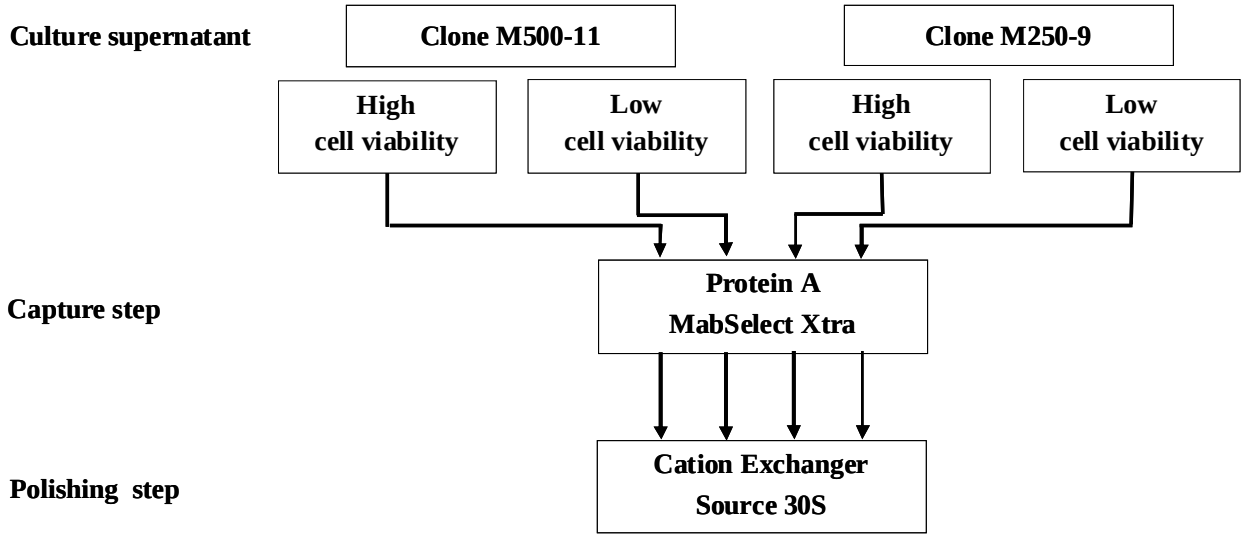
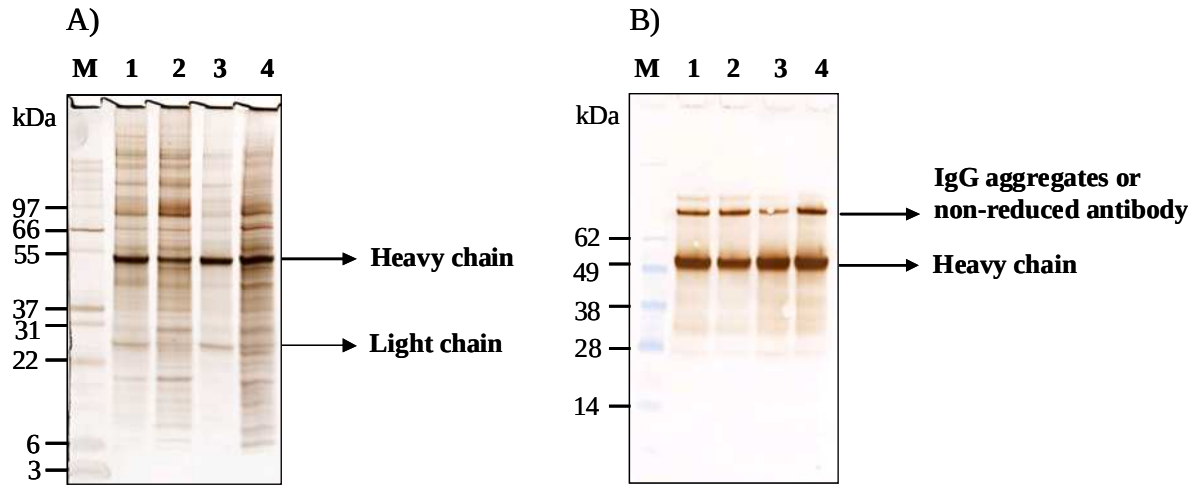


Fig. 1. Experimental design



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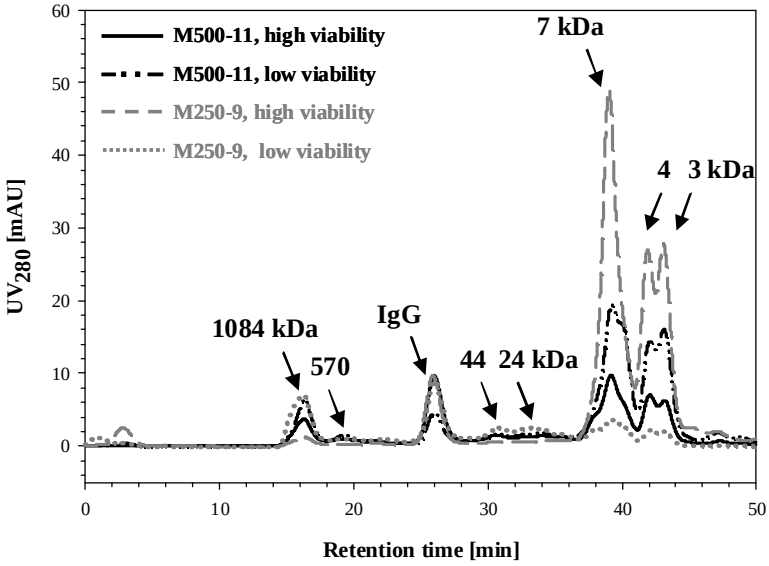

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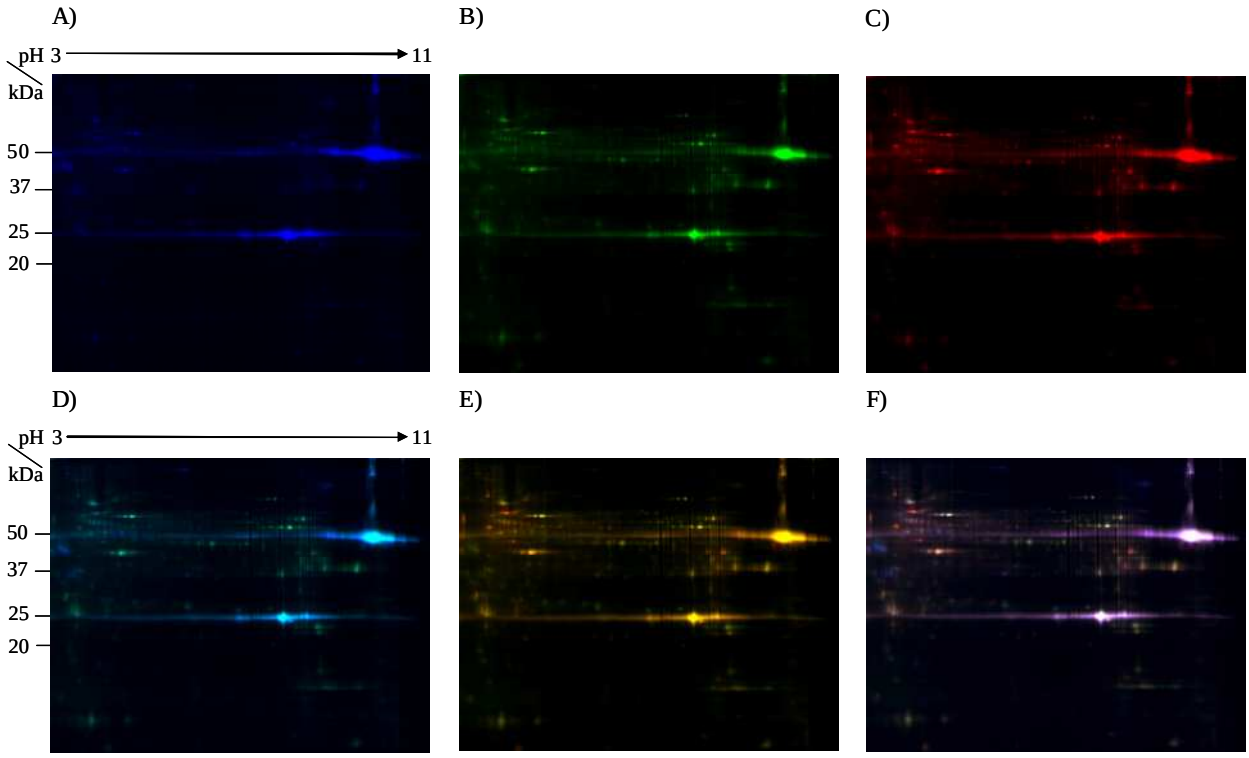


Fig. 4. Comparison of clones using 2-D DIGE. A) Clone M500-11 at high viability labeled with Cy2; B) clone M500-11 at low viability labeled with Cy 3; C) clone M250-9 at low viability labeled with Cy5; D) overlay of images A & B, comparison of the same clone at different viabilities; E) overlay of images B & C, comparison of different clones; F) overlay of images A & B & C. The analysis was prepared using ImageQuant Software. kDa, molecular mass (Mr) x 10-3.

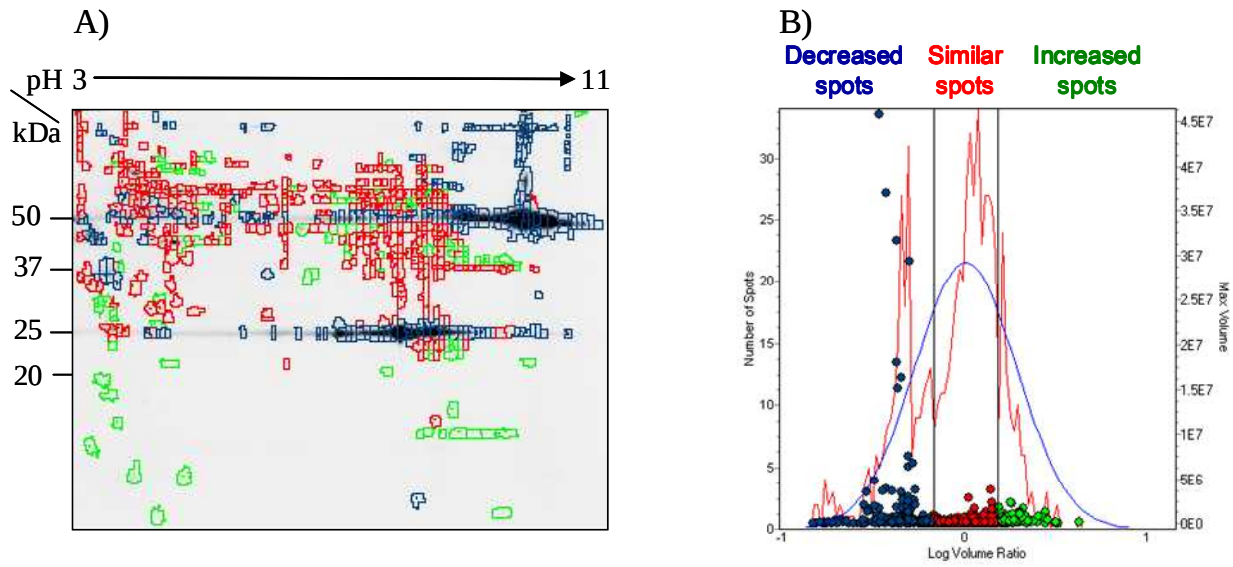


Fig. 5. Comparison of clone M500-11 at high viability labeled with Cy2 and low viability labeled with Cy3. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins that occur in higher concentration in clone at high viability, green spots (increased spots) represent proteins that occur in higher concentration in clone at low viability, red spots (similar spots) show proteins with the same concentration in both clones; B) Histogarm shows spot frequency plotted against log volume ratio.

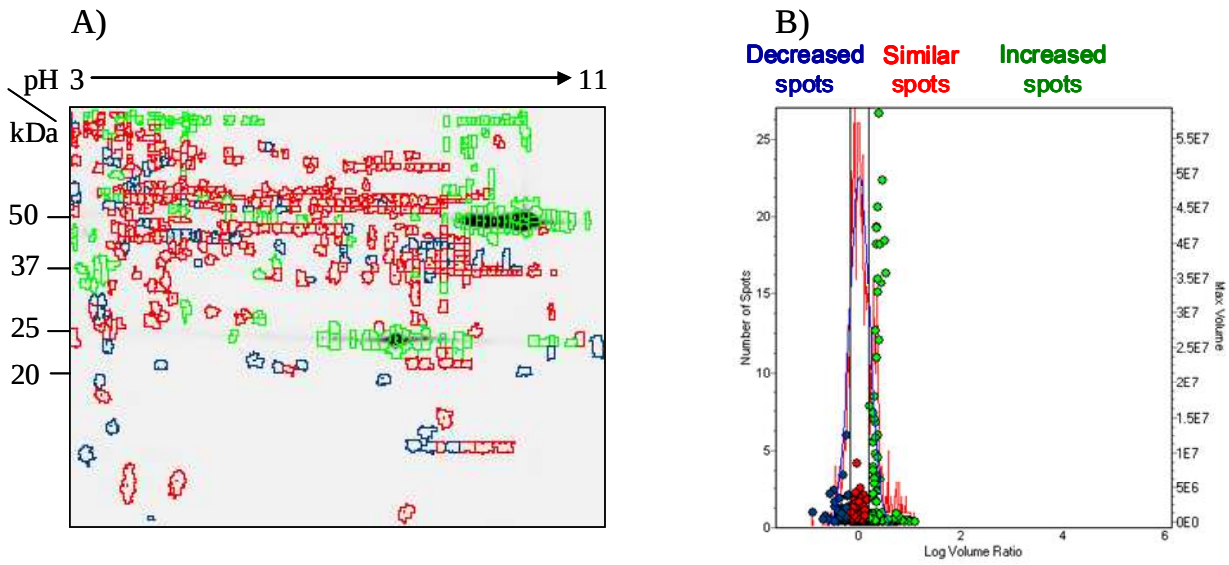
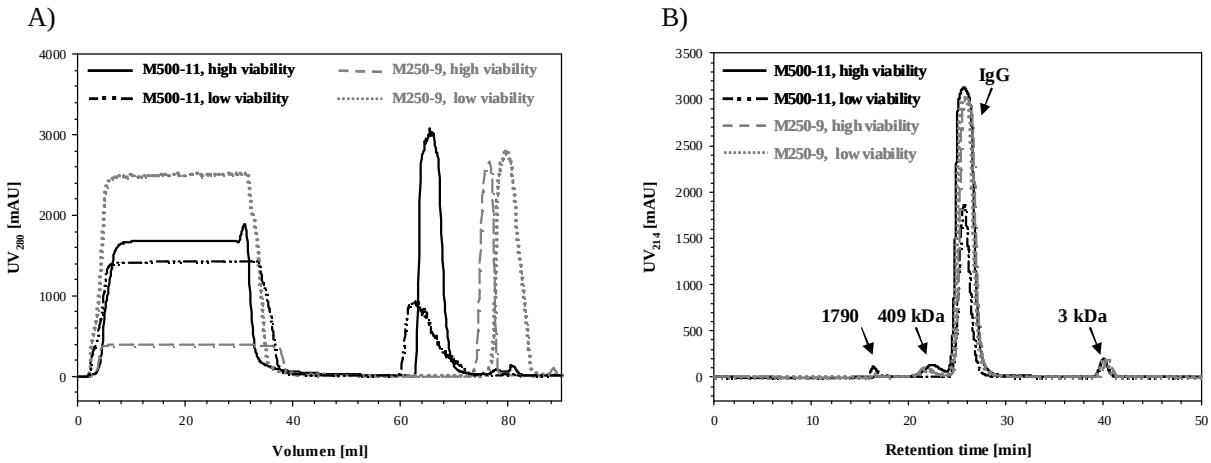


Fig. 6. Comparison of clones M500-11 at low viability labeled with Cy2 and M250-9 at low viability labeled with Cy3. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins in higher concentration in clone M500-11, green spots (increased spots) represent proteins in higher concentration in clone M250-11, red spots (similar spots) show proteins in the same among in both clones; B) Histogarm shows spot frequency plotted against log volume ratio.



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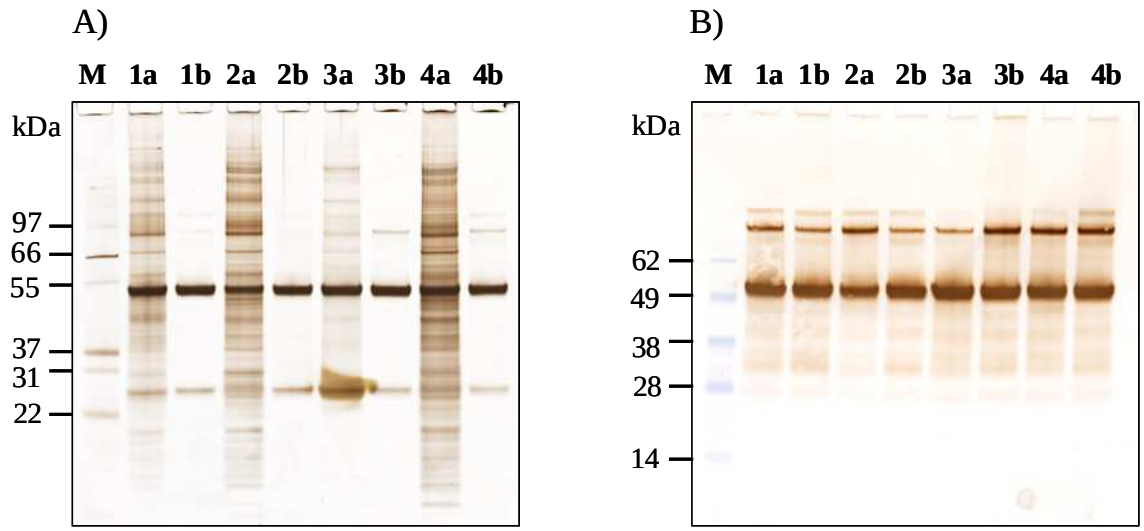
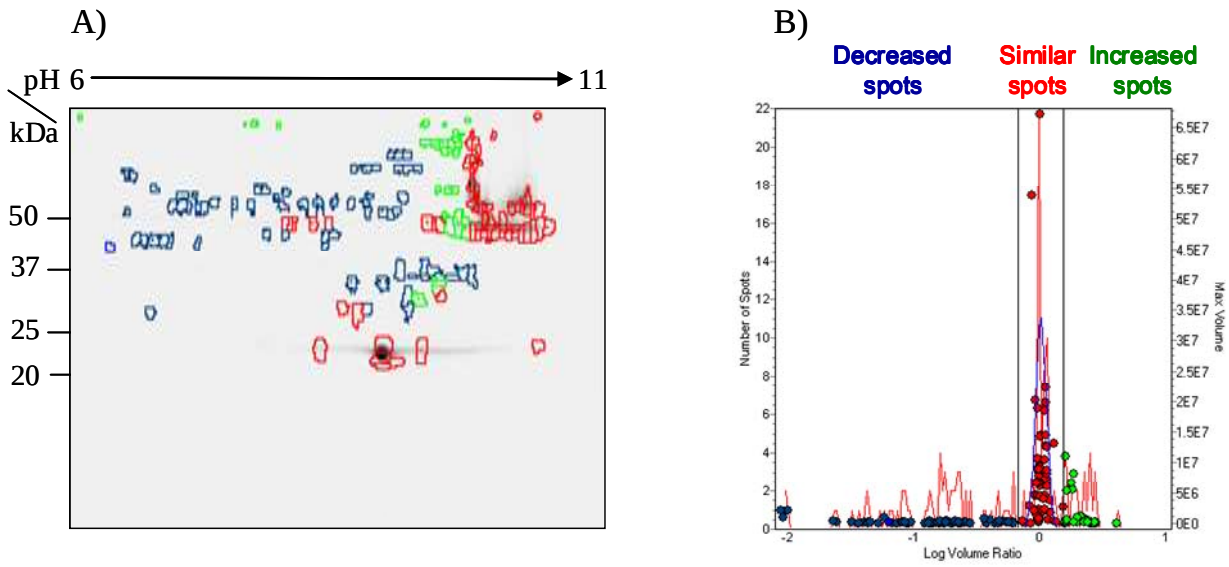


Fig. 8. **SDS-PAGE (A) and Western blot (B) analysis of elution pools from affinity chromatography runs in comparison to load materials. (M) Molecular weight markers; (1) clone M500-11 high viability; (2) clone M500-11 low viability; (3) clone M250-9 high viability; (4) clone M250-9 low viability; a) culture supernatant; b) purified fraction. kDa, molecular mass (Mr) x 10-3. The analyses were normalized to 0.5 µγ IgG.**



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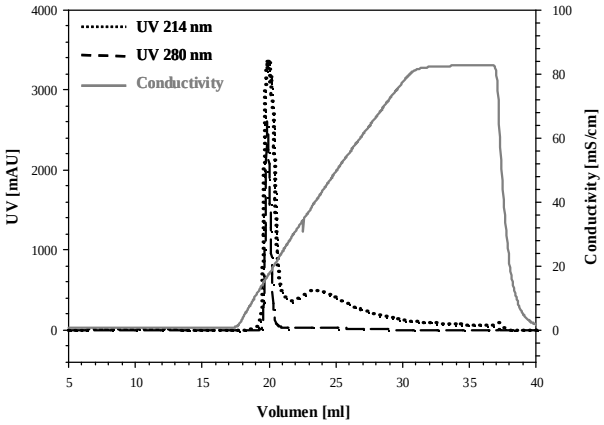
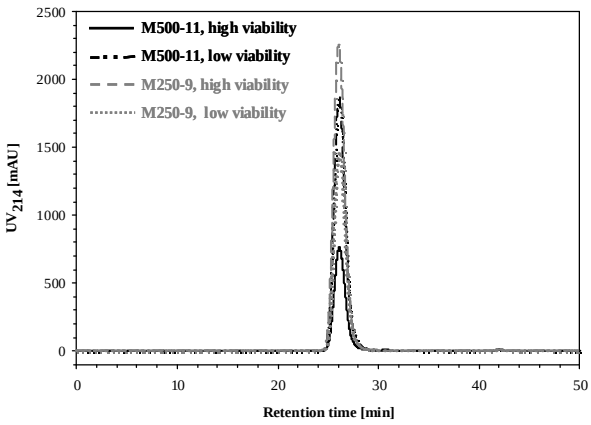
A)



B)



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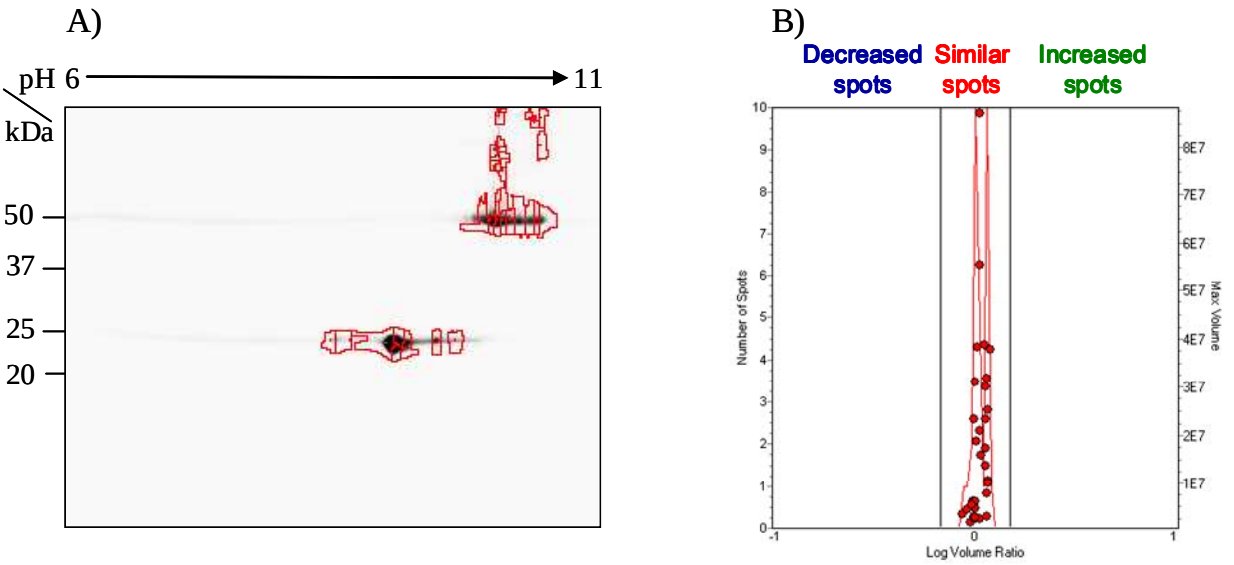

Fig. 11. Comparison of IgG1 fraction after polishing step from clones M500-11 at low viability labeled with Cy5 and M250-9 at low viability labeled with Cy3. Both samples were separated one a single gel and analyzed using DeCyder Software. A) Red spots (similar spots) are proteins in the same concentration in both samples; B) Histogarm shows spot frequency plotted against log volume ratio.

For submission to Journal of Chromatography A

2-D DIGE for comparison of affinity and non-affinity based downstream process of recombinant monoclonal antibody

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Abstract

Staphylococcus Protein A (sPA) affinity chromatography as capture step is state of art for antibody purification. Due to economic reasons sequences purification sequences for antibody purification without sPA have been investigated. Here we applied two dimensional fluorescence difference gel electrophoresis (2-D DIGE) for analyzing two different purification sequences, one consisting of sPA and cation ion exchanger (CEC), the other one of CEC, anion ion exchanger (AEC) in flow-through mode and hydrophobic interaction chromatography (HIC). Recombinant IgG_1 antibody against Rhesus D antigen expressed in Chinese hamster ovary cells was applied as a model system. SDS-PAGE and size exclusion chromatography show that both processes were efficient. The 2-D DIGE revered that Protein A as capture step was much more efficient than CEC and the affinity based process yielded in a product with less spots. We conclude that 2-D DIGE is a complementary tool for downstream process development and deeper understanding of purification processes.

Keywords: 2-D DIGE; IgG; Recombinant antibody; *Staphylococcus* ProteinA chromatography; Affinity chromatography, Mixed-mode chromatography

1 Introduction

Staphylococcus Protein A (sPA) affinity chromatography is a standard method for purification of recombinant antibodies. Reason for this popularity is the high selectivity, high dynamic binding capacity [1,2] and the high yield [3]. The culture supernatant can be directly passed over the column without any further pretreatment. Usually a three step process consisting of sPA and the additional chromatographic steps are used in manufacturing of recombinant antibodies [4,5]. Recently two steps purification has been proposed, where after sPA capture step a mixed mode ligand was used for polishing step. Actually more steps were performed because virus inactivation and condition and concentration by ultra-diafiltration were not counted [6]. The sPA capture step is very efficient, where 99.5% of CHO proteins can be removed [7]. Leaching of Protein A ligand is not really a problem. With modern media it is possible to reduce leached Protein A below 4 ppm during further purification [8]. Ishihara and Kadoya [9] reported, that the loading time and contact duration between proteases from culture supernatant and the Protein A media influence the ligand leakage. Additionally, sPA is very stable. Recently, we showed 50 cycles [10], others have demonstrated a shelf life up to 300 cycles [5] without significant loss of performance. Currently, many companies try to replace sPA despite its excellent performance. One reason is the economical pressure on the cost of manufacturing [11,12]. Follman and Fahrner [7] used factorial screening to successfully replace sPA as capture step. They described three non-affinity based column sequences with CHO proteins removal below 2 ng/mg antibody, the level comparable to affinity based process. This is an easy task in case, when the pI of the antibody is very high. The IgG can be bound at a very high pH and very high selectivity is obtained. Most of the host cell proteins are found in flow-through. When the pI of the antibody is low, it is more difficult to find an alternative method to sPA based purification [13].

Usually two additional chromatographic steps are required to clear host cell proteins to the required level. For non-affinity based process tracking of impurities is more important than for process with sPA as capture step, since the efficiency is so high. The conventional analytical techniques used in DSP are sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, size exclusion chromatography (SEC), analytical Protein A and enzyme-liked immunosorbent assay (ELISA). Those methods were chosen for the strategy of the process analytical technology [14] provided by Food and Drug Administration, as a framework for innovative pharmaceutical development, manufacturing and quality assurance[15]. Two dimensional (2-D) electrophoresis has

not been applied, because the gel-to-gel variation of this analysis is very high and samples comparison very difficult. The 2-D fluorescence difference gel electrophoresis (2-D DIGE), described by Orange et al. [16] overcomes those issues. Three different samples tagged with fluorescence markers (Cy2, Cy3 and Cy5), that bind to the side bone of lysine, can be pooled together and separate simultaneously on one isoelectric focusing (IEF) and SDS-PAGE gel. Standardization, high accuracy, reproducibility, efficiency and high sensitivity (up to 125 pg protein/ spot) [16,17] are also advantages of this method. This technique was already successfully applied for selecting of stabile cell lines, characterizing differential expressions profiles, verifying post-translational modifications and discovering biomarkers [18]. We utilized 2-D DIGE to expedite DSP development of recombinant anti-Rh(D) IgG_1 [19,20], as model system. Affinity based process consisting of sPA chromatography using a new-generation resin, MabSelect Xtra, and cation ion exchanger (CEC) using Source 30S was compared to a three step process consisting of a CEC using SP Sepharose Fast Flow (FF), Q Sepahrose FF in flow-through mode and hydrophobic interaction chromatography (HIC) using Phenyl Sepharose FF. The non-affinty based sequence was suggested by Follman and Fahrner. Each step was analyzed by SDS-PAGE, Western blot, analytical SEC, analytical Protein A and 2-D DIGE. 2-D DIGE was applied for comparison of different steps during a process or different column sequences. We focused on the application of 2-D DIGE for development of downstream process (DSP).

2 Material and methods

Culture supernatant

Parental dihydrofolate reductase (dhfr)- deficient CHO DG44 (Invitrogen, Carlsbad, CA, US) cells were transfected with two vectors, encoding the heavy chain and light chain of an IgG against RhD antigen (gift from Professor Robert Raison, University of Technology Sydney, Australia, with slight modification to the light chain expression vector). Stable cell pools obtained after selection were used to isolate single cell clones and subjected to gene amplification using step-wise increments of methothrexate (MTX). Clone M250-9 and M500-11 were high producer clones derived from an additional round of single cell cloning after amplification at 250nM and 500nM MTX respectively [21], and were adapted to in-house proprietary protein-free chemically defined media. Batch bioreactor cultures were conducted with each of the clones and harvested at two time points: one at high viability (>90%) and the other at low viability (~ 40%).

The culture broth was clarified by centrifugation at 4000 rpm (Beckman GS-6R, Palo Alto, CA, USA) for 10 min followed by filtration (0.45 + 0.2 μm, Sartobrand 150, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The supernatant was then frozen at -20ºC until further use. Prior purification, the samples were again filtrated using a 0.22 μm filter and concentrated using Tangential Flow Filtration (Millipore, Billerica, MA, US) with regenerated cellulose membranes, cut off 10 kDa (Millipore, Billerica, MA, US).

Preparative chromatography

Preparative chromatography was performed using ÄKTA*explorer 100* (GE Healthcare, Uppsala, Sweden) with detection by measurement of the absorbance at 214 nm and 280 nm. If not given otherwise all chemicals were purchased from Merck, Vienna, Austria. All buffers were filtered using 0.22 μm regenerated cellulose filters (Millipore Corporation, Billerica, MA, USA) and degassed prior to use. Before loading, all samples were also filtrated using 0.22 μm filters. All experiments were performed at room temperature.

For the affinity based process, 4 ml MabSelect Xtra (GE Healthcare, Uppsala, Sweden) and 0.6 ml Source30S (GE Healthcare, Uppsala, Sweden), were packed into Tricorn™10/50 column and Tricorn™5/50 column (GE Healthcare, Uppsala, Sweden), wit bead high of 5.5 cm and 3 cm, respectively. The MabSelect Xtra column was first equilibrated with 5 column volumes (CVs) of Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.2 mM Ha_2HPO_4, 1.8 mM KH_2PO_4,

pH 7.4) followed by loading of the filtered (0.22µm) sample. Unbound sample was washed out with 7 CVs of PBS and adsorbed IgG was then eluted using a step gradient of 0.1 M glycine, pH 3.5 and collected in fractions of 1 ml which were immediately neutralized using 1 M Na_2CO_3 to a final pH of 9. The column was reequilibrated with 5 CVs of PBS. The linear flow rate was 61 cm/h with residence time of 5 min, except for elution when a linear flow rate of 76 cm/h was applied.

For the second purification step, the elution pool of the sPA chromatography was diafiltrated into 10 mM CH_3COONa, pH 5.0 using PD10 column (GE Healthcare, Buckinghamshire, UK) containing Sephadex™ G-25 M resin. The procedure was carried out adhered to 'PD-10 desalting column' instruction manual: The column was equilibrated using 25 ml equilibration buffer. The sample, with the total volume of 2.5 ml, was loaded and proteins were eluted with 3.5 ml equilibration buffer. Samples prepared on this way were loaded into Source 30S column. Again the column was first equilibrated using 10 mM CH_3COONa, pH 5.0. The sample was loaded and unbound material was washed out with 7 CVs of equilibration buffer. IgG was eluted with a 20 CVs linear gradient using 10 mM Na_2HCOOH, 1 M NaCl, pH 5.0 buffer. The column was regenerated using 5 CVs of 1 M NaCl and finally reeqilibrated with 5 CVs of 10 mM CH_3COONa, pH 5.0. All steps were performed at the liner flow rate of 76 cm/h with residence time of 8.6 min.

The non-affinity based process described by Follman and Fahrner was used with minor modifications due to protein precipitation. 15.9 ml SP Sepharose Fast Flow (FF), 16.3 ml Q Sepharose FF and 15.7 ml Phenyl Sepharose FF Low Sub were loaded into Tricorn™10/200 column with bead high of 20.2 cm, 20.7 cm and 20.0 cm, respectively. All runs were performed using the linear flow rate of 300 cm/h with residence time of 4 min. For the capture step, sample was adjusted to pH 6.9 and conductivity 4.6 mS/cm with 1% HCl and distilled water. SP Sepharose FF column was equilibrated with 5 CVs of 20 mM MES, 50 mM NaCl, pH 5.5. After loading, the unbound substances were washed out with 5 CVs of the equilibration buffer. Adsorbed proteins were eluted using 10 CVs linear gradient until 80% of 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 1 M NaCl, pH 5.5. Regeneration of the column was carried out using 5 CVs of 1 M NaCl and finally the column was reequilibrated with 5 CVs of equilibration buffer. To avoid binding of IgG to the Q Sepharose FF column, the elution faction was adjusted to pH 6.5 and 5.5 mS/cm using 0.1 M NaOH and distilled water. Before loading, the column was equilibrated with 5 CVs of 25 mM Tris, 50 mM NaCl, pH 7.6. The flow-through product pool was collected. The column was regenerated with 7 CVs of 1 M NaCl and eventually reequilibrated with 5 CVs of equilibration buffer. To prepare the product pool for HIC, the conductivity was increased until 57.4 mS/cm using 2 M Na_2SO_4 and pH

was adjusted to 6.2 with 1% HCl. Column equilibration was carried out using 50 mM MES, 0.6 M Na_2SO_4, pH 6.2. Sample was loaded and unbound material was washed with 7 CVs of equilibration buffer. Hydrophobic interactions between adsorbed proteins and column ligands were released with step gradient of 50 mM MES, pH 6.0 and product pool was collected. The column was regenerated with 5 CVs of 0.5 M NaOH and reequilibrated with 5 CVs of elution buffer.

Total protein concentration

Total protein concentration was determined by absorbance at 280 nm using U2001 UV/Vis Spectrophotometer (Hitachi, Pleasanton, CA, US). The protein concentration was calculated using Beer-Lambert equation with 1.2 as molar extinction coefficient.

Analytical Protein A

For the IgG quantification the CIM® Protein A HLD Disk Monolithic Column (BIA Separations, Klagenfurt, Austria) was applied using the protocol evaluated by Tscheliessnig and Jungbauer [22] with minor modifications. Analyses were carried out on a high pressure liquid chromatography (HPLC) system, Agilent 1100 Series (Waldbronn, Germany). The 0.34 ml column with column high of 3 mm was equilibrated over 2 min with 30 mM phosphate buffer, 1 M NaCl, pH 7.4. The buffer was prepared by titration of Na_2HPO_4 and NaH_2PO_4 to the specific pH. 100 µl of filtrated sample (0.22 µm) was loaded into the column and unbound proteins were washed out during 0.5 min with equilibration buffer. IgG was eluated using 0.5 M acetic acid, pH 2.6 over 2 min. The column was reequlibrated for 2 min with equilibration buffer. The calibration curve was prepared using human IgG_1 (myeloma) purchased by Calbiochem (Darmstadt, Germany).

Analytical SEC

Analytical SEC experiments were performed on a high pressure liquid chromatography (HPLC) system, Agilent 1100 Series (Waldbronn, Germany) in isocratic mode using PBS buffer at flow rate 0.5 ml/min. 50 µl of filtrated (0.22 µm) samples were loaded into a Superdex™ 200 HR10/30 column (GE Healthcare, Uppsala, Sweden). Detection was carried out by measurement of the absorbance at 280 nm. The molecular mass calibration was performed using polyclonal IgG was obtained from Octapharma, Vienna, Austria.

SDS-PAGE and Western blot

Electrophoretic Separation was carried out using the NOVEX System (Invitrogen, Carlsbad, CA, US). Samples were combined with 4X NuPAGE® LDS Sample Buffer (106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM Phenol Red, pH 8.5), reduced with 0.1% DTT, heated at 100°C for 10 min and loaded on the NuPAGE® Novex 4–12% Bis-Tris Gels (1.0 mm). Runs were performed using NuPAGE® MES SDS Running Buffer (50 mM MES, 50 mM Tris base, 35 mM SDS, 1 mM EDTA, pH 7.3) at 200 V, 400 mA, 40 W for 50 min. The gels were stained using silver staining as described by Heukeshoven [23] or blotted to a 0.2 µm nitrocellulose membrane at 400 V, 200 mA for 2 hours. The transfer was facilitated using blotting buffer composed of 50 mM $NaB_4O_7 \times 10\ H_2O$, 0.1% (v/v) SDS, 10% (v/v) methanol. Membranes were blocked with 3% bovine serum albumin, 0.1% Tween20 in PBS for 2 hours or over night. After three 5-minute wash steps with 0.1% Tween 20 in PBS, the membrane was incubated with a 1:1000 dilution of the anti-human IgG Fc specific conjugated to horseradish peroxidase in 0.1 % Tween20 in PBS. After further two 5-minute washing steps the IgG heavy chain was detected by color reaction using 1.7 mM 3,3'-diaminobenzidine tetrahydrochloride, 30% H_2O_2 in PBS buffer.

2-D DIGE

2-D DIGE was performed using Ettan DALT System (GE Healthcare, Uppsala, Sweden). If not given otherwise all chemicals were purchased from Merck, Vienna, Austria.

Sample preparation included desalting using the 2-D Clean Up Kit and labeling using CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3 and Cy5) obtained from GE Healthcare (Uppsala, Sweden). The desalting assay was prepared followed the '2-D Clean-Up Kit' procedure for sample volumes of 1-100 µl containing 100 µg protein per sample. 300 µl precipitant was mixed by vortexing with 1-100 µl protein sample and incubated on ice for 15 min. After adding of 300 µl co-precipitant and mixing, the tube was centrifuged at 12 000 × g, 4ºC for 5 min and the supernatant was removed. The pellet was washed with 40 µl of co-precipitant and incubated on ice for 5 min. The sample was centrifuged for 5 min and the wash solution was removed. The pellet was dispersed in 25 µl distilled water by vortexing (5-10 seconds). The next wash step was carried out using 1 ml of wash buffer, pre-chilled for at least 1 h at -20°C, and 5 µl wash additive. After mixing and fully dispersing the pellet, the protein solution was incubated on ice for at least 30 min and vortexed for 30 seconds once every 10 min. The tube was centrifuged at 12 000 × g for 5 min and the supernatant was removed

and discarded. The pellet was air dried briefly for no more than 5 min and resuspended in an appropriate volume of labeling buffer (8 M urea, 4 % CHAPS (Sigma, St. Louis, MO, US), 30 mM Tris, pH 8.5).

The desalted samples, each containing 50 µg total protein, were labeled by a 30 min on ice incubation with 2 µl of 200 pmol/µl marker solutions in 99.8% anhydrous dimethylformamide (USB Corporation, Cleveland, OH, US) with. The reaction was stopped by adding 1 µl 10 mM L-Lysine. Three samples labeled with Cy2, Cy3 and Cy5, respectively were pooled together and mixed with rehydration buffer (2 M thiourea, 6 M urea, 4% CHAPS, 20 mM DTT (GE Healthcare, Uppsala, Sweden) and 0.25% appropriate IPG Buffer (GE Healthcare, Uppsala, Sweden)). For complex culture supernatant samples, 50 µg of labeled protein, and for purified samples, 30 µg of labeled protein were loaded into the loading cup on the anode of the rehydrated Immobiline™ DryStrip precast IEF gels with pH range between pH 3-11 with none-linear gradient (NL) or 6-11 with linear gradient. The isoform separation was performed using the Ettan IPGphor II IEF System (GE Healthcare, Uppsala, Sweden) with 50 µA/strip and total kVh of 65 for pH 3-11NL or 50 kVh for pH 6-11 strips at 20°C. For the second dimension, the IPG strips were denatured and reduced in equilibration buffer I (5 mM Tris/HCl pH 6.8, 6 M urea, 2% SDS, 30 % glycerol, 2% DTT) and subsequently alkylated with equilibration buffer II (5 mM Tris/HCl pH 6.8, 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide (Sigma, St. Louis, MO, US)). The incubation time in each buffer was 15 min. Prepared Immobiline™ DryStrip gels were placed on the top of the 12.5 % tris-glycine gels and sealed using an agarose sealing buffer. The second dimension used an Ettan DALT*six* Large Vertical System (GE Healthcare, Uppsala, Sweden) at 2.5 W per gel, 600 V, 400 mA, for 45 min to allowed migration of proteins form the IPG strip to the gel. The run was continued at 100 W, 600 V, and 400 mA for ~ 4 hours at room temperature. All gels were scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare, Uppsala, Sweden) with a resolution of 100 µm and photomultiplier tube set in the 400-530 V range. For Cy2 excitation wavelength of 488 nm and 520 nm band-pass (BP) 40 emission filter was applied. For Cy3 the setting was: excitation wavelength of 532 nm and 580 nm BP 30 emission filter and for Cy5 excitation wavelength of 633 nm and 670 nm BP 30 emission filter were set up. For comparison of protein abundances in different samples, ImageQuant and DeCyder Software (GE Healthcare, Uppsala, Sweden) were applied.

3 Results and discussion

Anti-Rh(D) IgG_1 was used to study 2-D DIGE for DSP development. The antibody had pI 9 and was over expressed in Chinese hamster ovary (CHO) cells. Cell culture was conducted with a serum free medium and harvested at low cell viability (~ 40%).

Two steps purification processes based on sPA as capture step was compared to three step sequence consisting of CEC, AEC in flow through mode and HIC (Fig. 1). This non-affinity based process suggested by Follman and Fahrner as sequence with high CHO protein removal. Although for non-affinity based purification a subtle refinement of conditions is required, we directly applied their conditions to our antibody. Thus the process resulted in a low yield of polishing step. Focus of our work was testing of 2-D DIGE for DSP development. The processes were characterized with conventional analytical methods used in DSP development, like SDS-PAGE, Western blot and SEC, in comparison to 2-D DIGE. IgG content was measured using by CIM disk affinity monolith.

The flow-scheme of the investigated processes is shown in Fig. 1. Chromatograms of preparative runs are presented in Fig. 2 and the run parameters are summarized in

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Table 1. The first process consisted of sPA using a new-generation resin, MabSelect Xtra, as capture step followed by a polishing step employing Source 30S. The high efficiency of capture step is demonstrated by SDS-PAGE (Fig. 3A). Additionally, no differences between capture step and polishing step were detected. IgG was identified using Western blot (Fig. 3B) developed with human heavy chain specific antibody. SEC results (Fig. 3C) indicated that high molecular weight compounds were removed from product pool during polishing step.

Culture supernatant and sPA and CEC elution fractions had been directly compared using 2-D DIGE and analyzed with the ImageQuant Software. This software indentifies protein spots and compares the spots intensity of up to three samples run simultaneously in a single 2-D gel. Fig. 4 shows qualitative comparison of culture supernatant labeled with Cy2 (Fig. 4A) and product pool after polishing step labeled with Cy3 (Fig. 4B). CHO proteins and over expressed IgG (heavy chain: ~50 kDa, pH 9-10; light chain: ~25 kDa, pH 6-7) were detected in culture supernatant. Pure IgG was found in CEC fraction. Superimposing of images A and B (Fig. 4C) allowed direct comparison of proteins pattern in both samples. Blue spots corresponded to CHO proteins, which were present in culture supernatant, but not in CEC fraction. These proteins were removed during the purification process. IgG isoforms presented in the same concentration in both samples developed turquoise color.

Fig. 5 demonstrates semiquantitative analysis of capture step and polishing performed using Difference In-Gel Analysis (DIA), a tool of DeCyder Software. sPA and CEC pools were labeled with Cy5 and Cy3 respectively and separated simultaneously in a single 2-D gel. The quantitative analysis based on detection of 225 spots and assumption, that spots with volume ration between -1.5 and 1.5 were identical. Blue spots (decreased spots) show proteins that occur only in sPA fraction and red spots (similar spots) represents proteins that are in the same concentration in both samples. Histogram demonstrates the number of decreased and similar spots. 83 spots (37 %) were defined as decreased and 143 (63 %) as similar. This clearly shows the efficiency of the CEC step. In contrast to SDS-PAGE, with 2-D DIGE we are able to demonstrate the purification effect of polishing step after affinity chromatography step.

Then we have investigated the non-affinity based process. Preparation of samples for capture step included pH and conductivity adjustment to pH 6.9 and 4.6 mS/cm, respectively to allow binding of IgG to the CEC column. Comparison of culture supernatant before and after conditioning step using SEC (Fig. 6) showed a very different protein pattern in these samples. High molecular weight proteins were degraded and low molecular weight proteins were aggregated during this step. This

results in many green spots in molecular weight between 10 and 100 kDa on the 2-D DIGE gel (Fig. 7). For this analysis culture supernatant before and after conditioning were marked with Cy2 and Cy5 respectively and resolved on a single gel. Comparison of purification steps by SDS-PAGE (Fig. 8A) and SEC (Fig. 8C) indicated that the capture step was very efficient, which is explained by the high pI of the antibody. Product pools of all purification steps are identical by SDS-PAGE. Western blot (Fig. 8B) was used to identify IgG based on the heavy chain bands. Due to SEC results, the intermediate and polishing steps removed impurities with molecular weight smaller than IgG.

The efficiency of the capture step is also confirmed by 2-D DIGE using ImageQuant Software (Fig. 9). Culture supernatant after conditioning step was tagged with Cy3 (Fig. 9A) and CEC elution pool was tagged with Cy5 (Fig. 9B). In Fig. 9C images A and B were overlaid, where red spots represented CHO proteins removed during the capture step, green spots were IgG isoforms that occur in higher concentration in elution pool (~50 kDa, pH 9-10) and yellow spots indicated IgG isoforms that occur in the same concentration in both samples (~25 kDa, pH 6-7). High purity of product was obtained after capture step.

Further we compared intermediate purification (AEC) and polishing step (HIC) with 2-D DIGE in pH range 6 -11 and used DeCyder Software for quantitative evaluation (Fig. 10). Between 89 resolved spots 90% were defined as identical (red spots), 5 spots (5.6%) as decreased (blue spots), and 4 spots (4.5%) as increased (green spots). It seems that during polishing step proteins may have be either depredated or modified.

Than we compared the capture step of the affinity and non-affinity based processes (Fig. 11A-B). A completely different protein pattern is observed reflecting the different selectivity of Protein A and CEC. Blue spots (decreased spots) indicated proteins in higher concentration in sPA elution pool, green spots (increased spots) indicated proteins in higher concentration in CEC elution fraction. Red spots are proteins that occur in the same concentration in both samples. Totally 102 spots were identified, thereof 47 (46.1%) were similar, 16 (15.7%) are in higher concentration in Protein A eluate and 39 spots (38.2%) were in higher concentration in CEC. In SP Sepharose FF more impurities were detected than in sPA.

For product quality control we compared the polishing steps of these two processes (Fig. 11C-D). Blue spot (decreased spots) show proteins that occur in higher concentration in CEC elution pool, green spots (increased spots) represents proteins that occur in higher concentration in HIC elution pool. Red spots correspond to proteins that are in the same concentration in both samples. Totally,

61 spots were detected, where 4 spots (6.6%) were in higher concentration in affinity based process and 16 spots (26.2%) were in higher concentration in non-affinity based process.

The identical protein pattern could not be obtained by the two different processes. The affinity based process was more efficient, but we have not optimized the non-affinity based sequence. We just took the protocol, which was described by Follman and Fahrner.

In this work we demonstrate, that 2-D DIGE is a valuable tool to facilitate DSP development. High sensitivity of this method allowed detection of low abundance impurities and determination of minor differences between impurities patterns in product fractions. This technique could be utilized for characterization of feed stocks as well as product quality control. Tracking of impurities during processes, especially for non-affinity based sequences, discovering of protein modifications and visualization of isoforms results in better understanding of purification platforms. This deeper knowledge also contributes to process development significantly increase processes robustness. Due to economical pressure and the speed to market, 2-D DIGE constitutes a complementary tool for faster and targeted DSP development.

4 Conclusion

The 2-D DIGE is a useful complementary method to expedite DSP development. We applied this technique for tracking of impurities during purification processes and comparison of affinity based and non-affinity based column sequences. Two steps affinity based process composed of sPA as capture step followed by CEC and non-affinity based process consists of CEC, AEC in flow-through mode and HIC were investigated using Anti-Rh(D) IgG_1 expressed in CHO as model system. Non-affinity based sequence was suggested by Follman and Fahrner without optimization, which resulted in very low yield in HIC step. The impurities pattern in sPA elution fraction and purification effect of CEC as polishing step was shown. High impurities clearance on CEC as capture step was achieved due to high pH of IgG. Comparison of intermediate and polishing step of non-affinity based process demonstrated contaminants removal and protein modification on HIC column. Furthermore, various impurities pattern was observed, when capture and polishing steps of both processes were compared. Non-affinity based process was less efficient than the process with sPA as capture step. That shows the requirement of optimization for HIC step.

We showed, that 2-D DIGE is a very helpful task for visualization of purification steps, tracking of impurities and process comparison. Consequent applying of this technique for DSP leads to targeted DSP projects and robust processes.

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Table 1
Characterization of affinity and non-affinity based processes.

Process	Purification step	Volume (ml)	IgG concentration (mg/ml)	Total protein concentration (mg/ml)	Yield (%)
Affinity based	Concnetrated culture supernatant	28.5	2.02	1.92	100.0
	Capture step, Protein A	8.50	3.93	5.10	58.1
	Polishing step, CEX	1.70	1.16	1.76	38.4
Non-affinity based	Conditioned culture supernatant	769.90	0.04	1.54	100.0
	Capture step, CEX	37.00	0.62	0.66	70.0
	Intermediate step, AEX	83.00	0.14	0.18	67.8
	Polishing step, HIC	15.00	0.05	0.33	7.5

Legend to figures

Fig. 1. Experimental design.

Fig. 2. Purification of Anti-Rh(D) IgG_1 using A) affinity based process; B) non-affinity based process. Chromatograms of preparative runs. A) Capture step: Protein A chromatography with MabSelect Xtra, polishing step: CEC with Source30S; B) Capture step: CEC with SP Sepharose FF, intermediate step in flow-through mode with Q Sepharose FF, polishing step: HIC with Phenyl Sepharose 6 FF (low sub).

Fig. 3. Affinity based process analyzed using A) SDS-PAGE; B) Western blot; C) SEC. Lanes: (M) Molecular weight markers; (1) culture supernatant; (2) concentrated culture supernatant; (3) capture step, Protein A eluate; (4) polishing step, CEC eluate. kDa, molecular mass (M_r) x 10^{-3}.

Fig. 4. Affinity based process analyzed using 2-D DIGE. A) Culture supernatant labeled with Cy2; B) CEC elution fraction labeled with Cy3; C) Overlay of images A & B. The analysis was performed using ImageQuant Software. kDa, molecular mass (M_r) x 10^{-3}.

Fig. 5. Comparison of capture step and polishing step of affinity based process using 2-D DIGE. sPA eluate was labeled with Cy5 and CIX eluate was labeled with Cy3. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins that occur in higher concentration in sPA eluate; red spots (similar spots) represent proteins that occur in the same concentration in both samples; B) Histogram shows spot frequency plotted against log volume ratio.

Fig. 6. Comparison of culture supernatant before and after conditioning for CEC capture step using SEC.

Fig. 7. Comparison of culture supernatant before and after conditioning for CEC capture step using 2-D DIGE. Sample before conditioning was labeled with Cy2 and sample after conditioning was with Cy5. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Blue spots (decreased spots) represent proteins that occur in higher concentration in sample before conditioning; green spots (increased spots) represents proteins that occur in higher concentration in samples after conditioning; red spots (similar spots) show proteins that are in the same concentration in both samples; B) Histogram shows spot frequency plotted against log volume ratio.

Fig. 8. Non-affinity based process analyzed using A) SDS-PAGE; B) Western blot; C) SEC. Lanes: (1) culture supernatant; (2) capture step, CEC eluate; (2) intermediate step, AEC eluate; (3) polishing step, HIC eluate. kDa, molecular mass (M_r) x 10^{-3}.

Fig. 9. Capture step of non-affinity based process analyzed using 2-D DIGE. A) Conditioned culture supernatant labeled with Cy5; B) CEC elution fraction labeled with Cy3; C) overlay of A & B. The analysis was performed using ImageQuant Software. kDa, molecular mass (M_r) x 10^{-3}.

Fig. 10. Comparison of intermediate purification (AEC) and polishing step (HIC) of non-affinity based process. AEC flow-through fraction was labeled with Cy3 and HIC elution fraction was labeled with Cy5. Both samples were separated on a single gel and analyzed using DeCyder Software. A) Red spots (similar spots) represent proteins that occur in the same concentration in both samples; blue spots (decreased spots) represents proteins that occur in higher concentration in AEC flow-through; green spots (increased spots) represent proteins that occur in higher concentration in HIC elution pool; B) Histogram shows spot frequency plotted against log volume ratio.

Fig. 11. Comparison of affinity and non-affinity based processes with 2-D DIGE. The analysis was performed using DeCyder Software. A-B) Capture step: sPA elution fraction was labeled with Cy3 and CEC elution fraction was labeled with Cy5. Both samples were separated on a single gel. A) Blue spots (decreased spot) represents proteins that occur in higher concentration in sPA pool; green spots (increased spots) represents protein that occur in higher concentration in CEC pool; red spots (similar spots) represents proteins in the same concentration in both samples; B) Histogram shows spots frequency plotted against log volume ratio. C-D)

Polishing step: CEC elution fraction was labeled with Cy5 and HIC elution fraction was labeled with Cy3. Both samples were separated on a single gel. C) Blue spots (decreased spot) represents proteins that occur in higher concentration in CEC pool; green spots (increased spots) represents protein that occur in higher concentration in HIC pool; red spots (similar spots) represents proteins in the same concentration in both samples; D) Histogram shows spots frequency plotted against log volume ratio.

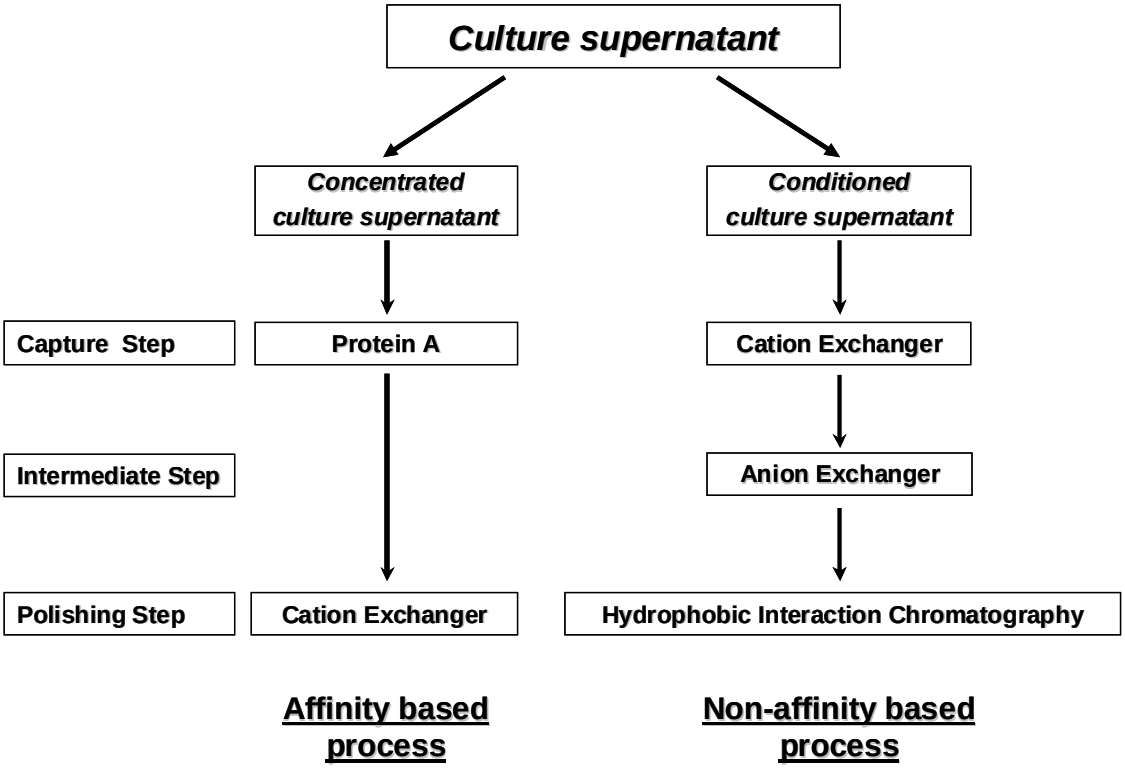

Culture supernatant
Concentrated culture supernatant
Conditioned culture supernatant
Capture Step
Protein A
Cation Exchanger
Intermediate Step
Anion Exchanger
Polishing Step
Cation Exchanger
Hydrophobic Interaction Chromatography
Affinity based process
Non-affinity based process

Fig. 1.

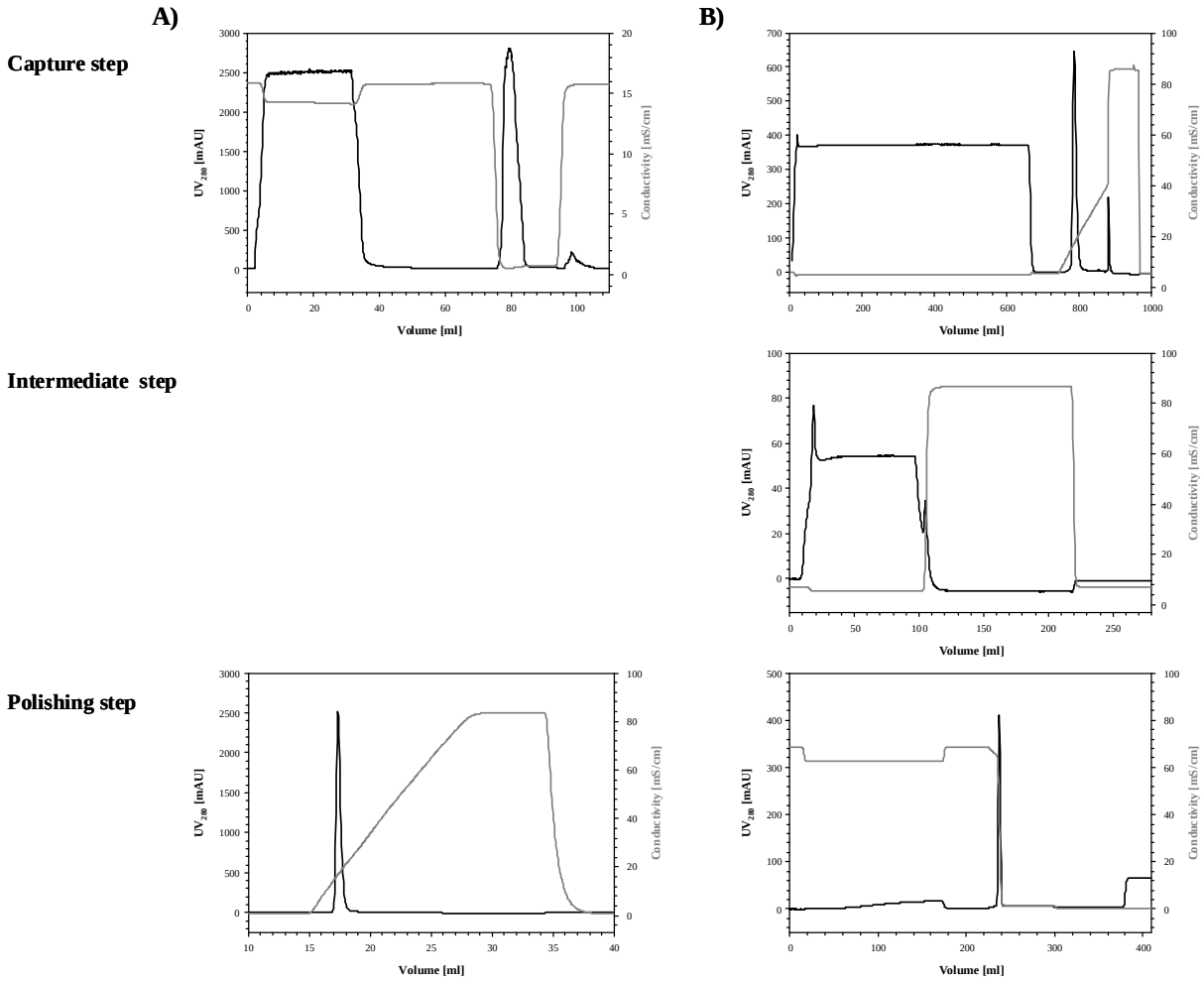

A)
B)
Capture step
Intermediate step
Polishing step
UV280 [mAU]
Conductivity [mS/cm]
Volume [ml]

Fig. 2.

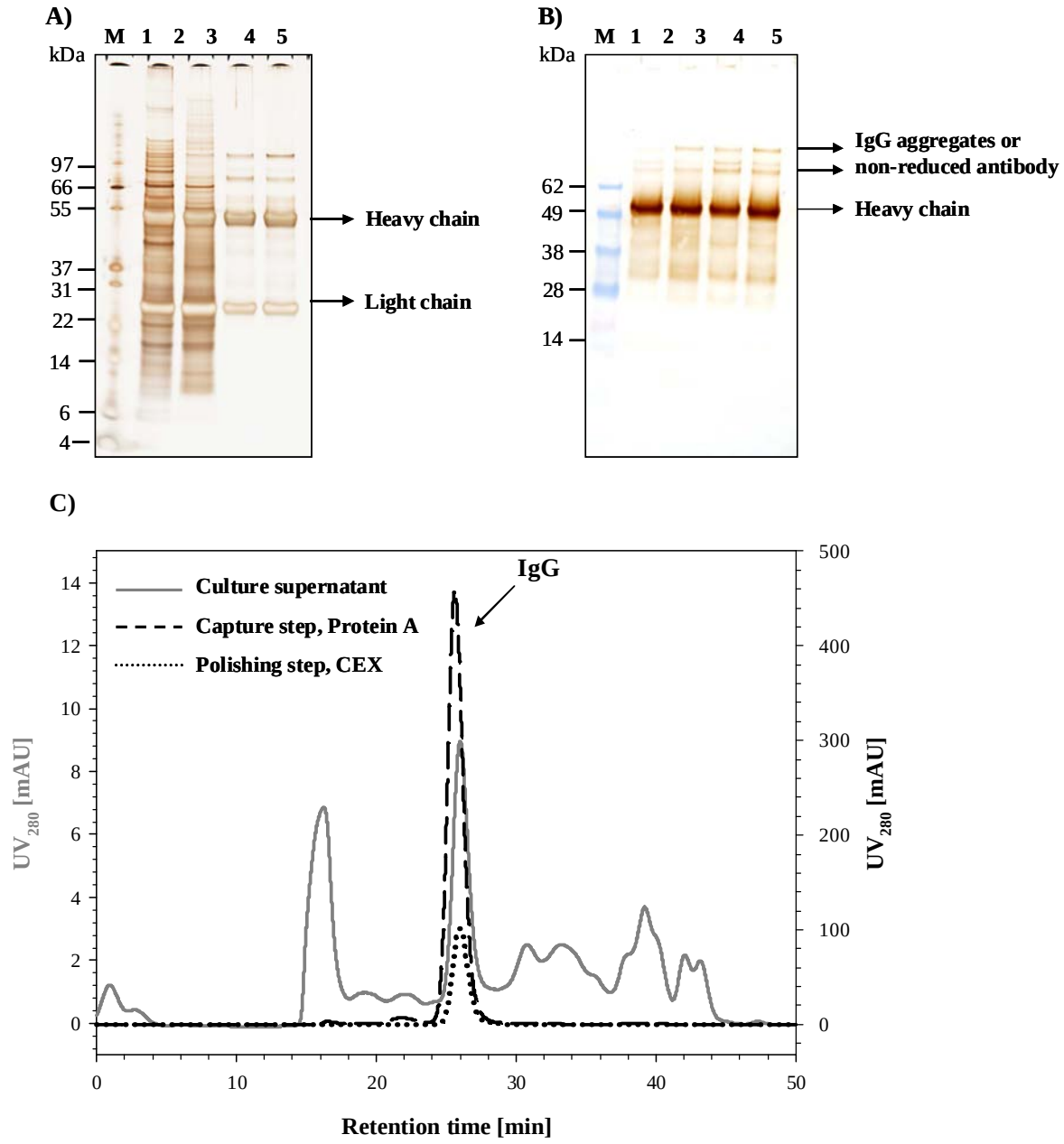

A)
M 1 2 3 4 5
kDa
97
66
55
37
31
22
14
6
4
Heavy chain
Light chain
B)
M 1 2 3 4 5
kDa
62
49
38
28
14
IgG aggregates or
non-reduced antibody
Heavy chain
C)
IgG
Culture supernatant
Capture step, Protein A
Polishing step, CEX
UV280 [mAU]
UV280 [mAU]
Retention time [min]

Fig. 3.

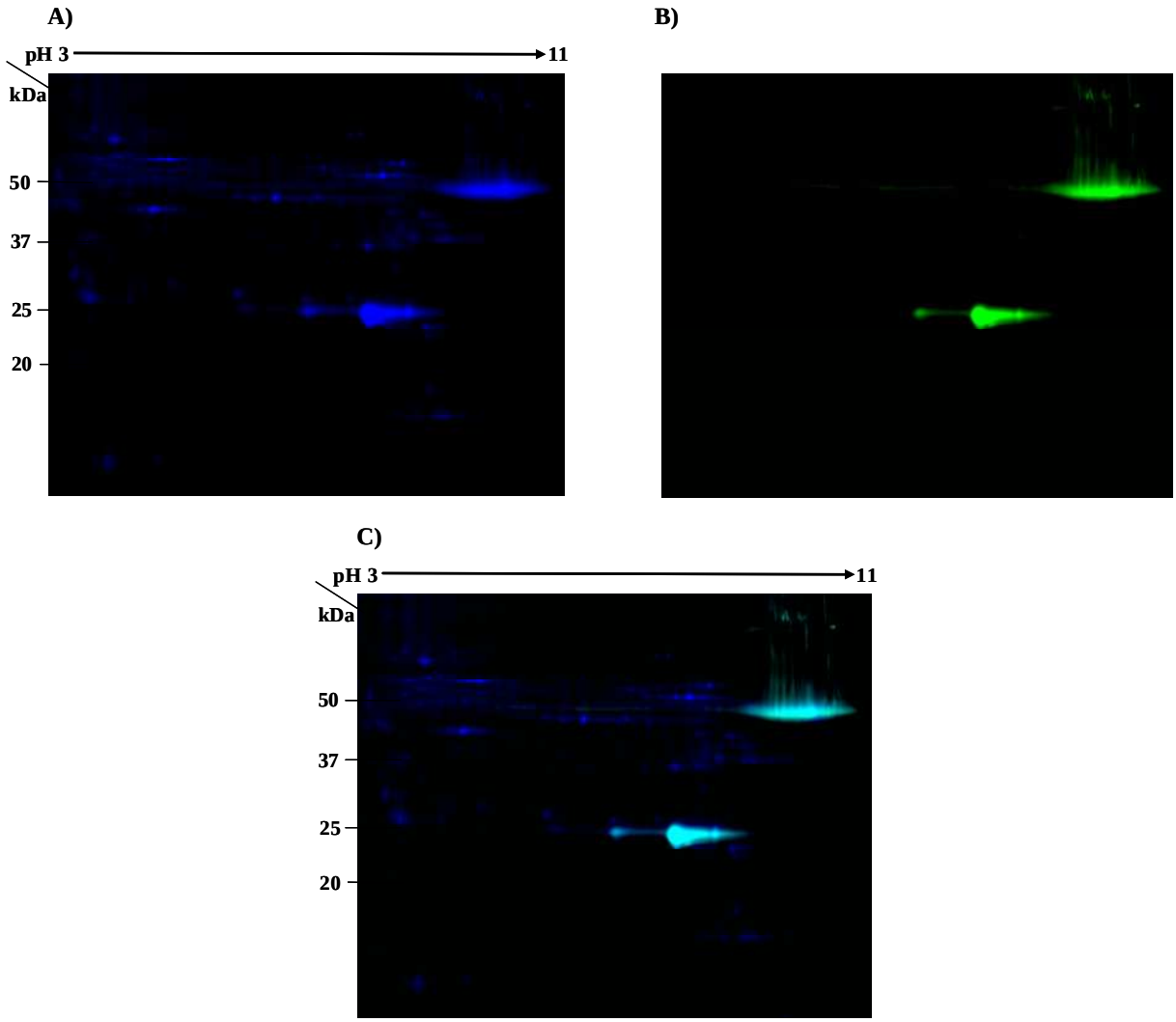


Fig. 4.

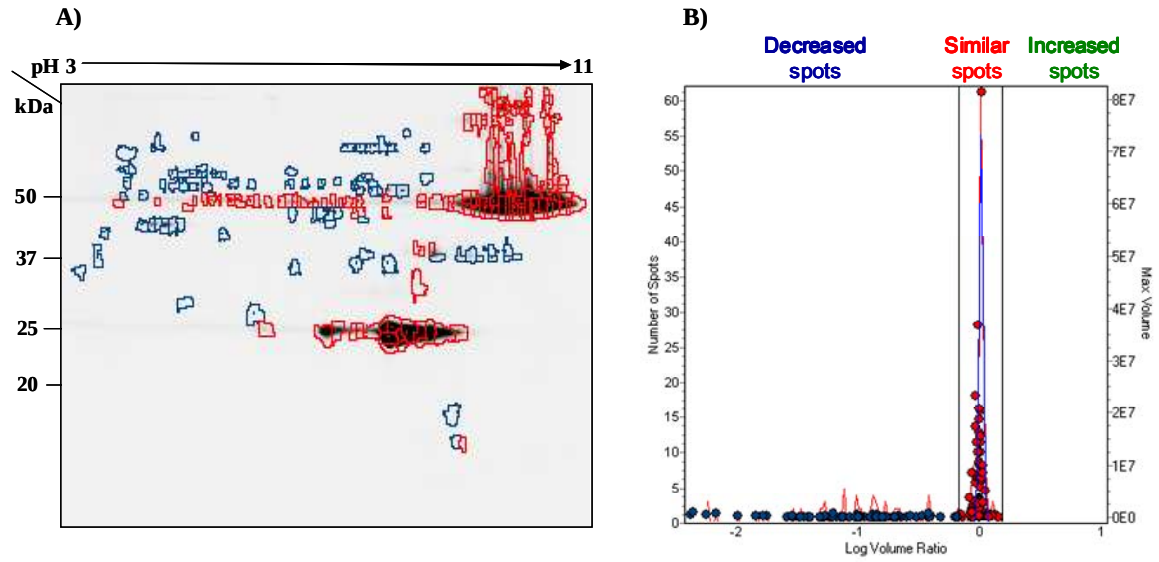

A)
pH 3
11
kDa
50
37
25
20
B)
Decreased spots
Similar spots
Increased spots
Number of Spots
Max Volume
Log Volume Ratio
60
55
50
45
40
35
30
25
20
15
10
5
0
8E7
7E7
6E7
5E7
4E7
3E7
2E7
1E7
0E0
-2
-1
0
1

Fig. 5.

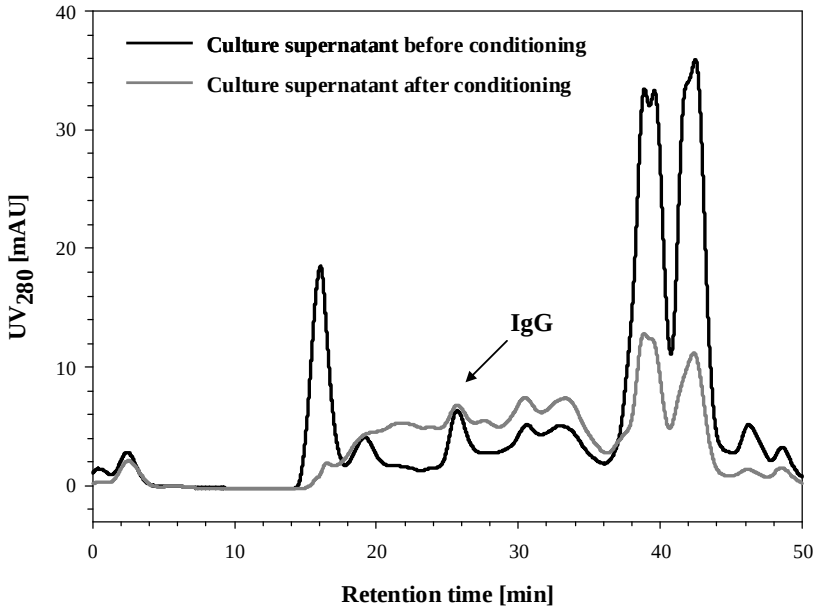

Culture supernatant before conditioning
Culture supernatant after conditioning
IgG
UV280 [mAU]
Retention time [min]
0
10
20
30
40
50

Fig. 6.

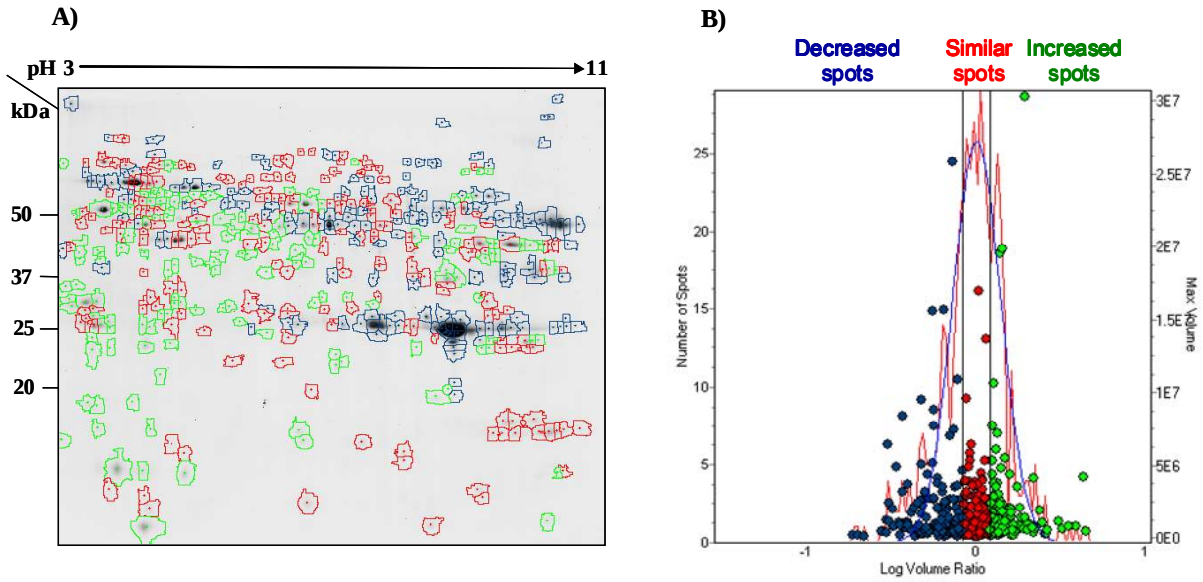

A)
pH 3
11
kDa
50
37
25
20
B)
Decreased spots
Similar spots
Increased spots
Number of Spots
Max Volume
Log Volume Ratio
0
5
10
15
20
25
-1
0
1
3E7
2.5E7
2E7
1.5E7
1E7
5E6
0E0

Fig. 7.

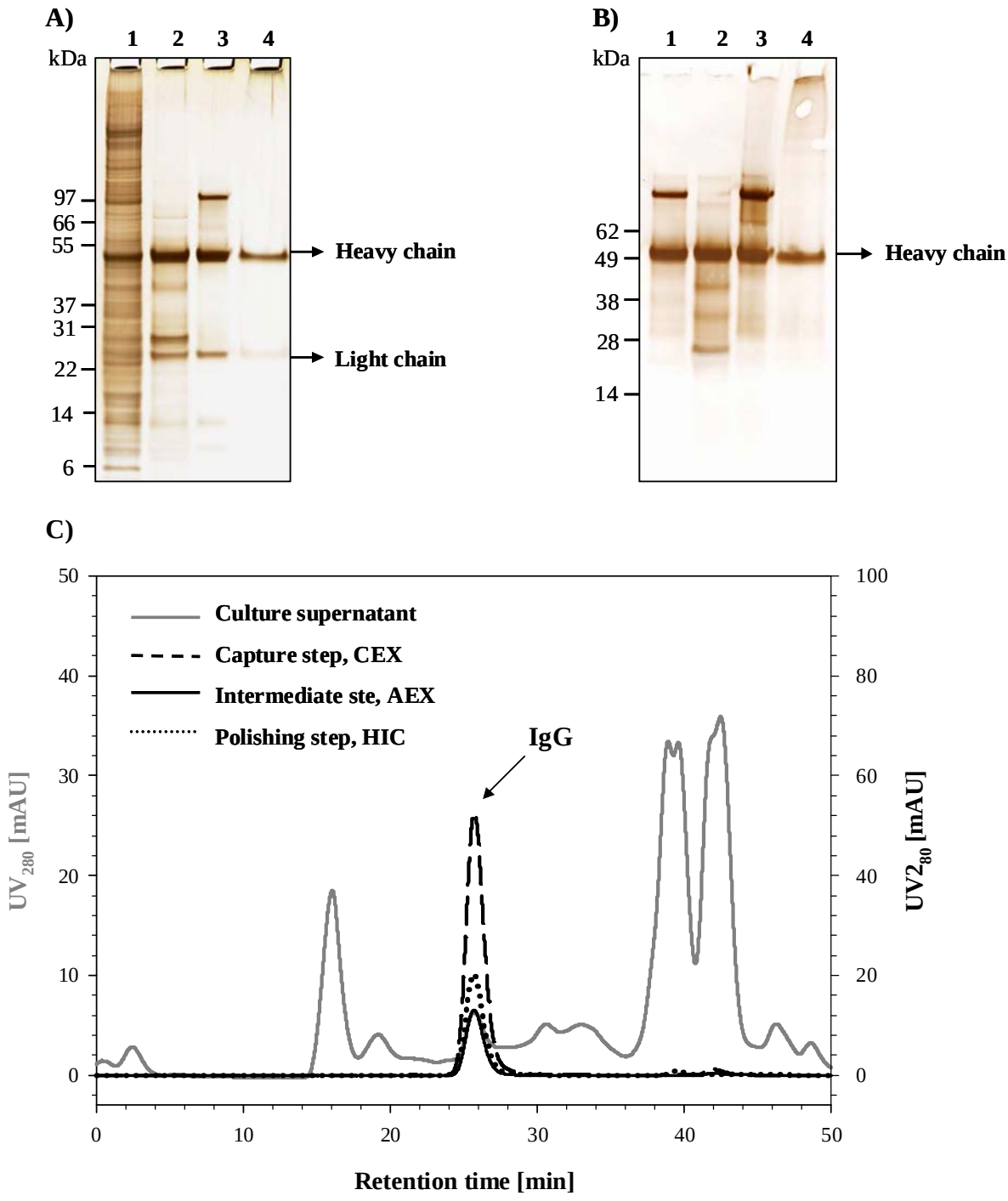

A)
1 2 3 4
kDa
97
66
55
37
31
22
14
6
Heavy chain
Light chain
B)
1 2 3 4
kDa
62
49
38
28
14
Heavy chain
C)
Culture supernatant
Capture step, CEX
Intermediate ste, AEX
Polishing step, HIC
IgG
UV280 [mAU]
UV280 [mAU]
Retention time [min]

Fig. 8.

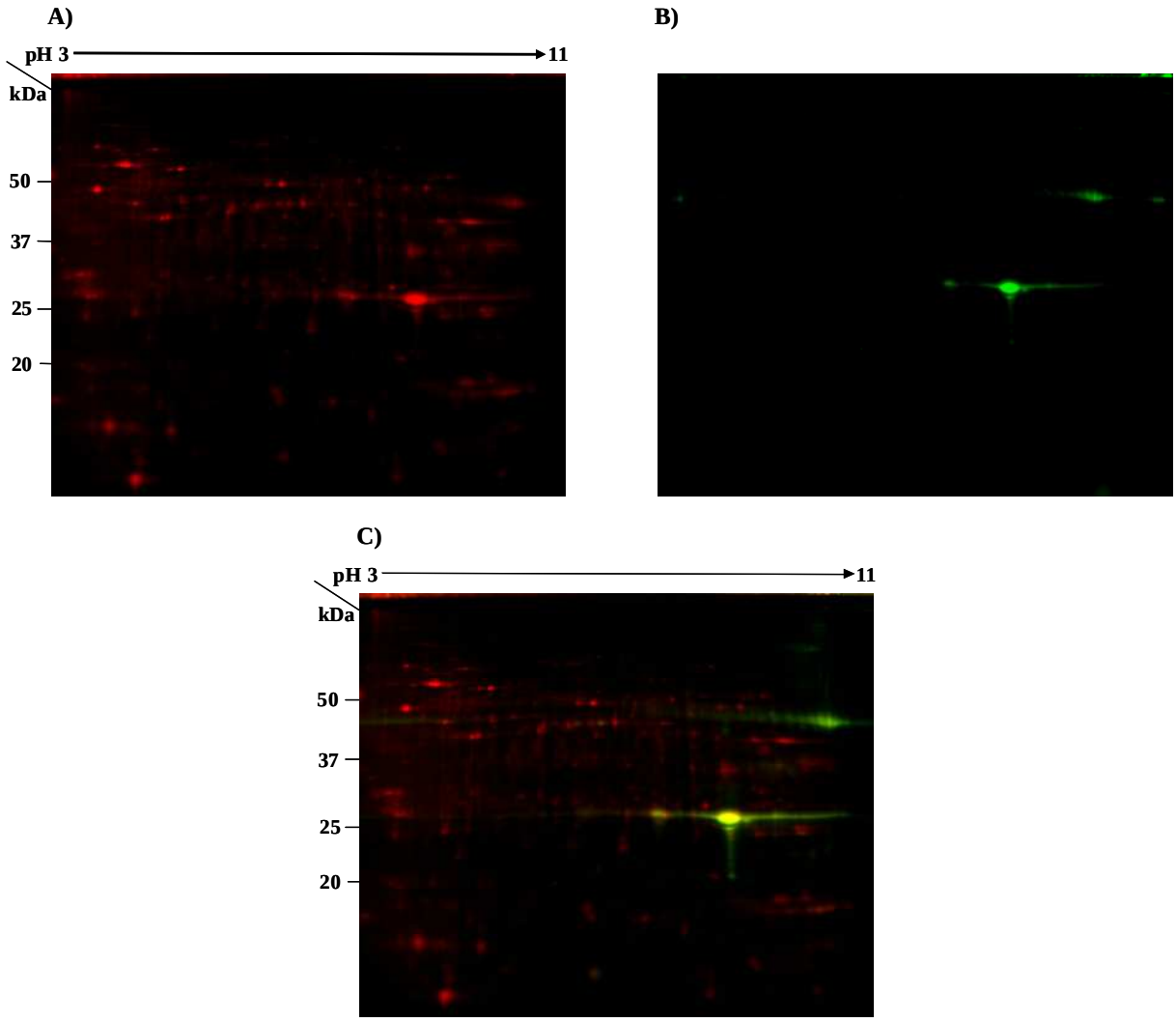
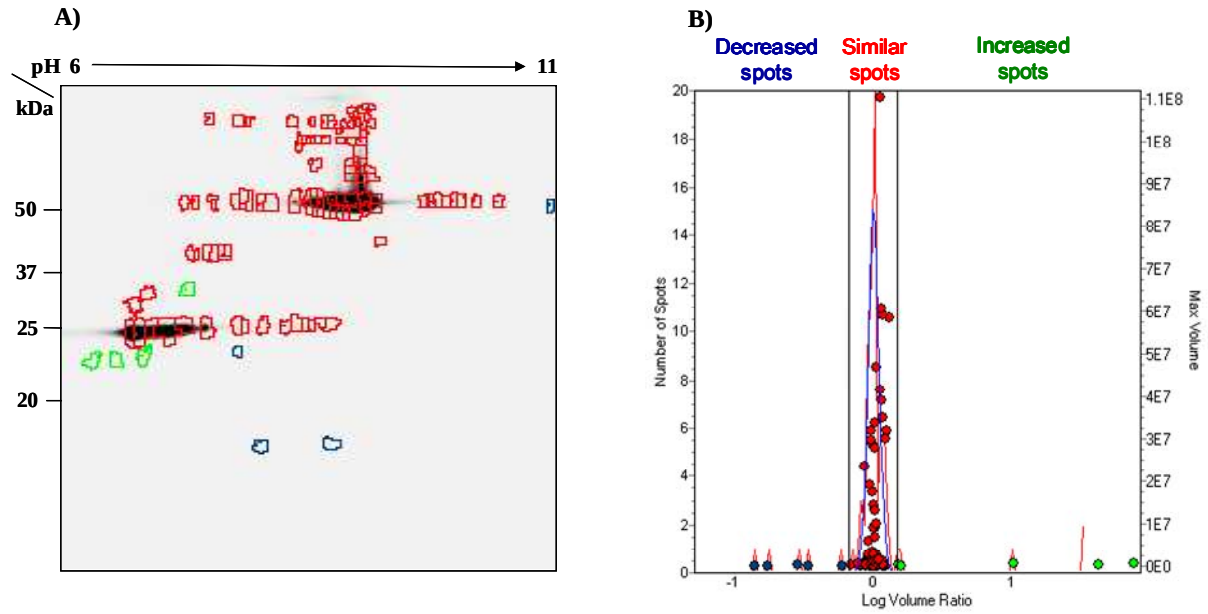


Fig. 9.


A)
pH 6
11
kDa
50
37
25
20
B)
Decreased spots
Similar spots
Increased spots
Number of Spots
Max Volume
Log Volume Ratio
1.1E8
1E8
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8E7
7E7
6E7
5E7
4E7
3E7
2E7
1E7
0E0

Fig. 10.

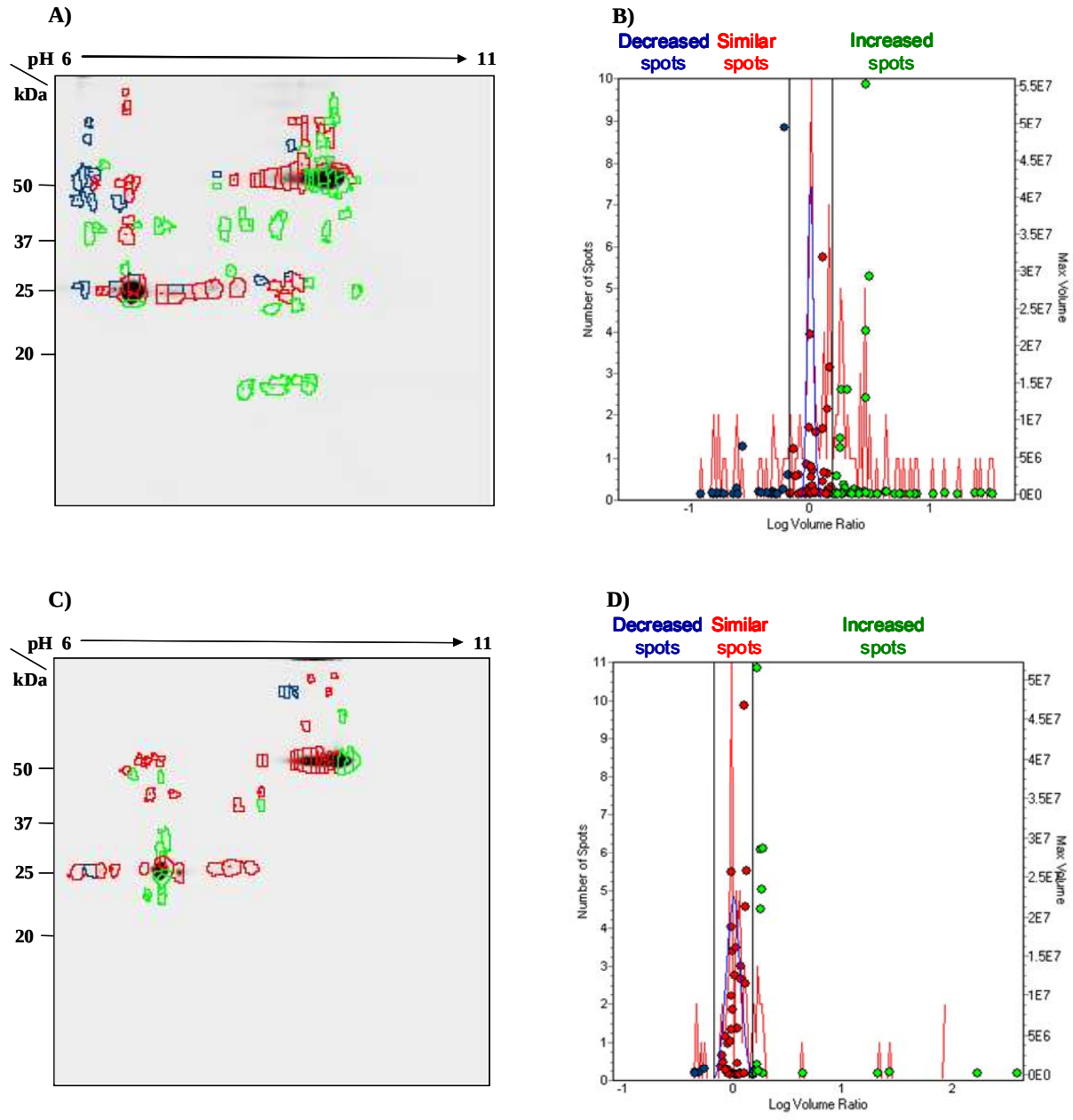
A)
pH 6
11
kDa
50
37
25
20
B)
Decreased spots
Similar spots
Increased spots
Number of Spots
Max Volume
Log Volume Ratio
C)
pH 6
11
kDa
50
37
25
20
D)
Decreased spots
Similar spots
Increased spots
Number of Spots
Max Volume
Log Volume Ratio

Fig. 11.

For submission to Electrophoresis

Use of 2-D differential gel electrophoresis to facilitate development in downstream process

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Abstract

For therapeutic antibody production, protein A affinity chromatography is often replaced by non-affinity based purification sequences, which are considered as more economical. A new non-affinity based three column process for the production of Fc-fusion protein was developed. Best results were obtained by a sequence consisting of an anion ion exchange chromatography using Q sepharose extrem load (QXL) resin for the capture step, hydrophobic interaction chromatography (HIC) with Phenyl-650M (P-650M) column and Q sepharose fast flow (QFF) for the polishing step. The specifications of glycosilations, aggregation level, critical impurities like monocyte chemoattractant protein-1 (MCP-1) and host cell proteins (CHOP) were met. An overall yield of 41% could be achieved. In this study, 2-D difference gel electrophoresis (DIGE) was applied for the monitoring the individual steps of the purification process. The characterization of product isoforms and mapping of host cell proteins provided an increased understanding of the purification processes. 2-D DIGE technology enabled a detailed comparison of the product derived from different process steps and clearly demonstrated the removal of isoforms within the process.

Keywords: 2-D difference gel electrophoresis, Fc-fusion protein, antibody-fusion protein, monocyte chemoattractant protein-1, non-affinity downstream process

Abbreviations: CHOP, chinese hamster ovary host cell proteins; **DIGE**, difference gel electrophoresis; **HMW**, high molecular weight; **MCP-1**, monocyte chemoattractant protein-1; **N/A**, not applicable; **NANA**, N-acetylneuraminic acid; **PSFF**, phenyl sepharose fast flow;
P-650M, phenyl-650M; **QXL**, Q sepharose extreme load; **QFF** Q sepharose fast flow; **SA,** silalic acid; **UNO S**, UNOsphere S

1 Introduction

2-D differential gel electrophoresis (DIGE) is an established method for high resolution protein analysis from complex biological samples [1]. This approach allows the simultaneous co-separation of up to three separate samples on a single gel. Three samples are labeled with spectrally resolvable CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3, Cy5), pooled and analyzed on single 2-D gel. This enables the detection and quantification of small differences in protein abundance between different samples. Cy2 is commonly used as an internal standard to minimize gel-to-gel variation and significantly increase accuracy, reproducibility and statistical confidence. The prime applications of this method are studies on protein expression levels in mammalian cells [2-4], microorganisms [5-7] and plant cells [8, 9]. 2-D DIGE is a helpfull tool for understanding of the pathogenesis mechanism [10-12] and for the identification of biomarkers [13, 14] to develop novel drug targets or to monitor therapeutic processes. This approach was used in the field of neuroscience [15], cancer proteomics [16-18], haematology [19], osteoporosis [20], Parkinson´s-, Alzheimer´s-pathology. In this study, the non-affinity based three column process for a new pharmaceutical Fc-fusion protein approved for a rheumatoid arthritis treatment [21-35] was investigated.

Fc-fusion protein is produced by recombinant DNA technology in a mammalian cell expression system [41]. Due to the Fc portion of human immunoglobulin G1 with hinge, CH2, and CH3 domains [41] this protein can be successfully purified using protein A column. The current purification process includes additional three steps for further removal of CHOP, monocyte chemoattractant protein-1 (MCP-1), DNA, low glycosilated products, potential contaminants including endotoxin and viral particles according to drug specification. MCP-1, called also CCL2 [42, 43] is an important impurity due to pro-inflammatory effects in human [44, 45]. MCP-1 is of importance for nonalcoholic fatty liver disease [46], multiple sclerosis [47], human heart [48] and prostate cancer [49, 50]. Additional, the pathogenic effect in bowel diseases [51], atherosclerosis [52] and chronic lung diseases, like panbronchiolitis, asthma, cystic fibrosis and bronchitis [53] was reported. For the new process several column sequences containing anion ion exchange chromatography, cation ion exchanger and hydrophobic interaction chromatography in the flowthrough/chase and bind/elute mode were tried out. Additional, filtration step using negatively charged 70 D Zeta Plus® Depth Filter was tested.

To facilitate development, DIGE can be employed to identification and characterization the impurities. 2-D DIGE is also useful in the optimization of chromatography steps. The

influence of culture supernatants, containing protein glycoforms with different sialic acid content, different host cell protein levels, and different salt and pH levels on the outcome of unit operations can be evaluated using this approach. Additionally, DIGE can be used to compare individual steps in a sequence and steps from two different purification schemes. The technique can also be applied to compare different process flow schemes and for drug substance quality control.

It is the first study to apply 2-D DIGE for downstream process development. Tracking of impurities, detection of critical contaminates and monitoring of removal of isoforms during the process is demonstrated.

2 Materials and methods

Two dimensional gels were developed using the Ettan DALT System (Amersham Biosciences, Uppsala, Sweden). Samples preparation included desalting using 2-D Clean Up Kit (Amersham Biosciences, Uppsala, Sweden) and labelling using CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3 and Cy5). 50 µg proteins were labeled with 2 µl of 200 pmol/µl marker solutions. The 1st dimension used an Ettan IPGphor Cup Loading Manifol with Immobiline™ DryStrip precast IEF gels with pH range between pH 3-10 and 4-7. The samples were loaded to the loading cup on the cathode of the rehydrated IPG strips. The protein separation took place at 20°C, 50 µA/strip and total kVh of 63. For the 2nd dimension, the IPG strips are denatured and reduced in Equilibration Buffer I (375 mM Tris/HCl pH 8.8, 6 M urea, 2% SDS, 30 %glycerol, 1% DTT) and subsequently alkylated with Equilibration Buffer II (375 mM Tris/HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide). The incubation time in each buffer was 15 minutes. Prepared Immobiline™ DryStrip gels were placed on the top of the precast 4-20% tris-glycine gels (Jule, Milford, CT, US) and sealed using an agarose sealing buffer. The 2nd dimension used an Ettan DALT*twelve* Large Vertical System (Amersham Biosciences, Uppsala, Sweden). Fluorescence detection was achieved with the Typhoon Trio Variable Mode Imager (Amersham Biosciences, Uppsala, Sweden). All gels were scanned using a Typhoon Trio Variable Mode Imager at 100 µm resolution, with PMTs set in the 400-530 V range. For the comparison of protein abundance in different samples ImageQuant software (Amersham Biosciences, Uppsala, Sweden) was applied. CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3 and Cy5), IPG-Buffers were supplied by Amersham Biosciences (Uppsala, Sweden). 99.8% anhydrous DMF came from Aldrich (St. Louis, MO, US). CHAPS, DTT, 0.1% SDS solution, urea, thiourea, iodoacetamide, glycerol, methanol, amino acids were

produced by Sigma (St. Louis, MO, US). Equilibration Buffers (I, II) for DIGE came from BioRad (Hercules, CA, US).

Chromatography experiments used either a UV-1 and chart recorder system (Amersham Biosciences, Uppsala, Sweden) or a Vision Workstation (Applied Biosystem, Foster City, CA, US). The 70D Zeta Plus BioCap™ (CUNO, Palatine, IL, US) filtration experiments were monitored using a UV-1 and chart recorder system. Anion exchanger resins (QXL, QFF) and phenyl sepharose FF were purchased from Amersham Biosciences (Uppsala, Sweden). Phenyl-650M was produced by Tosoh Corporation (Montgomeryville, PA, US) and UNO S came from BioRad (Hercules, CA, US). HEPES, sodium salts, sodium hydroxide, ammonium sulphate and other chemicals for buffer preparation were purchased from VWR (West Chester, PA, US).

3 Results

The goal of the studies was to develop a non-affinity based three-column downstream process for Fc-fusion protein. Four column sequences were tested, as presented in Table 1. The 1st sequence could not be completed, because of precipitation problem. A pH below of the fusion protein pI was required to bind the protein on a cation ion exchanger, namely UNOsphere S (UNO S). This process sequence will not be discussed in this paper. Additional, filtration steps using negatively charged 70 D Zeta Plus® Depth Filters were added to the sequence 3, including Q sepharose extreme load (QXL), phenyl sepharose fast flow (PSFF) in flowthrough/chase mode and Q sepharose fast flow (QFF), to achieve the desired removal of impurities according to the specifications. Sialic acid (SA) molar ratio, high molecular weight (HMW) level, MCP-1 and CHOP reduction are quality requirements, which have to be met in the process development. In addition, at least 50% total yield was required.

Figure 1 presents the overall yield of investigated processes. The highest yield was achieved using sequence 4 consisting of QXL, Phenyl-650 M (P-650M) in bind/elute mode and QFF. Use of the P-650M column enabled the highest total yield. The yield of this step is not higher than the step yield of PSFF used in sequence 3 and reached approximately 60%. The advantage of this hydrophobic interaction matrix is a very high MCP-1 removal of at least 3 log in flowthrough fraction (Table 4). Thus, the yield of the polishing step could be increased to 94% and overall yield reached 41%. The process yield of the current Fc-fusion protein downstream sequence is in the range between 35 and 40%, so this sequence may be considered as an alternative to the affinity based purification treatment.

Analysis of SA molar ratio and aggregation-products showed also the potential of this sequence. These results are presented in Table 2 and Table 3. All processes achieved the required glycosilation of SA molar ration ≥ 9 NANA. In addition, sequence 3 and 4 met the HMW specification of ≤ 2%. This was a problem in the sequence 2 after UNO S column. Although, the precipitation problem from the sequences 1 was resolved stabilizing proteins with 5% sucrose, the aggregation effects across the column could not be avoided. Due to HMW level of 17% the further optimization work on this sequence was stopped.

The MCP-1 and CHOP removal was an issue of the QXL, PSFF and QFF sequence. To resolve this problem 70 D Zeta Plus® Depth Filters were tested. Table 4 and Table 5 show the results. Using the negatively charged depth filter, MCP-1 reduction of about 3 log and CHOP reduction to below the detection level were achieved. The MCP-1 concentration after the filtration step was 0.6 ng/mL and met the Fc-fusion protein specification of ≤ 9.5

ng/mL. Because of very good potential of these treatment further investigations of Fc-fusion protein binding to the matrix using a feed solution with a fusion protein concentration of 48 mg/mL adjusted to pH 7.0, 7.5 or 8.0 were carried out. Additionally, the influence of a wash step on the yield was established. The results are summarized in Figure 2. Fc-fusion protein did not bind to the filter. Step yields of 95-101% were achieved. The wash step increased the step yield by 13.7-22.8%. The investigations of required wash volume indicated that one filter volume is sufficient for product recovery.

MCP-1 removal was also achieved in sequence 4. MCP-1 reduction of 5 log was achieved. The highest reduction of 3 log was reached using P-650M column. The concentration of this protein in final product was 1.6 ng/mL and also met the biopharmaceutical specification. The advantage of QXL, P-650M, QFF sequence in comparison to QXL, PSFF, QFF with additional filtration is a fewer number of steps. In this case, time and material are saved. Sequence 2 had a MCP-1 level of 400 ng/mL. This column sequence shows low potential for the manufacturing application.

The existing issue of QXL, P-650M, QFF sequence is CHOP reduction. The CHOP content in the final product was 1.1×10^5 ng/mL. The alternative for removal of troublesome impurity could be using of 70D Zeta Plus BioCap™, which reduces this protein level to less than the limit of detection. In order to minimize the number of running steps and increasing of process yield to $\geq$ 50% other possibilities were investigated. The influence of harvest conductivity on the QXL dynamic binding capacity and CHOP removal was explored. The cell culture broth was adjusted to 12 mS/cm, 13 mS/cm and 14 mS/cm and loaded onto the QXL column. In addition different elution conditions were tested. Elution buffers with a low salt concentration 25 mM HEPES, 250 mM NaCl pH 8.0 and with a high salt content 25 mM HEPES, 325 mM NaCl pH 8.0 were examined. The SA enhancement from 7.5 to 8.3 NANA (in average) was achieved on the QXL column. No influence of load material conductivity and elution conditions on the SA level of the product pool was identified. The HMW level of the QXL elution fraction was also not affected. The MCP-1 reduction was dependent on the conductivity of the harvest material. The MCP-1 removal increased with higher conductivity of the load material, as shown in Figure 3. The relationship between yield and CHOP reduction in different load and elution conditions is shown in Figure 4. With increasing salt content in the elution buffer, higher yields but lower CHOP reductions were achieved. Additionally, the yield was affected by the conductivity of load material - the lower the conductivity, the higher the yield. Additional optimization studies are required to optimize the QXL running conditions.

The reduction of protein impurities across the purification process was monitored using 2-D DIGE and confirmed by analytical assays. The reduction of glycoforms with low sialic acid content was also visually demonstrated using 2-D DIGE as shown below. A typical DIGE gel of an entire proteome from a harvest sample in the pI range between 3 and 11 is shown in Figure 5. The over expressed fusion protein can be seen as spots in the pI range 4.0 and 7.0 at 50 kDa. The molecular mass of the target antibody-fusion protein is ~90 kDa, and due to the reducing conditions of the gel, the protein spots are visible in the 45-50 kDa range. In addition to the over expressed target protein, numerous host cell proteins are visible in Figure 5. The pI and molecular weight range of the target protein was confirmed using a smaller pI range gel with a purified target protein sample. The results are shown in Figure 6. The high resolution gel shows 17 distinct glycoforms between pI 4.5 and 6.0. Several glycoforms with pIs between 6.0 and 7.0 that are present in Figure 5 are not present in Figure 6. This comparison demonstrates the removal of glycoforms with low sialic acid content (pIs from 6.0 to 7.0) by the purification process. Spots in the molecular weight region between 80 and 90 kDa represent either non-reduced protein or protein aggregates. The application of 2-D DIGE for the monitoring of purification process development is shown using QXL, PSFF and QFF sequence as an example. Samples from the harvest, QXL and QFF product pools were labeled with Cy2, Cy5 and Cy3, respectively, and were analyzed under identical conditions on a single 2-D gel. The result is presented in Figure 7. The analysis of the samples across the downstream process shows the reduction of impurities. During the first purification step a high removal of contaminants was observed, especially in the basic range of the gel. The QXL column removes negatively charged proteins and other molecules. In contrast to the harvest sample, the QFF product pool has very low impurity levels. An ELISA assay of the QFF product pool resulted in a host cell protein level of 1.2 µg/mL. This impurities are visible in pI between 4.5 and 5.0 at approximately 37 kDa and as 4 slightly spots between 5.5 and 6.5 at ~40 kDa. This spots were not detected in the Fc-fusion protein reference material. Overlaying the images using Image Quant Software makes it possible to investigate small differences in protein abundance between samples. The results are shown in Figure 8. Superimposing the blue image of the harvest sample on the QXL elution sample generates pink spots if the same proteins are present in both samples. Spots in blue represent proteins only present in the harvest sample as shown in Figure 8A. Similarly, the blue image of the harvest sample was compared with the green image of QFF elution pool. Superimposing the images produced turquoise spots for proteins contained in both samples as shown in Figure 8B. In Figure 8C, the QXL and QFF elution pools were compared. Proteins present

only in the QXL elution pool are visible as red spots. Using 2-D DIGE, the impurity removal across the purification sequence is clearly visible. The critical impurities of this process are detected. Proteins visible in Figure 8C as yellow distinct two spots at pI ~5.0 and molecular mass ~37 kDa, a slightly spot at pI ~4.5 with the same molecular mass and 4 spots in the pI range between 5.5 and 6.5 at ~40 kDa could not be removed during this purification process. The information about the pI and molecular mass of impurities can be very helpful for the downstream process development. In addition, the establishment of mass spectrometry methods may lead to identification and further characterization of critical contaminants. Additionally, the elimination of glycoforms with low sialic acid content was shown. From the ~20 isoforms visible in the harvest sample, only 17 are present in the QFF sample. The glycoforms removed by the downstream process are the blue spots with a molecular mass of ~50 kDa in the pI range between 6.0 and 7.0 as shown in Figure 8B.

Initial DIGE feasibility experiments have demonstrated the potential to monitor the downstream process. The characterization and mapping of host cell proteins provides an increased understanding of the purification system. The tracking of the removal of glycoforms through the process is also a helpful process development tool.

4 Discussion

In this paper the first application of 2-D DIGE for the development of an improved next generation downstream process was reported. Although 2-D gel electrophoresis has been used in the past for the characterization of individual steps in a downstream process [54], the method has not been applied to purification process design. One reason for this is the low comparability of 2-D gels. DIGE overcomes this drawback and may be an excellent tool for downstream process development. In addition the tracking of removal of post-translational modifications through the purification sequence is possible with this method. The specification of biopharmaceutical product declares the protein glycosilation as SA molar ratio. It gives information about the average of high and low glycosilated isoforms. Using 2-D DIGE, it is possible to detect each isoform and to investigate the ratio of each species due to the spot size. High sensitivity of this method, which allows detection of 125 pg of single protein [55], affords monitoring of multiple charged isoforms during the treatment. Specific determination of protein glycosilation increases product quality and maintain the robustness of the process. Due to high sensitive fluorescence detection, an investigation of CHOP reduction during the process and detection of low-abundance contaminates could be carried out. The monitoring of the process and tracking of changes during the treatment is very important for downstream process, which insures the drug quality.

Often, purification systems are refined after approval of the biopharmaceuticals. Minimization of process steps, testing of new purification media, using of simples buffer formulations are issues of downstream process development which should increase product yield maintaining the desired product quality and ensuring a robust process. Investigation to replace the affinity chromatography with non-affinity processes to minimize operation costs and avoid a risk of immunogenic or other physiological responses in human as result of ligand leached from the basic matrix were carried out [56]. For the conventional ion exchange chromatography new alternatives are tested. These columns can be replaced using disposable, easy to scale up charged membrane filters. Simplification of use, minimization of utility consumption and operating costs are further advantages of this method.

Fc-fusion protein process development involved the investigation of chromatography step to identify a three-column non-affinity downstream process. The best result was achieved using QXL, P-650M (bind/elute mode), QFF. The fusion protein yield of this process was 41% with glycosylation of final product 9.3 NANA and HMW ration of 1.7%. The MCP-1

removal accorded to the Fc-fusion protein's specification and was ≤ 9.5 ng/ml. The additional advantage of this process was volume reduction in comparison to flowthrough/chase mode. The existing issue is the CHOP removal. The way to resolve this problem could be applying of 70 D Zeta Plus® Depth Filter after QFF column, which removes CHOP under detection range. However, in order to minimize the number of purification steps in the sequence, optimization of QXL capture step was started. To find the best running conditions, further investigations are required.

We applied 2-D DIGE to monitor a non-affinity based Fc-fusion protein downstream process development. The reduction of impurities across chromatography columns is clearly visible on the 2-D gels and agrees with the analytical results. The reduction of Fc-fusion protein low sialic acid isoforms was demonstrated. The 2-D DIGE method offers significant benefits to process development in term of increased throughput, ease of use, reproducibility, and accurate quantification of protein.

5 References

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Table 1. Potential downstream process

Process	Column order
Process 1	QXL - UNO S - PSFF
Process 2	QXL - PSFF - UNO S
Process 3	QXL - PSFF - QFF - S-filter
Process 4	QXL - P-650M - QFF

Table 2. Sialic acid enhancement across three column process

Process	Sialic Acid (NANA)				
	Load	Step 1	Step 2	Step 3	Filtration
Process 2: QXL - PSFF - UNO S	7.9	8.3	9.3	9.4	N/A
Process 3: QXL - PSFF - QFF - S-filter	7.8	8.3	8.8	9.7	9.5
Process 4: QXL - P-650M - QFF	7.4	8.1	9.4	9.1	N/A

Table 3. HMW level across three column process

Process	HMW Reduction (%)				
	Load	Step 1	Step 2	Step 3	Filtration
Process 2: QXL - PSFF - UNO S	27.5	28.2	3.0	16.8	N/A
Process 3: QXL - PSFF - QFF - S-filter	24.3	24.9	1.4	1.9	2.2
Process 4: QXL - P-650M - QFF	24.2	26.5	1.6	1.7	N/A

Table 4. MCP-1 reduction across three column process

Process	MCP-1 Reduction (log)				
	Step 1	Step 2	Step 3	Filtration	Final
Process 2: QXL - PSFF - UNO S	1.5	0.0	0.3	N/A	1.8
Process 3: QXL - PSFF - QFF - S-filter	1.6	0.0	0.3	3.3	5.2
Process 4: QXL - P-650M - QFF	1.3	2.9	0.7	N/A	4.9

Table 5. CHOP reduction across three column process

Process	CHOP Reduction (log)				
	Step 1	Step 2	Step 3	Filtration	Final
Process 2: QXL - PSFF - UNO S	0.8	1.9	0.2	N/A	3.0
Process 3: QXL - PSFF - QFF - S-filter	0.5	2.7	0.6	< dr a)	< dr a)
Process 4: QXL - P-650M - QFF	0.3	2.3	0.7	N/A	3.3

a) < dr, below detection range

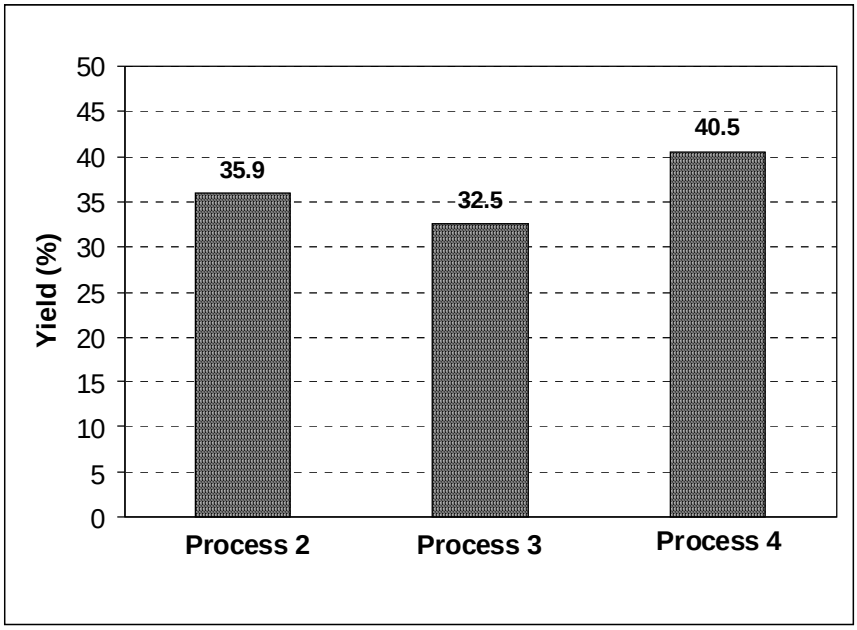

Figure 1. Overall yield comparison

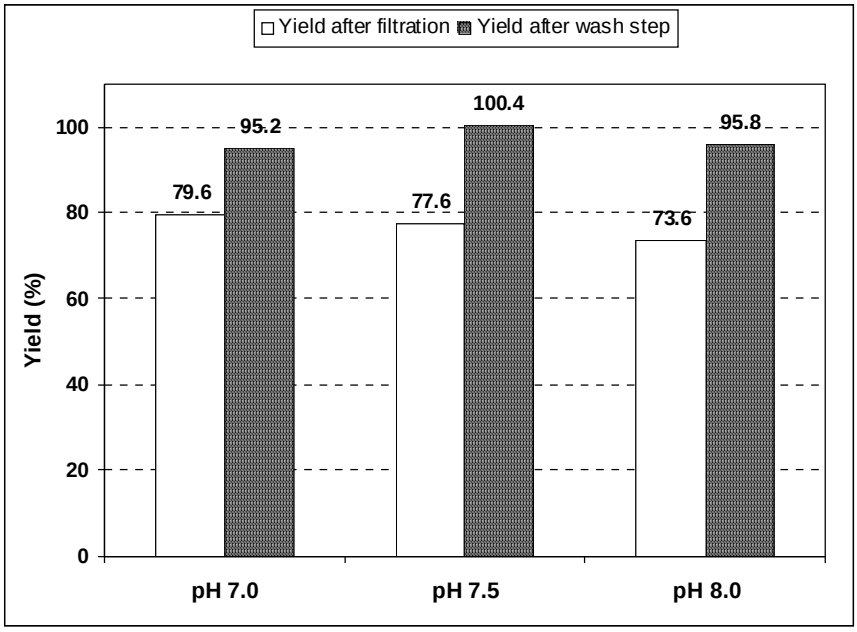

Figure 2. Influence of wash step on the filtration yield using 70D Zeta Plus BioCap™

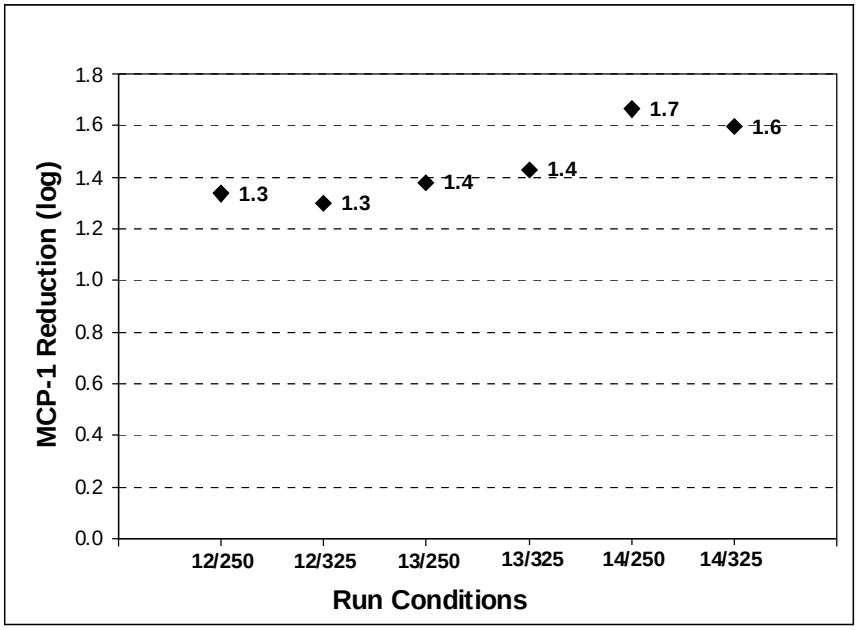

Figure 3. Influence of harvest conductivity and elution conditions on the MCP-1 reduction during QXL step

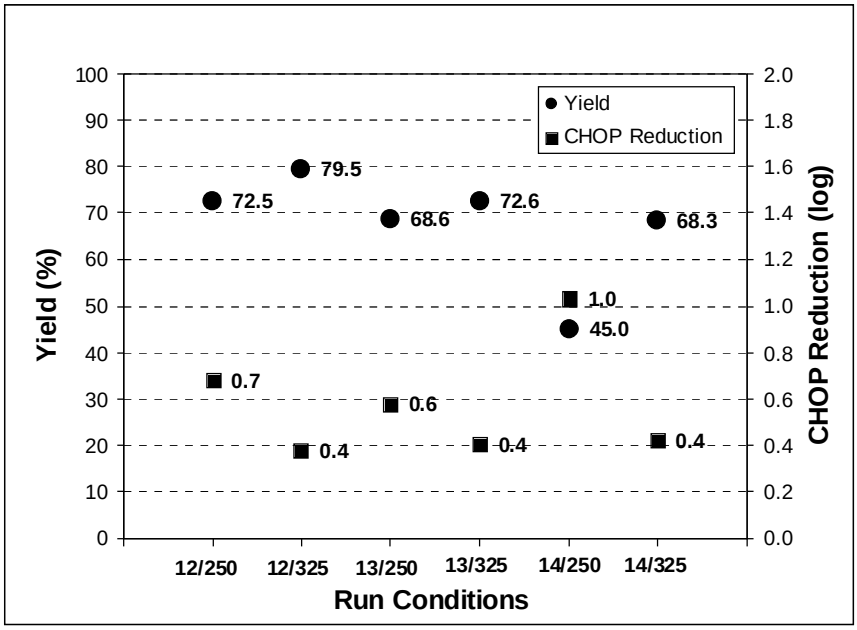

Figure 4. Inluence of harvest conductivity and elution conditions on the yield and CHOP reduction during QXL step

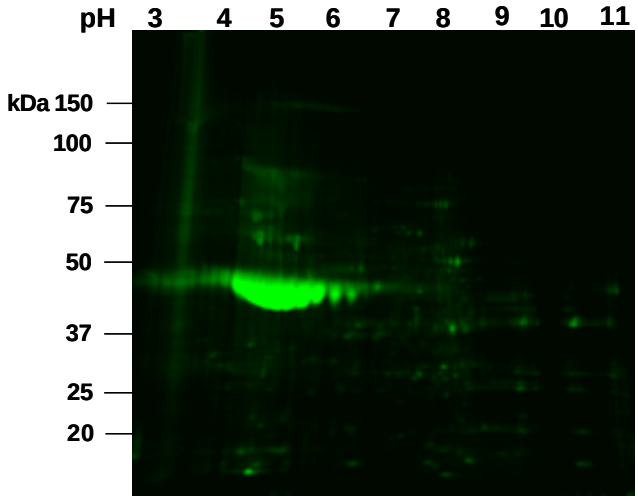

Figure 5. Proteome in harvest material

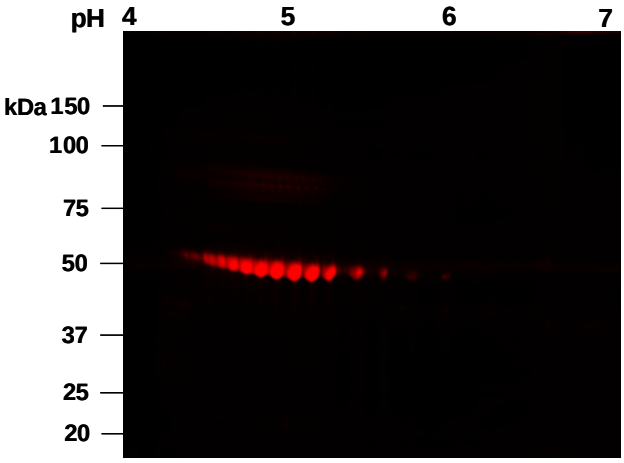

Figure 6. Analysis of purified reference material

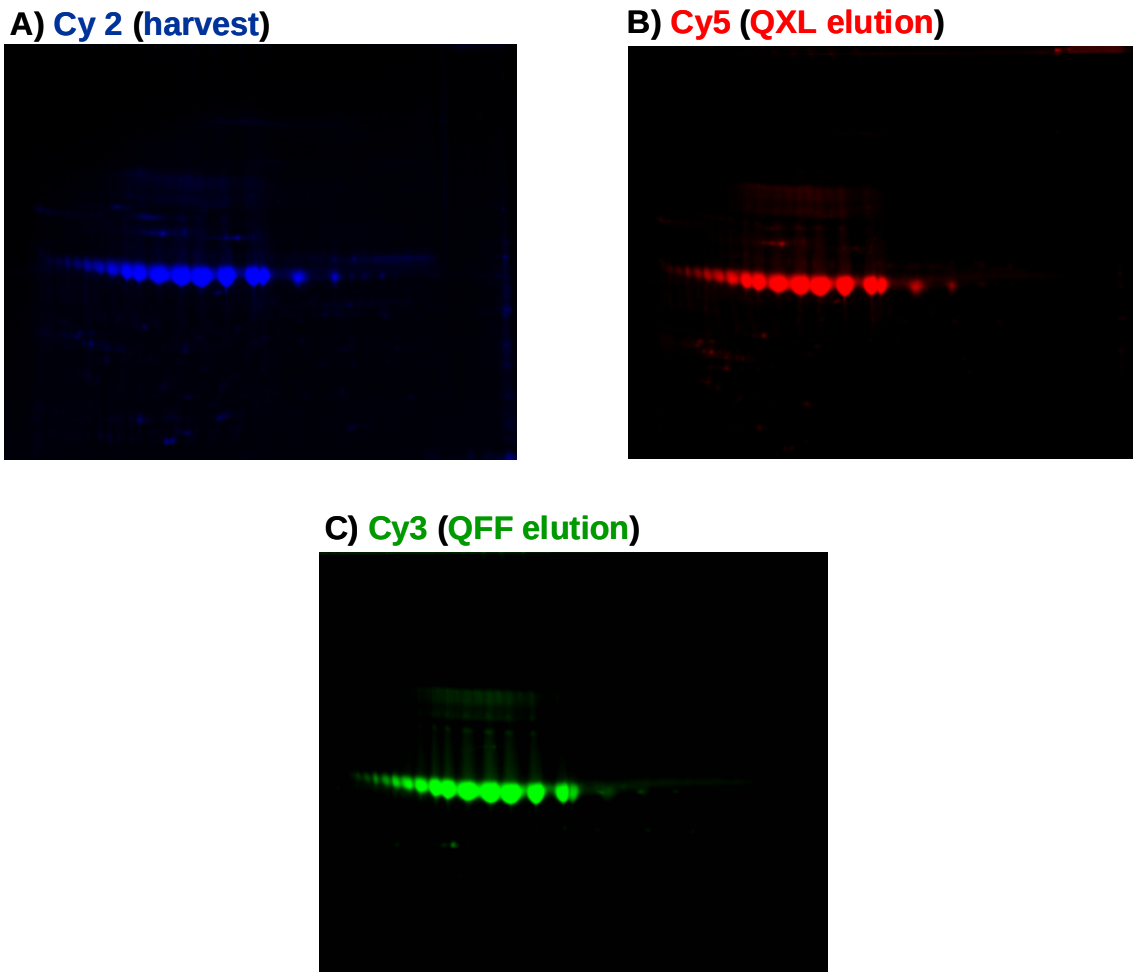

Figure 7. Comparison of different purification steps using 2-D DIGE

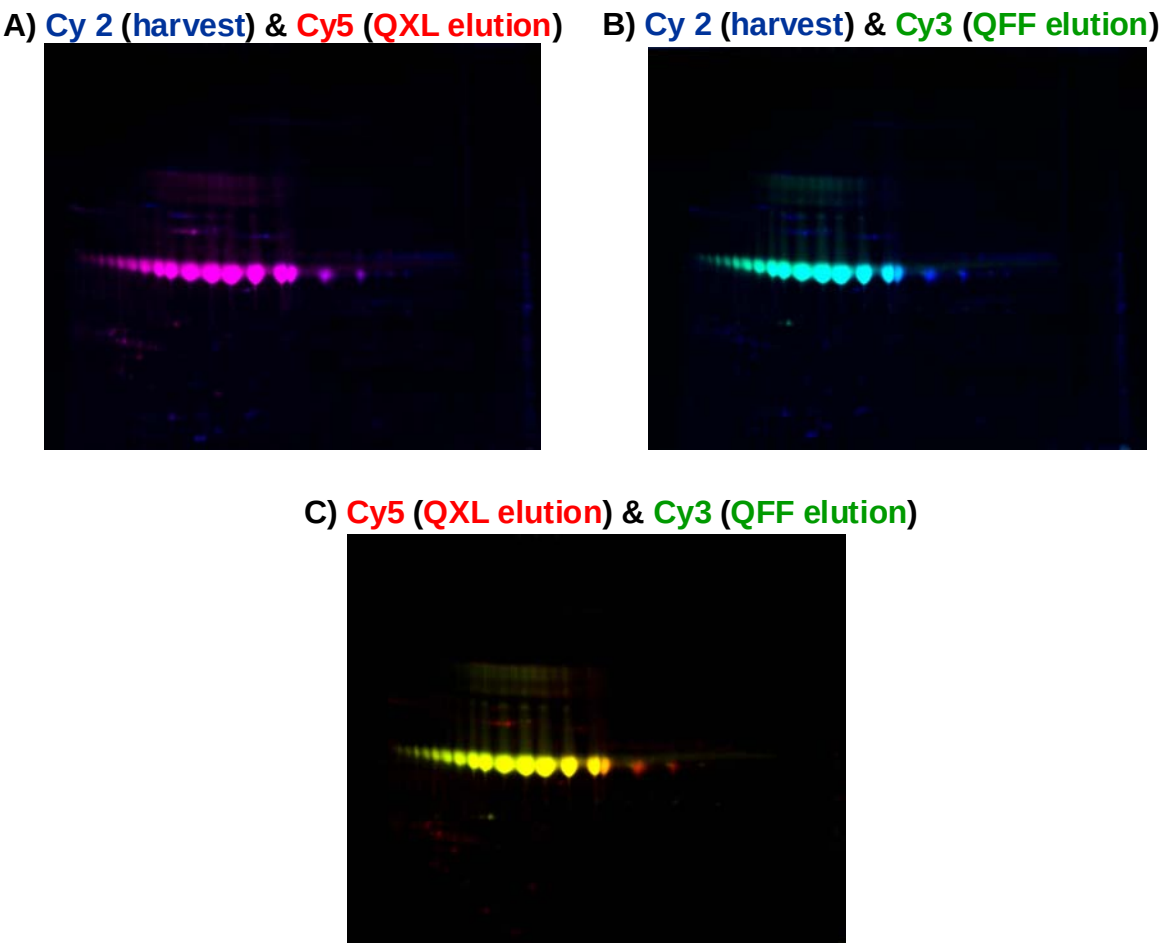

Figure 8. Direct comparison of different purification steps