Downstream process development and preliminary formulation development for the bispecific antibody NF-CU$_{N297Q}$ for a clinical phase I/IIa trial

Dissertation

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Summary

Over the last two decades, monoclonal antibodies improved the treatment options for various cancers significantly. However, the production process for these protein-based therapeutics is considerably more demanding than that for small molecule drugs. This is particularly true for complex proteins such as bispecific antibodies.
The purpose of this thesis was to develop a production process and a preliminary formulation buffer for the bispecific FLT3×CD3 antibody NF-CU$_{N297Q}$, which was developed for the treatment of acute myeloid leukemia (AML) and is intended to be applied in a clinical phase I/IIa trial. Thus, the main challenges for the production- and formulation development are to produce a sufficient amount of the active pharmaceutical ingredient (API) with very high quality and to ensure long-term stability of the product.
Good manufacturing practice (GMP)-compliant production processes are usually developed and performed by the pharmaceutical industry. The special aspect of this thesis is the realization of GMP-compliant late-stage development of a protein-based pharmaceutical drug at a small spin-off company operating at an university campus.

As a first step, specifications for critical quality attributes (CQA) were established and justified. If mandatory requirements from regulatory authorities existed for certain CQAs, these specifications were adopted for the development process. If no specific requirements were demanded, commonly applied specifications were adopted from similar production processes. If commonly applied specifications were not feasible, specifications were deducted from experimental characterizations. One example for this approach is the determination of the tolerable amount of product-related impurities such as aggregates, light chain dimers and light chains in the final product formulation. The required amount of the API for a clinical phase I/IIa trial including all necessary validations was discussed and determined theoretically.
The second task was to find an economic approach for the development of a production process which fulfills the previously developed specifications and complies with GMP requirements. At the beginning of process development, a suitable Chinese hamster ovary (CHO)-based expression cell line, a so-called primary seed bank (PSB), was generated and a scalable standard fermentation protocol was successfully adopted. A variety of purification applications was tested for their suitability to fulfill the previously determined specifications. In conclusion, a production process consisting of affinity chromatography using KappaSelect followed by anion exchange- and hydrophobic interaction chromatography and nanofiltration was developed. The small-scale model (scale 1:100) of the process fulfills the requirements for product quality and quantity and complies with GMP requirements.
A preliminary protein formulation buffer for the purified bispecific antibody was selected using thermodynamic and kinetic stability studies in carefully chosen test formulations. The evaluation of the melting temperature and the extent of aggregation of the API after a distinct time period of thermal stress resulted in a formulation buffer consisting of Histidine, Trehalose and Tween® 20. This buffer indicates stability and a long shelf-life of bispecific antibody NF-CU$_{N297Q}$.

The performed process- and preliminary formulation development are central prerequisites for GMP-compliant production. Bispecific antibody NF-CU$_{N297Q}$ produced with this GMP-process and formulated in the developed buffer will be suitable for application in a clinical phase I/IIa trial for the treatment of AML. This is an important prerequisite for the successful proof of safety and efficacy of this pharmaceutical drug.

Zusammenfassung

In den letzten beiden Jahrzehnten hat die Anwendung von monoklonalen Antikörpern die Behandlungsmöglichkeiten von diversen Krebsarten deutlich verbessert. Verglichen mit niedermolekularen Wirkstoffen ist der Produktionsprozess für diese proteinbasierten Wirkstoffe allerdings sehr viel anspruchsvoller. Dies gilt insbesondere für komplexe Proteine wie bispezifische Antikörper.
Das Ziel dieser Arbeit bestand darin, einen Produktionsprozess und eine vorläufige Proteinformulierung für den bispezifischen FLT3×CD3 Antikörper NF-CU$_{N297Q}$ zu entwickeln. Der Antikörper ist zur Behandlung der akuten myeloischen Leukämie (AML) vorgesehen und soll in naher Zukunft in einer klinischen Phase I/IIa Studie erprobt werden. Die Hauptherausforderung der Prozess- und Formulierungsentwicklung ist somit die Produktion einer ausreichenden Menge von biologisch aktivem Wirkstoff mit sehr hoher Reinheit, sowie die Sicherstellung einer langen Haltbarkeit.
Die Entwicklung und Durchführung von *good manufacturing practice* (GMP)-konformen Produktionsprozessen wird üblicherweise durch die pharmazeutische Industrie durchgeführt. Der besondere Aspekt dieser Arbeit bestand darin, die GMP-konforme Spätphasenentwicklung eines protein-basierten Wirkstoffes in einem kleinen Biotechnologieunternehmen auf einem universitären Campus durchzuführen.

Zunächst wurden Spezifikationen für kritische Qualitätsmerkmale (*critical quality attributes* = CQA) festgelegt und begründet. Wurden Spezifikationen für bestimmte CQA von regulatorischen Behörden vorgegeben, so wurden diese übernommen. Falls keine verbindlichen Vorgaben existierten, so wurden übliche Spezifikationen von vergleichbaren Produktionsprozessen übernommen. Falls auch die Übernahme üblicher Spezifikationen nicht praktikabel war, so wurden eigene Spezifikationen anhand von experimentell erhobenen Daten gesetzt. Ein Beispiel für diesen Ansatz ist die Ermittlung der maximal zulässigen Konzentration von produktspezifischen Verunreinigungen wie Aggregaten, dem Dimer der leichten Kette und der leichten Kette im finalen Produkt. Die erforderliche Menge von biologisch aktivem Wirkstoff für eine klinische Phase I/IIa Studie inklusive aller notwendigen Validierungen wurde theoretisch ermittelt.
Die zweite Aufgabe bestand darin, einen günstigen und effizienten Ansatz für die Entwicklung eines Produktionsprozesses zu finden, welcher darüberhinaus die zuvor festgelegten Spezifikationen erfüllt und GMP-konform durchführbar ist. Zu Beginn der Prozessentwicklung wurde eine geeignete *Chinese hamster ovary* (CHO)-basierte Expressions-Zelllinie, eine sogenannte *primary seed bank* (PSB), generiert und ein Standard-Fermentationsprotokoll erfolgreich adaptiert. Anschließend wurde eine Reihe von Aufreinigungsmethoden auf ihre Eignung hinsichtlich des Erreichens der zuvor festgelegten Spezifikationen getestet. Der resultierende Aufreinigungsprozess setzt sich aus den drei chromatographischen Methoden Affinitätschromatographie mittels KappaSelect, Anionenaustausch- und hydrophober Interaktionschromatographie, sowie einer Nanofiltration zusammen. Ein *small-scale model* des Produktions-

prozesses (Maßstab 1:100) wurde entwickelt und erfüllt die Anforderungen an die Produktqualität und -quantität. Darüberhinaus ist der Produktionsprozess GMP-konform durchführbar.
Für den gereinigten bispezifischen Antikörper wurde anschließend eine vorläufige Proteinformulierung entwickelt. Hierzu wurden thermodynamische und kinetische Stabilitätsstudien in sorgfältig ausgewählten Testformulierungen durchgeführt. Die Schmelzpunktbestimmung und die Bestimmung des Aggregatanteils nach kontinuierlicher thermischer Belastung führten zu einem Formulierungspuffer, welcher sich aus Histidin, Trehalose und Tween® 20 zusammensetzt. Dieser Puffer gewährleistet Stabilität und eine lange Haltbarkeit des bispezifischen Antikörpers NF-CU$_{N297Q}$.

Die durchgeführte Prozessentwicklung und die vorläufige Formulierungsentwicklung sind zentrale Vorraussetzungen für die GMP-konforme Produktion des bispezifischen Antikörpers. Der mit dem entwickelten Prozess GMP-konform hergestellte formulierte bispezifische Antikörper NF-CU$_{N297Q}$ ist zur Anwendung in einer klinischen Phase I/IIa Studie geeignet. Somit ist eine wesentliche Voraussetzung für den Nachweis der Sicherheit und Wirksamkeit dieses Wirkstoffes erfüllt.

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Introduction

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1.1 General introduction

1.1.1 Therapeutic monoclonal antibodies

Already in the late 19^{th} century, Paul Ehrlich proposed the potential existence of so-called "magic bullets" capable of selectively targeting a disease-causing organism and releasing a specific toxin. With the development of hybridoma technology by Köhler and Milstein in 1975, the generation of "magic bullets" comprising antibodies against defined target antigens became tangible (Köhler and Milstein, 1975).
Nowadays, monoclonal antibodies (mAbs) play an important role in the treatment of cancer, autoimmune and inflammatory diseases. With a revenue of 75 billion US-dollars in 2013, antibody-related therapeutics are responsible for half of the sales of all biological products (Ecker et al., 2015). Blockbusters with the highest revenue include Adalimumab, Etanercept and Infliximab directed against tumor necrosis factor α (TNFα). Those drugs have been developed for the treatment of autoimmune and inflammatory diseases. Rituximab directed against CD20, Trastuzumab, which binds to human epidermal growth factor receptor 2 (HER2/neu), and Bevacizumab directed against the vascular epidermal growth factor (VEGF) contribute to the treatment of various cancers types. In addition, numerous new products are currently evaluated in clinical trials and expect approval by regulatory authorities.

Human antibodies are globular proteins, which can be subdivided into five structurally similar subclasses: IgA, IgD, IgE, IgG and IgM. Immunoglobulin G1 (IgG1) is the most abundant subclass in serum and almost all monoclonal antibody therapeutics are based on this structure. Therefore, the following description refers to the IgG1-subclass.
The different functions of antibodies can be allocated to different parts of the molecule. The antigen-binding site is located at the N-terminus of each Fab-fragment (fragment antigen binding), whereas the effector functions are triggered via the C-terminal Fc-fragment (fragment crystallizable) (see figure 1.1 A). When the Fc-fragment binds to its receptors, antibody dependent cell-mediated cytotoxicity (ADCC) via the type III Fcγ-receptors (FcγRIII; CD16) on Natural killer (NK)-cells or antibody-dependent cellular phagocytosis (ADCP) via type I Fcγ-receptors (FcγRI; CD64) on monocytes and macrophages or type II Fcγ-receptors (FcγRII; CD32) on neutrophil granulocytes is induced. The Fc-part is also capable of activating the complement system resulting in complement dependent cytotoxicity (CDC). Two Fab- and one Fc-fragment are connected via a highly flexible hinge region to form the symmetrical "Y"-like shape of antibodies (see figure 1.1 A). IgG1-antibodies consist of two identical heavy chains of about 50 kDa each and two identical light chains of about 25 kDa each (Fleischman et al., 1963). Dimerization of two heavy chains is stabilized via two disulfide bonds in the hinge region. Dimerization of heavy and light chain is stabilized via a disulfide bond between the hinge region and the C-terminus of the light chain. Each chain can be subdivided into immunogobulin domains consisting of about 110 amino acids arranged via seven or nine strands to a compact β-barrel structure including one intramolecular disulfide bond (Edmundson et al., 1976). The heavy chain is subdivided into four domains, whereas the light chain is subdivided into two domains.

Each chain comprises one variable domain at the N-terminus (V_L for the light chain and V_H for the heavy chain). Those domains consist of three hypervariable complementary determining regions (CDR) embedded into four framework regions (FR). The other immunoglobulin domains can be regarded as constant (C_L for the light chain and C_H1, C_H2 and C_H3 for the heavy chain). C_L associates to C_H1 and stabilizes dimerization of heavy and light chain. C_H2 is the effector domain, which binds to Fcγ-receptors on immune cells and to complement factor C1q. C_H3 stabilizes dimerization of the heavy chains and binds to the neonatal Fc-receptor (FcRn).

Monoclonal antibodies are usually of mouse origin, which also holds true for some of the first therapeutic antibodies. Due to immunogenic reactions of patients against those murine antibodies, different molecule sections were replaced by human IgG1-structures (see figure 1.1). In a first approach, the variable domains remain from murine origin, whereas the constant domains are human-derived (Morrison et al., 1984). One prominent example for these chimerized antibodies is Rituximab. A further advancement was the generation of humanized antibodies, which consist of murine CDRs in human antibody framework regions (Jones et al., 1986) (for example Trastuzumab). Finally, the problems associated with murine mAb sections were completely circumvented by production of fully human antibodies. Those antibodies are generated via phage-display or via immunization of mice with human antibody genes (McCafferty et al., 1990; Marks et al., 1991). One example for this approach is Adalimumab.

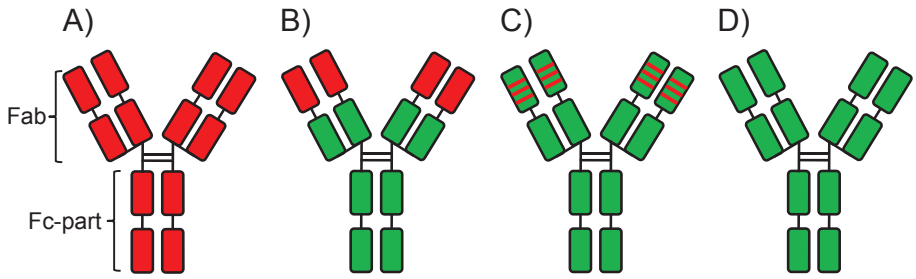


Figure 1.1: **IgG-Antibodies with decreasing murine part ratios for reduction of immunogenic reactions**
A: fully murine IgG antibody; Fab = Fragment antigen binding; Fc = Fragment crystallizable; B: chimerized antibody with variable murine domains and constant human domains; C: humanized antibody with murine complementary determining regions in a human antibody framework; D: human IgG antibody

1.1.2 Improvement strategies for monoclonal antibodies

Due to the fact that mAbs were exhibiting only limited success in the treatment of various diseases, different improvement strategies were proposed.
As originally suggested by the "magic bullet" concept, one possibility for improvement of efficacy is the generation of antibody-drug conjugates (ADC). For example the α-CD33-mAb Gemtuzumab-Ozogamicin (Mylotarg®) for treatment of AML is conjugated to the toxin Calicheamicin. However, this antibody was never approved by EMA (European medicines agency) and the approval by the Food and drug ad-

ministration (FDA) was withdrawn because of severe side effects in 2010.
An alternative approach to improve efficacy of mAbs is via the optimization of the Fc-part. A higher affinity towards Fcγ-receptors can be achieved by the exchange of distinct amino acids in the C_H2-domain (Shields et al., 2001; Lazar et al., 2006). Another approach to increase affinity of the Fc-part to its receptors is to modify antibody glycosylation (Umaña et al., 1999).
A third possibility to improve antibody efficacy is to generate bispecific antibodies. The main rationale for this approach is to recruit T-cells as effector cells; T-cells do not express Fc-receptors and must therefore be recruited via an antibody-fragment directed against an activating T-cell epitope. Nowadays a huge repertoire of different bispecific antibody formats is proposed (Nuñez-Prado et al., 2015). If T-cell recruitment is intended, the integration of a functional Fc-part in bispecific antibodies should be avoided due to the possibility of Fcγ-receptor positive cells and T-cells attacking each other via Fcγ-receptor/CD3-mediated activation. However, the approved antibody Catumaxomab (Removab®), a rat-mouse hybrid antibody directed against the epithelial adhesion molecule (EpCam) and CD3, still comprises a functional Fc-part. The high cytokine release induced by this antibody might be the result of this mechanism. The second bispecific antibody approved to date (11/2015) is Blinatumomab (Blincyto®), a bispecific single-chain fragment variable (scFv) antibody directed against CD19 on tumor cells and CD3 on T-cells for the treatment of B-ALL (Bargou et al., 2008; Ribera et al., 2015). The mode of action of T-cell recruiting bispecific antibodies is explained in figure 1.2.

1.1.3 Acute myeloid Leukemia and the target antigen FLT3

Leukemias are malignant diseases of the hematopoietic system and were first described by Rudolf Ludwig Karl Virchow in 1845. Leukemic diseases can be characterized by an accumulation of leukocyte precursor cells in bone marrow and blood. All cell lineages of the hematopoietic system can be affected, resulting in anemia, leukopenia or thrombocytopenia (NCI, 2013). Leukemic diseases may have a variety of different causes, which can be subdivided into endogenous (e.g. genetic predisposition) and exogenous causes (e.g. radiation, smoking, chemicals).
Leukemias can be subdivided into acute and chronic subtypes. Acute leukemias are characterized by malignant proliferation of cells in early differentiation stages. This results in a severe lack or absence of mature white blood cells. Chronic leukemias are characterized by malignant proliferation of cells in late differentiation stages.
Furthermore, leukemias can be subdivided into myeloid and lymphoid subtypes according to the origin of malignant cells. Myeloid leukemias derive from granulocyte-, macrophage- or monocyte precursors, whereas lymphoid leukemias derive from B- or T-cell precursors (Medicinenet, 2015).
Acute myeloid leukemia (AML) is a relatively rare disease with 4 new cases per 100,000 people each year. The incidence increases with age, median age at diagnosis is 67 and 5-year survival rate is 25.9 % (NIH, 2015).
First-line treatment of AML usually consists of chemotherapy and can be divided into two phases: induction and consolidation therapy. The goal of induction therapy is to achieve a complete remission by reducing the number of leukemic cells to an undetectable level; the goal of consolidation therapy is to eliminate any residual

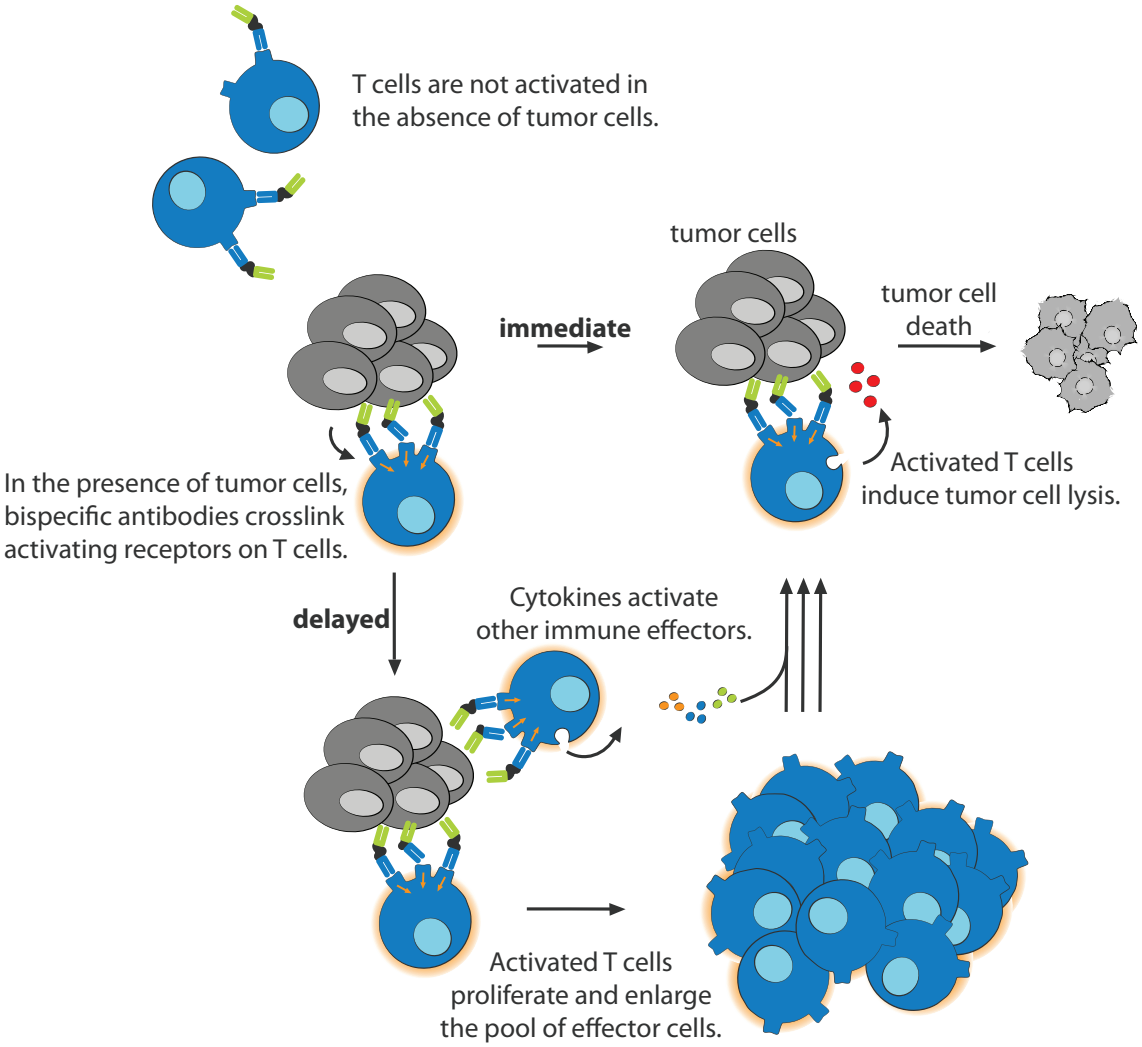


Figure 1.2: **Mode of action of bispecific α-CD3 antibodies**
The bispecific antibody binds to target molecules on tumor cells and at the same time to activating receptors expressed on T-cells. The cross-linkage of the receptors on the T-cell leads to T-cell activation and, during the immediate response, to direct killing of tumor cells by T-cells. During the delayed response, the activation of T-cells leads to T-cell proliferation and thereby to an enlarged pool of T-effector cells. In addition, activated T-cells secrete pro-inflammatory cytokines, which recruit other immune cells. Both mechanisms boost the inflammatory anti-tumor response and strongly contribute to the killing of tumor cells. (© Synimmune GmbH)

disease. Induction therapy starts with a treatment cycle of Cytarabine and an Anthracycline (most often Daunorubicin). Consolidation therapy starts with high-dose Cytarabine. For patients with high risk of relapse, autologous or allogeneic stem cell transplantation is recommended (DGHO, 2015).

Experimental treatment options with biologicals included antibodies directed against CD33 or the IL-3-receptor (CD123). The approval for α-CD33-antibody Gemtuzumab-Ozogamicin was withdrawn because of severe side effects, as stated earlier. CD33 is a pan-myeloid marker, which is also expressed on benign monocytes (Griffin et al., 1984). CD123 was also investigated as treatment option for AML (Jin et al., 2009), but the antigen is also expressed on dendritic cells and mast cells (Dahl et al., 2004).

Perhaps the most promising target for immunotherapy of AML is *fms*-like tyrosine kinase 3 (FLT3), a type III receptor tyrosine kinase also known as CD135. FLT3 is almost exclusively expressed on the surface of multipotent hematopoietic progenitors in the bone marrow (Matthews et al., 1991). Expression of FLT3 was detected on the surface of malignant cells in 70-100 % of AML cases (Birg et al., 1992; Drexler, 1996; Rosnet et al., 1996). The FLT3-molecule consists of five extracellular immunoglobulin-like domains, a short transmembrane domain and a cytoplasmatic region consisting of an ATP-binding domain and a phosphotransferase domain. After binding the natural FLT3 ligand, the receptor homodimerizes and the cell receives signals for survival, proliferation and differentiation (Lyman and Williams, 1995; Gabbianelli et al., 1995). 30 % of AML patients exhibit an internal tandem duplication mutation of FLT3 (ITD). This mutation results in permanent activation and signal transduction of the receptor resulting in unregulated proliferation of affected cells (Yokota et al., 1997).

1.1.4 The bispecific antibody NF-CU$_{N297Q}$

Bispecific FLT3×CD3 antibody NF-CU$_{N297Q}$ was developed for the treatment of AML at the Department of Immunology of the University of Tübingen (Durben, 2012; Durben et al., 2015). The format of the antibody is termed FabSc (see figure 1.3). This notation addresses the fact that the molecule consists of a Fab-fragment and a single-chain fragment variable (scFv) antibody fragment. The Fab-fragment is identical with the Fab-fragment of 4G8SDIEM, a chimeric mAb directed against FLT3, which was previously developed at the Department of Immunology (Hofmann et al., 2012). The single chain fragment is derived from the humanized α-CD3-antibody UCHT1 in V_L-V_H-orientation. The connection between Fab and scFv-fragment consists of the hinge-region and C_H2-domain-backbone of IgG1. In order to circumvent dimerization of two heavy chains, two site-directed mutagenesis' were performed in the hinge region; C226S and C229S. In addition, the C_H3 domain was excluded from the molecule. For prevention of glycosylation, the site-directed mutagenesis N297Q was performed in the C_H2 domain. In mAbs, the carbohydrates at position N297 are covered between two heavy chains. In the FabSc-format, heavy chains are not paired and carbohydrates would be exposed; mutagenesis N297Q prevents glycosylation and therefore reduces the likelihood of immunological reac-

tions. In order to prevent binding to Fcγ-receptors, the following modifications were performed in the C_H2 domain: E233P; L234V; L235A; ΔG236; D265G; A327Q and A330S. The C_H2 domain was extended by the first three amino acids of the C_H3 domain (G341, Q342 and P343) to ensure a correct tertiary structure. The notation NF-CU_{N297Q} results from N-terminal FLT3-antigen, C-terminal single-chain derived from UCHT-1 with site-directed mutagenesis N297Q. The amino acid sequence of NF-CU_{N297Q} can be found in appendix 5.2 at page 138.
The disadvantages of the FabSc-format compared to IgG-derived mAbs are the reduced productivity and the fact that Protein A is not suitable for initial purification. The last issue can be solved using other affinity media such as KappaSelect. The advantages of the FabSc-format compared to the bispecific scFv-format are the somewhat longer serum half-life, a higher productivity and a reduced tendency to aggregate (Durben et al., 2015). The latter is very important to prevent unspecific activation of T-cells due to cross-linkage of CD3.

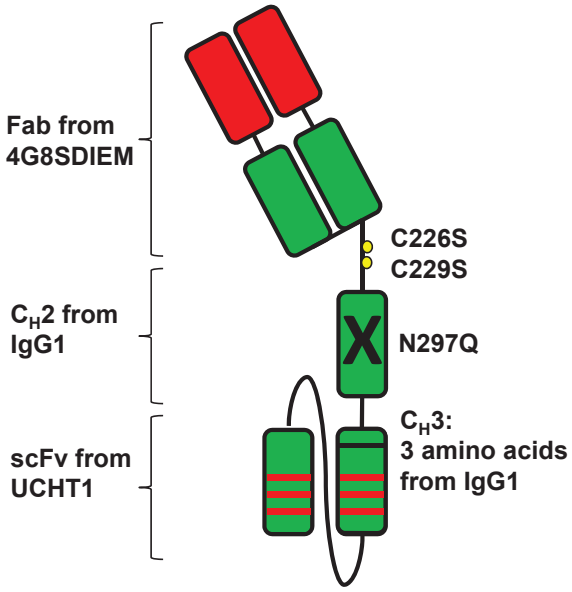


Figure 1.3: **The bispecific antibody NF-CU_{N297Q}**
red: murine part; green: human part; The X in the C_H2 domain represents the site-directed mutagenesis' E233P; L234V; L235A; ΔG236; D265G; A327Q and A330S.

1.2 Background and context of the thesis

The monoclonal α-FLT3 antibody 4G8 was generated at the Department of Internal Medicine (II) of the University clinic of Tübingen (Rappold et al., 1997). The chimerized and Fc-optimized version 4G8SDIEM (WO/2011/076922) and the bispecific antibody NF-CU_{N297Q} (WO/2013/092001) were generated and initially characterized at the Department of Immunology (University of Tübingen) (Hofmann et al., 2012; Durben et al., 2015). 4G8SDIEM will be applied in the minimal residual disease (MRD) state of AML. In contrast to that, the bispecific antibody will be applied in a progressive state of the disease.

The spin-off company Synimmune GmbH was founded for good manufacturing practice (GMP)-compliant development, clinical evaluation and commercialization of these two promising antibodies. The company with ten full time employees was funded by the German ministry of education and science (*Bundesministerium für Bildung und Forschung*); Grant number GO-Bio 03/6070.
Synimmune GmbH was responsible for process- and formulation development, GMP-production, quality control and preparation of clinical trials. The development tasks were performed in close collaboration with the Department of Immunology (University of Tübingen). GMP-production was performed at the GMP-facility of the university clinic in Tübingen.
The production process for 4G8SDIEM was adopted from a standard production protocol for monoclonal antibodies. The antibody was formulated in a standard formulation buffer for monoclonal antibodies.
Because of the different antibody format of NF-CU$_{N297Q}$ (FabSc-format), these approaches used for production of 4G8SDIEM were not feasible for the bispecific antibody. Thus, the challenge of this thesis was to define quality and quantity objectives (specifications) for the bispecific antibody, which fulfill the requirements for a clinical phase I/IIa trial. In addition, an economic approach for process- and formulation development had to be found and successfully performed.
The special aspect of this thesis is the realization of good manufacturing practice (GMP)-compliant late-stage development of a protein-based pharmaceutical drug at an university-related setting. GMP-compliant production processes are usually developed and performed by the pharmaceutical industry.

1.3 Process development objectives for clinical phase I/IIa trial

"The goal of manufacturing process development for the drug substance[1] is to establish a commercial manufacturing process capable of consistently producing drug substance of the intended quality." states ICH[2]-Guideline Q11 "Development and Manufacture of Drug Substances" (ICH-Q11, 2012). Not only for commercial processes, but also for clinical application in a phase I/IIa trial, quality objectives for biotechnological products such as NF-CU_{N297Q} must be defined. Those quality objectives (specifications) play a major role in the conduction of the development process.
Regulatory authorities use the term "critical quality attributes" (CQA) to characterize a product in terms of quality and therefore patient safety. ICH-Guideline Q11 defines CQAs in the following way: "A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. Potential drug substance CQAs are used to guide process development. The list of potential CQAs can be modified as drug substance knowledge and process understanding increase." (ICH-Q11, 2012).
One of the main difficulties of process development is to define CQA-specifications for the future drug substance and drug product[3]. In most cases, regulatory authorities do not dictate specified values, but demand high quality and scientific and risk-evaluated justification of specifications (ICH-Q6B, 1999). Thus, the task is to develop a process capable of producing large amounts of high-quality product after developing justified specifications for a scientifically based product-release by the qualified person. ICH-Guideline Q11 subdivides CQAs in properties or characteristics that affect identity, purity, biological activity and stability of the active pharmaceutical ingredient (API). The following description refers to biologicals which are produced in chinese hamster ovary (CHO)-cells.

1.3.1 Product Identity

The identity of the product must be verified in order to minimize the risk of confusion with similar products. Identity of biologicals can be verified with various methods. A SDS-PAGE and isoelectric focusing electrophoresis may be performed and the results should be compared to theoretical values. In addition, the biological properties can be used for identification. Binding of mAbs to their target antigen can be analyzed via flow cytometric analysis.

[1] Bulk of formulated active pharmaceutical ingredient (API) in formulation buffer

[2] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; a project which combines regulatory requirements from Europe, USA and Japan

[3] A finished dosage form of formulated active pharmaceutical ingredient (API) in formulation buffer

1.3.2 Product Purity

Product purity CQAs can be subdivided into product-related and process-related impurities.
Product-related impurities are aggregated or fragmentary parts of the molecule. Due to their API-specific properties, they should be characterized elaborately. In mAbs, aggregates, light chain dimers or monomeric light chains might occur.
Due to the fact that biologicals are produced in living cells such as chinese hamster ovary (CHO)-cells, process-related impurities such as host cell proteins (HCP) and host cell DNA (HCDNA) might be present in the final product. If mAbs are purified via biospecific interaction chromatography, leached affinity ligands from the purification process such as Protein A or KappaSelect can be found in the final product. Another example for process-related impurities are endotoxins, which are an indicator for contamination with gram-negative bacteria. Other possible contaminants include viruses or transmissible spongiform encephalopathy (TSE) proteins, so-called prions. In contrast to product-related impurities, process-related impurities do not need to be characterized because a CHO-based fermentation followed by biospecific interaction chromatography can be regarded as well known.

1.3.2.1 Product-related impurities

Aggregates / Multimers

Protein aggregation is a common phenomenon during production of biologicals, but the mechanism of aggregation is still poorly understood (Wang, 2005). Aggregates[1] can be subdivided into soluble/unsoluble, covalent/non-covalent, reversible/non-reversible, native/denatured types (Cromwell et al., 2006). At the beginning of aggregate formation, antibodies may partially be unfolded, leading to exposure of more hydrophobic regions of the molecule which promotes intermolecular interactions leading to aggregation (Kiese et al., 2008).
Aggregates increase immunogenicity of the API and might lead to reduced efficacy due to enhanced generation of neutralizing antibodies (Ahmadi et al., 2015).
Due to the fact that aggregates are more hydrophobic than monomers, aggregate removal can be achieved via implementation of hydrophobic interaction chromatography (HIC) (Lu et al., 2009; Suda et al., 2009).
Common specifications for aggregates in biotechnological products range below 5 % (Chon and Zarbis-Papastoitsis, 2011), an aggregate content below 1 % is more favorable due to possible immunogenic reactions and should be pursued.
Due to the fact that the biological properties of aggregates might be API-specific, further characterization is advised. The aggregate content can be monitored via size exclusion chromatography (SEC) or field flow fractionation (FFF). The performance of flow cytometric analysis on cells presenting the target antigens or potency assays might be suitable methods for biological characterization.

[1] the term aggregate will be applied for multimers as well

Fragments

The presence of the antibody fragments light chain dimer (LCD) and light chain (LC) in the cell culture supernatant of mAbs has long been known (Knittler et al., 1995). This might be the result of a faster translation of the light chain gene compared to the longer heavy chain gene (Schlatter et al., 2005). In addition, the reducing conditions inside the endoplasmatic reticulum may result in formation of light chain dimers from free light chains (Knittler et al., 1995). This contamination is not critical, as long as the mAbs are purified via Protein A, which binds the heavy chain of a mAb. When using KappaSelect as affinity ligand for antibody fragments, LCD/LC are purified along with the monomeric IgG. There is not much known about the effects of LCD/LC in patients except for the fact that they might have a negative influence on renal function (Matsuura et al., 1999).
For evaluation of theoretical properties on chromatographic media such as ion-exchange resins, the experimental determination of the isoelectric point (IEP) might be helpful.
Due to the fact that the biological properties of these mAb-fragments are API-specific, further characterization is highly recommended. The LCD/LC content can be monitored via size exclusion chromatography (SEC) or field flow fractionation (FFF). The performance of flow cytometric analysis on cells presenting the target antigens or potency assays might be suitable methods for biological characterization.
In contrast to light chains, the presence of free Heavy chains in the cell culture supernatant is not likely because heavy chains do not fold correctly in the absence of light chains and remain bound to the immunoglobulin binding protein in the endoplasmatic reticulum (Schlatter et al., 2005; Vanhove et al., 2001). Unfolded heavy chain polypeptides are subjected to degradation by the proteasome (Fagioli et al., 2001).

1.3.2.2 Process-related impurities

Host cell proteins (HCP)

Potential risks associated with HCP-contamination are toxicity, immunogenicity and influence on efficacy of the API. Induced α-HCP-antibodies may also react to endogenous human protein homologs. Furthermore, HCPs can act as adjuvant to increase the rate of formation of anti-API antibodies (de Zafra et al., 2015).
The following properties can be utilized for reduction of HCP content: molecular size can be exploited with ultra- and diafiltration (UF/DF) and isoelectric point (IEP)-distribution can be utilized for purification via ion-exchange chromatography. 10 % of CHO host cell proteins are smaller than 25 kDa, 40 % distribute from 25 to 50 kDa, 35 % can be found in the range of 50 to 75 kDa and 10 % distribute in the range of 75 to 100 kDa. 30 % of proteins have an IEP lower than 5. 35 % of the IEPs distribute in the range of 5 to 6, 15 % in the range of 6 to 7 and 20 % of the HCPs have an IEP greater than 7 (Jin et al., 2010).
Commonly accepted specifications for CHO HCPs in biotechnological products are below 100 parts per million (ppm) (Chon and Zarbis-Papastoitsis, 2011).

Host cell DNA (HCDNA)

The FDA describes the risk of Host cell DNA as follows: "Residual DNA might be a risk to your final product because of oncogenic and/or infectivity potential. There are several potential mechanisms by which residual DNA could be oncogenic, including the integration and expression of encoded oncogenes or insertional mutagenesis following DNA integration. Residual DNA also might be capable of transmitting viral infections if retroviral proviruses, integrated copies of DNA viruses, or extrachromosomal genomes are present." (FDA-Guidance for Industry, 2010). Due to the relatively low IEP with $\leqslant 5$, DNA can efficiently be removed from proteins with high IEPs such as mAbs via ion-exchange chromatography. Commonly accepted specifications for CHO HCDNA in biotechnological products are below 10 ng/dose (Chon and Zarbis-Papastoitsis, 2011; WHO-Biologicals, 2010).

Leached affinity ligand

Biospecific interaction chromatography (also known as affinity chromatography) is usually performed as initial purification step for mAbs. The most widely used affinity ligands for mAb purification are Protein A and G, a potential alternative for antibody fragments is Protein L or camel body-derived KappaSelect. Despite the fact that the protein ligand is covalently linked to its resin, leaching might occur upon acidic elution of the API. The potential risk of affinity ligand contamination in the final product is the same as for HCPs.
For evaluation of theoretical properties on chromatographic media such as ion-exchange resins, the experimental determination of the isoelectric point (IEP) might be helpful. Commonly accepted specifications for protein ligand in biotechnological products are below 10 ppm (Chon and Zarbis-Papastoitsis, 2011).

Endotoxins

Endotoxins such as lipopolysaccharides (LPS) are released after disruption of the cell wall of gram-negative bacteria and can therefore be used as surrogate readout for contamination of the product with gram-negative bacteria.
Endotoxins act as pyrogens and can induce inflammation or severe septic shocks (Opal, 2010). Due to the relatively low IEP of $\leqslant 5$, LPS can easily be removed from proteins with high IEP such as mAbs via ion-exchange chromatography (see HCDNA). Demanded specifications are 5 IU/h/kg (Ph.Eur. 7.0 part 2.6.14. "Bacterial endotoxins").

Viral and TSE safety

Viral contamination of the API may be the result of contaminated cell lines or raw materials. One potentially hazardous virus is the Lymphocytic Choriomeningitis Virus (LCMV) which infects hamster and human cells. Enveloped viruses are usually pH-sensitive and larger than 30 nm and can be inactivated or removed via low pH-values and nanofiltration respectively. Non-enveloped viruses exhibit isoelectric points of 3 to 7 and can therefore be removed effectively from mAbs with high IEPs by ion exchange chromatography (Michen and Graule, 2010). The capacity to clear

infectious viruses within a production process is assessed via validation of virus removal on a small-scale model (ICH-Q5A (R1), 1999).
The European medicines agency (EMA) states that a conversion of the demanded sterility assurance level (SAL) of 10^{-6} for sterile products to viral safety is justifiable (EMA-CPMP/BWP/268/95, 1996)

Transmissible spongiform encephalopathy (TSE) might occur via transmission of prions of animal origin into the product. Prions are suspected to be a possible cause for Creutzfeldt–Jakob disease. All raw materials must be produced animal-component free. Analytical techniques for identification of prions do not exist so far (10/2015) and therefore their absolute absence is demanded (EMA-2011/C-73/01, 2011).

1.3.3 Biological activity and stability of the product

The preservation of biological activity is a main issue during production and purification of the API. Binding properties of mAbs can be monitored via flow cytometric analysis on cells presenting the target antigens. In the case of CD3-specific antibodies such as NF-CU$_{N297Q}$, T-cell proliferation can be monitored via specific cell proliferation assays. Efficacy can be monitored with potency assays. In general, the most effective way to preserve the activity of the API is to avoid harsh conditions such as extreme pH-, conductivity- or temperature conditions.
Stability studies for biologicals are essential to assess sensitivity to factors that might cause aggregation and degradation. Those processes might have a negative influence on biological activity and therefore product safety. A high stability is directly linked to a long shelf-life.
As stated in ICH-Guideline Q5C, determination of expiration date should be based on real-time/real-temperature studies (ICH-Q5C, 1995). Nevertheless, the use of accelerated stability studies with increased temperature is common. These studies should be performed with carefully chosen test buffers and can be performed as part of the formulation development.

1.3.4 Required amount of active pharmaceutical ingredient (API)

The required amount of API for clinical application or market demands depends on the efficacy and safety profiles of the API and must be defined.

1.4 Strategies to achieve development objectives

1.4.1 Molecular biology and Cell banking

In order to address viral safety, the parental cell line must be analyzed according to ICH-Guideline Q5A to verify the absence of viral contaminants (ICH-Q5A (R1), 1999). The most commonly used parental cell line is a Chinese hamster ovary (CHO) cell line. In order to address TSE-safety, the plasmid used for transfection must be produced animal-component free.
The generation of a primary seed bank (PSB) must be performed under controlled conditions in an access-controlled laboratory. All activities related to the generation of the PSB should be documented. The handling of other cell culture, bacteria or viruses is prohibited. Storage temperature must be recorded periodically. The PSB should be thawed periodically to verify the suitability for production (EU-GMP-Part 2, 2005). Origin, history and production of parental cell lines must be documented (ICH-Q5D, 1997). The PSB is the direct precursor of the master cell bank (MCB) and the working cell bank (WCB). The MCB/WCB must be produced under GMP-compliant conditions. Raw materials must be documented and must be animal component-free (EMA-2011/C-73/01, 2011).

1.4.2 Fermentation

In order to prevent microbial or viral contamination, process scale fermentation must be performed in a clean room (EU-GMP-Part 1, 2003). In order to address TSE-safety in process scale fermentation, cell culture medium must be animal components free. The following parameters should be recorded periodically and controlled as appropriate in order to ensure a reproducible and robust process: cell density and viability, temperature, gas composition, glucose, glutamine and pH. Antibody concentration should be monitored in the late-phase of fermentation.

1.4.3 Chromatographic purification strategies

The classical approach for separation of biopharmaceutical products from impurities is to apply chromatographic techniques. The first partition chromatography was performed by Martin and Synge in 1952 and can be regarded as precursor of today's chromatographic techniques (Martin, 1952). Nowadays, a typical purification protocol for mAbs starts with biospecific interaction chromatography (affinity chromatography) for purification of the API from the cell culture supernatant. The degree of purity achieved after this purification step is usually not sufficient for biopharmaceuticals (Tugcu et al., 2008). This initial purification is followed by so-called polishing methods such as ion exchange chromatography or hydrophobic interaction chromatography.

1.4.3.1 Biospecific interaction chromatography (Affinity chromatography)

The most commonly used chromatography medium for initial purification of mAbs is Protein A. If this ligand is not applicable, several alternatives such as Protein

G, Protein L or KappaSelect can be evaluated (Hnasko and McGarvey, 2015). The advantage of these affinity media is their high selectivity and capacity for mAbs, which makes them the ideal tool for initial purification from cell culture supernatant. With the application of affinity chromatography, the HCP and HCDNA content can be reduced very effectively. This purification strategy has no direct influence on the aggregate content. Protein A and G can reduce the content of light chain dimer (LCD) and light chain (LC), whereas Protein L and KappaSelect purify LCD/LC along with the monomeric IgG. Elution is performed at low pH and might have an influence on biological activity, this should be investigated.

1.4.3.2 Ion exchange chromatography (IEX)

Ion-exchange chromatography is a very effective polishing approach for the removal of process- and product-related impurities.

Anion exchange chromatography (AIEX) is characterized by a cationic ligand on the chromatographic resin. When a protein sample is applied to an equilibrated column with a certain pH, all impurities with IEPs below this pH exhibit a negative net charge and therefore bind to the resin. All impurities with higher IEPs than the applied pH exhibit a positive net charge, do not bind and flow through the column. Figure 1.4 shows a compendium of the distribution of IEPs of process-related impurities (or better: isoelectric areas for the sum of molecules) from chapter 1.3.2.2 starting at page 12.

Cation exchange chromatography (CIEX) is characterized by an anionic ligand on the chromatographic resin. When a protein sample is applied to an equilibrated column with a certain pH, all impurities with IEPs below this pH exhibit a negative net charge, do not bind and therefore flow through the column. All impurities with higher IEPs than the applied pH exhibit a positive net charge and bind to the resin.

Most of the impurities exhibit acidic IEPs, thus a basic IEP of the desired molecule is favorable for ion-exchange chromatography. The IEPs of the leached affinity ligand or antibody fragments such as LCD or LC must be determined experimentally for a prediction of retention properties on ion-exchange resins. Aggregates can be separated from the monomer as well; due to their larger size, they carry more charged residues than the monomer. In conclusion, in a buffer with a pH lower than the IEP of the monomer, the aggregate exhibits an IEP which is apparently higher. In contrast, in a buffer with a pH higher than the IEP of the monomer, the aggregate exhibits an IEP which is apparently lower.
The conductivity should be low to maintain ionic interactions. The length of ionic interactions (debye length k^{-1}) depends on the electrolyte concentration according to equation 1.1.

$$k^{-1} = \sqrt{\frac{\epsilon_r \epsilon_0 RT}{2F^2 C_0}} \tag{1.1}$$

k^{-1}	debye length
ϵ_0	permittivity of free space
ϵ_r	dielectric constant
R	universal gas constant
T	absolute temperature in kelvins
F	Faraday constant
C_0	concentration of the electrolyte (the conductivity dependents directly on this concentration)

However, if conductivity is too low, the risk of aggregation increases. As long as no extreme pH- or conductivity-values are applied, the biological activity should be unaffected in ion exchange chromatography.

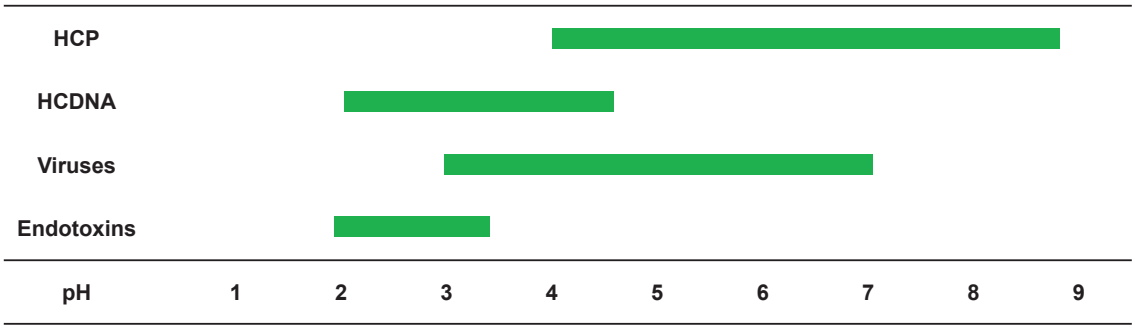


Figure 1.4: **Isoelectric points (IEP) of process-related impurities**
Process-related impurities exhibit different isoelectric points (or better: isoelectric areas for the sum of molecules). These properties can be utilized for separation of these process-related impurities from the active pharmaceutical ingredient (API) via ion-exchange chromatography. Especially when the IEP of the API is high, the separation can be very efficient. HCP = Host cell proteins; HCDNA = Host cell DNA

1.4.3.3 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography (HIC) is an attractive tool for removal of aggregates and molecule fragments, which are usually more hydrophobic than the protein monomers and therefore bind stronger to the hydrophobic ligand of the resin (Suda et al., 2009; Lu et al., 2009). No predictions about the properties of process-related impurities are possible. However, hydrophobicity decreases with difference of applied pH and IEP due to formation of charged residues, which are hydrophilic. HIC is an entropy-dependent separation method. Thus, this method is very temperature-sensitive. When proteins bind to the resin, entropy increases due to the release of water molecules from the hydration shell. Effects on biological activity must be evaluated, but in general HIC is not as harsh as reversed phase chromatography due to the absence of organic solvents.

1.4.3.4 Multimodal chromatography (MMC)

A combination of two different chromatographic techniques is termed multimodal chromatography (MMC). The most common combination is the use of HIC and CIEX[1]. A prediction of retention properties of the API and the impurities is extremely difficult and should be evaluated experimentally. This method is demanding, but effective when sufficiently controlled.

1.4.4 Viral and TSE safety

The most important guideline addressing viral safety is ICH-Guideline (ICH-Q5A (R1), 1999). The Guideline states: "Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

- selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;
- assessing the capacity of the production processes to clear infectious viruses;
- testing the product at appropriate steps of production for absence of contaminating infectious viruses."

Master and working cell banks (MCB/WCB) must be tested for viral contamination according to this ICH-Guideline and a meaningful certificate of origin is required for raw materials. Testing of the drug product will be performed in the process validation phase of the clinical phase III process. End product testing is not required for products of CHO-origin due to the fact that this cell line is regarded as well-known (ICH-Q5A (R1), 1999).
In order to ensure viral safety in the production process, at least two purification techniques, pH-inactivation and nanofiltration, are commonly used to remove enveloped viruses. pH-inactivation is usually performed after affinity chromatography at pH 3.5 for 1 h and inactivates especially enveloped viruses efficiently (Brorson et al., 2003). Nanofiltration is usually performed with a 20 nm filter and reduces viral load. A suitable technique for removal of non-enveloped viruses is ion exchange chromatography (Michen and Graule, 2010); due to the acidic IEP of viruses (see figure 1.4) they can be separated from the API, especially when the IEP of the API is basic (see section 1.4.3.2).
The capacity to clear infectious viruses within a production process is assessed via validation of virus removal on a small-scale model at the end of the process development phase (viral clearance study) (ICH-Q5A (R1), 1999). Within this validation, viruses are spiked to different steps of the process to verify the removal properties. The validation of virus removal of a certain relevant model virus must be justified. Usually enveloped and non-enveloped viruses are included into the study.

No animal-derived raw material should be used in the process (except for the use of the CHO-based production cell line). Thus, no TSE proteins would be introduced into the process.

[1] when using the term MMC in this thesis, it refers to the combination of HIC and CIEX

1.4.5 Formulation development

The choice of an appropriate storage buffer, i.e. formulation buffer, is a critical aspect for protein stability and therefore shelf-life.
Storage forms can be subdivided into liquid, lyophilized and frozen forms. The liquid dosage form is the least stressful condition for the API and the effort of development is relatively low. On the other hand, this is the storage form with the shortest shelf-life. The lyophilized storage form ensures a very long shelf-life and the storage conditions are not very stressful for the API. The effort to develop such a dosage form is very high. The frozen storage form has a very long shelf-life, but the conditions might be stressful for the protein. The effort to develop and store those dosage forms is relatively high.
Diverse modifications might occur during storage of the API; oxidation and aggregation is likely. The main challenge of formulation development is to develop a buffer in which these kind of modifications occur to a very low extent and/or very late. Suitable buffer components, pH, conductivity and osmolality are parameters to consider. Sugars may be added as stabilizers and as protectants against stressful influences. Surfactants such as Polysorbates (e.g. Tween® 20) may be added as stabilizers as well. Additional formulation components may include amino acids, inorganic salts and antioxidants.
As already mentioned earlier, ICH-Guideline Q5C states that determination of expiration date should be based on real-time/real-temperature studies (ICH-Q5C, 1995). Nevertheless, the use of accelerated stability studies for determination of thermodynamic and kinetic stability of a protein in certain formulation buffers is common (Weiss et al., 2009). The thermodynamic stability of the protein should be investigated using thermal shift assays such as differential scanning fluorimetry. The kinetic stability of the protein can be investigated by determination of the aggregate content after a distinct period of thermal stress.

1.5 Aim and objective of the thesis

The purpose of this thesis was to develop a production process and a preliminary protein formulation buffer for bispecific antibody NF-CU$_{N297Q}$. The subsequent GMP-compliant production is a prerequisite for a clinical phase I/IIa trial for the treatment of AML.

The first task was to develop justifiable specifications for critical quality attributes (CQA), which had to be identified, evaluated and, if required, further characterized to define quality objectives. In addition, the required amount of the Active pharmaceutical ingredient (API) had to be defined.
The second task was to find an economic approach for the development of a production process which fulfills the previously determined specifications and complies with GMP requirements. This task subdivides into development of a production cell line, fermentation- and purification development.
The third task was to develop a preliminary protein formulation buffer for the bispecific antibody to ensure stability.

The special aspect of this thesis is the realization of late-stage development of a protein-based pharmaceutical drug at a small spin-off company operating at a university campus for a First-in-man study. This development is usually performed by the pharmaceutical industry.
Bispecific antibody NF-CU$_{N297Q}$ produced under GMP-conditions with the developed process must be suitable for application in a clinical phase I/IIa trial for the treatment of AML.

Chapter 2

Material and Methods

Contents

2.1 Material

2.1.1 Equipment

7500 Fast Real-Time PCR system	Life technologies, Darmstadt
ÄKTA purifier 100	GE Healthcare, München
CCD Camera Fusion SL	Vilber Lourmat, Eberhardzell
Cell harvester	Inotech Biosystems, Rockville, MD/USA
Centifuge 5417 C	Eppendorf, Hamburg
Centrifuge Labofuge 400R	Thermo Scientific, Waltham, MA/USA
Centrifuge Megafuge 1.0	Thermo Scientific, Waltham, MA/USA
CO_2-Incubator Haereus	Thermo Scientific, Waltham, MA/USA
Criterion™ Cell Electrophoresis	Bio-Rad, München
Electrophoresis power supply power pac 1000	Bio-Rad, München
ELISA reader Spectra Max 340	Molecular Devices, Ismaning
FACS Calibur	BD Biosciences, Heidelberg
Freezer -20 °C	Liebherr, Biberach an der Riss
Freezer -80 °C	Colora Messtechnik, Frankenthal
Gammacell 1000 elite	MDS Nordion, Ottawa, Can
Gene Pulser® II	Bio-Rad, München
Incubator Cellstar	Nunc, Wiesbaden
Laminar Airflow Cabinet	Integra Biosciences, Biebertal
Liquid Nitrogen Tank Cryofreezer	Cryo Anlagenbau, Wilnsdorf
Magnetic Stirrer MR 2002	Heidolph instruments, Schwabach
Microbeta2 Counter	Perkin Elmer, Rodgau
Microscope Axiovert 135	Carl Zeiss Microscopy, Jena
Mini-PROTEAN Tetra Electrophoresis System	Bio-Rad, München
Multi Channel Pipette	Abimed, Langenfeld
Multistepper StepMate	Abimed, Langenfeld
Nanodrop™ 1000	Thermo Fisher Scientific, Schwerte
Orbital Shaker Celltron	Infors HT, Bottmingen/Basel, CH
pH-/conductivity-meter Seven Multi	Mettler-Toledo, Giessen
Pipetboy Pipetus	Hirschmann Laborgeräte, Eberstadt
Pipettes 2, 10, 20, 200, 1000 µL	Gilson, Middleton, WI/USA
Refrigerator	Liebherr, Biberach an der Riss
SevenMulti™ with pH- and Conductivity Expansion Units	Mettler Toledo, Giessen
Scale AC 2115	Sartorius, Göttingen
Scale LC 821	Sartorius, Göttingen
Thermoblock	Liebisch, Bielefeld
Thermocycler PTC-100	MJ Research, St.Bruno, CA
Thermoshaker ELISA Plates	Biosan, Riga, LV
UHPLC Dionex™ UltiMate™ 3000 BioRS	Thermo Scientific, Waltham, USA
Climate Cabinet Unichromat 3000	Uniequip Laborgerätebau, Planegg
Water Bath Assistent WTE 3185	Karl Hecht, Sondheim v.d. Rhön

Water Bath Memmert	Memmert, Schwabach
Wave Reactor Biostat Cultibag RM 20 Optical	Sartorius, Göttingen

2.1.2 Glas and plastic supplies

Amicon Ultra-4 Centrifugal Filter Units MWCO 30 kDa	Millipore, Schwalbach
Beakers (glass)	Schott, Mainz
Beakers (plastic)	Vitlab, Großostheim
Bottletop Filters (0.22 µm)	Millipore, Schwalbach
Cell Culture Flasks 75 cm^2	Greiner Bio-One, Frickenhausen
Cell Culture Flasks 75 cm^2	TPP, Trasadingen, CH
Cell Culture Plates 6, 24, 48, 96 well	TPP, Trasadingen, CH
Cell Culture Plates 96 well	Greiner Bio-One, Frickenhausen
Combination Cap (Luer Lock)	Fresenius Kabi, Bad Homburg
Combitips 1.25, 2.5, 5 mL	Eppendorf, Hamburg
Counting Chamber (Neubauer)	Brand, Weinheim
Cryo Vials 2 mL	TPP, Trasadingen, CH
Cultibag RM 2, 20 L optical	Sartorius, Göttingen
Discofix C-3	B-Braun, Melsungen
Electroporation Cuvettes 4 mm	PEQLAB Biotechnologie, Erlangen
ELISA Plates Nunc F96 Maxisorp	Nunc, Langenselbold
Erlenmeyer Flasks (plastic) 125, 250, 500 mL	Thermo Fisher Scientific, Schwerte
FACS Tubes 0.5 mL	Sarstedt, Nümbrecht
Fast Optical 96-well Reaction Plate	Applied Biosystems, Singapore
HPLC Vials 300 µL	Thermo Scientific, Waltham, MA/USA
Hypodermic Needles 18 G×1.5 inch	BD Biosciences, Heidelberg
Infusion Extender (0.3 x 4.1 mm)	Oriplast, Neunkirchen
Measuring Cylinders (glass)	Isolab Laborgeräte, Wertheim
Nalgene™ Cryo 1 °C Freezing Container	Thermo Fisher Scientific, Schwerte
Pasteur Pipettes	Wilhelm Ulbrich, Bamberg
PCR Softtubes 0.5 mL	Biozym Scientific, Hessisch Oldendorf
PE Tubes 50 mL	Greiner Bio-One, Frickenhausen
Pipette Tips with Filters 10, 20, 200, 1000 µL	Biozym Scientific, Hessisch Oldendorf
Pipette Tips with Filters 10, 20, 200, 1000 µL	Starlab, Ahrensburg
Polypropylene (PP) Tubes 15 mL	TPP, Trasadingen, CH
Printet Filter Mats A	PerkinElmer, Rodgau
Reaction Tubes 1.5, 2 mL	Eppendorf, Hamburg
Round Bottom Plates, 96 well	Greiner Bio-one, Frickenhausen
Round Bottom Tubes 5 mL	BD Falcon, Heidelberg
Serological Pipettes 1, 2, 5, 10, 25 mL	TPP, Trasadingen, CH
Serological Pipettes 1, 2, 5, 10, 25, 50 mL	BD Falcon, Heidelberg
Sterile Filters 0.22 µm, diameters 4, 13, 33 mm	Millipore, Schwalbach
Syringes (plastic) 2, 5, 10, 20, 30, 50 mL	BD Biosciences, Heidelberg
Tubespin Bioreactor 50, 600 mL	TPP, Trasadingen, CH
Tubing 0.125/0.25 Inch (inner/outer diameter)	Saint-Gobain Performance Plastics, Lindau

Virus Filters Planova 15 N, 20 N, BioEX	Asahi Kasei, Köln

2.1.3 Chemicals and reagents

[^{3}H-methyl]-Thymidine	Hartmann Analytic, Braunschweig
10 x IEF Anode Buffer	Bio-Rad, München
10 x IEF Cathode Buffer	Bio-Rad, München
10 x Tris/Glycine/SDS Buffer	Bio-Rad, München
Acetic acid	Carl Roth, Karlsruhe
Ammoniumsulfate	Merck, Darmstadt
β-Mercaptoethanol	Carl Roth, Karlsruhe
Biocoll separating solution	Biochrom, Darmstadt
Bovine Serum Albumin (BSA)	Carl Roth, Karlsruhe
Calibration buffers for conductivity 12.88 mS/cm, 1413 µS/cm	Mettler-Toledo, Giessen
Calibration buffers for pH 4.01, 7.00, 9.21	Mettler-Toledo, Giessen
Criterion™ Precast Gel IEF pH 3-10, 1.0 mm	Bio-Rad, München
CDM4PERMAb™	GE Healthcare, München
Citric Acid	Sigma-Aldrich Chemie, München
ClonaCell™CHO ACF Supplement (40x)	Stemcell, Köln
Coomassie staining "Roti-blue 5x"	Carl Roth, Karlsruhe
Coomassie R250 (Brilliant blue R)	Life Technologies, Darmstadt
Copper sulfate ($CuSO_4$)	Carl Roth, Karlsruhe
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie, München
Dulbecco's Phosphate Buffered Saline (DPBS)	Life Technologies, Darmstadt
Efficient Feed A and B	Life Technologies, Darmstadt
Ethanol	AppliChem, Darmstadt
FACS-Clean, FACS-Flow, FACS-Rinse	BD Biosciences, Heidelberg
Fetal Calf Serum (FCS)	Perbio Science, Bonn
Gel filtration standard (151-1901)	Bio-Rad, München
Geneticin sulfate (G 418)	Biochrom, Berlin
Glycine	Carl Roth, Karlsruhe
HEPES	Carl Roth, Karlsruhe
Histidine	Carl Roth, Karlsruhe
Hydrochloric acid 36 %	Merck, Darmstadt
IEF Sample Buffer	Bio-Rad, München
Iscove's Modified Dulbecco's Medium (IMDM)	Lonza, Verviers, BE
Isoelectric Focusing Calibration Kit High Range pI (pH 5 - 10.5)	GE Healthcare, München
Isopropanol	Carl Roth, Karlsruhe
Laemmli Sample Buffer	Bio-Rad, München
L-Glutamine 200 mM	Lonza, Verviers, BE
MEM non-essential amino acids	C.C.Pro, Neustadt
Methanol	Merck, Darmstadt
Mini-Protean TGX 10 %-Gels	Bio-Rad, München
Monosodium dihydrogenphosphate	Merck, Darmstadt

PageRuler™ Prestained Protein Ladder	Thermo Fisher Scientific, Schwerte
Penicillin	Cambrex, Verviers, BE
pH-gradient Buffer A (pH 5.6)	Thermo Fisher Scientific, Schwerte
pH-gradient Buffer B (pH 10.2)	Thermo Fisher Scientific, Schwerte
Phosphoric acid 85%	AppliChem, Darmstadt
Phytohemagglutinin	Life Technologies, Darmstadt
Roswell Park Memorial Institute Medium-1640 (RPMI)	Life Technologies, Darmstadt
Sodium acetate	Carl Roth, Karlsruhe
Sodium azide	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium pyruvate	C.C.pro, Neustadt
Sodium succinate	Fluka Chemie, Buchs
Sodium hydroxide 40 %	Carl Roth, Karlsruhe
Streptavidin-monoHRP	BD Biosciences, Heidelberg
Streptavidin-polyHRP	Immunotools, Friesoythe
Streptomycin	Cambrex, Verviers, Belgien
Sulfosalicylic acid	Carl Roth, Karlsruhe
TMB Microwell Peroxidase System (2-C)	Medac, Wedel
Trehalose$\times 2\,H_2O$	Carl Roth, Karlsruhe
Trichloroacetic Acid	Carl Roth, Karlsruhe
Tris-Base	Sigma-Aldrich Chemie, München
Tris-HCl	Sigma-Aldrich Chemie, München
Trypan-Blue 0,4 %	Sigma-Aldrich Chemie, München
Tween® 20 (Polysorbate 20)	Merck, Darmstadt
Tween® 80 (Polysorbate 80)	Merck, Darmstadt
Ultima Gold™ High Flashpoint LSC-cocktail	PerkinElmer, Rodgau
Water for injection (Ampuwa®)	Fresenius Kabi, Bad Homburg

2.1.4 Chromatography columns and media

Hiload™ -16/60-Superdex™ 200 pg	GE Healthcare, München
Hiprep™ -26/10-Desalting Column	GE Healthcare, München
Hiscreen™ -4.7 mL-Capto MMC	GE Healthcare, München
Hiscreen™ -4.7 mL-Capto Q	GE Healthcare, München
Hiscreen™ -4.7 mL-Capto SP ImpRes	GE Healthcare, München
Hiscreen™ -4.7 mL-Phenyl Sepharose High Performance	GE Healthcare, München
HiTrap™ -1 mL-Butyl Sepharose 4 Fast Flow	GE Healthcare, München
HiTrap™ -1 mL-Butyl Sepharose High Performance	GE Healthcare, München
HiTrap™ -1 mL-Butyl-S Sepharose 6 Fast Flow	GE Healthcare, München
HiTrap™ -1 mL-Capto MMC	GE Healthcare, München
HiTrap™ -1 mL-Capto Q	GE Healthcare, München
HiTrap™ -1 mL-Capto S	GE Healthcare, München
HiTrap™ -1 mL-Capto SP ImpRes	GE Healthcare, München
HiTrap™ -1 mL-KappaSelect	GE Healthcare, München

HiTrap™ -1 mL-Octyl Sepharose 4 Fast Flow	GE Healthcare, München
HiTrap™ -1 mL-Phenyl Sepharose 6 Fast Flow (high sub)	GE Healthcare, München
HiTrap™ -1 mL-Phenyl Sepharose 6 Fast Flow (low sub)	GE Healthcare, München
HiTrap™ -1 mL-Phenyl Sepharose High Performance	GE Healthcare, München
HiTrap™ -1 mL-Protein L	GE Healthcare, München
HiTrap™ -1 mL-Protein G	GE Healthcare, München
KappaSelect, 25 mL	GE Healthcare, München
CaptureSelect IgG-C_H1 Affinity Matrix	Life Technologies, Darmstadt
MediaScout® Columns 10 mL	Atoll, Weingarten
Tricorn 5/20 columns	GE Healthcare, München
MAbPac™ SCX-10 RS, 5 µm BioLC™ 2.1x50 mm	Thermo Scientific, Waltham, MA/USA
Superdex™ 200 Increase 10/300 GL	GE Healthcare, München
TOSOH TSK-Gel® G3000 SWXL 7,8x300; 5 µm	Tosoh Biosciences, Darmstadt

2.1.5 Buffers and media

2.1.5.1 Cell culture

IMDM-complete medium	IMDM 10 % FCS (heat inactivated 1 h 56 °C) 4 mM L-Glutamine 100 U/ml Penicillin 100 µg/ml Streptomycin 1 x Sodium pyruvate 1 x MEM-NEAA 50 µM β-Mercaptoethanol
RPMI-complete medium	RPMI 10 % FCS (heat inactivated 1 h 56 °C) 4 mM L-Glutamine 100 U/ml Penicillin 100 µg/ml Streptomycin 1 x Sodium pyruvate 1 x MEM-NEAA 50 µM β-Mercaptoethanol
Cryopreservation medium 4 °C	45 % fresh cell culture medium 45 % conditioned medium (supernatant of centrifuged cells) 10 % DMSO
CDM4PERMAb + 4 mM L-Glutamine	CDM4PERMAb 4 mM L-Glutamine
Efficient Feed B + 40 mM L-Glutamine	Efficient Feed B

	40 mM L-Glutamine
Trypan blue solution	0.1 % Trypan blue in DPBS

2.1.5.2 Chromatography

Buffer recipies adjusted to 20 °C ± 2 °C

KappaSelect

0.1 M Glycine (pH 2.5)	0.1 M Glycine in water (bidest) adjust pH with HCl (36 %)

Anion exchange chromatography (AIEX)

50 mM Tris (pH 9.3 Cond. 0.5 mS/cm)	45 mM Tris Base 5 mM Tris-HCl in water (bidest)
50 mM Tris (pH 7.2 Cond. 4 mS/cm)	5 mM Tris Base 45 mM Tris-HCl in water (bidest)
50 mM Tris (pH 8.0 Cond. 3 mS/cm)	18.45 mM Tris Base 31.55 mM Tris-HCl in water (bidest)
50 mM Tris (pH 8.0 Cond. 80 mS/cm)	18.45 mM Tris Base 31.55 mM Tris-HCl 1 M NaCl in water (bidest)
150 mM Tris (pH 9.60)	145 mM Tris Base 5 mM Tris-HCl in water (bidest)

Cation exchange chromatography (CIEX)

20 mM Phosphate (pH 6.22)	20 mM NaH_2PO_4 adjust pH with NaOH (10 M) in water (bidest)
20 mM Phosphate (pH 8.5)	20 mM NaH_2PO_4 adjust pH with NaOH (10 M) in water (bidest)
20 mM Phosphate (pH 7.3 Cond. 3.0 mS/cm)	20 mM NaH_2PO_4 adjust pH with NaOH (10 M)

	adjust Cond. With NaCl (1 M) in water (bidest)
20 mM Phosphate (pH 7.3 Cond. 80 mS/cm)	20 mM NaH_2PO_4 adjust pH with NaOH (10 M) 1 M NaCl in water (bidest)
20 mM Phosphate (pH 7.3 Cond. 150 mS/cm)	20 mM NaH_2PO_4 adjust pH with NaOH (10 M) 2 M NaCl in water (bidest)
20 mM Phosphate (pH 7.3 Cond. 15 mS/cm)	20 mM NaH_2PO_4 adjust pH with NaOH (10 M) 123 mM NaCl in water (bidest)
150 mM HEPES (pH 8.00)	150 mM HEPES adjust pH with NaOH (10 M) in water (bidest)

Hydrophobic interaction chromatography (HIC)

1 M Ammonium sulfate / 50 mM Tris (pH 8.00 Cond. 130 mS/cm)	1 M Ammonium sulfate 18.45 mM Tris Base 31.55 mM Tris-HCl in water (bidest)
50 mM Tris (pH 8.00 Cond. 3.0 mS/cm)	18.45 mM Tris Base 31.55 mM Tris-HCl in water (bidest)
50 mM Tris (pH 8.00 Cond. 18.5 mS/cm)	81.3 mM Ammonium sulfate 18.45 mM Tris Base 31.55 mM Tris-HCl in water (bidest)
1 M Ammonium sulfate (Cond. 130 mS/cm)	1 M Ammonium sulfate

Multimodal chromatography (MMC)

100 mM Acetate / 50 mM Phosphate / 20 mM Succinate (pH 5.0)	100 mM Sodium acetate 50 mM NaH_2PO_4 20 mM Sodium succinate adjust pH with HCl (36 %)
100 mM Acetate / 50 mM Phosphate / 20 mM Succinate	100 mM Sodium acetate 50 mM NaH_2PO_4

(pH 8.5)	20 mM Sodium succinate adjust pH with NaOH (10 M)
25 mM Phosphate (pH 7.00 Cond. 4.3 mS/cm)	25 mM NaH_2PO_4 adjust pH with NaOH (10 M) in water (bidest)
25 mM Phosphate (pH 7.00 Cond. 80 mS/cm)	25 mM NaH_2PO_4 adjust pH with NaOH (10 M) 1 M NaCl in water (bidest)
25 mM Phosphate (pH 7.00 Cond. 20 mS/cm)	25 mM NaH_2PO_4 adjust pH with NaOH (10 M) 173 mM NaCl in water (bidest)
25 mM Phosphate (pH 7.00 Cond. 63 mS/cm)	25 mM NaH_2PO_4 adjust pH with NaOH (10 M) 687 mM NaCl in water (bidest)

2.1.5.3 SDS-PAGE

Coomassie staining solution	20 % Roti Blue 5x 20 % Methanol 60 % water (bidest.)
Destain solution	water (bidest.)
Laemmli Sample Buffer (non-reduced)	Laemmli Sample Buffer
Laemmli Sample Buffer (reduced)	10 % β-Mercaptoethanol in Laemmli Sample Buffer
Electrophoresis buffer	10 % 10 x Tris/Glycine/SDS Buffer in water (bidest.)

2.1.5.4 ELISA

Wash buffer	0.05 % Tween® 20 in DPBS
Blocking solution	0.05 % Tween® 20 10 % BSA

	in DPBS
Dilution buffer	2.5 % BSA in DPBS
Stop solution	1 M Phosphoric acid

2.1.5.5 Isoelectric focusing electrophoresis

Fixation solution	4 % Sulfosalicylic acid 12.5 % Trichloroacetic acid 30 % Methanol in water (bidest.)
Conditioning solution	4 % Sulfosalicylic acid 27 % Isopropanol 30 % Acetic acid in water (bidest.)
Staining solution	4 % Sulfosalicylic acid 27 % Ethanol 10 % Acetic acid 0.04 % Coomassie R250 (Brilliant blue R) 0.5 % $CuSO_4$ in water (bidest.)
Destaining solution	4 % Sulfosalicylic acid 40 % Methanol 10 % Acetic acid in water (bidest.)

2.1.5.6 Flow cytometric analysis

FACS buffer	0.01 % NaN_3 1 % FCS (heat inactivated) in DPBS

2.1.6 Plasmids

Plasmid pGH2-1353 (see figure 2.1) was designed for transfection of 25-CHO-S cells to express antibody NF-CU_{N297Q}. Design and synthesis of the plasmid was performed by Dr. Ludger Grosse-Hovest, head of R&D at Synimmune GmbH and Entelechon GmbH respectively. The DNA sequence coding for the bispecific antibody was codon-usage-optimized for hamster cells. The Plasmid was produced under animal-component-free conditions.

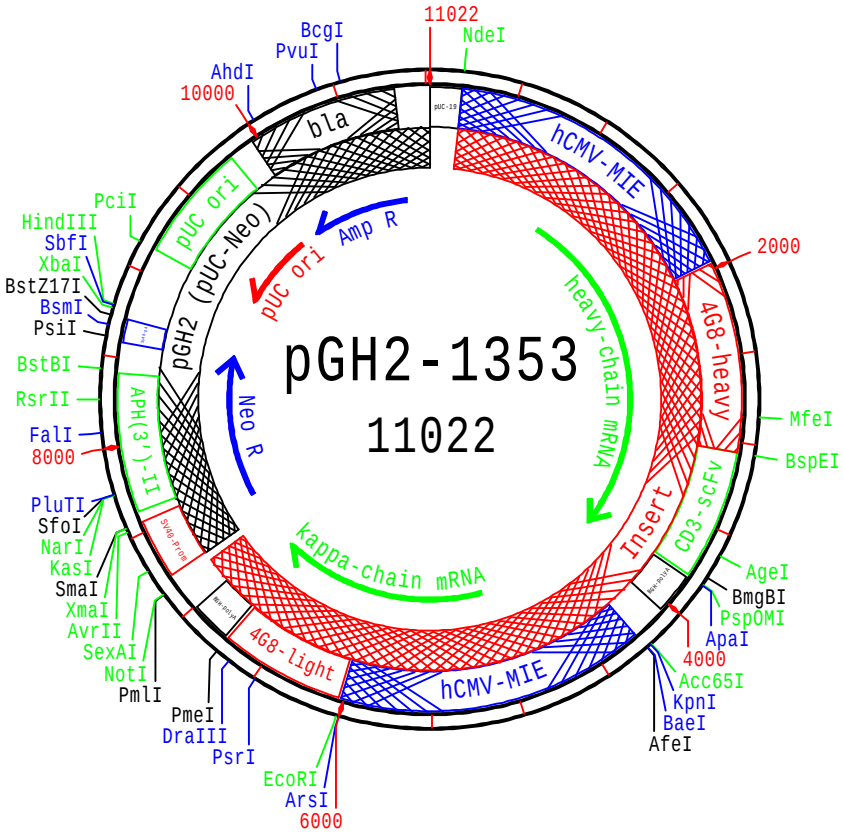


Figure 2.1: **Plasmid chart of expression vector pGH2-1353** The plasmid backbone of the 11022 bp plasmid pGH2-1353 is based on the plasmid pUC-Neo shown as an black inner arch. pUC-Neo carries a beta-lactamase operon (bla; Ampicillin resistance), an origin of replication (pUC-ori), and a neomycin resistance cassette. The expression of the neomycin resistance gene (APH(3')-II) is controlled by the SV40 promoter and polyA-region. The expression of the light and heavy chain of the bispecific antibody fragment is independently controlled by the human Cytomegalovirus Major Immediate-Early Promoter-Enhancer element (hCMV-MIE) and the termination signal of the bovine growth horme (BGH). 4G8-light = cDNA for the variable and constant domain of the light chain of antibody 4G8; 4G8-heavy = cDNA for the variable, constant C_H1, modified hinge and modified C_H2 domain of antibody 4G8; CD3-scFv = variable heavy and light chain domains of antibody UCHT1 linked by a Glycin-Serine linker. Restriction enzyme sites for uniquely cutting enzymes and the sites of the fusion of restriction enzyme sites used for cloning, are indicated. Design and synthesis of the plasmid was performed by Dr. Ludger Grosse-Hovest, head of R&D at Synimmune GmbH and Entelechon GmbH respectively. (© Synimmune GmbH)

2.1.7 Antibodies

Antibodies used for this thesis are listed in table 2.11.

Table 2.11: **Antibodies**

Notation	**Specificity**	**Species**	**Conjugate**	**Source of supply**
GaH-Fc	human Fc part	goat (polyclonal)	none	Dianova, Hamburg
GaH-Fc-PE	human Fc-part	goat (polyclonal)	R-Phycoerythrin	Dianova, Hamburg
GaM-IgG-Biot	mouse IgG	goat (polyclonal)	Biotin	Dionova, Hamburg
NF-CU$_{N297Q}$ (SP2/0)	FLT3 x CD3	mouse/human	none	M. Durben, Tübingen
NP-C16$_{N297Q}$ (SP2/0)	PSMA x CD16	mouse/human	none	F. Vogt, Tübingen
NP-CU$_{N297Q}$ (SP2/0)	PSMA x CD3	mouse/human	none	F. Vogt, Tübingen
MaH-κL-PE	human κ light chain	mouse (monoclonal)	R-Phycoerythrin	Biolegend, San Diego, USA

2.1.8 Cell lines

Table 2.12: **Cell lines**

Name	Description	Source of supply
25-CHO-S	ovarian cancer cell line of chinese hamster (CHO-K1)	Celonic AG, Basel, CH
Jurkat	human T-cell lymphoma	D. Schendel, München
NALM-16	human acute lymphoblastic leukemia	DSMZ, Braunschweig

2.1.9 Kits

Table 2.13: **Kits**

Kit	Supplier
ADCC Reporter Bioassay, Core Kit	Promega, Mannheim
CaptureSelect™ Kappa Select Leakage ELISA Kit	Life technologies, Darmstadt
CHO HCP ELISA Kit, 3G (F550)	Cygnus technologies, Southport, NC/USA
PrepSEQ® Residual DNA Sample Preparation Kit	Life technologies, Darmstadt
PROTEOSTAT® Thermal Shift Stability Assay Kit	Enzo Life Sciences, Lörrach
ResDNASEQ® Quantitative CHO DNA Kit	Life technologies, Darmstadt

2.1.10 Software

Table 2.14: **Software**

Software	Supplier
CellQuestPro	Becton-Dickinson, Heidelberg
Chromeleon 7.2	Thermo Scientific, Braunschweig
Modde 10.0	Umetrics, Frankfurt
MS Office	Microsoft, Unterschleißheim
PLA-2.1	Stegmann Sytems, Rodgau
Sigma Plot V12.0	SPSS, München
TeXnicCenter 2.02	ToolsCenter.org (Open Source Project)
Unicorn 5.11/5.31	GE Healthcare, München

2.2 Methods

2.2.1 Cell culture

All cell lines and human peripheral blood mononuclear cells (PBMC) were cultivated at 37° C, 5% CO_2 and 90 % rel. humidity. For cultivation of CHO cells basal medium CDM4PERMAb supplemented with 4 mM L-Glutamine and feed medium (Efficient Feed B) supplemented with 40 mM L-Glutamine were used. NALM-16 cell line and human PBMC were cultivated in IMDM-complete medium; Jurkat cell line was cultivated in RPMI-complete medium.

2.2.1.1 Thawing of cryopreserved cells

CHO cells: One cryo vial containing cryopreserved cells was thawed in a water bath at 37 °C. Subsequently, the cell suspension was transferred into a 15 mL PP tube. 10 mL of cold (4 °C) basal medium was added slowly (approximately 1 min.). The suspension was centrifuged (210 × g for 5 min.) and the supernatant was discarded. The cells were resuspended in 25 mL pre-warmed (37 °C) basal medium and were transferred into a 125 mL disposable Erlenmeyer flask. After 24 h of cultivation, cell culture medium was exchanged by centrifugation (210 × g for 5 min.) by 25 mL of new pre-warmed (37 °C) basal medium.
NALM-16 and Jurkat cells: One cryo vial containing cryopreserved cells was thawed in a water bath at 37 °C. The cell suspension was transferred into a 75 cm^2 cell culture flask with 10 mL of pre-warmed (37 °C) cell culture medium.

2.2.1.2 Isolation of human PMBC

PMBC were extracted from heparinized blood from human donors (mandatory premise: ethics approval). 12.5 mL blood was diluted with 12.5 mL DPBS and carefully applied to 12.5 mL "biocoll separating solution". The preparation was centrifuged at 670 × g for 30 min. without brake. PBMC were extracted with a 10 mL serological pipette and the same volume of DPBS was added. The PBMC solution was centrifuged at 210 × g for 10 min. and PBMC were resuspended in 25 mL DPBS. The cells were washed two times with 25 mL DPBS via centrifugation at 210 × g for 10 min. and were subsequently resuspended in IMDM-complete medium to obtain desired cell density.

2.2.1.3 Viable Cell count

Viability of cells was assessed by trypan blue staining. Trypan blue solution was added to cell suspension in a 96-well-plate in a ratio of 1:2. Subsequently, the amount of viable cells was determined with a Neubauer counting chamber and an inverse microscope. The amount of viable cells per large square multiplied with 10^4 and the dilution factor results in the viable cell density per mL cell culture medium.

2.2.1.4 Cultivation and standard fermentation protocol for antibody production

CHO cells were cultivated in suspension cell culture in disposable Erlenmeyer flasks on a shaker with 150 rpm (rotation diameter d = 2.5 cm). In expansion phase, viable cell density was maintained between 0.5 and 3.0 x 10^6 viable cells per mL. In late-logarithmic phase at 3 - 6 x 10^6 viable cells per mL, 10 % (V/V) feed medium was added to maintain a high cell density in stationary growth phase of 6 – 8 x 10^6 viable cells per mL. In intervals of 2 days, additional 10 % (V/V) feed medium was added to the cell culture. Cells were harvested when cell viability fell below 80 % after three feed additions. Cells were cultivated in Erlenmeyer flasks (V = 125 mL, 250 mL and 500 mL) and Tubespin bioreactors (V = 600 mL).
NALM-16 and Jurkat cells were cultivated in cell culture flasks (A = 75 cm^2). Viable cell density was maintained between 0.5 and 2.0 x 10^6 viable cells per mL.

2.2.1.5 Fermentation in a 10 L wave-reactor

Cultivation of CHO-cells was performed in a Cultibag RM 2 L and 20 L optical. Inoculum for Cultibag RM 2 L optical was cultivated according to standard fermentation protocol for antibody production until expansion phase. 3 x 10^8 viable cells in expansion phase were inoculated into 300 mL basal medium in a Cultibag RM 2 L optical. Cultivation conditions were 37 °C, 5 % CO_2, rocking angle of 6° and rocking rate of 20 rpm. In expansion phase, cell viability was maintained between 0.5 and 3.0 x 10^6 viable cells per mL. Expansion phases were 300 mL to 900 mL in Cultibag RM 2 L optical and 900 mL to 2000 mL to 3750 mL and to 6700 mL in Cultibag RM 20 L optical. In late-logarithmic phase of 3 - 6 x 10^6 viable cells per mL, 10 % (V/V) feed medium was added to maintain a high cell density in stationary growth phase of 6 – 8 x 10^6 viable cells per mL. In intervals of 2 days, additional 10 % (V/V) feed medium was added to cell culture. Cells were harvested when cell viability fell below 80 % after three feed additions.

2.2.1.6 Cell harvesting

Cell culture supernatant was centrifuged twice at 6000 × g for 30 min. Subsequently, the supernatant was filtered through a 0.22 µm filter and sodium azide was added to a final concentration of 0.02 %. pH was adjusted to 7 – 8 with 40 % NaOH.

2.2.1.7 Cryopreservation

Cell suspension containing 10^7 cells was centrifuged (210× g for 5 min.). Cells were resuspended in 1 mL cold cryopreservation medium (4 °C) and transferred to a 2 mL cryo vial. The cryo vial was transferred to a Nalgene™ Cryo 1 °C Freezing Container and stored at -80 °C. 2 days to 2 months later the cryo vial was transferred to the vapor phase of liquid nitrogen (-140 °C).

2.2.1.8 Stable Transfection of Chinese hamster ovary (CHO) cells for antibody production

For stable transfection via electroporation 2 x 10^7 25-CHO-S cells were washed three times with 25 mL of fresh, cold (4 °C) basal medium (centrifugation at 210× g for 5 min.). Cells were resuspended in 200 µL fresh basal medium (4 °C) and transferred to a cuvette for electroporation which was pre-cooled on ice. 10 µg of linearized plasmid DNA was added to the suspension. Electroporation was performed with U = 230 V and C = 975 µF. Subsequently, the cell suspension was transferred to 12 mL pre-warmed cell culture medium and a 1:2 serial dilution until 1:128 was performed. 50 µL was pipetted into each well of 96-well-plates. 24 h later 50 µL of cell culture medium containing 2 mg/mL selection antibiotic G418 was added to each well. Two to four weeks later, the supernatant of growing cells was assessed in an ELISA for the presence and concentration of produced antibody. High-producer clones were expanded to 3 mL in 6-well-plates and subsequently diluted to 1 cell per well in a 96-well plate containing basal medium with 1 x ClonaCell™CHO ACF Supplement. Two to four weeks later, the supernatant of growing monoclonal cells was assessed in an ELISA for the presence and concentration of produced antibody. High-producer clones were expanded to 3 mL in 6-well-plates and subsequently diluted to 1 cell per well in a 96-well plate containing basal medium with 1 x ClonaCell™CHO ACF Supplement. Two to four weeks later, the supernatant of growing cells was assessed in an ELISA for the presence and concentration of produced antibody. High-producer clones were expanded to 3 mL in 6-well-plates and subsequently to 10 mL in TPP-Bioreactors (V = 50 mL) until standard cultivation was performed.

2.2.2 Purification

2.2.2.1 pH- and conductivity-measurement of buffers

For pH and conductivity-measurement, a SevenMulti™ with pH and conductivity expansion units was used. The device was used according to manufacturer's instructions.

2.2.2.2 Liquid chromatography system ÄKTA purifier 100

The Fast protein liquid chromatography (FPLC) system ÄKTA purifier 100 was operated with control software Unicorn 5.11 according to manufacturer's instructions. The operation temperature for the chromatography applications was set to 21 ± 1 °C in a climate cabinet.
Prior to use, chromatography columns were equilibrated with at least three column volumes of indicated buffer A. Standard detection methods were the measurement of optical absorbance at $\lambda = 280$ nm (OD_{280}) and conductivity as well as the measurement of the system pressure before the column. The eluted peaks were fractionated by peak detection (OD_{280}) and collected into 5 mL round bottom tubes.
Biospecific interaction chromatography (affinity chromatography) for initial purification from cell culture supernatant (pH 7-8) included chromatographic resins such as Protein A, G and L, CaptureSelect IgG-C_H1 and KappaSelect. Polishing applications included Anion exchange chromatography (AIEX), Cation exchange

chromatography (CIEX), Hydrophobic interaction chromatography (HIC) and Multimodal chromatography (MMC). A detailed list of the applied columns and resins can be found in table 2.1.4 at page 26.

Columns with a bed volume of V = 1 mL and bed height of h = 2.4 cm were used for **Determination of dynamic binding capacity at 10 % breakthrough** for affinity media ($DBC_{10\,\%}$) and for **Evaluation of polishing strategies**. If not stated differently, the columns were operated with a flow rate of 1 mL/min. and are referred as "Hitrap™ 1 mL columns". A 5 mL sample loop was used for sample application.

For characterization and separation of the monomer and the product-related impurities, a "Hiload™ -16/60-Superdex™ 200 pg" Size-exclusion chromatography (SEC)-column was used. This column was operated with a flow rate of 1.2 mL/min. A 5 mL sample loop was used for sample application.

Two scalable models of the production process with process-realistic flow rates and protein loading capacity were used: the **buffer verification model** and the **small-scale model**. The buffer verification model was used to verify previously evaluated chromatography conditions. The small-scale model was used to simulate a scaled-down production process in the scale of 1:100. This means, that instead of 100 L cell culture supernatant only 1 L is purified and the volume of the small-scale columns is ~ 10 mL instead of 1 L. The column dimensions of the production process and the two process models are found in table 2.15.
The linear flow rates in the production process are standard values according to manufacturer's instructions (see table 2.15). For abidance of process-realistic flow rates, the following premises must be applied for both process models:
The chromatography columns of the buffer verification model are equipped with the half bed height of the process scale columns. Thus, in order to keep residence time constant, the linear flow rate must be decreased by the factor of two (see equations 2.1 and 2.2). The chromatography columns of the small-scale model are equipped with the same bed height as the process scale columns. Therefore, the linear flow rate must be kept constant to achieve the same residence time .

$$\text{residence time [h]} = \frac{\text{bed height [cm]}}{\text{linear flow rate [cm/h]}} \tag{2.1}$$

$$\text{linear flow rate [cm/h]} = \frac{\text{volumetric flow rate [mL/h]}}{\text{cross sectional area of column [cm}^2\text{]}} \tag{2.2}$$

The 1 L KappaSelect column of the production process purifies a maximum of 12950 mg KappaSelect binding protein (KSBP) per production batch (see discussion part 4.2 at page 116). This results in 60.9 mg KSBP for the buffer verification model and 121.7 mg KSBP for the small-scale model when the protein load is scaled according to column volumes. After application of a security factor, the applied

amount of protein was 70.5 mg for the buffer verification model and 130 mg for the small-scale model. Column dimensions, flow rates and protein binding properties for process scale, buffer verification model and small-scale model are summarized in table 2.15. The small-scale model was only applied for KappaSelect, AIEX and HIC.
For the buffer verification model, a 150 mL superloop was used for sample application. For the small-scale model, a direct load via the system pumps was performed for sample application.

Table 2.15: **Column dimensions, flow rates and protein binding properties of Process scale, Buffer verification model and Small-scale model;** The small-scale model was only applied for KappaSelect, AIEX and HIC; The applied amount of KappaSelect binding protein to the process scale KappaSelect column will be discussed in discussion part 4.2 at page 116; AC = Affinity Chromatography; AIEX = Anion exchange chromatography; CIEX = Cation exchange chromatography; HIC = Hydrophobic interaction chromatography; MMC = Multimodal chromatography

Characteristics	**Process scale**	**Buffer verification model**	**Small-scale model**
Column volume [mL]	1000	4.7	9.4
Bed height [cm]	20	10	20
Cross sectional area [cm^2]	50	0.466	0.466
Appl. of KSBP theory [mg]	12950	60.9	121.7
Appl. of KSBP with security factor [mg]	N/A	70.5	130
Lin. flow rate [cm/h]			
AC: KappaSelect	400	200	400
AIEX/CIEX/MMC	150	75	150 (only AIEX)
HIC: Phenyl HP	120	60	120
Vol. flow rate [mL/min.]			
AC: KappaSelect	333.33	1.55	3.11
AIEX/CIEX/MMC	125	0.59	1.18 (only AIEX)
HIC: Phenyl HP	100	0.47	0.94

2.2.2.3 Nanofiltration

Nanofiltration was performed with the support of "Asahi Kasei" Corporation. Three tangential flow filters were tested: Planova™ 15 N, Planova™ 20 N and Planova™ BioEx with filtration surface of 0.001 m^2 each. A filtration device was set up according to figure 2.2.

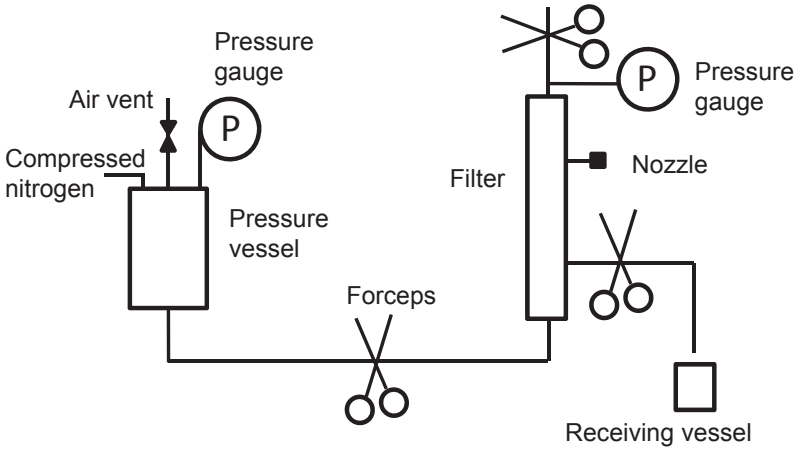


Figure 2.2: **Filtration procedure - Nanofiltration**
A tangential flow filtration (TFF) was performed. Filtration liquid was placed into the pressure vessel and was filtrated into the receiving vessel with 98 kPa trans-membrane pressure (TMP) for Planova™ 15/20 N and with 330 kPa TMP for Planova™ BioEx.

Prior to use, all filters were flushed with 20 mL water for injection with a maximum of 98 kPa trans-membrane pressure (TMP) with compressed nitrogen and a leakage test was performed (bubble point test, see below). Subsequently, filters were flushed with 20 mL DPBS with a maximum of 98 kPa. Antibody solution was placed into the pressure vessel and was filtrated with indicated maximum TMP of filters: Planova™ 15 N and 20 N with a maximum TMP of 98 kPa and Planova™ BioEx with a maximum TMP of 330 kPa. Filtration was performed with constant pressure. The filtrated volume was assessed with a measuring cylinder every 10 min. for Planova™ 15/20 N and every 5 min. for Planova™ BioEx. The required filtration volume was calculated for each filter individually. After use, all filters were flushed with 20 mL water for injection with a maximum of 98 kPa TMP and a leakage test (bubble point test) was performed.

Bubble point test:
After flushing the test filters with water for injection, the pressure vessel was filled with nitrogen gas and a pressure of 98 $\pm$ 5 kPa was applied to the filtration membrane. For the next 20 sec., the filter membrane was visually inspected for emerging nitrogen bubbles. The test is regarded as passed if no bubbles emerge.

2.2.3 Characterization of the active pharmaceutical ingredient and product-related impurities

2.2.3.1 Determination of antibody concentration

For determination of antibody concentration, the protein absorbance at 280 nm was used. According to the Beer-Lambert law, the concentration can be calculated with the equation for molar extinction (see 2.3). A useful conversion is equation 2.4 for specific extinction, which results in a concentration in [g/L].

$$E = lg\left(\frac{I_0}{I}\right) = \epsilon * c * d \tag{2.3}$$

$$E = E_{1\,cm}^{0.1\,\%} * c^{'} * d \tag{2.4}$$

$$E_{1\,cm}^{0.1\,\%} = \epsilon / M \tag{2.5}$$

E	molar extinction
I_0	reference intensity
I	sample intensity
ϵ	molar extinction coefficient [L/(mol*cm)]
c	concentration [mol/L]
d	path length [cm]
$E_{1\,cm}^{0.1\,\%}$	specific extinction [L/(g*cm)]
$c^{'}$	concentration [g/L]
M	molar mass [g/mol]

The specific extinction for antibody solutions of a concentration of 1 g/L and a path length of 1 cm is 1.37 L/(g*cm).

2.2.3.2 ELISA for antibody concentration

Coating reagent:
GaH-Fc; dilution 1:1000 in DPBS

Biotinylated reagent:
GaM-IgG-Biot; dilution 1:10.000 in dilution buffer

Standard solution:
NF-CU_{N297Q} (SP2/0); dilution according to work list in DPBS

50 µL of diluted capture antibody was applied to each well of an ELISA Plate "Nunc F96 Maxisorp" and incubated over night at 4 °C. The content of the wells was discarded and 100 µL of blocking solution was added to each well. After an incubation period of 1 h at room temperature, the content of the wells was discarded and the plate was washed three times with wash buffer. 50 µL of standard or sample was added to each well indicated on work list. After 1 h of incubation at room temper-

ature, the content of the wells was discarded and the plate was washed four times with wash buffer. Detection antibody was diluted in dilution buffer and 50 µL was added per well. After 1 h of incubation at room temperature, the content of the wells was discarded and the plate was washed four times with wash buffer. Steptavidin-polyHRP was diluted 1:10000 in dilution buffer and 100 µL was added to each well. After 30 min. incubation at room temperature, the content of the wells was discarded and the plate was washed six times with wash buffer. The 1:1-mixture of "TMB Microwell Peroxidase System (2-C) solution" was prepared and 100 µL was added to each well. The plate was incubated for 5 min. in the dark and 50 µL of 1 M Phosphoric acid was added. OD_{450} was measured with an ELISA plate reader. A standard curve was calculated using 4-Parameter logistic-fit-analysis.

2.2.3.3 Isoelectric focusing electrophoresis

Samples were diluted 1:2 in "IEF Sample Buffer". 1 to 5 µg protein was applied to each well of "Criterion™ Precast Gel IEF pH 3-10, 1.0 mm". 20 µL of reconstituted "Isoelectric Focusing Calibration Kit High Range pI (pH 5 - 10.5)" was applied to the gel as a IEP standard. Running buffer for the anode was 1:10 diluted "10 x Anode Buffer". Running buffer for the cathode was 1:10 diluted "10 x Cathode Buffer". Operating conditions were U = 100 V for 60 min., U = 250 V for 60 min. and U = 500 V for 30 min. in an electrophoresis cell. Gel was fixed with Fixation solution for 30 min. followed by conditioning with Conditioning solution for 5 min. Gel was stained with 'Staining solution for 2 h and destained with Destaining solution for 1 h. The gel was photographed using a CCD camera.

2.2.3.4 SEC / UHPLC

Size exclusion chromatography (SEC) was performed using a UHPLC-system "Dionex™ UltiMate™ 3000 BioRS" equipped with two columns: "TOSOH TSK-Gel G3000 SWXL 7,8x300; 5 µm" or "Superdex 200 Increase 10/300 GL". The Superdex column was introduced later and elicits better resolution of antibody aggregates, monomers and fragments. A maximum of 10 µg or 50 µL of sample was applied per run. The system was operated at 500 µL/min. with DPBS. Proteins were detected via OD at $\lambda = 220$ nm (OD_{220}). Chromatograms were compared to an external gel filtration standard (Bio-Rad). Data acquisition and analysis was performed via control software Chromeleon 7.2.

2.2.3.5 CIEX / UHPLC

Cation Exchange chromatography was performed using a UHPLC-system "Dionex™ UltiMate™ 3000 BioRS" equipped with column "MAbPac SCX-10 RS, 5 µm". 10 µL sample containing 10 µg protein was diluted 1:2 in water and again 1:3 in "pH gradient buffer A (pH 5.60)". A maximum of 60 µL of diluted sample was applied per run. The system was operated at 500 µL/min with "pH gradient buffer A (pH 5.60) and B (pH 10.2)". Proteins were detected via OD at 280 nm. Data acquisition and analysis was performed via control software Chromeleon 7.2.

2.2.3.6 SDS-PAGE

Samples were diluted 1:2 in "laemmli sample buffer". When performing a SDS-PAGE with reducing conditions, 10 % β-mercaptoethanol was added. Samples were incubated at 75 °C for 5 min. in a thermo cycler. 2 µg of sample was applied to each well of a "Mini-PROTEAN® TGX™ 10 %-Gel". 5 µL of "PageRuler™ Prestained Protein Ladder" was applied to the gel as protein standard. Operating conditions were U = 200 V for 30 min. in an electrophoresis cell with Tris/Glycine/SDS buffer. Gels were stained in coomassie staining solution for 1 h followed by destain solution for 2 h.

2.2.4 Process-related impurities

2.2.4.1 Host cell protein ELISA for CHO cells

A commercially available ELISA kit was used: "CHO HCP ELISA kit, 3G (F550)". All required solutions were supplied with the kit. ELISA was performed according to the manufacturer's instructions.
Pre-coated 96-well plates were incubated with 100 µL anti-CHO:HRP and 50 µL sample or standard for 2 h at 180 rpm on a microtiter plate shaker. The content of the wells was discarded and the plate was washed five times with washing solution. 100 µL TMB substrate was added and after an incubation period of 30 min., 100 µL stop solution was added. OD_{450} was measured with an ELISA plate reader. A standard curve was calculated using 4-Parameter logistic-fit-analysis.

2.2.4.2 ELISA for determination of leached KappaSelect ligand

A commercially available ELISA kit was used: "CaptureSelect™ Kappa Select Leakage ELISA Kit". The manufacturer's instructions were modified (see below).

Coating reagent:
Goat IgG anti-KappaSelect affinity ligand; dilution 1:200 in DPBS

Biotinylated reagent:
Biotinylated Goat IgG anti-KappaSelect affinity ligand; dilution 1:200 in dilution buffer

Standard solution:
CaptureSelect™ human Ig kappa affinity ligand, dilution according to work list in protein-L purified NF-CU_{N297Q} polished via AIEX-medium CaptoQ.

50 µL of diluted coating reagent was applied to each well of an ELISA Plate "Nunc F96 Maxisorp" and incubated over night at 4 °C. The content of the wells was discarded and the wells were washed five times with wash buffer. Subsequently 250 µL of blocking solution was added to each well and the plate was incubated at room temperature for 30 min. on a microtiter plate shaker. The content of the wells was discarded and the plate was washed one time with wash buffer. Standard and samples were incubated at 95 °C for 15 min. and centrifuged at 12,000 ×g for

5 min. 100 µL of standard or samples was added to each well indicated on work list. After 1 h of incubation at room temperature, the content of the wells was discarded and the plate was washed five times with wash buffer. Detection antibody was diluted in dilution buffer and 100 µL was added per well. After 1 h of incubation at room temperature, the content of the wells was discarded and the plate was washed five times with wash buffer. Steptavidin-monoHRP was diluted 1:2500 in dilution buffer and 100 µL was added to each well. After 30 min. of incubation at room temperature, the content of the wells was discarded and the plate was washed five times with wash buffer and two times with water (bidest.). The 1:1-mixture of "TMB Microwell Peroxidase System (2-C) solution" was prepared and 100 µL was added to each well. The plate was incubated for 5 min. in the dark and 50 µL of 1 M Phosphoric acid was added. OD_{450} was measured with an ELISA plate reader. A standard curve was calculated using 4-Parameter logistic-fit-analysis.

2.2.4.3 Host cell DNA quantitative PCR for CHO cells

Two commercially available kits were used: "PrepSEQ® Residual DNA Sample Preparation Kit" and "ResDNASEQ® Quantitative CHO DNA Kit"

DNA extraction:
DNA extraction was performed according to manufacturer's instructions. Samples were diluted according to work list in DPBS containing additional 0.35 M NaCl. Protein residues are lysed via Protein K. DNA is concentrated via magnetic particles and eluted for analysis.

QPCR:
Quantitative PCR (QPCR) and evaluation was performed according to manufacturer's instructions. QPCR war performed on a 7500 Fast Real-Time PCR system.

2.2.5 Biological activity

2.2.5.1 Flow cytometric analysis

Target cells NALM-16 or Jurkat were cultivated with high viability. Cells were washed three times in FACS buffer (centrifugation at 670 × g for 3 min.) and resuspended to a cell density of 10^6 cells/mL in FACS buffer. 100 µL of suspension was transferred to each well of a round bottom plate, centrifuged (670 × g for 3 min.) and incubated with 50 µL of antibody solution for 30 min. at 4 °C. Cells were washed three times with FACS buffer (see above) and incubated with secondary antibody linked to a chromophore for 30 min. at 4 °C. Cells were washed three times (see above) and resuspended in 200 µL FACS buffer and transferred to FACS tubes 0.5 mL.
Flow cytometry was performed on a "FACSCalibur flow cytometer". Data acquisition and evaluation was performed using "CellQuestPro software". Binding of NF-CU_{N297Q} to FLT3 was investigated using NALM-16 cells and binding to CD3 was investigated using Jurkat cells. The bispecific antibody was detected using GaH-Fc-PE or MaH-κL-PE antibodies.

2.2.5.2 Inactivation of target cells

Target cells were inactivated via radiation with 120 Gy using a ^{137}Cs source in a "Gammacell 1000 elite" irradiation chamber.

2.2.5.3 T-cell proliferation Assay

T-cell proliferation induced by the FLT3×CD3 antibody was measured using a [^{3}H]-Thymidine incorporation assay. 10^5 PBMC in 50 µL IMDM-complete medium were seeded into each well of a 96-well plate. 10^5 inactivated target cells NALM-16 in 50 µL IMDM-complete medium were added to each well. Subsequently, the antibody was diluted in IMDM-complete medium and 50 µL of each dilution was added to wells indicated on work list. The 96-well plate was incubated for 72 h and then pulsed with 0.5 µCi/well [^{3}H]-Thymidine. After 20 h cells were harvested on Printet Filtermat A and precipitated radioactivity was determined in a liquid scintillation counter.

2.2.5.4 Potency Assay (Promega)

This Assay was performed to determine the potency of NF-CU$_{N297Q}$ and product-related impurities. A commercially available kit was used: "ADCC Reporter Bioassay, Core Kit". The Potency assay was performed according to the manufacturer's instruction with NALM-16-cells as target cells. The effector to target ratio was 1:2 (30,000 NALM-16 and 60,000 effector cells per assay).
The effector cells of this kit are Jurkat cells transfected with a Fc-receptor which triggers the NFAT-activation path. A luciferase gene is linked to the NFAT-gene and is co-expressed with NFAT. When adding luciferin and Adenosintriphosphate (ATP), activation can be measured via luminescence intensity.
NF-CU$_{N297Q}$ does not work via classical ADCC because it does not bind to Fc-receptors. However, Jurkat cells are T-cell-derived cells and therefore express a T-cell-receptor and CD3, which also utilizes the NFAT-activation pathway. Thus, this assay can be used to assess NF-CU$_{N297Q}$ activity.
Analysis and calculation of EC$_{50}$-values was performed via "PLA-software 2.1".

2.2.6 Stability and formulation development

Experiments for optimization of product stability were planned, performed and evaluated in an Design of experiment-approach (Fisher, 1990). The usual one factor at a time-approach was not suitable due to the fact that the investigated factors influence each other and the responses. Data analysis was performed via software "Modde 10.0".

2.2.6.1 Differential scanning fluorimetry

This assay is applied to determine the melting temperature T$_{m1}$, or half-maximal denaturation temperature, of NF-CU$_{N297Q}$ in a certain buffer. A commercially available kit was used for this assay: "ProteoStat® Thermal Shift Stability Assay Kit" Fluorescence of the dye at 610 nm is induced when bound to hydrophobic residues

of a protein. Thus, the protein of interest is stressed by increasing temperature which induces unfolding and therefore presentation of hydrophobic residues. Suitable formulation buffers contribute to a higher resistance of a protein to temperature-induced unfolding. The experiments were performed in a "7500 Fast Real-Time PCR system". One part "10 x assay buffer" was diluted in 9 parts water for injection. One part "1000 x ProteoStat® TS Detection Reagent" was diluted in 99 parts "1 x Assay buffer". For each reaction 22.5 µL NF-CU_{N297Q} [1 mg/mL] in a certain buffer was mixed with 2.5 µL 10 x ProteoStat® TS Detection Reagent. The following reaction plates were used: "Fast optical 96-well reaction plate". Temperature was increased from 25 °C to 100 °C with 1 °C/min. Fluorescence was assessed at 610 nm. Fluorescence was plotted against temperature (see figure 2.3). The maximum of the first derivative of this plot represents the temperature, where half of the protein is unfolded and is referred to as melting temperature T_{m1}.

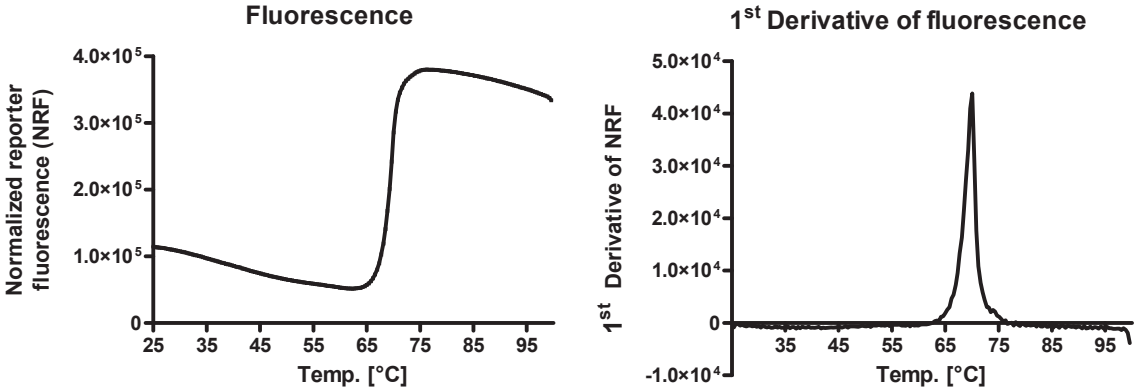


Figure 2.3: **Differential scanning fluorimetry - Example formulation buffer**
Buffer composition: NF-CU_{N297Q} [1 mg/mL] in 20 mM Histidine pH 6.2, 0.075 % Tween® 20, 50 g/L Trehalose, 75 mM NaCl; T_{m1} = 70.1 °C

2.2.6.2 Thermal stress test

This assay was used to determine the amount of aggregation of a protein in a certain buffer resulting from long-term constant thermal stress. Study temperature was 40 °C for a time period of one week for formulation screening experiment and for four weeks for formulation optimization experiment. Samples were stressed in an incubator "cellstar" and determination of aggregate and monomer content was assessed via "Sec / UHPLC" with column "Superdex 200 Increase 10/300 GL".

Chapter 3

Results

Contents

An expression cell line and a fermentation protocol were developed for the efficient production of bispecific antibody NF-CU$_{N297Q}$. Subsequently, product-related impurities were identified and characterized. Process-related impurities were not characterized because the CHO-based production process followed by biospecific interaction chromatography can be regarded as well-known. Different purification strategies were evaluated for their suitability of effectively separating the active pharmaceutical ingredient (API) from product- and process-related impurities. A purification process was developed and the purification was verified with a so-called small-scale model (1:100) of the production process. The performance of nanofilters was evaluated to find a suitable filter for the production process. Finally, a suitable protein formulation buffer was developed, which indicates high stability and a long shelf-life of bispecific antibody NF-CU$_{N297Q}$.

3.1 Cell line - Primary seed bank

The parental cell line 25-CHO-S, a CHO-K1 cell line adapted to suspension cell culture, was stably transfected with plasmid pGH2-1353 and selection was performed using the aminoglycoside antibiotic G418. Plasmid pGH2-1353 was designed for transfection of 25-CHO-S cells for expression of bispecific antibody NF-CU$_{N297Q}$. High-producer clones were identified by ELISA for antibody concentration at semi-confluent growth in 96-well plates. After sequential subcloning and two single cell cloning procedures, clone "T1/16/6-B5//E2/P2-G5/P1-A6" (designated "L2A6") was chosen for the generation of a primary seed bank (PSB). A production titer of 35 mg/L KappaSelect binding protein (KSBP) was obtained in the cell culture supernatant of shaker flasks. For characterization of KSBP see results part 3.3.1 at page 50.

Separate reports were generated for the plasmid construction (by Dr. Ludger Große-Hovest) and for the generation of a PSB (by Gregor Neumann). The presentation of those reports would exceed the scope of this thesis. The reports are administered by Dr. Ludger Große-Hovest, Head of R&D at Synimmune GmbH.

3.2 Fermentation

Basal cell culture medium CDM4PERMAb and a fed-batch protocol for antibody production was adopted from the fermentation process of 4G8SDIEM, the first product of Synimmune GmbH. In the late-logarithmic growth phase of PSB-clone "L2A6", 10 % (V/V) feed medium Efficient feed B supplemented with 40 mM L-Glutamine was added to cell suspension. 2 and 4 days later, additional 10 % (V/V) Efficient feed B supplemented with 40 mM L-Glutamine was added.
This procedure resulted in a KSBP concentration of 165 mg/L (with 55 % monomer content) in cell culture supernatant produced in shaker flasks. In addition, in a 10 L wave-reactor fermentation a KSBP-titer of 110 mg/L (with 55 % monomer) was achieved. This concentration is sufficient for production of the bispecific antibody as discussed in chapter 4.2 at page 116.

3.3 Development of a purification procedure

3.3.1 Identification and Characterization of product-related impurities

After purification of bispecific antibody NF-CU$_{N297Q}$ from cell culture supernatant with KappaSelect affinity chromatography medium, 4 Peaks appear in an elution profile of size-exclusion chromatography (SEC)-separation on "Hiload™ -16/60-Superdex™ 200 pg"' (see figure 3.1). The proteins of the 4 Peaks were termed KappaSelect binding protein (KSBP).
P1 as the first peak is assumed to be an aggregate. P2 as the largest peak is assumed to be the monomer and P3 and P4 seem to be light chain dimer (LCD) and light chain (LC), respectively (Knittler et al., 1995; Schlatter et al., 2005). In order to verify these assumptions, SDS-PAGE and an isoelectric focusing electrophoresis was performed. In addition, the proteins were further characterized by flow cytometric analysis on cells expressing the antigen of interest. Finally, a T-cell proliferation assay and a potency assay were performed to proof the absence of any biological activity of the product-related impurities P1, P3 and P4.

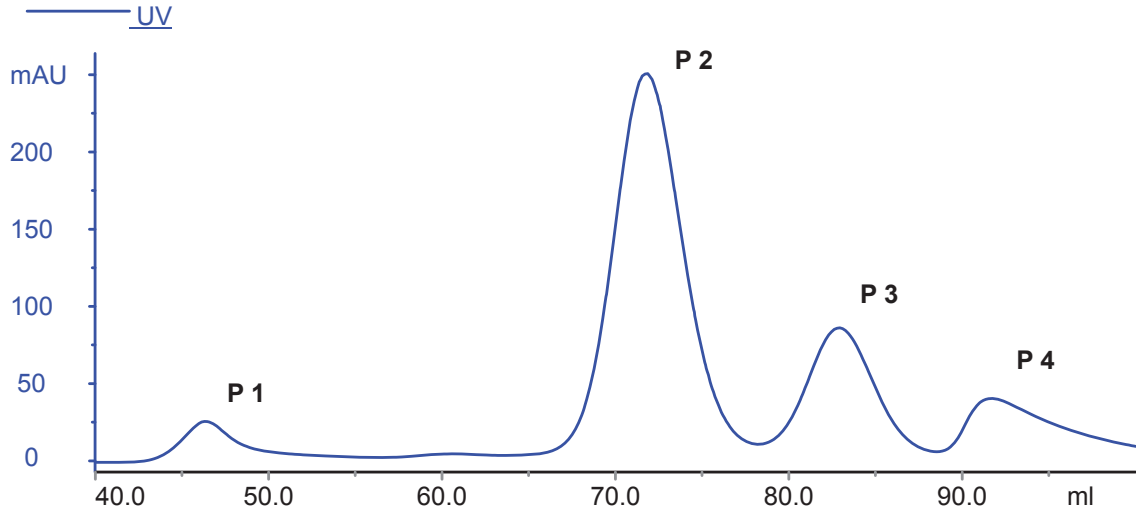


Figure 3.1: **Product-related impurities - Preparative SEC-separation**
ÄKTA Purifier 100
Column: Hiload™ -16/60-Superdex™ 200 pg;
Protein (KSBP): 5 mg in 2 mL;
Buffer: DPBS
Flow rate: 1.20 mL/min
P1: Aggregate; P2: Monomer; P3: LCD; P4: LC

3.3.1.1 SDS-PAGE

The theoretical molecular weight of the monomer, LCD and LC was calculated with the online bioinformatics resource portal "Expasy" (http://web.expasy.org/compute_pi/ (date of request: 01.07.2015)). For amino acid sequence of NF-CU$_{N297Q}$ see chapter 5.2 at page 138. For calculation of the molecular weight (MW) of the

monomer, the heavy and light chain sequences were plotted in series. For calculation of the MW of LCD, the LC sequence was plotted twice. A calculation of the MW of the aggregate is not possible. Theoretical and experimentally determined MW of KSBP are summarized in table 3.1
At the SDS-PAGE gel under unreduced condition (see figure 3.2 A), P1 is hardly visible. This may be due to the fact that this aggregate is too large to migrate into the gel. The apparent molecular weight of P2 is 23 kDa heavier than the calculated weight of the monomer, the MW for P3 and P4 are in the range for LCD and for LC.
At the SDS-PAGE gel under reduced condition (see figure 3.2 B), P1 shows bands at 70 and 30 kDa, which might represent heavy and light antibody chains indicating that the aggregate consists at least partly of aggregated monomer. P2, P3 and P4 exhibit the correct sizes for heavy and light chains respectively. Thus, the reduced samples indicate that P1 is an aggregate, P2 is the monomer, P3 is LCD and P4 is LC. The reason why LC appears to be smaller under unreduced conditions can be explained by the presence of one intramolecular disulphide bond per antibody domain. This results in an incomplete unfolding of the protein and therefore a smaller apparent size. In conclusion, the nature of the product-related impurities has been clearly defined.

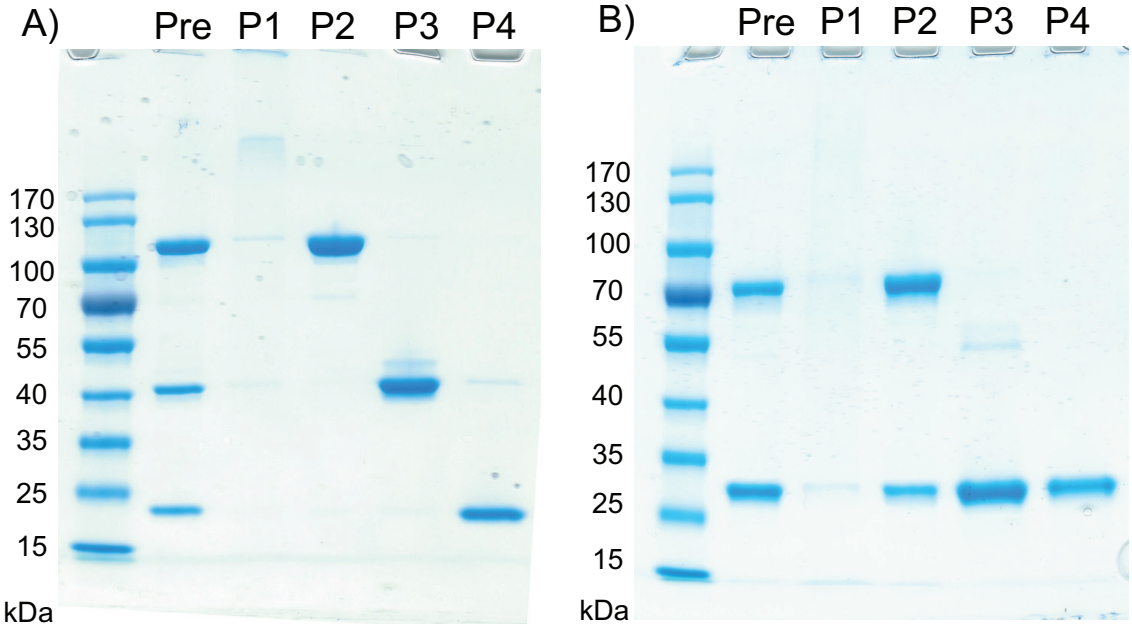


Figure 3.2: **Product-related impurities - SDS-PAGE**
SEC fractions of figure 3.1 at page 50 (P1-4) and purified KSBP (Pre); A = unreduced condition; B = reduced condition; molecular weights of the samples are summarized in table 3.1; marker: "PageRuler™ Prestained Protein Ladder"

3.3.1.2 Isoelectric focusing electrophoresis

Theoretical isoelectric points (IEPs) were calculated with the online bioinformatics resource portal "Expasy" (http://web.expasy.org/compute_pi/ (date of request: 01.07.2015)). For amino acid sequence of NF-CU$_{N297Q}$ see appendix part 5.2 at page

Table 3.1: **Product-related impurities - SDS-PAGE**
Molecular weight of SEC fractions of figure 3.1 at page 50 (P1-4) in kDa; experimental and theoretical data of KappaSelect binding protein (KSBP)

	Unreduced condition		Reduced condition	
Fraction	**Theoretical**	**Experimental**	**Theoretical**	**Experimental**
P1 = Aggregate	N/A	N/A	N/A	~ 70/30
P2 = Monomer	87	~ 110	63.8/23.4	~ 70/30
P3 = LCD	46.7	~ 40	23.4	~ 30
P4 = LC	23.4	~ 20	23.4	~ 30

138. For calculation of the IEP of the monomer, the heavy and light chain sequences were plotted in series. For calculation of the IEP of LCD, the LC sequence was plotted twice.
Experimental IEPs were determined using isoelectric focusing electrophoresis (see figure 3.3). The IEP of the aggregate could not be determined, this may be due to the fact that the aggregate is too large to migrate into the gel. Experimentally determined IEPs for the monomer, LCD and LC accord with the calculated IEPs (see table 3.2).
Those results confirm the nature of the product-related impurities. The knowledge of the IEP of the monomer and the product-related impurities is important for evaluation of purification strategies, especially for ion exchange chromatography (IEX).

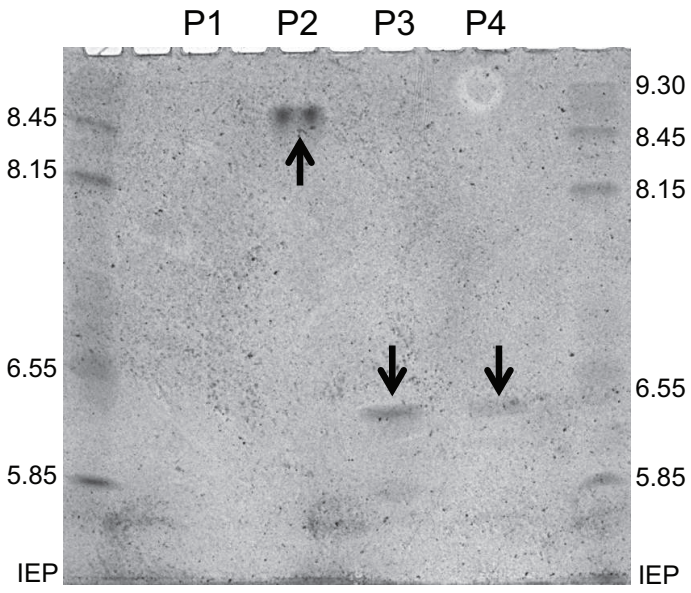


Figure 3.3: **Product-related impurities - Isoelectric focusing electrophoresis - determination of isoelectric points (IEP)**
SEC fractions of figure 3.1 at page 50 (P1-4); IEPs of the samples are summarized in table 3.2; marker: "Isoelectric Focusing Calibration Kit High Range pI (pH 5 - 10.5)"

Table 3.2: **Product-related impurities - Isoelectric focusing electrophoresis - calculated IEP**
IEPs of SEC fractions of figure 3.1 at page 50; experimental and theoretical data of KappaSelect binding protein (KSBP)

Fraction	Theoretical IEP	Experimental IEP
P1 = Aggregate	N/A	N/A
P2 = Monomer	8.5	∼ 8.5
P3 = LCD	6.2	∼ 6.2
P4 = LC	6.2	∼ 6.2

3.3.1.3 Flow cytometric analysis

Flow cytometric analysis of the aggregate, monomer, LCD and LC was performed on NALM-16 cells for the FLT3 specificity and on Jurkat cells for the CD3 specificity.
Figure 3.4 A and B show that only the monomer binds to FLT3 and CD3 at low concentrations (starting at 100 ng/mL). Aggregates, LCD and LC only bind at high concentrations (starting at 10 µg/mL). At very high concentrations, the aggregate exhibits enhanced binding to CD3 and especially to FLT3. LCD binds with a slightly higher affinity to FLT3 and CD3 than LC.

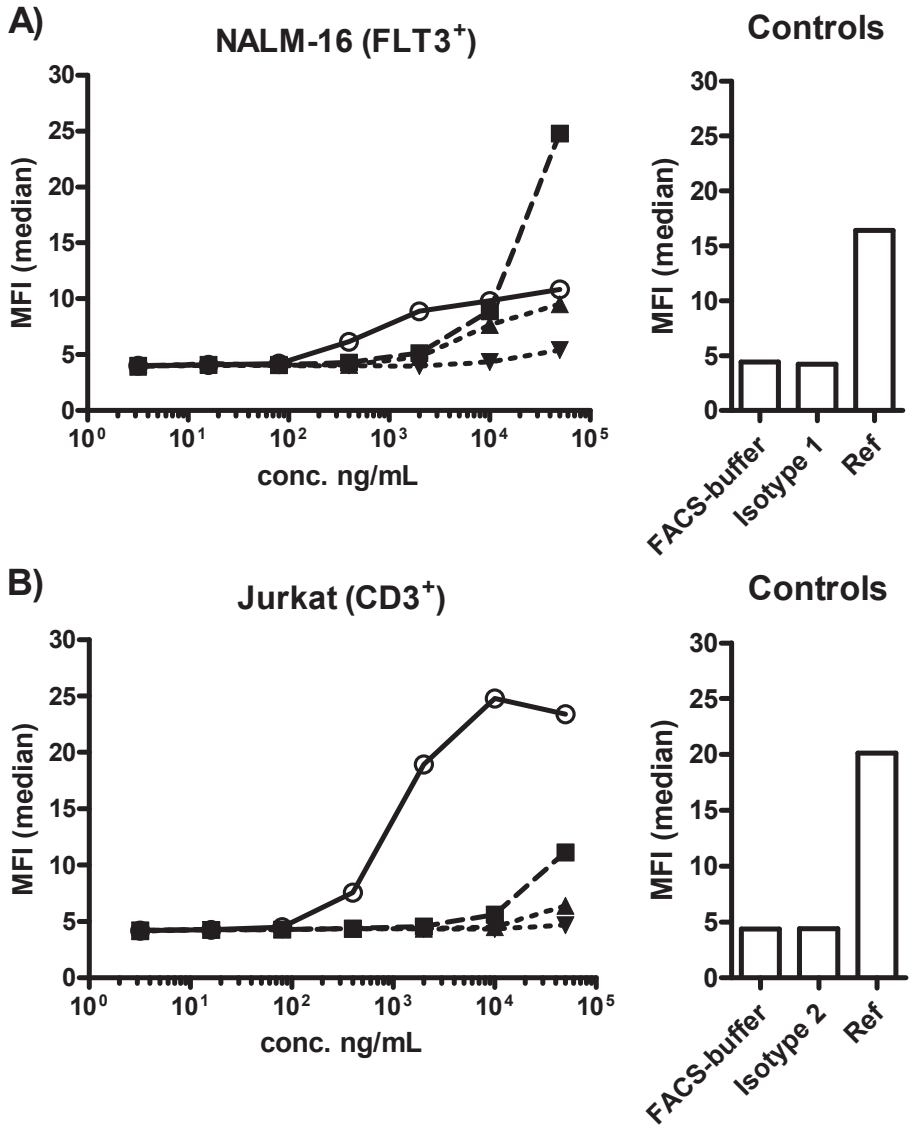


Figure 3.4: **Product-related impurities - Flow cytometric analysis**
Titration of SEC fractions of figure 3.1 at page 50 on **A)** NALM-16 (FLT3$^+$) and **B)** Jurkat (CD3$^+$) cells; Aggregate (■); Monomer (○); LCD (▲); LC (▼); Isotype 1 = NP-CU$_{N297Q}$ (SP2/0) 50 µg/mL; Isotype 2 = NP-C16$_{N297Q}$ (SP2/0) 50 µg/mL; Reference = NF-CU$_{N297Q}$ (SP2/0) 50 µg/mL; Detection: MaH-κL-PE

3.3.1.4 T-cell proliferation assay

The T-cell proliferation assay was performed with the aggregate, monomer, LCD and LC and was divided into two experiments. The first experiment was performed with freshly isolated PBMC of a healthy donor and target cells NALM-16 (see figure 3.5 A). This experiment was performed to see if and to what extent product-related impurities induce proliferation of T-cells in the presence of target cells expressing FLT3. The second experiment was performed with PBMC to see if and to what extent product-related impurities induce proliferation of T-cells in the absence of target cells (see figure 3.5 B). No activation should be detectable in the absence of

target cells.
In the first experiment, it can be seen that only the monomer induces a strong dose-dependent proliferation of PBMC at lower concentrations (starting at 1 ng/mL). Aggregates, LCD and LC only induce proliferation of PBMC at higher concentrations (starting at 100 ng/mL). In the second experiment, again only the monomer induces proliferation of PBMC (starting at 4 ng/mL). Aggregates, LCD and LC only induce proliferation of PBMC at higher concentrations (starting at 400 ng/mL); they exhibits only very weak biological activity.

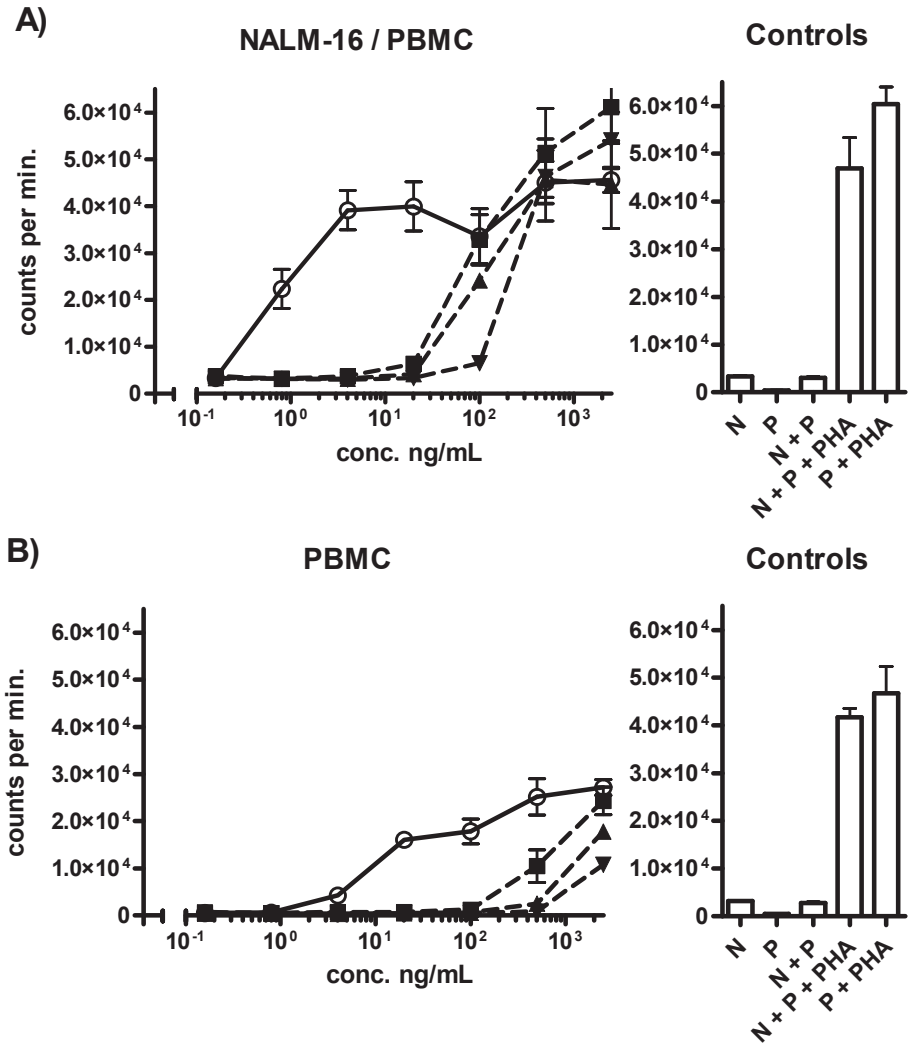


Figure 3.5: **Product-related impurities - T-cell proliferation assay**
Titration of SEC fractions of figure 3.1 at page 50 with 100,000 target cells / 100,000 PBMC per well (**A**) and without target cells / 100,000 PBMC per well (**B**); Aggregate (■); Monomer (○); LCD (▲); LC (▼); N = NALM-16; P = PBMC; PHA = 10 µg/mL phytohemagglutinin

3.3.1.5 Potency Assay (Promega)

The aggregate, monomer, LCD and LC were titrated in a potency assay to determine the potency compared to a reference standard (NF-CU_{N297Q} (SP2/0)). In figure 3.6 A and B it can be seen that the aggregate, LCD and LC are two to three log-values

less potent than the monomer and the reference. EC_{50}-values were calculated and compared to the potency of an appropriate reference (see table 3.3). The aggregate exhibits a EC_{50} of 99.4 ng/mL, which corresponds to a potency of 0.8 % compared to reference. The monomer exhibits almost the same EC_{50} as the reference which corresponds to a relative potency of 90 %. EC_{50} of LCD and LC could not be calculated because no plateau-phase was reached. Thus, the aggregate, LCD and LC exhibit only weak biological activity.

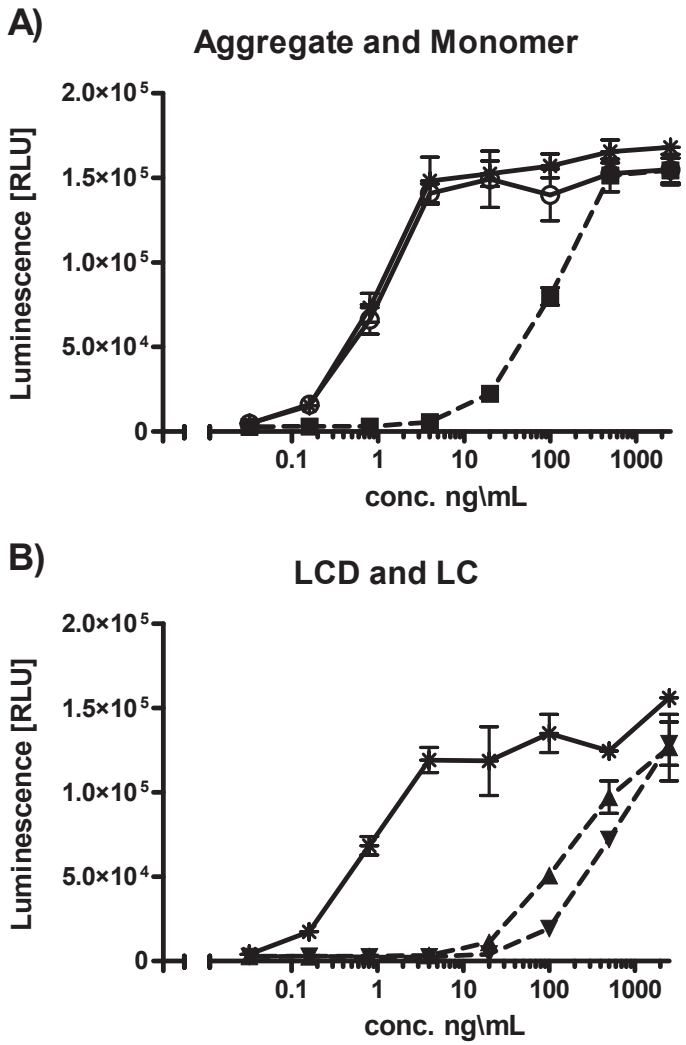


Figure 3.6: **Product-related impurities - Potency assay (Promega)**
Titration of SEC fractions of figure 3.1 at page 50; Aggregate (■); Monomer (○); LCD (▲); LC (▼); Reference (✻) = NF-CU_{N297Q} (SP2/0); E:T = 2:1; E = modif. Jurkat; T = NALM-16

Table 3.3: **Product-related impurities - Potency Assay - EC_{50}-values of KSBP**; Ref 1 corresponds to Aggregate and Monomer experiment, whereas Ref 2 corresponds to LCD and LC experiment

	EC_{50} [ng/mL]	**% of Ref**
Aggregate	99.3	0.8
Monomer	0.9	90
LCD	N/A	N/A
LC	N/A	N/A
Ref 1	0.8	100
Ref 2	0.8	100

3.3.1.6 Summary and Conclusion

The product-related impurities of bispecific antibody NF-CU_{N297Q} can be subdivided into multimers and fragmentary parts of the antibody. Those impurities are purified along with the monomer when using KappaSelect as affinity chromatography medium. The four KappaSelect binding proteins (KSBP) -aggregate, monomer, light chain dimer (LCD) and light chain (LC)- were identified via SDS-PAGE and isoelectric focusing electrophoresis. The product-related impurities exhibit almost no biological activity, whereas the monomer binds towards FLT3 and CD3, activates T-cells in a cell-proliferation assay and exhibits almost the same potency as a suitable reference standard. In addition, the monomer activates PBMC in the absence of target cells to a small extent.
Despite the fact that a contamination with product-related impurities seems to be uncritical due to their minimal biological activity, they should be removed from the monomer for safety reasons and to minimize unintended immunological reactions.

3.3.2 Evaluation of purification strategies

A variety of different purification strategies was evaluated for their suitability of separating the active pharmaceutical ingredient (API) from product- and process-related impurities. Different chromatographic techniques were applied and intensively discribed in the following part:

- Biospecific interaction chromatography - Affinity chromatography (AC) in chapter 3.3.2.1 at page 59 f.
- Anion exchange chromatography (AIEX) in chapter 3.3.2.2 at page 61 ff.
- Cation exchange chromatography (CIEX) in chapter 3.3.2.3 at page 66 ff.
- Hydrophobic interaction chromatography (HIC) in chapter 3.3.2.4 at page 71 ff.
- Multimodal chromatography (MMC) in chapter 3.3.2.5 at page 78 ff.

In order to fulfill regulatory requirements, only chromatographic media with a so-called regulatory support file were used. Those media are certified to be used under Process development- and GMP-conditions.

The performance of affinity media was assessed in pilot experiments. A characterization of binding properties of bispecific antibody NF-CU_{N297Q} towards different affinity media was performed.

For the development of suitable polishing applications, the performance of the four methods AIEX, CIEX, HIC, MMC was evaluated. Hitrap™ columns (V = 1 mL) were used for evaluation of conditions such as pH, conductivity and binding properties on an ÄKTA system. Analysis of the removal of product-related impurities light chain dimer (LCD) and light chain (LC) was performed via SEC (Tosoh) / UHPLC.
The next step was to verify the determined conditions in a so-called **buffer verification model** on Hiscreen™ columns (V = 4.7 mL). At this step, criteria for process realistic flow rates and protein-loading capacities were met (see discussion part 2.2.2.2 at page 37).
Extended analysis of the polishing methods was performed in chapter 3.3.2.6 at page 83 ff. The analysis by size exclusion chromatography was improved by the use of a column with better resolution than the Tosoh column; SEC (Superdex) / UHPLC. With this method, the evaluation of aggregate-, LCD- and LC-content was possible. Further analytical techniques included the evaluation of the monomer yield, SDS-PAGE, flow cytometric analysis and a T-cell proliferation assay. The required effort for performance of the methods was also included in the evaluation.

3.3.2.1 Biospecific interaction chromatography - Affinity chromatography (AC)

A variety of different affinity chromatography media were evaluated for their binding properties towards NF-CU_{N297Q}. Protein A or Protein G did not allow binding at all and other media such as "CaptureSelect IgG-CH_1", which binds to the CH_1-domain of antibodies, were exhibiting very low binding capacities. KappaSelect and Protein L showed very good binding properties. Protein L binds to the framework regions of the variable domain of the light chain. Therefore, an influence on the tertiary structure of the complementary determining regions might be possible. KappaSelect binds towards the constant domain of the light chain. Thus, KappaSelect was chosen for further investigation.
An established method for determination of the binding capacity of a certain protein to an affinity medium is the determination of the dynamic binding capacity at 10 % breakthrough ($DBC_{10\,\%}$). $DBC_{10\,\%}$ is determined with previously purified protein in DPBS with the same residence time as for the performance of the production process scale. A linear flow rate of 400 cm/h is recommended for affinity chromatography process applications with KappaSelect. For a column with a volume of 1 L and a bed height of 20 cm, this linear flow rate results in a residence time of 3 min. (see equations 2.2 and 2.1 at page 38). Thus, this residence time was applied on a Hitrap™ column; a CV of 1 mL and a bed height of 2.4 cm results in a volumetric flow rate of 0.33 mL/min. Previously purified KSBP containing ∼ 6 % aggregates, ∼ 55 % monomer, ∼ 22 % LCD and ∼ 17 %LC was applied onto the column (see figure 3.7).
Marker M1 marks the condition where KSBP is applied onto the column. The OD_{280} at the marker M1 is 498 mAU, the OD_{280} at marker M2 is 49.8 mAU (10 % of marker M1). The volume between the two markers M1 and M2 multiplied with the feed concentration represents the amount of KSBP where a 10 % breakthrough through the column can be observed. This amount is termed $DBC_{10\,\%}$. The volume between the two markers is 11.8 mL, the feed concentration was 1.57 mg/mL. This results in a $DBC_{10\,\%}$ of 18.5 mg KSBP per mL chromatography medium.

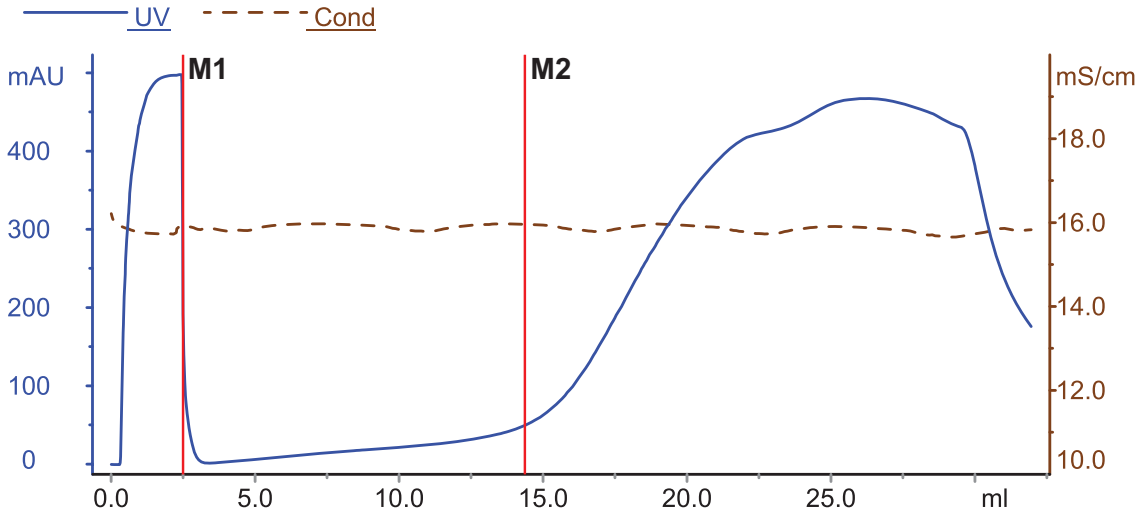


Figure 3.7: **Affinity Chromatography - $DBC_{10\,\%}$ of KappaSelect binding protein (KSBP) on KappaSelect; $DBC_{10\,\%}$ = 18.5 mg/mL resin**
ÄKTA Purifier 100
Column: HiTrap™ -1 mL-KappaSelect;
Protein (KSBP) conc.: 1.57 mg/mL;
Buffer: DPBS
Flow rate: 0.33 mL/min
Marker M1: OD_{280} of bypass mode = 498 mAU; V = 2.4 mL
Marker M2: OD_{280} at $DBC_{10\,\%}$ = 49.8 mAU; V = 14.2 mL

3.3.2.2 Anion Exchange Chromatography (AIEX)

A modern and commonly applied chromatography medium from GE Healthcare was used for anion exchange chromatography. Capto Q is a strong anion exchange resin based on quaternary ammonia structure (see figure 3.8).

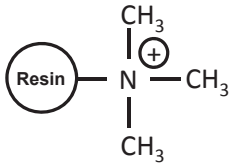


Figure 3.8: **Capto Q - ligand**

In order to remove as many HCPs, HCDNAs, Viruses and Endotoxins from the active pharmaceutical ingredient (API) as possible, a slightly lower pH than the isoelectric point (IEP) of the API should be applied. With this condition, the mentioned impurities exhibit a negative net charge and bind to the resin, whereas the monomer flows through the column without binding; the so-called flow-through mode (FT mode). However, as a first step, a bind-elute mode with pH-gradient elution was performed for assessment of a suitable pH-value for the FT mode. The conductivity of the applied buffer should be as low as possible to increase the binding capacity of the resin. However, conductivity was not lowered below 2 mS/cm to avoid protein precipitation.

Bind-elute mode with pH-gradient Elution:
(see figure 3.9 at page 63)
The IEP of the monomeric antibody is 8.5, the IEP of LCD/LC is 6.2. Thus, a slightly higher pH than the IEP of the monomer was used to bind the monomer and LCD/LC. Binding-pH was 9.3. Subsequently, pH was decreased to 7.2 with a gradient of 20 column volumes (CV). A suitable buffer ion for this pH-value is Tris due to its pKa-value of 8.2. In addition, it is a cationic buffer ion and therefore does not interfere with the AIEX-matrix.
The first peak of figure 3.9 A marks the monomer, the second peak marks LCD and LC (verified via SEC Tosoh in figure 3.9 B). pH and conductivity right in between the two peaks were determined (marker M) in order to apply this condition as starting condition for the next experiment.
Condition: 50 mM Tris pH 8.00, conductivity 3 mS/cm

Flow-through mode:
(see figure 3.10 at page 64)
With the selected conditions (see above), the monomer should flow through the column without binding and LCD/LC should bind. In order to verify the binding of LCD/LC to the resin, buffer A containing 1 M NaCl was used to elute LCD/LC. This elution step will not be performed in process scale.
In contrast to theoretical assumptions, almost the whole KSBP passed through in flow-through mode (FT mode) (Peak 1). Peak 2 consists of LCD and LC as expected. Despite the fact that this method seems to be not suitable for removal of

LCD and LC on a Hitrap™ column, is has to be taken into account that separation properties are also dependent on flow rate and protein concentration. The separation property can differ enormously under process-realistic flow rate and protein load on a Hiscreen™ column. Due to the fact that the determined conditions are very favorable for removal of process-related impurities (relatively high pH and low conductivity), this method was applied in the buffer verification model.

Buffer verification model[1]:
(see figure 3.11 at page 65)
For the buffer verification model of AIEX, 11 mL KappaSelect eluate in 0.1 M Glycine pH 2.5 containing the required 70.5 mg KappaSelect binding protein (KSBP) was used. This solution was shifted to the required conditions of pH 8.00 and conductivity of 3 mS/cm using 5 mL 150 mM Tris pH 9.60 and 4 mL water for injection. Thus, 20 mL pH- and conductivity-adapted solution containing 70.5 mg of KSBP was applied onto a Hiscreen™ column with a flow rate of 0.59 mL/min.
The first peak contains almost pure monomer, whereas the second and third peak consist only of LCD and LC. The second peak probably occurs because of a change of the buffer properties. Note that the buffer at Peak 1 consists of Glycine (which does not buffer pH at the pH-value of 8), the monomer and the buffer ion Tris. Buffer conditions at Peak 2 exhibits the same conductivity, but the buffer consists only of Tris as buffer ion and LCD/LC. Thus, the buffer at Peak 2 has a higher buffer capacity than buffer at Peak 1. Those little differences in buffer composition probably lead to an elution of LCD/LC. In addition, due to the fact that a superloop was used for sample application, pressure increases up to 0.5 bar after application of the protein solution and flushing of the superloop. Thus, this may lead to a premature elution property of LCD/LC as well.
Regardless of these considerations, the procedure is capable of clearly separating the monomer from the contaminants. In addition, the premature elution of LCD/LC does not matter, because only Peak 1 is fractionated, Peak 2 and 3 will not be processed further during the production process. The third peak occurs because of reduced binding capacity resulting from increased salt concentration and was only applied to verify the binding of LCD/LC to the resin. This step will not be performed in the production process.

[1]For explanation of flow rate and amount of KSBP see part 2.2.2.2 at page 37

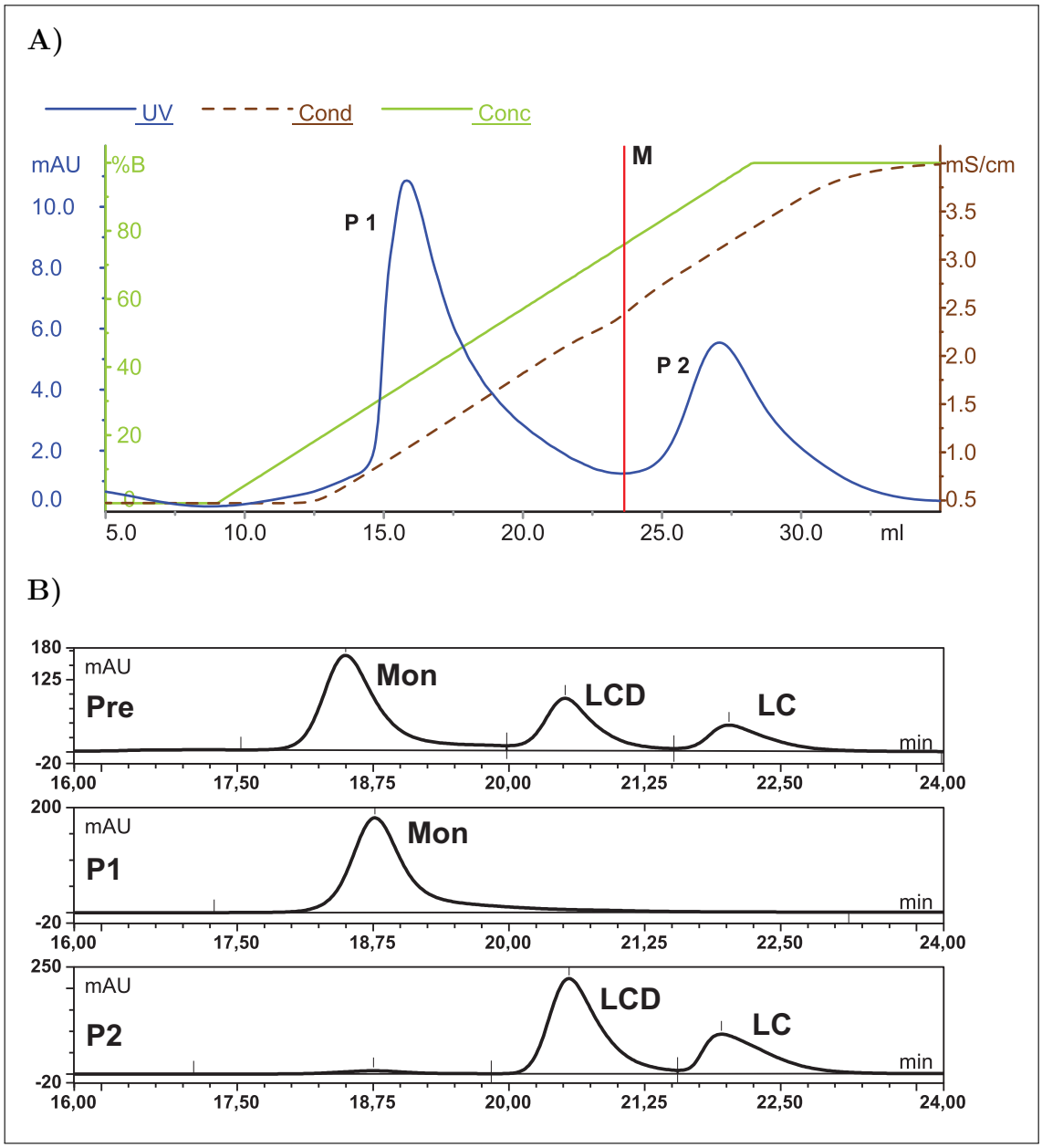


Figure 3.9: **AIEX - Evaluation of buffer conditions - Bind-elute mode with pH-gradient Elution**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Capto Q; Protein amount: 1 mg;
Buffer A: 50 mM Tris pH 9.3; Buffer B: 50 mM Tris pH 7.2;
Gradient: 20 CV; Flow rate: 1 mL/min
Marker M: pH 8.00 Cond. 3.0 mS/cm
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

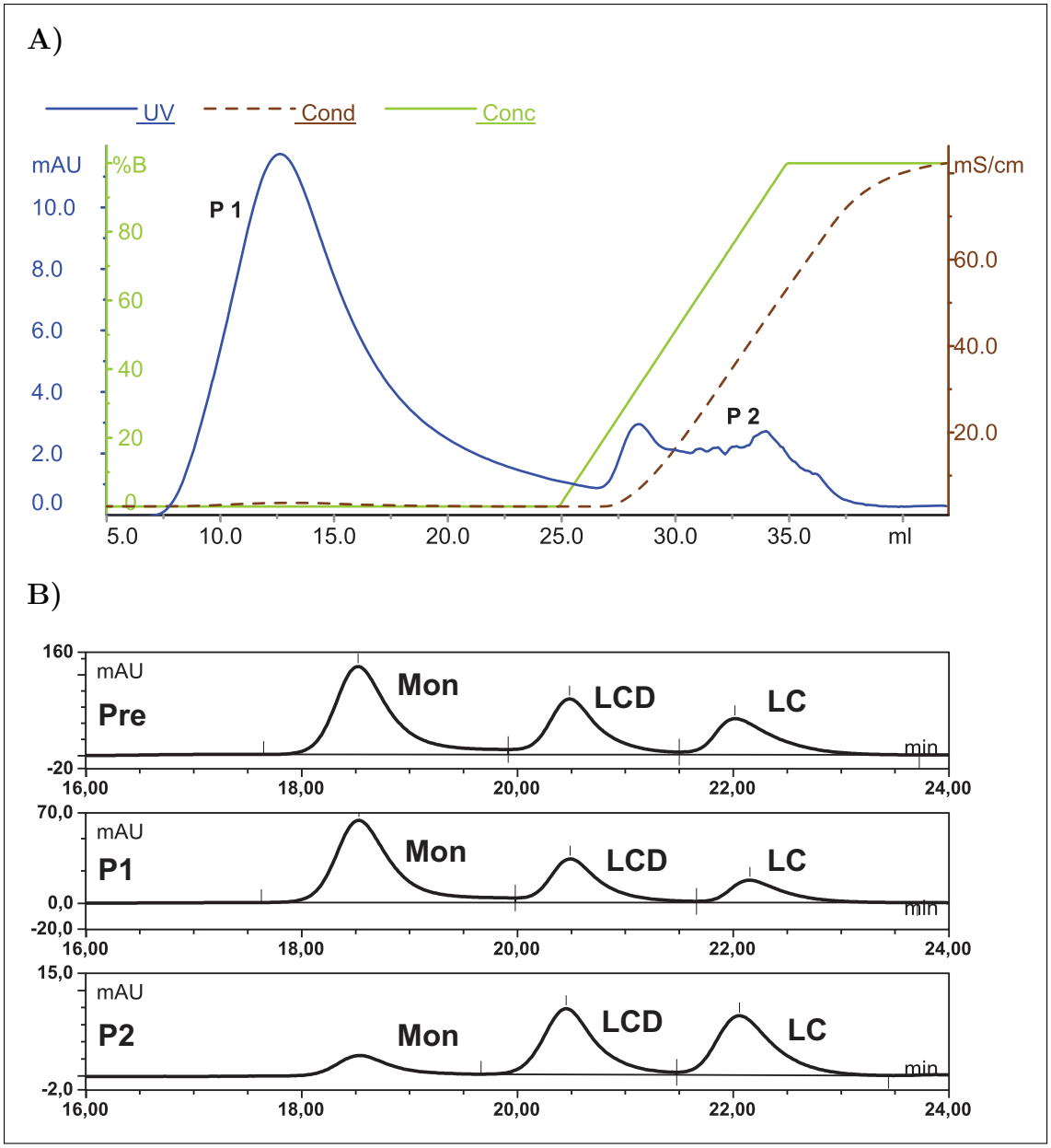


Figure 3.10: **AIEX - Evaluation of buffer conditions - flow-through mode and salt gradient**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Capto Q; Protein amount: 1 mg;
Buffer A: 50 mM Tris pH 8.0 Cond. 3.0 mS/cm; Buffer B: 50 mM Tris pH 8.0 Cond. 80 mS/cm ;
Gradient: 20 CV; Flow rate: 1 mL/min
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

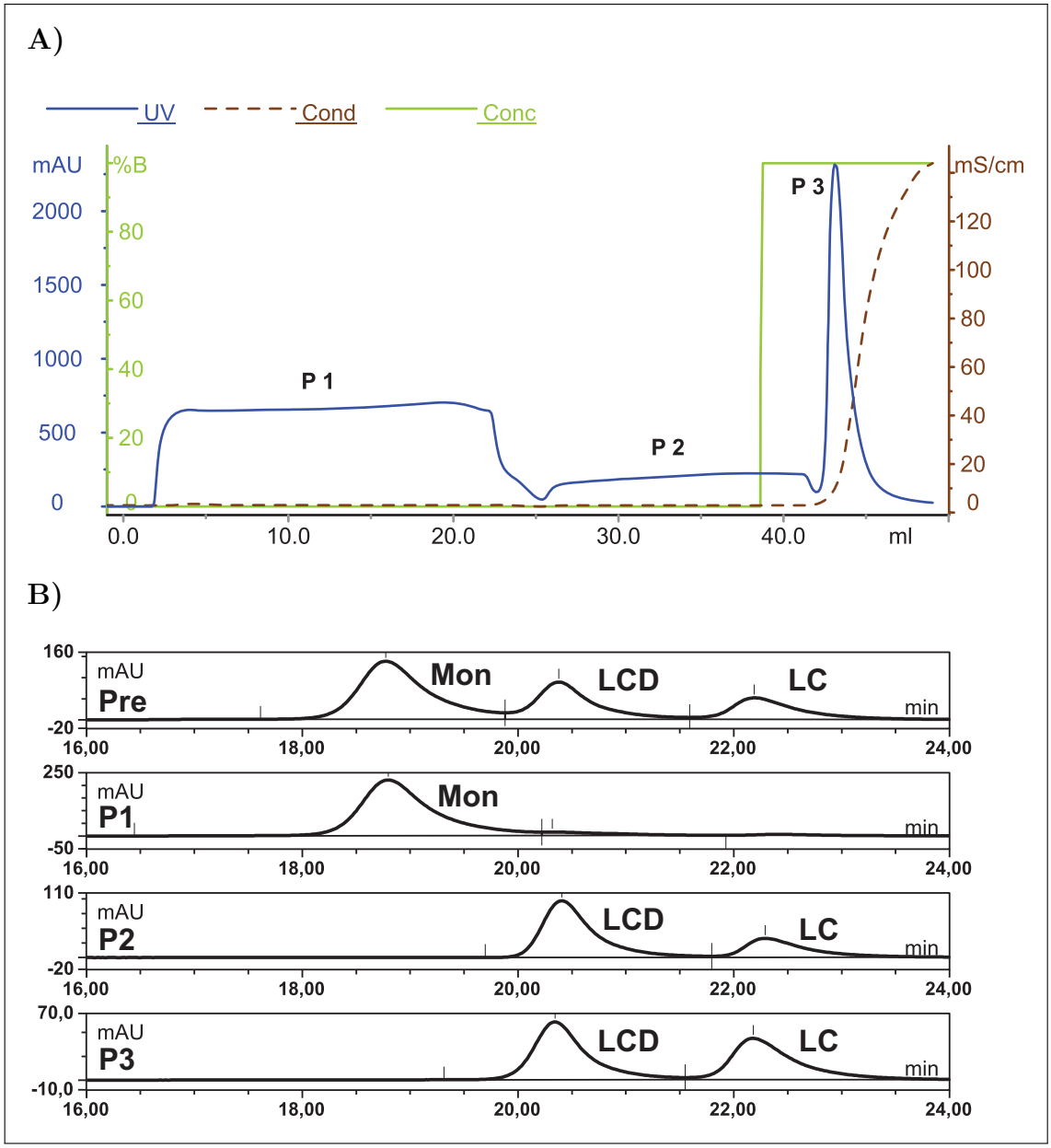


Figure 3.11: **AIEX - Buffer verification model**
A: ÄKTA Purifier 100
Column: Hiscreen™ -4.7 mL-Capto Q;
Protein amount: 70.5 mg in 20 mL;
Buffer A: 50 mM Tris pH 8.0 Cond. 3.0 mS/cm; Buffer B: 50 mM Tris pH 8.0 Cond. 80 mS/cm ;
Gradient: -; Flow rate: 0.59 mL/min
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

3.3.2.3 Cation exchange chromatography (CIEX)

A modern and commonly applied chromatography medium from GE Healthcare was used for cation exchange chromatography. Capto SP ImpRes is a strong cation exchange resin based on sulfopropyl structure (see figure 3.12).

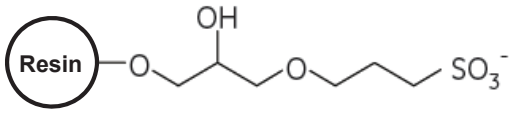


Figure 3.12: **Capto SP ImpRes - ligand**

As in AIEX, in order to remove as many HCPs, HCDNAs, Viruses and Endotoxins from the API as possible, a pH slightly below the IEP of the API should be applied. With this condition, the mentioned impurities exhibit an anionic net charge and do not bind to the resin, whereas the antibody binds to the resin; the so-called bind-elute mode (BE mode). For conductivity, the same rules as for AIEX can be applied.

Bind-elute mode with pH-gradient Elution:
(see figure 3.13 at page 68)
The IEP of the monomeric antibody is 8.5, the IEP of LCD/LC is 6.2. Thus, a slightly lower pH than the IEP of LCD/LC was used to bind the monomer and LCD/LC. Binding-pH was 6.22. Subsequently, pH-value was increased to 8.5 with a gradient of 20 column volumes (CV). A suitable buffer is Phosphate due to its pKa-value of 7.2. In addition, it is an anionic buffer ion and therefore does not interfere with the CIEX-matrix.
LCD/LC flow through the column. pH and conductivity were determined immediately before Peak 2 (see marker M), which represents the monomer. The determined condition was applied as starting condition for the next experiment.
Condition: pH 7.3, conductivity 3 mS/cm

Bind-elute mode with salt-gradient Elution:
(see figure 3.14 at page 69)
With the selected conditions (see above), LCD/LC flow through the column while the monomer binds to the resin. For elution of the monomer, an increase of conductivity is more favorable than an increase of pH because it is less stressful for the monomer and easier to control in process scale.
After binding of the monomer to the resin with buffer A, conductivity was slowly increased by a gradient of 20 CV to an elution buffer containing buffer A and 1 M NaCl. The following suitable elution condition was determined (marker M):
Condition: 20 mM Phosphate pH 7.3, conductivity 15 mS/cm (adjusted with NaCl)

Buffer verification model[1]:
(see figure 3.15 at page 70)
As in the AIEX-experiment, KappaSelect eluate in 0.1 M Glycine pH 2.5 was used as starting material. 11 mL of this eluate contained the required 70.5 mg KappaSelect

[1]For explanation of flow rate and amount of KSBP see part 2.2.2.2 at page 37

binding protein (KSBP).
This solution was shifted to the desired conditions of pH 7.30 and conductivity of 3 mS/cm using 7 mL 150 mM HEPES pH 8.0 and 12 mL water for injection. Thus, 30 mL pH- and conductivity-adapted solution containing 70.5 mg KSBP was applied onto a Hiscreen™ column with a flow rate of 0.59 mL/min.
The verification of buffer conditions worked out well. Peak 1 represents LCD/LC, Peak 2 is the monomer. Peak 3 is not verifiable via SEC.

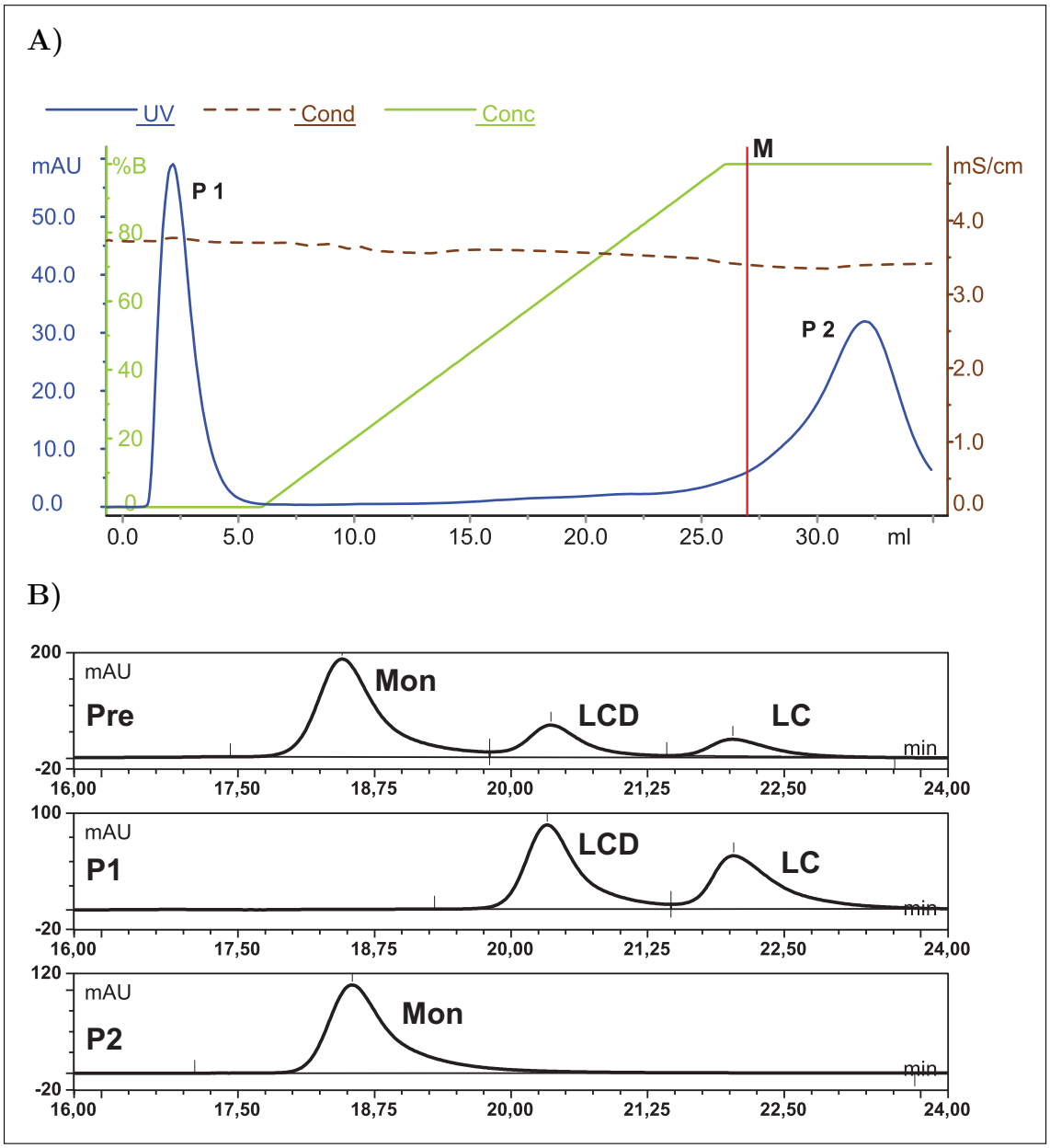


Figure 3.13: **CIEX - Evaluation of buffer conditions - Bind-elute mode with pH-gradient Elution**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Capto SP ImpRes; Protein amount: 1 mg;
Buffer A: 20 mM Phosphate pH 6.22;
Buffer B: 20 mM Phosphate pH 8.5;
Gradient: 20 CV; Flow rate: 1 mL/min
Marker M: pH 7.4 Cond. 3.5 mS/cm
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

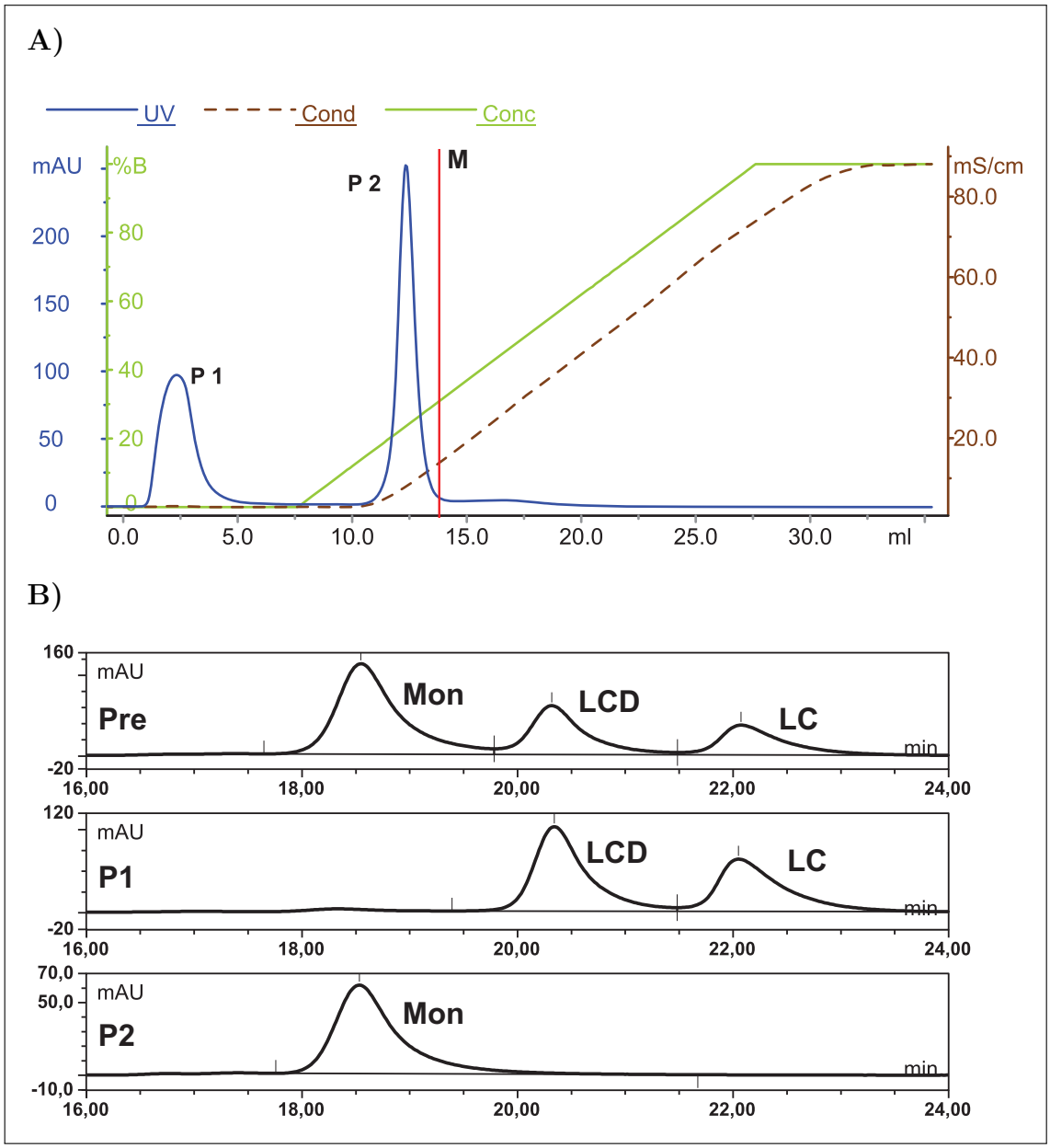


Figure 3.14: **CIEX - Evaluation of buffer conditions - Bind-elute mode with salt-gradient Elution**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Capto SP ImpRes; Protein amount: 1 mg;
Buffer A: 20 mM Phosphate pH 7.3 Cond. 3.0 mS/cm;
Buffer B: 20 mM Phosphate pH 7.3 Cond. 80 mS/cm ;
Gradient: 20 CV; Flow rate: 1 mL/min
Marker M: pH 7.3 Cond. 15 mS/cm
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

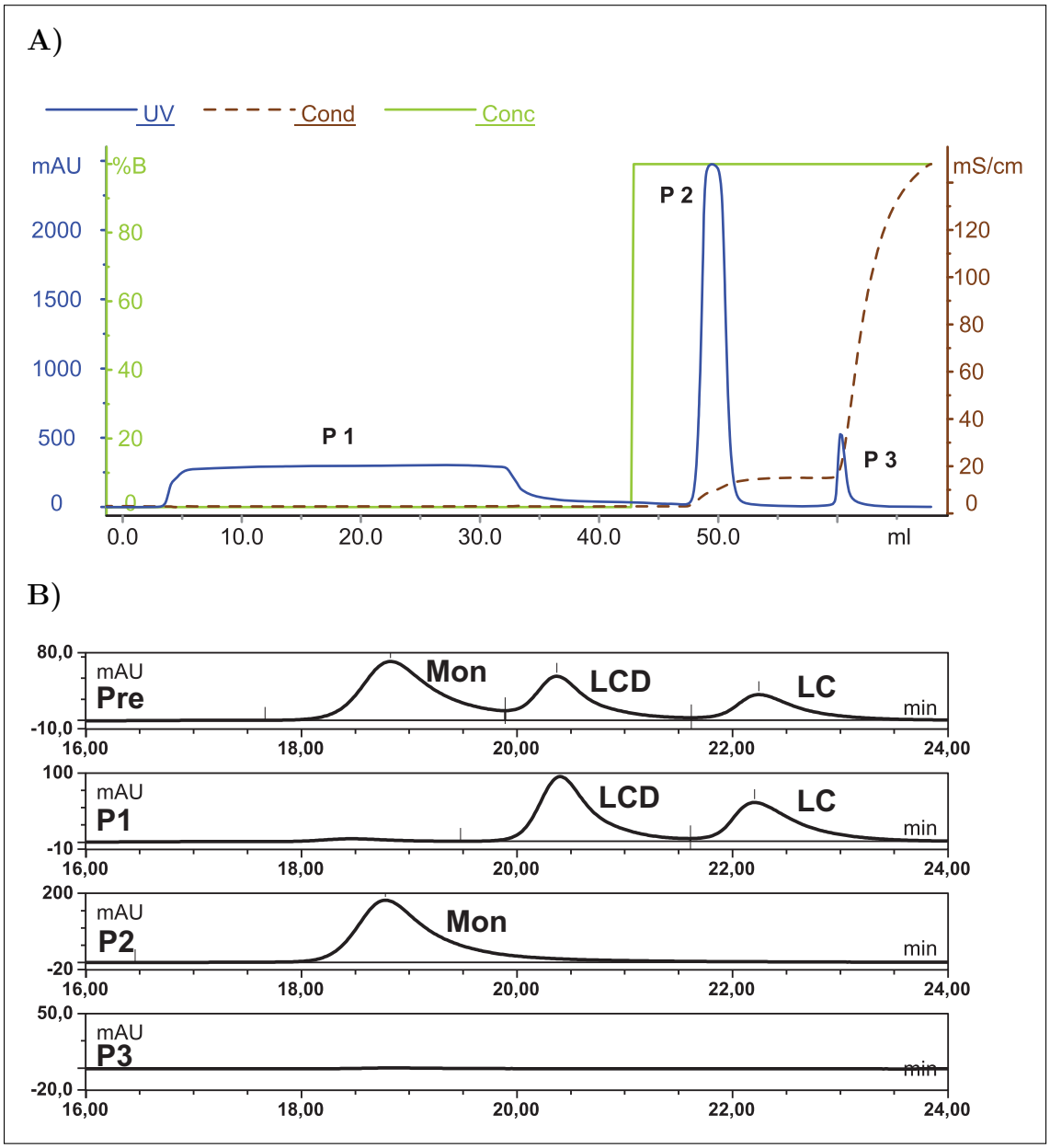


Figure 3.15: **CIEX - Buffer verification model**
A: ÄKTA Purifier 100
Column: Hiscreen™ -4.7 mL-Capto SP ImpRes;
Protein amount: 70.5 mg in 30 mL;
Buffer A: 20 mM Phosphate pH 7.30 Cond. 3 mS/cm;
Buffer B: 20 mM Phosphate pH 7.30 Cond. 15 mS/cm;
Buffer C: 20 mM Phosphate pH 7.30 Cond. 150 mS/cm ;
Gradient: -; Flow rate: 0.59 mL/min
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

3.3.2.4 Hydrophobic interaction chromatography (HIC)

A prediction of suitability of a certain hydrophobic chromatography medium for a distinct application is not possible. Thus, a variety of different chromatography media was evaluated for their suitability to separate the monomer from product-related impurities. Figure 3.16 arranges different media according to their relative hydrophobicity and average resolution. The following media were applied: "Butyl Sepharose 4 fast flow", "Butyl Sepharose High performance", "Butyl-S Sepharose 6 fast flow", "Octyl Sepharose 4 fast flow", "Phenyl Sepharose 6 fast flow (high sub)", "Phenyl Sepharose 6 fast flow (low sub)" and "Phenyl Sepharose high performance".

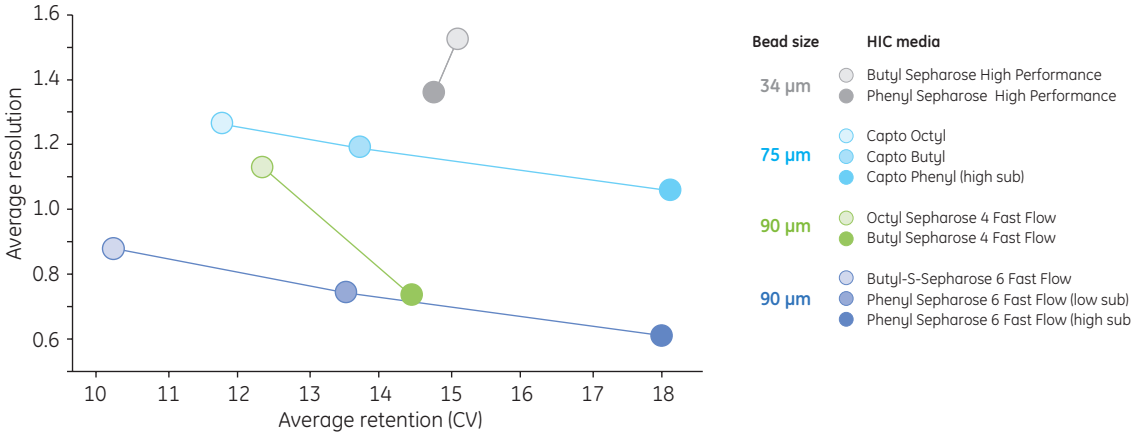


Figure 3.16: **HIC ligands and media**
Resolving power and hydrophobicity of various HIC media based on elution studies of the six model proteins: ovalbumin, α-chymotrypsinogen, ribonuclease, lactoferrin, lysozym, α-amylase.
(© GE Healthcare)

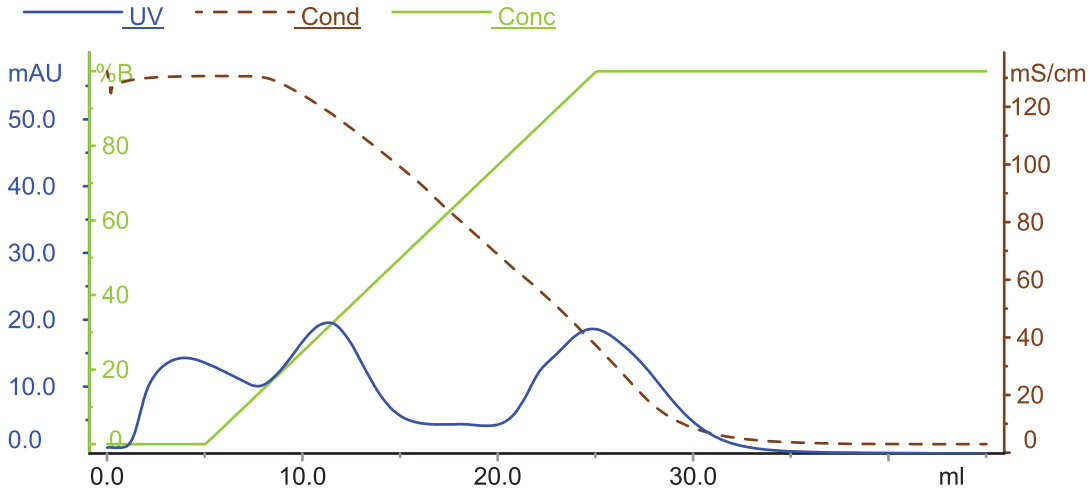
Bind-elute mode with salt-gradient Elution:
(see figure 3.17 at page 73-75)
On HIC media, proteins bind at high-salt conditions and elute at low-salt conditions. Hydrophobic proteins bind tighter than hydrophilic proteins. Thus, KSBP were bound by application of high salt buffer A. Conductivity was decreased by application a gradient (20 CV) to low salt buffer B resulting in an individual elution profile. Peaks were not assessed in SEC-analysis.
For the column with the best resolution, Phenyl Sepharose High Performance (Phenyl HP), the conductivity between the two peaks was determined (marker M) and used as starting condition for an experiment in flow-through mode.
Condition: 50 mM Tris pH 8.00 conductivity 18.5 mS/cm.

Flow-through mode:
(see figure 3.18 at page 76)
With the selected conditions (see above), the monomer flows through the column while LCD/LC bind to the resin. The elution with decreased salt concentration at the end of the experiment was applied to verify the binding of LCD/LC to the resin, this elution step will not be performed in the production process.

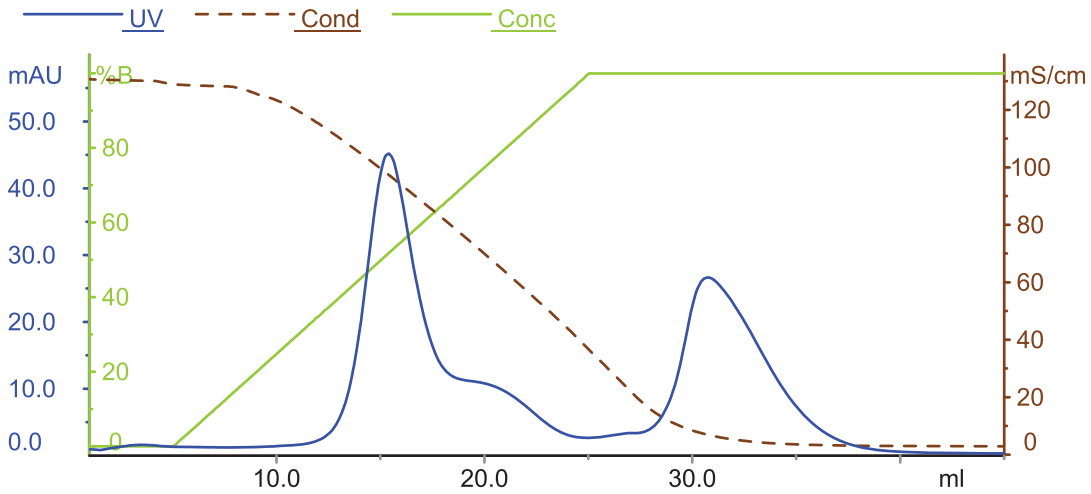
Buffer verification model[1]:
(see figure 3.19 at page 77)
As in the AIEX-experiment, KappaSelect eluate in 0.1 M Glycine pH 2.5 was applied as starting material. 11 mL of this eluate contained the required 70.5 mg KappaSelect binding protein (KSBP). This solution was shifted to the required conditions of pH 8.00 and conductivity of 18.5 mS/cm using 4.5 mL 150 mM Tris pH 9.60 and 1.5 mL 1 M $(NH_4)_2SO_4$. Thus, 17 mL pH- and conductivity adapted solution containing 70.5 mg KSBP was applied onto a Hiscreen™ column with a flow rate of 0.47 mL/min.
The first peak represents almost pure monomer, whereas the second and third peak consist of only LCD and LC. The second peak probably occurs because of a change of the buffer properties similar to the AIEX-experiment. In addition, the same pressure condition was occurring. This might be the reason why the larger molecule LCD leaches (Peak 2) and the smaller molecule LC still sticks to the column. Those complex interactions were avoided in further experiments by using a sample pump instead of a superloop for sample application.
Nevertheless, these interactions do not impact the process itself. Only Peak 1 is fractionated, Peaks 2 and 3 will not be processed further in production process. The third peak occurs because of reduced binding capacity resulting from decreased salt concentration and was only applied to verify the binding of LCD/LC to the resin. This elution step will not be performed in production process.

[1]For explanation of flow rate and amount of KSBP see part 2.2.2.2 at page 37

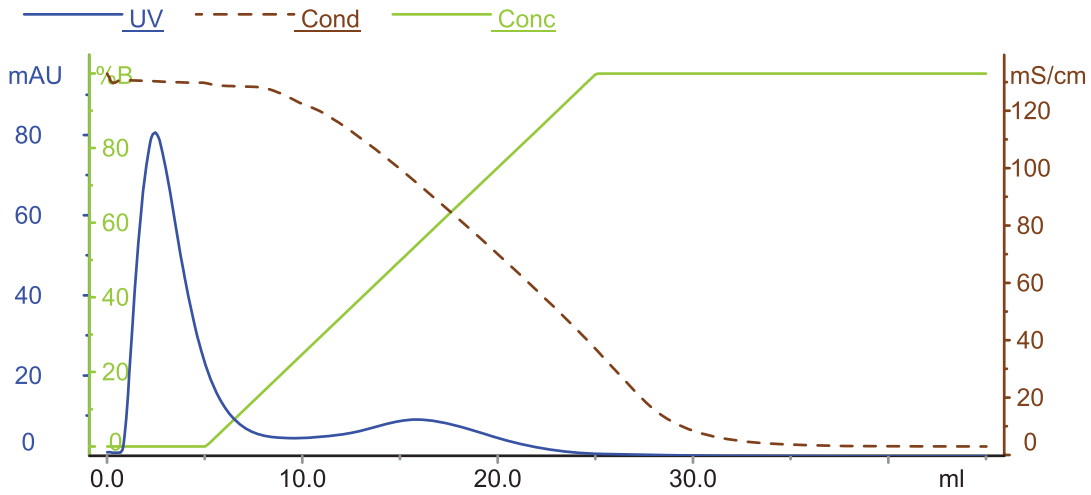
A) HiTrap™ -1 mL-Butyl Sepharose 4 Fast Flow



B) HiTrap™ -1 mL-Butyl Sepharose High Performance

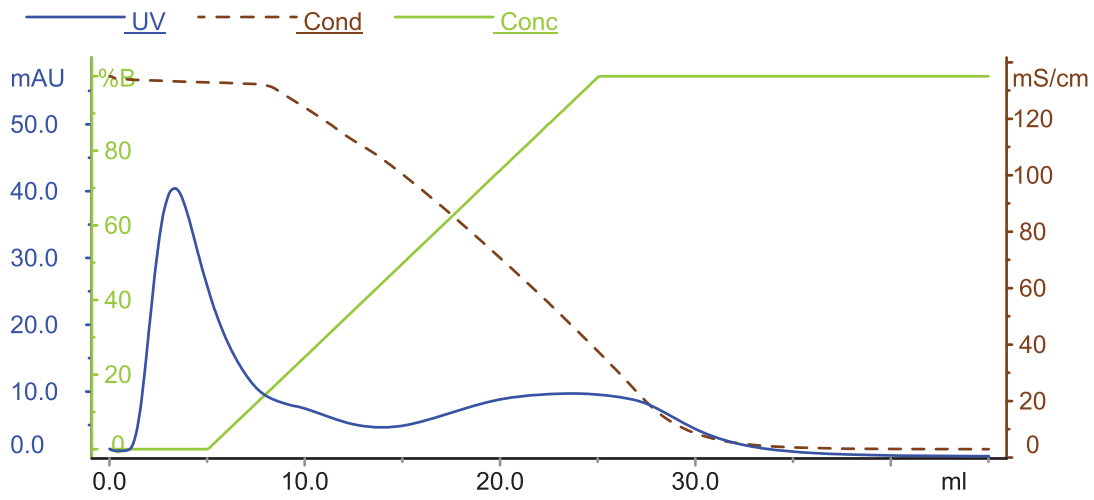


C) HiTrap™ -1 mL-Butyl-S Sepharose 6 Fast Flow

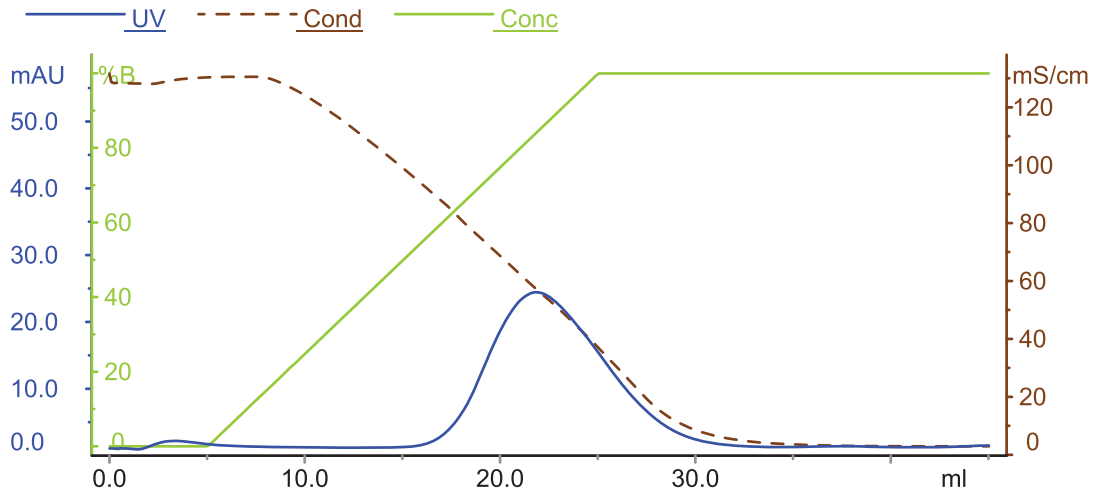


HIC - Media screening - Bind-elute mode with salt-gradient Elution 1/3

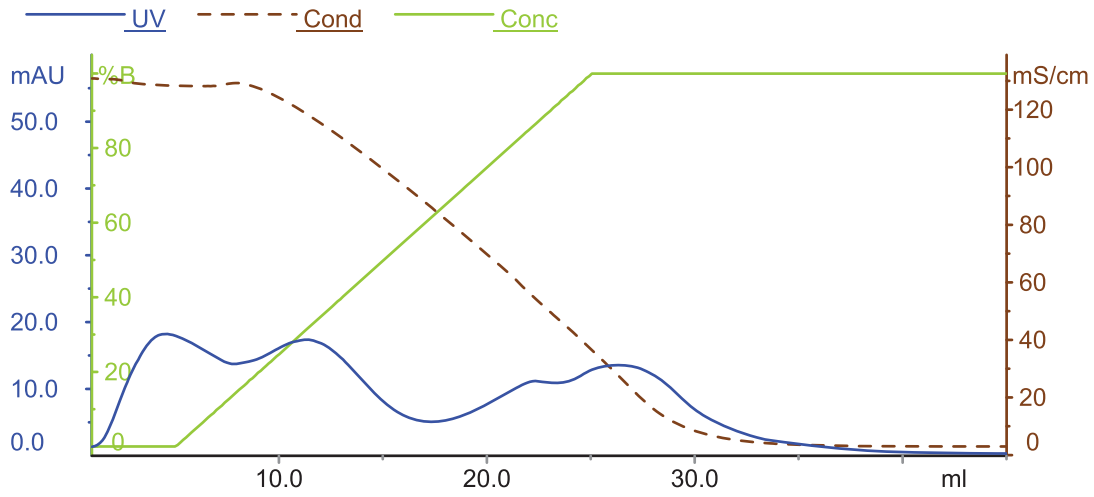
D) HiTrap™ -1 mL-Octyl Sepharose 4 Fast Flow



E) HiTrap™ -1 mL-Phenyl Sepharose 6 Fast Flow (high sub)



F) HiTrap™ -1 mL-Phenyl Sepharose 6 Fast Flow (low sub)



HIC - Media screening - Bind-elute mode with salt-gradient Elution 2/3

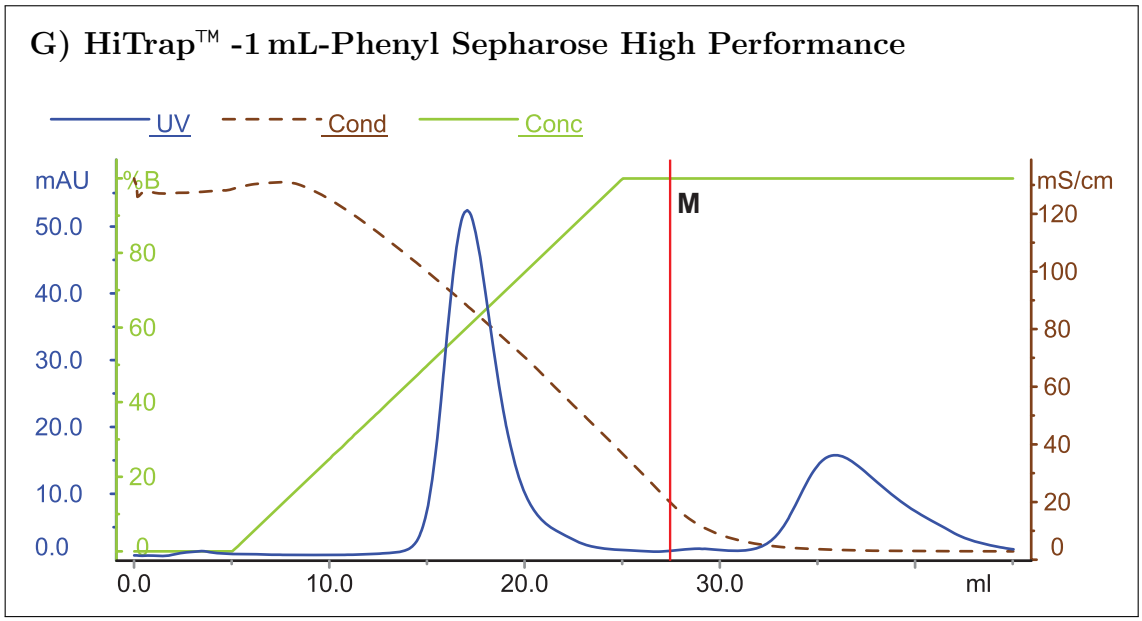


Figure 3.17: **HIC - Media screening - Bind-elute mode with salt-gradient Elution 3/3 ÄKTA Purifier 100**
Column: see A) to G);
Protein amount: 1 mg;
Buffer A: 1 M Ammonium sulfate/ 50 mM Tris pH 8.00 Cond. 130 mS/cm;
Buffer B: 50 mM Tris pH 8.00 Cond. 3.0 mS/cm ;
Gradient: 20 CV; Flow rate: 1 mL/min
Marker M: pH 8.00 Cond. 18.5 mS/cm

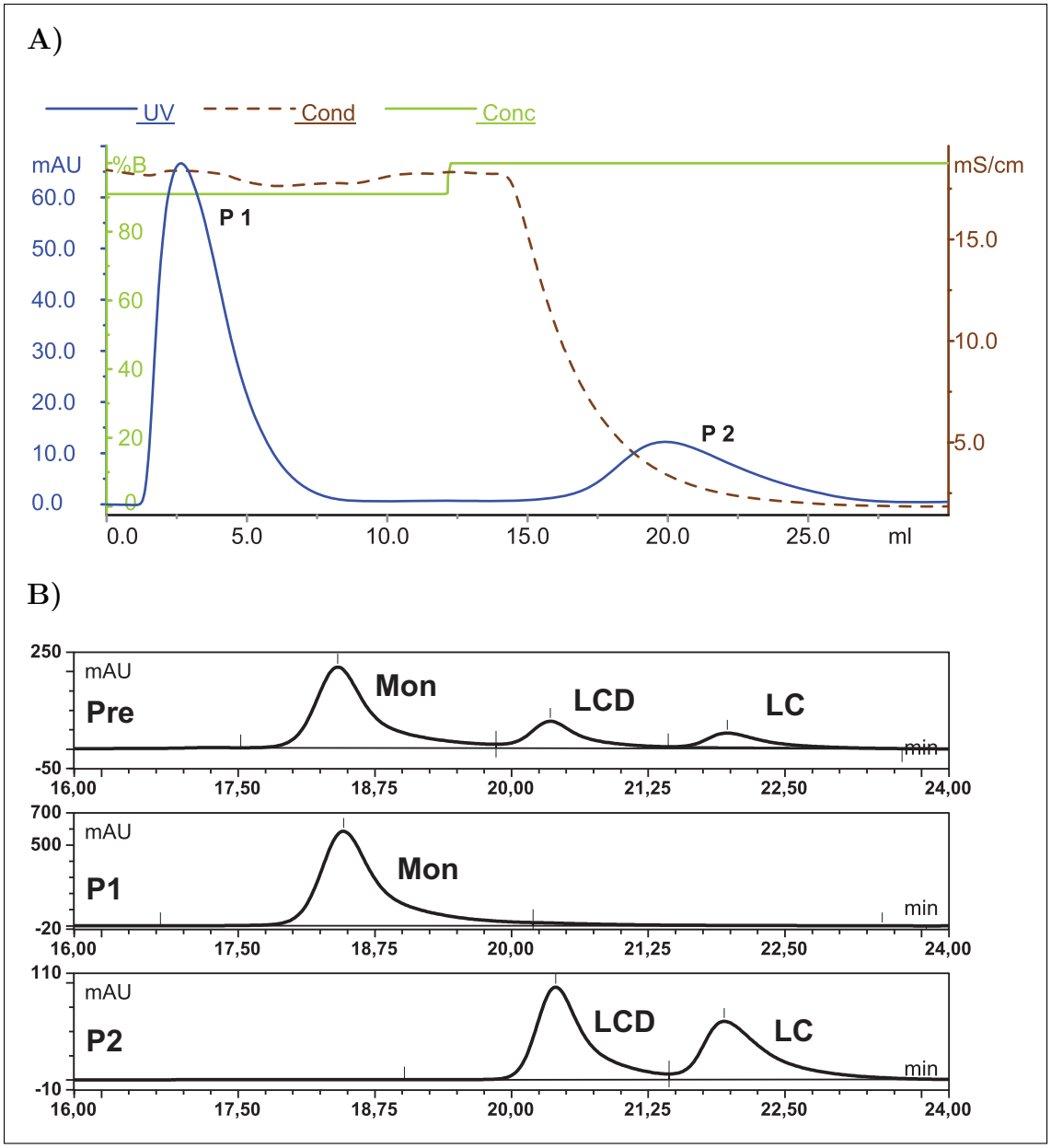


Figure 3.18: **HIC - Evaluation of buffer conditions - Flow-through mode**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Phenyl Sepharose High Performance; Protein amount: 1 mg;
Buffer A: 50 mM Tris pH 8.00 Cond. 18.5 mS/cm ;
Buffer B: 50 mM Tris pH 8.00 Cond. 3.0 mS/cm ;
Gradient: 20 CV; Flow rate: 1 mL/min
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7,8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

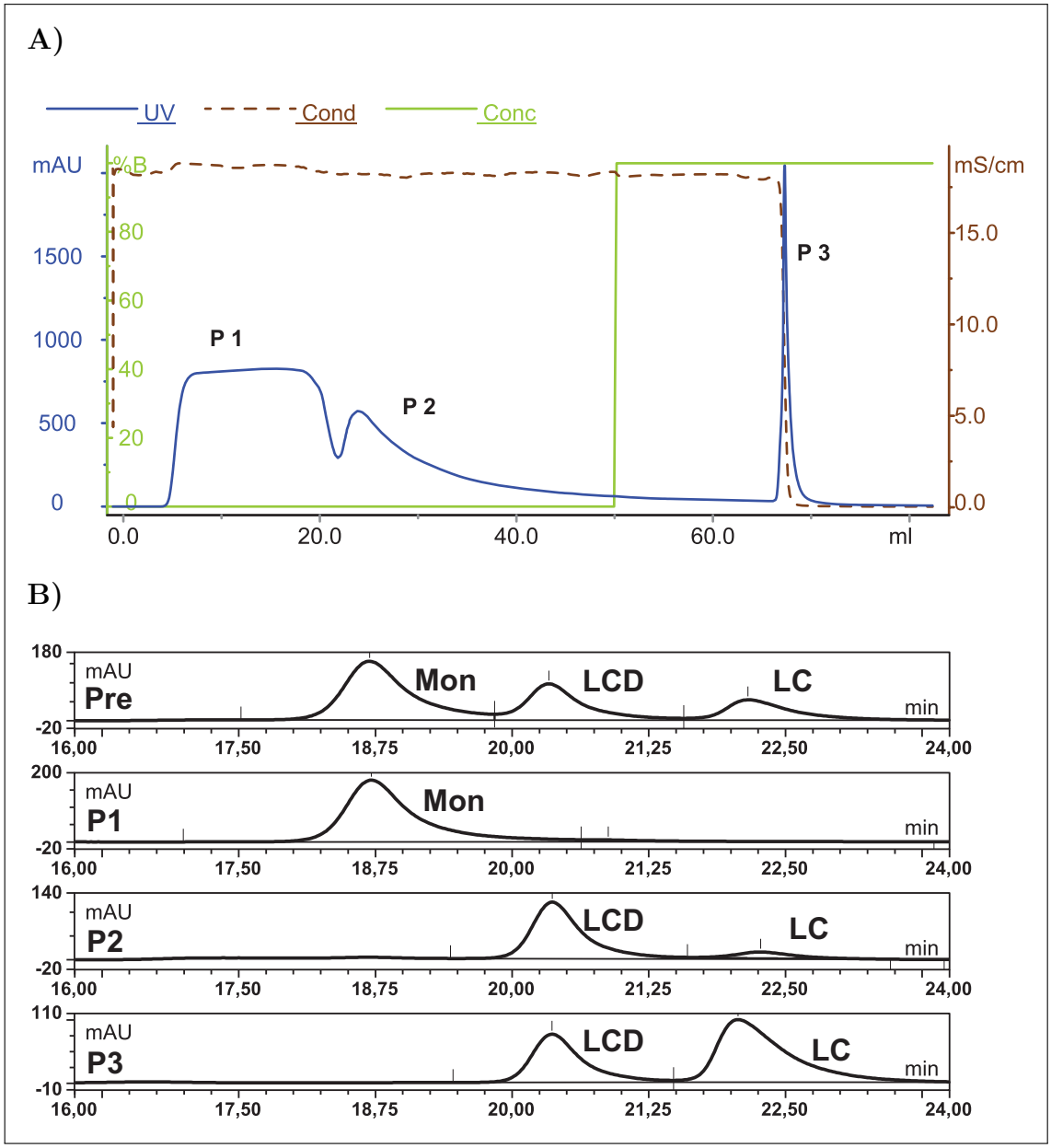


Figure 3.19: **HIC - Buffer verification model**
A: ÄKTA Purifier 100
Column: Hiscreen™ -4.7 mL-Phenyl Sepharose High Performance;
Protein amount: 70.5 mg in 17 mL;
Buffer A: 50mM Tris pH 8.00 Cond. 18.5 mS/cm
Buffer B: water (bidest.)
Gradient: -; Flow rate: 0.47 mL/min
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

3.3.2.5 Multimodal Chromatography (MMC)

A modern and commonly applied chromatography medium from GE Healthcare was used for multimodal chromatography. Capto MMC is a chromatography resin with a multimodal CIEX/HIC-ligand (see figure 3.20).

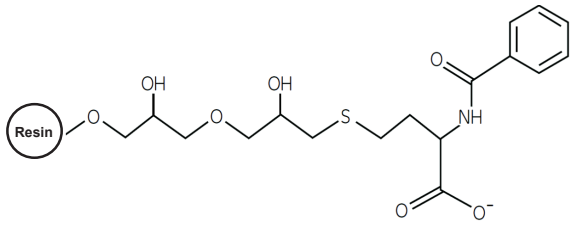


Figure 3.20: **Capto MMC - ligand**

As in AIEX and CIEX, in order to remove as many HCPs, HCDNAs, Viruses and Endotoxins from the API as possible, a pH slightly below the IEP of the API should be applied. With this condition, the mentioned impurities exhibit an anionic net charge and do not bind to the resin, whereas the antibody binds to the resin; the so-called bind-elute mode (BE mode). Due to their hydrophobic properties, multimodal media are much more salt tolerant than CIEX-media.

Bind-elute mode with pH-gradient Elution:
(see figure 3.21 at page 80)
The IEP of the monomeric antibody is 8.5, the IEP of LCD/LC is 6.2. Thus, a slightly lower pH than the IEP of LCD/LC was used to bind the monomer and LCD/LC. Binding-pH was 5.0. Subsequently, pH-value was increased to 8.5 with a gradient of 20 column volumes (CV). The following buffers were recommended by manufacturer GE Healthcare: 100 mM Acetate / 50 mM Phosphate / 20 mM Succinate pH 5.0 (buffer A) and pH 8.5 (buffer B).
The first peak marks the LCD and LC, the second peak represents the monomer. pH and conductivity right in between the two peaks were determined (marker M) in order to apply this condition as starting condition for the next experiment.
Condition: pH 7.0 conductivity 17 mS/cm.

Bind-elute mode with salt-gradient Elution:
(see figure 3.22 at page 81)
The selected condition (see above) was slightly altered. Buffer ion was Phosphate in order to simplify the buffer and conductivity was decreased to 4.3 mS/cm to increase the binding capacity of the monomer. With these conditions, LCD/LC flow through the column while the monomer binds to the resin. As in CIEX, the increase of conductivity is more favorable for elution of the monomer than an increase of pH because this condition is less stressful for the monomer and easier to control in process scale.
After binding of the monomer to the resin with buffer A, conductivity was increased by a gradient of 20 CV to an elution buffer containing buffer A and 1 M NaCl. The following suitable application and elution conditions were determined (markers M1 an M2):

Application: 25 mM Phosphate pH 7.00, conductivity 17 mS/cm
Elution: 25 mM Phosphate pH 7.00, conductivity 63 mS/cm
Because of the high conductivity required for elution, it can be concluded that the monomer exhibits a high binding affinity towards the resin.

Buffer verification model[1]:
(see figure 3.23 at page 82)
As in the AIEX-, CIEX- and HIC-experiments, KappaSelect eluate in 0.1 M Glycine pH 2.5 was used. 21 mL of this eluate contained the required 70.5 mg KappaSelect binding protein (KSBP). This solution was shifted to the required condition of pH 7.00 and conductivity 20 mS/cm using 24 mL 75 mM Phosphate pH 8.0 and 18 mL 0.2 M NaCl. Thus, 65 mL of pH- and conductivity adapted solution containing 70.5 mg of KSBP was applied onto a Hiscreen™ column with a flow rate of 0.59 mL/min.
The verification of buffer conditions did not show the expected results. Peak 1 represents LCD/LC and to some extent the monomer, Peak 2 represents the monomer. As already mentioned, the binding affinity of the monomer towards the resin is very high. However, because of the huge amount of monomer in the flow-through fraction, the binding capacity is low at the same time. This is a disadvantage of this method. Further optimization would be required, if this method would be applied.

[1]For explanation of flow rate and amount of KSBP see part 2.2.2.2 at page 37

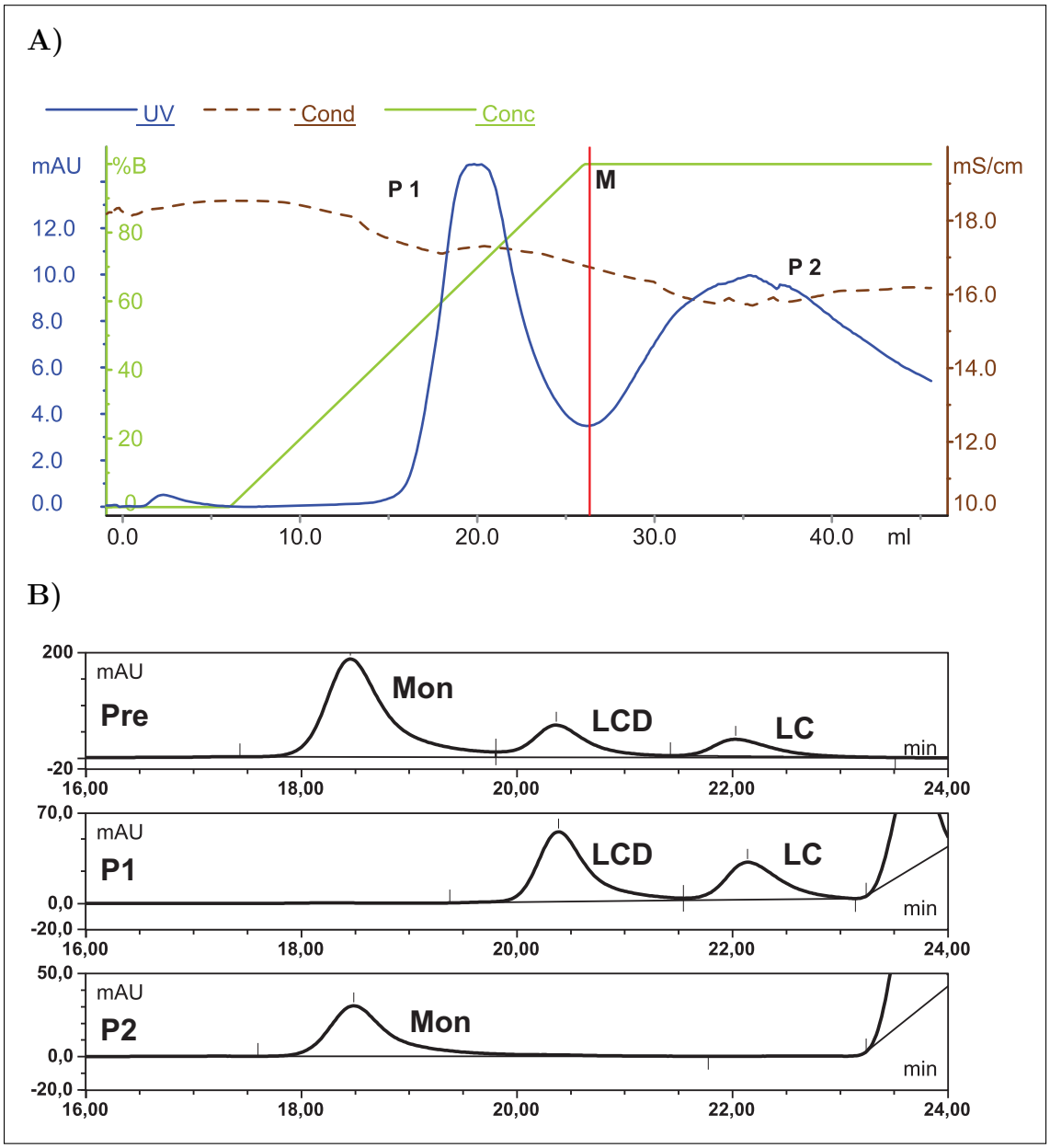


Figure 3.21: **MMC - Evaluation of buffer conditions - Bind-elute mode with pH-gradient Elution**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Capto MMC; Protein amount: 1 mg;
Buffer A: 100 mM Acetate / 50 mM phosphate / 20 mM Succinate pH 5.0;
Buffer B: 100 mM Acetate / 50 mM phosphate / 20 mM Succinate pH 8.5;
Gradient: 20 CV; Flow rate: 1 mL/min
Marker M: pH 7.0 Cond. 17.0 mS/cm
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain; Peak at 23.75 min. results from intrinsic absorption of Succinate at 220 nm

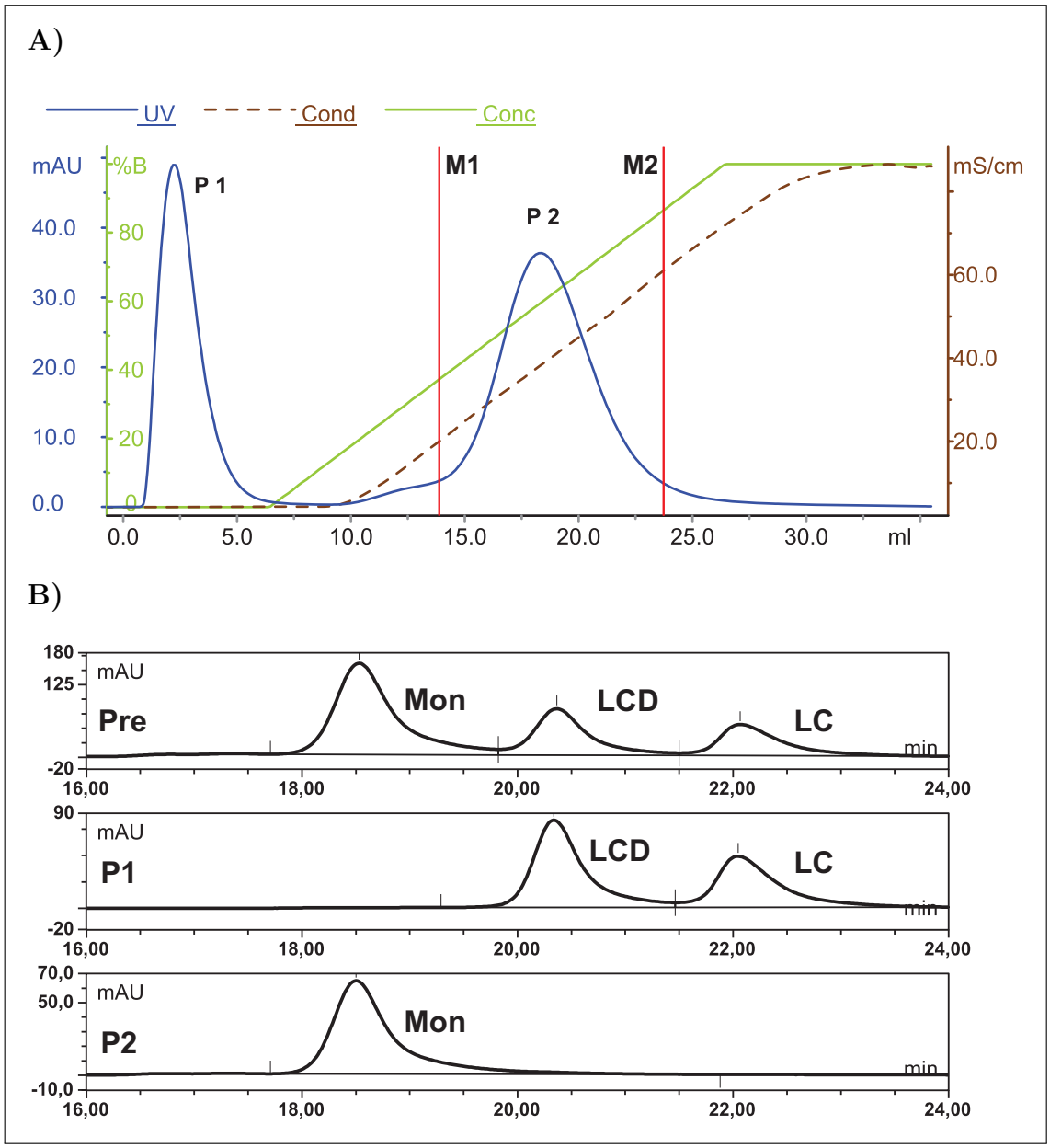


Figure 3.22: **MMC - Evaluation of buffer conditions - Bind-elute mode with salt-gradient Elution**
A: ÄKTA Purifier 100
Column: HiTrap™ -1 mL-Capto MMC; Protein amount: 1 mg;
Buffer A: 25 mM Phosphate pH 6.98 Cond. 4.3 mS/cm;
Buffer B: 25 mM Phosphate pH 6.98 Cond. 80 mS/cm ;
Gradient: 20 CV; Flow rate: 1 mL/min
Marker M1: pH 7.00 Cond. 20 mS/cm
Marker M2: pH 7.00 Cond. 63 mS/cm
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

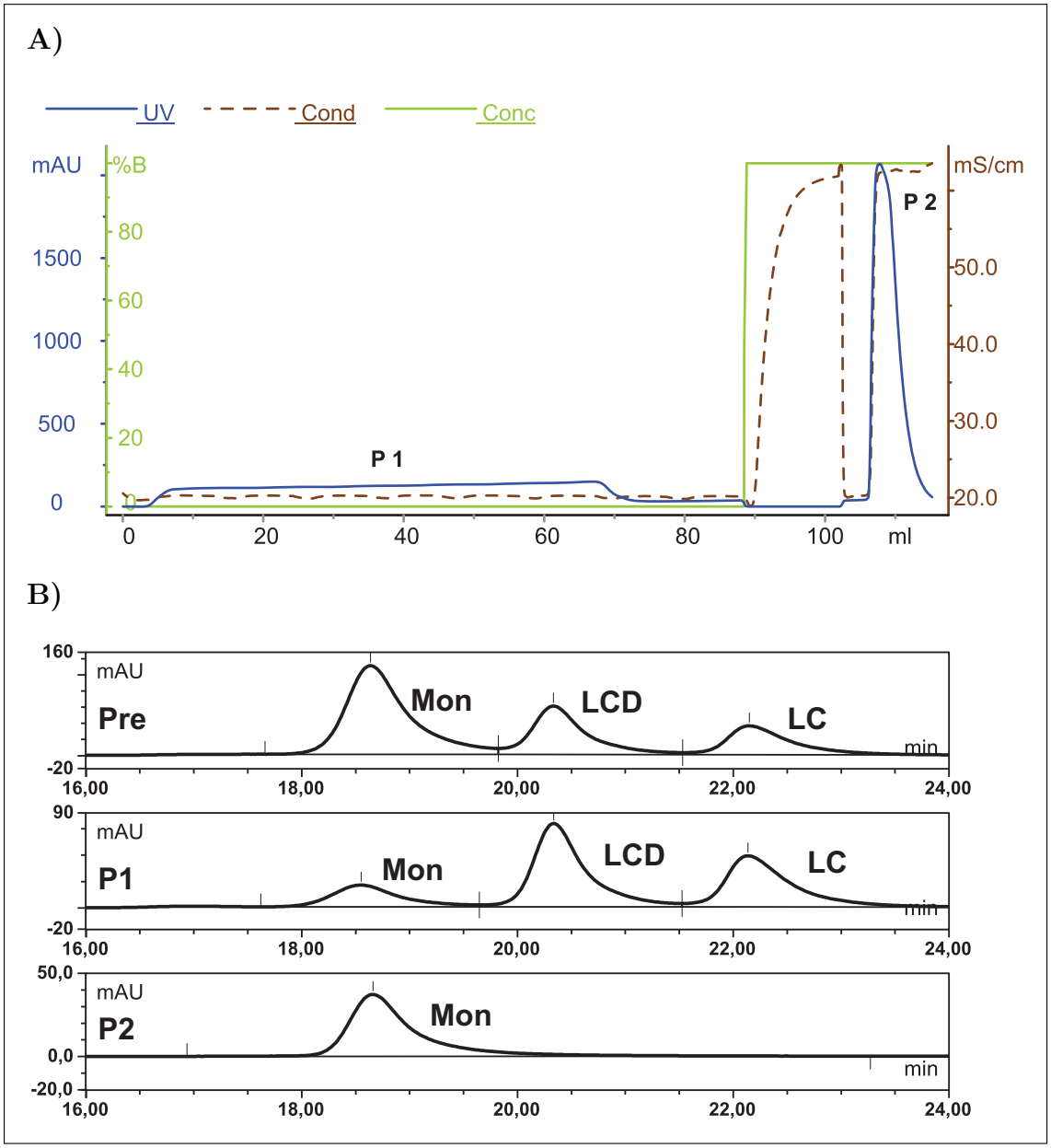


Figure 3.23: **MMC - Buffer verification model**
A: ÄKTA Purifier 100
Column: Hiscreen™ -4.7 mL-Capto MMC;
Protein amount: 70.5 mg in 65 mL;
Buffer A: 25 mM Phosphate pH 7.3 Cond. 20 mS/cm;
Buffer B: 25 mM Phosphate pH 7.3 Cond. 63 mS/cm;
Gradient: -; Flow rate: 0.59 mL/min
B: UHPLC BioRS 3000
Column: SEC TOSOH TSK-Gel® G3000SWXL 7.8x300; 5 µm
Analysis of load (Pre) and fractions of A; Mon = Monomer, LCD = Light chain dimer, LC = Light chain

3.3.2.6 Extended analysis

In the following, the buffer verification models of the 4 methods (AIEX, CIEX, HIC and MMC) were compared according to the following criteria. KappaSelect binding protein (KSBP) was analyzed before and after each polishing method.

Purity, yield and process effort:

- SEC Superdex (improved SEC analysis) for evaluation of the content of aggregates, monomer, LCD and LC in KSBP
- Evaluation of monomer yield
- SDS-PAGE for characterization of KSBP
- Evaluation of the required effort to perform the polishing method

Biological activity

- Flow cytometric analysis on FLT3- and CD3-positive cell lines
- T-cell proliferation assay

Purity, Monomer yield and required effort to perform the method

SEC Superdex (improved SEC analysis):
The four polishing methods were compared with SEC (Superdex) analysis (see figures 3.24 to 3.27) . The peak evaluation results from Chromeleon 7.2 software are summarized in table 3.4
AIEX is an effective method for reduction of the aggregate content. The enrichment of the monomer and LCD/LC removal is not very effective.
CIEX is enriching aggregates. The enrichment of monomer and removal of LCD/LC is very effective.
HIC is a very effective method for reduction of the aggregate content. In addition, the enrichment of monomer and the removal of LCD/LC is efficient.
MMC is also an effective method for reduction of the aggregate content, enrichment of monomer and removal of LCD/LC.
Thus, in regard to this analytical evaluation, AIEX, HIC and MMC are suitable for polishing of the active pharmaceutical ingredient (API). CIEX is not suitable due to its aggregate-enriching properties.

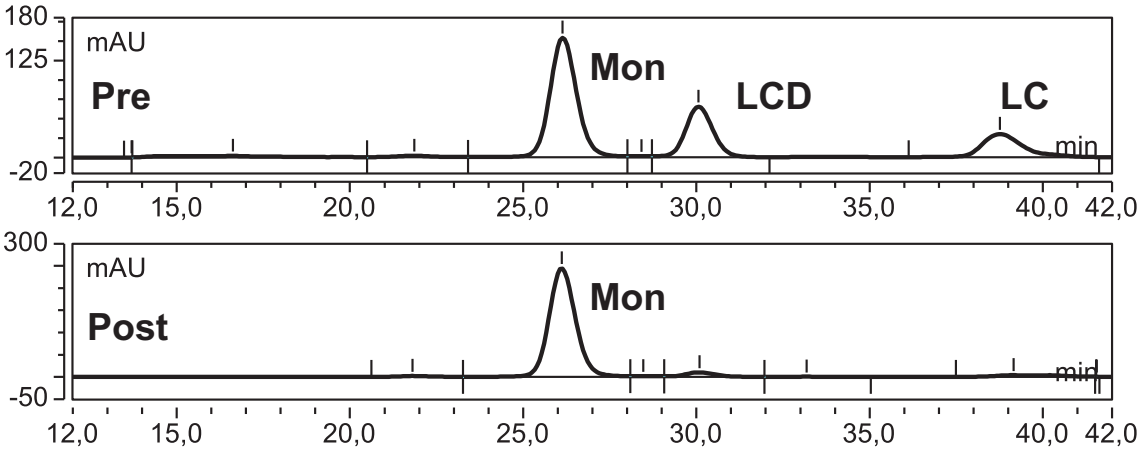


Figure 3.24: **Buffer verification model - SEC (Superdex) - AIEX UHPLC BioRS 3000**
Column: Superdex™ 200 Increase 10/300 GL
Analysis of load (Pre) and polishing application (Post); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

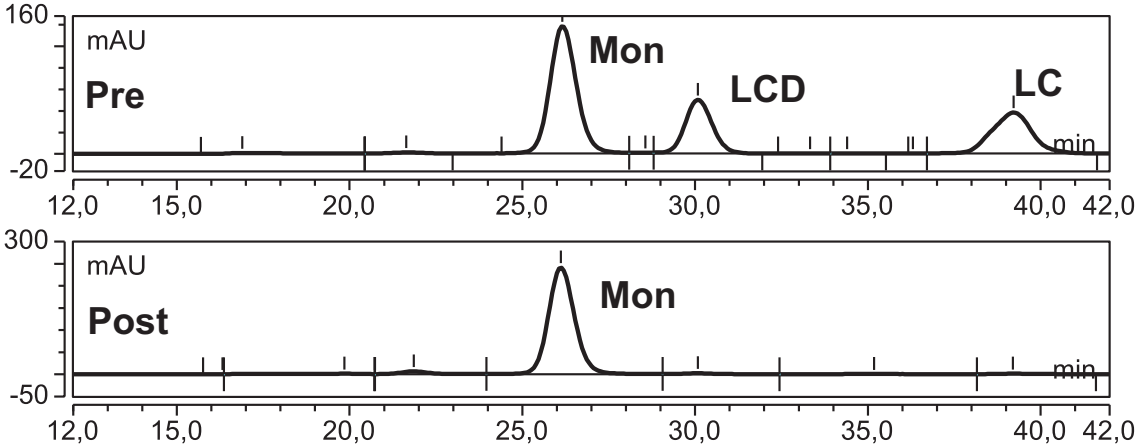


Figure 3.25: **Buffer verification model - SEC (Superdex) - CIEX**
UHPLC BioRS 3000
Column: Superdex™ 200 Increase 10/300 GL
Analysis of load (Pre) and polishing application (Post); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

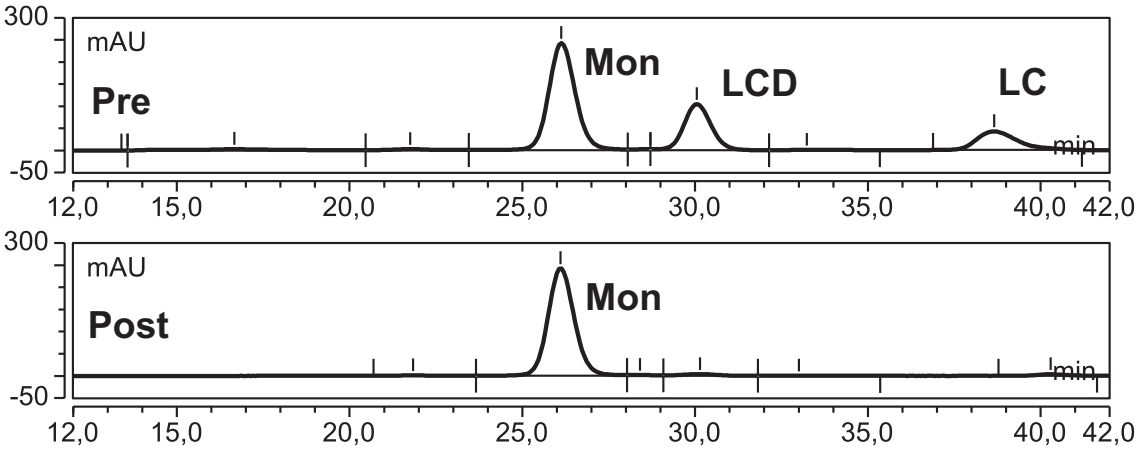


Figure 3.26: **Buffer verification model - SEC (Superdex) - HIC**
UHPLC BioRS 3000
Column: Superdex™ 200 Increase 10/300 GL
Analysis of load (Pre) and polishing application (Post); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

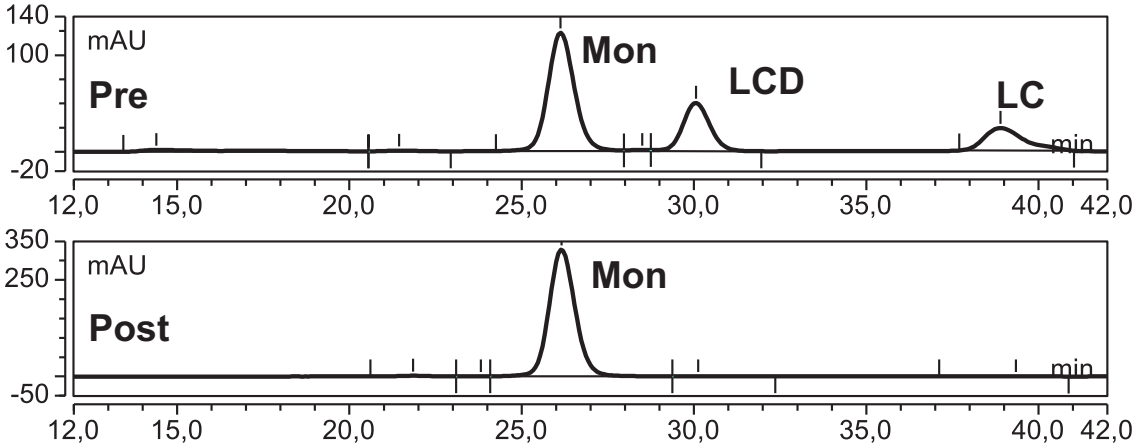


Figure 3.27: **Buffer verification model - SEC (Superdex) - MMC UHPLC BioRS 3000**
Column: Superdex™ 200 Increase 10/300 GL
Analysis of load (Pre) and polishing application (Post); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

Table 3.4: **Buffer verification model - Extended analysis - SEC Superdex**
KSBP analysis pre and post each polishing application

Sample	Aggregate [%]	Monomer [%]	LCD [%]	LC [%]
Pre AIEX	4.4	55.5	23.7	16.0
Post AIEX	0.8	91.3	4.2	3.0
Pre CIEX	1.3	51.5	22.1	24.7
Post CIEX	3.8	93.1	1.2	1.0
Pre HIC	3.7	56.3	24.5	14.8
Post HIC	0.6	94.7	2.2	1.5
Pre MMC	3.2	56.7	23.5	16.2
Post MMC	0.8	98.6	0.3	0.3

Monomer Yield:
Yield was determined by multiplying protein concentration with the monomer content (determined via SEC Superdex) before and after application of a chromatographic technique. The monomer yield is 89 % for AIEX, 98-100 % for CIEX, 90 % for HIC and only 75 % for MMC. The low yield for MMC was already seen in the purification chromatogram 3.23 at page 82; relevant amount of monomer was found in the flow-through fraction.

SDS-PAGE:
SDS-PAGE shows analogue bands compared to SEC-analysis (see figure 3.28). No additional or missing bands are visible (except for the fact that the aggregate fraction is missing on SDS-PAGE because of the heat-treatment of the samples in SDS-PAGE preparation). In regard to this analytical evaluation, all techniques are suitable for polishing of the API.

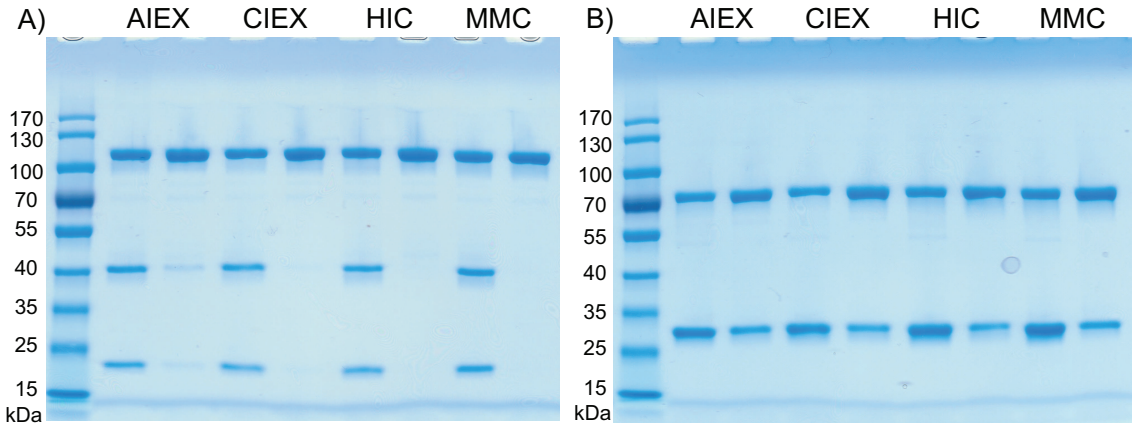


Figure 3.28: **Buffer verification model - SDS-PAGE**
Analysis of load (Pre) and polishing application (Post); A = unreduced condition; B = reduced condition; marker: "PageRuler™ Prestained Protein Ladder"

Required effort to perform the method:
Method effort is low for AIEX and HIC, because these methods can be performed in flow-through mode (FT mode). MMC requires a medium method effort due to the application of a bind-elute mode (BE mode). The effort for CIEX is very high due to the application of a BE mode and relatively high back pressure due to a small bead size (40 µm), this results in a low flow rate.

Biological activity:

Flow cytometric analysis on FLT3- and CD3-positive cell lines:
Flow cytometric analysis of monomer-normalized KSBP before and after each polishing method was almost identical for all four polishing methods compared to reference NF-CU$_{N297Q}$ (SP2/0) (see figures 3.29 and 3.30). In regard to this analytical evaluation, all techniques are suitable for polishing of the API.

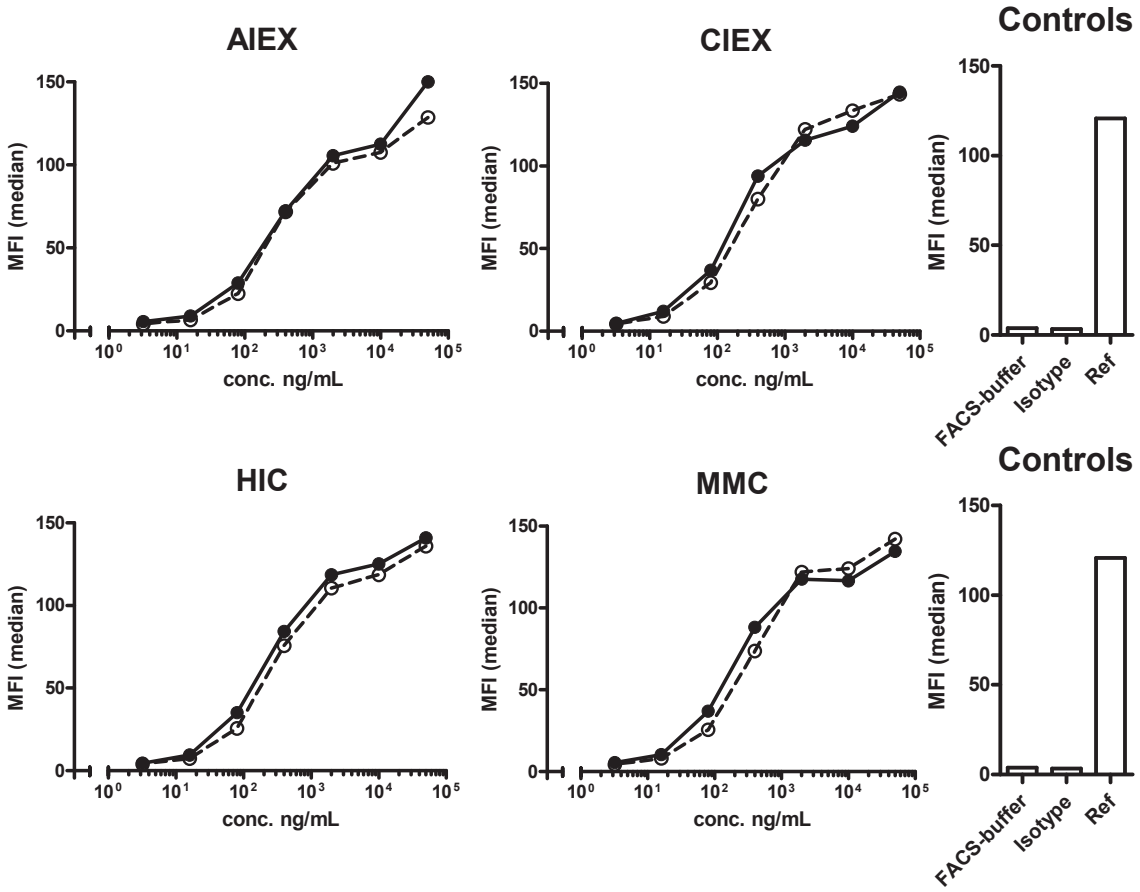


Figure 3.29: **Buffer verification model - Flow cytometric analysis - NALM-16 cells**
Titration of monomer-normalized samples pre (●) and post (○) polishing application on NALM-16 cells; Isotype = NP-CU$_{N297Q}$ (SP2/0) 10 µg/mL; Ref = NF-CU$_{N297Q}$ (SP2/0) 10 µg/mL; Detection: GaH-Fc-PE

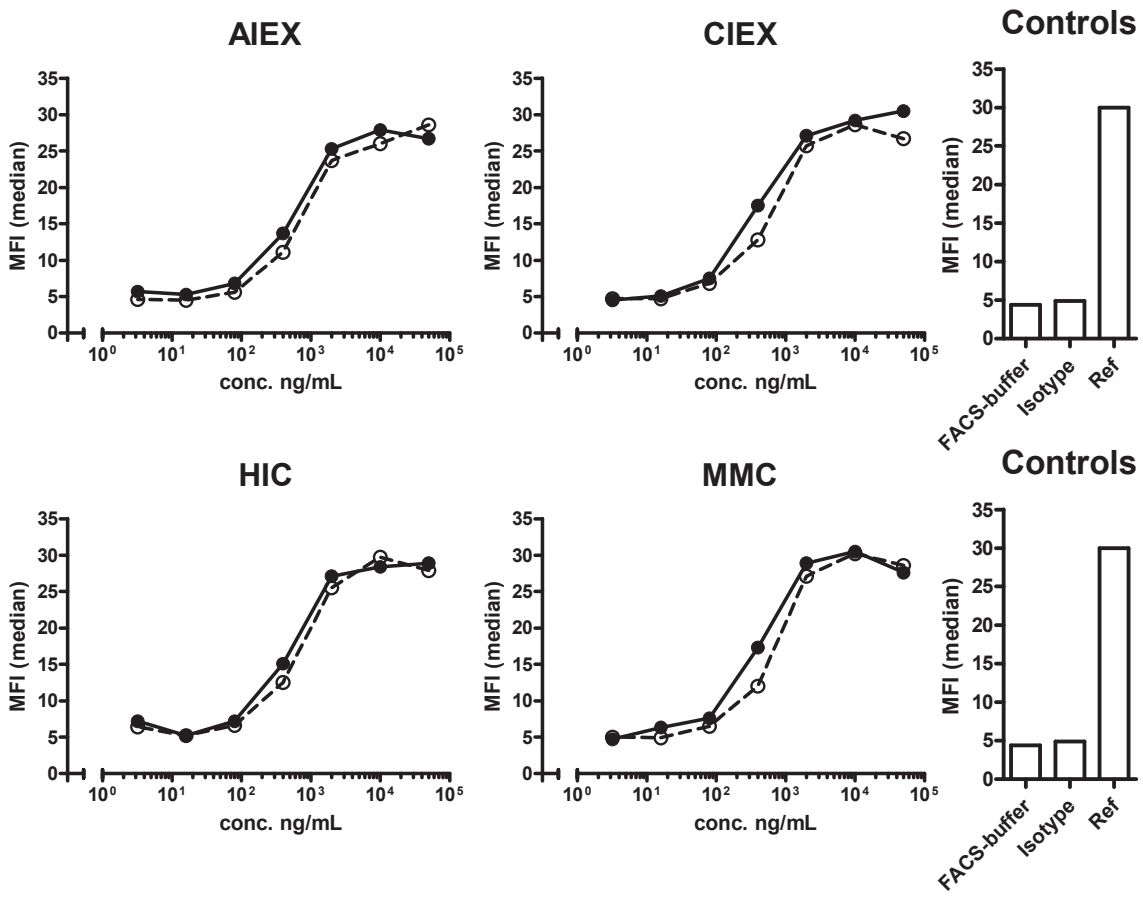


Figure 3.30: **Buffer verification model - Flow cytometric analysis - Jurkat cells**
Titration of monomer-normalized samples pre (●) and post (○) polishing application on Jurkat cells; Isotype = NP-C16$_{N297Q}$ (SP2/0) 10 µg/mL; Ref = NF-CU$_{N297Q}$ (SP2/0) 10 µg/mL; Detection: GaH-Fc-PE

T-cell proliferation assay:
Titration of monomer-normalized KSBP obtained in the T-cell proliferation assay before and after each polishing method was almost identical for all four polishing methods compared to reference NF-CU$_{N297Q}$ (SP2/0) (see figure 3.31). In regard to this analytical evaluation, all techniques are suitable for polishing of the API.

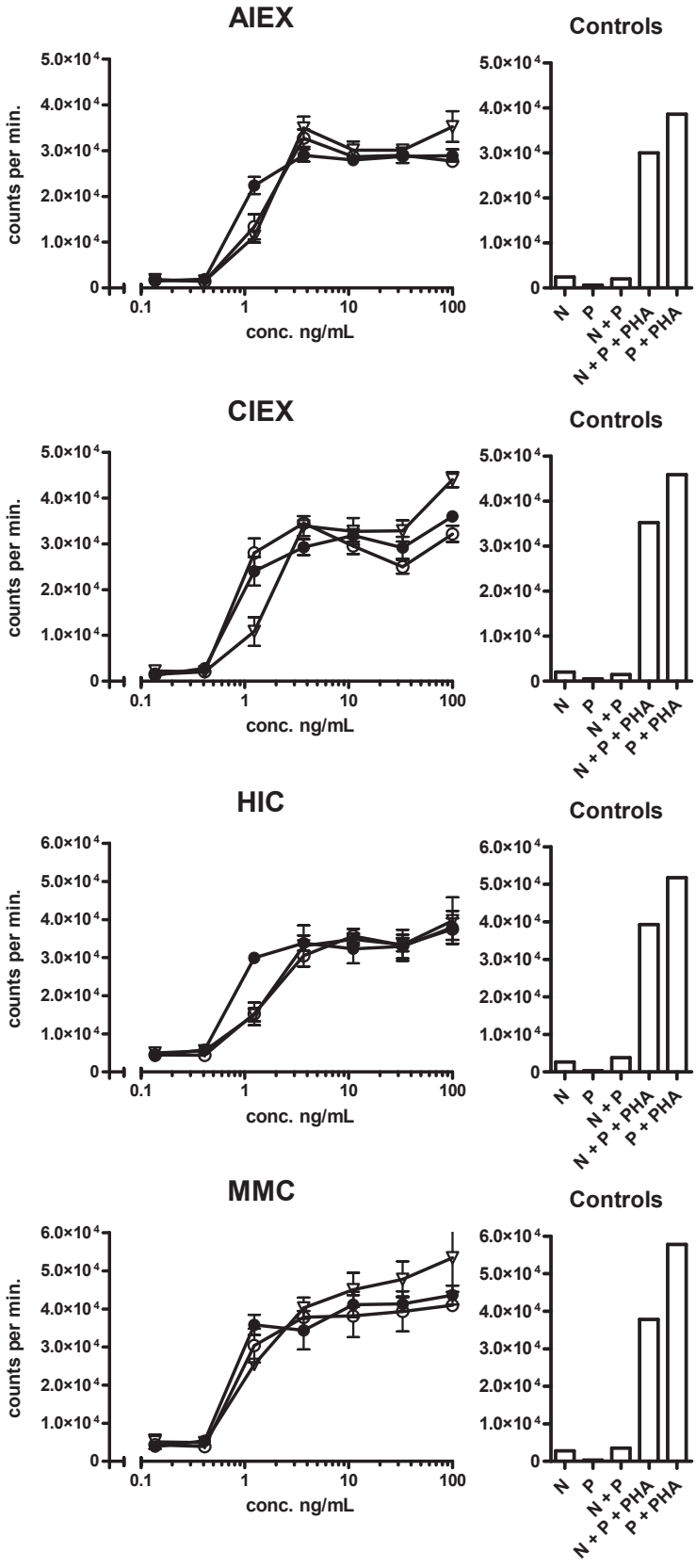


Figure 3.31: **Buffer verification model - T-cell proliferation assay**
Titration of monomer-normalized samples pre (●) and post (○) polishing application; Reference (▽) = NF-CU$_{N297Q}$ (SP2/0); E:T = 1:1; N = NALM-16; P = PBMC; PHA = 10 µg/mL phytohemagglutinin

3.3.2.7 Summary and Conclusion

The results for the extended analysis are summarized in table 3.5.
AIEX is an effective method for the enrichment of monomer, results in high monomer yield and no loss of biological activity is observed. Furthermore, this technique promises good theoretical properties for the removal of process-related impurities; the relatively high pH-value of 8 and the low conductivity of 3 mS/cm are very favorable for this application (see part 1.4.3 at page 15).
CIEX is not a suitable method due to the aggregate-enriching properties.
HIC is a very effective method for the enrichment of monomer and reduction of LCD/LC content and especially for the reduction of aggregate content. Monomer yield is high and no loss of biological activity can be observed.
MMC is not a suitable method due to a very low monomer yield.

The combination of AIEX and HIC is suitable because of the different separation mechanisms of these methods. The advantage of this combination is the effective removal of process-related impurities via AIEX and the effective removal of product-related impurities (especially aggregates) via HIC. Those methods were applied for the development of a small-scale model, a 1:100-scale model of the planned production process scale.

Table 3.5: **Summary of extended analysis for buffer verification model of AIEX, CIEX, HIC and MMC**

	AIEX	**CIEX**	**HIC**	**MMC**
Aggregate [%] (SEC)	0.8	**3.8**	**0.6**	0.8
Monomer [%] (SEC)	91.3	93.1	94.7	98.6
LCD/LC [%] (SEC)	7.2	2.2	3.7	0.6
Monomer Yield [%]	89	98-100	90	**75**
SDS-PAGE	Analogue bands to SEC			
Method effort	**Low**	**Very high**	**Low**	Medium
Flow cytometric analysis	Same binding pre and post method			
T-cell proliferation assay	Same proliferation pre and post method			

3.3.3 Development of a small-scale model of the production process

At the end of chapter 3.3.2, it was concluded that the combination of polishing methods AIEX (Capto Q) and HIC (Phenyl HP) is reasonable and suitable. For simulation of a production process, a small-scale model (1:100) must be developed. The theoretical background for performance of the methods such as scaling of columns and flow rates is explained in part 2.2.2.2 at page 37.

In order to verify the suitability of the combination of KappaSelect, AIEX (Capto Q) and HIC (Phenyl HP), three purifications with two different batches of supernatant were performed. For verification of process limitations, one purification was performed with 90 %, one with 110 % and one with 135 % load of the intended 130 mg of KSBP (see 2.2.2.2 at page 37).

In the buffer verification models of chapter 3.3.2, AIEX was performed in FT mode with a 50 mM Tris buffer pH 8.00 conductivity 3 mS/cm, HIC was performed in FT mode with a 50 mM Tris buffer pH 8.00 conductivity 18.5 mS/cm (adjusted with $(NH_4)_2SO_4$). Thus, the easiest way to combine these two polishing applications is to perform the AIEX method first, then add the required amount of 1 M $(NH_4)_2SO_4$ to increase conductivity to 18.5 mS/cm and then perform the HIC method.
The three verification batches were named AH1.1, AH1.2 and AH1.3. "AH1" represents the first possible combination of AIEX and HIC (see above). The ennumeration behind this abbreviation represents the batch number. For each batch, samples were collected after each column to perform analytical evaluation (in-process controls 1-3 (IPC-1-3)).

The following analytical evaluation was performed for IPCs of the three verification batches:

- SEC (Superdex) / UHPLC and monomer yield
- SDS-PAGE
- Analytical CIEX / UHPLC
- Flow cytometric analysis on FLT3- and CD3-positive cell lines
- T-cell proliferation assay
- Potency Assay (Promega)
- Process related impurities (HCP-, HCDNA-, LKS-removal)

For purification chromatograms and parameters see appendix part 5.3.1 at page 139 ff. and table 5.2 at page 139. A compendium of results for IPCs can be found in the appendix (see table 5.3 at page 143).

3.3.3.1 SEC (Superdex) / UHPLC and Monomer yield

(see appendix figure 5.4 at page 144, table 5.4 at page 145 and summarizing figure 3.32 at page 93)
With process AH1, it was possible to reduce the aggregate content from 6.6 % via 4.4 % to 0.3 %. The main aggregate clearance was achieved via HIC. Monomer content rises from 53.9 % to 97.2 % and LCD/LC content declines from 39.3 % to 1.8 %. The main LCD/LC clearance was achieved via AIEX.
Monomer yield was calculated from tables 5.2 at page 139 and 5.3 at page 143 with the following equation (amount in [mg]):

$$\text{Monomer yield [\%]} = \frac{\text{IPC-2 amount Monomer}}{\text{AIEX appl. amount Monomer}} * \frac{\text{IPC-3 amount Monomer}}{\text{HIC appl. amount Monomer}} * 100 \tag{3.1}$$

Monomer yield declines with rising KSBP-load. 100 % KSBP load corresponds to 130 mg KSBP (see chapter 2.2.2.2 at page 37). Monomer yield is 91.5 % at 90 % maximum load to 77 % at 135 % maximum load (see figure 3.32 D).

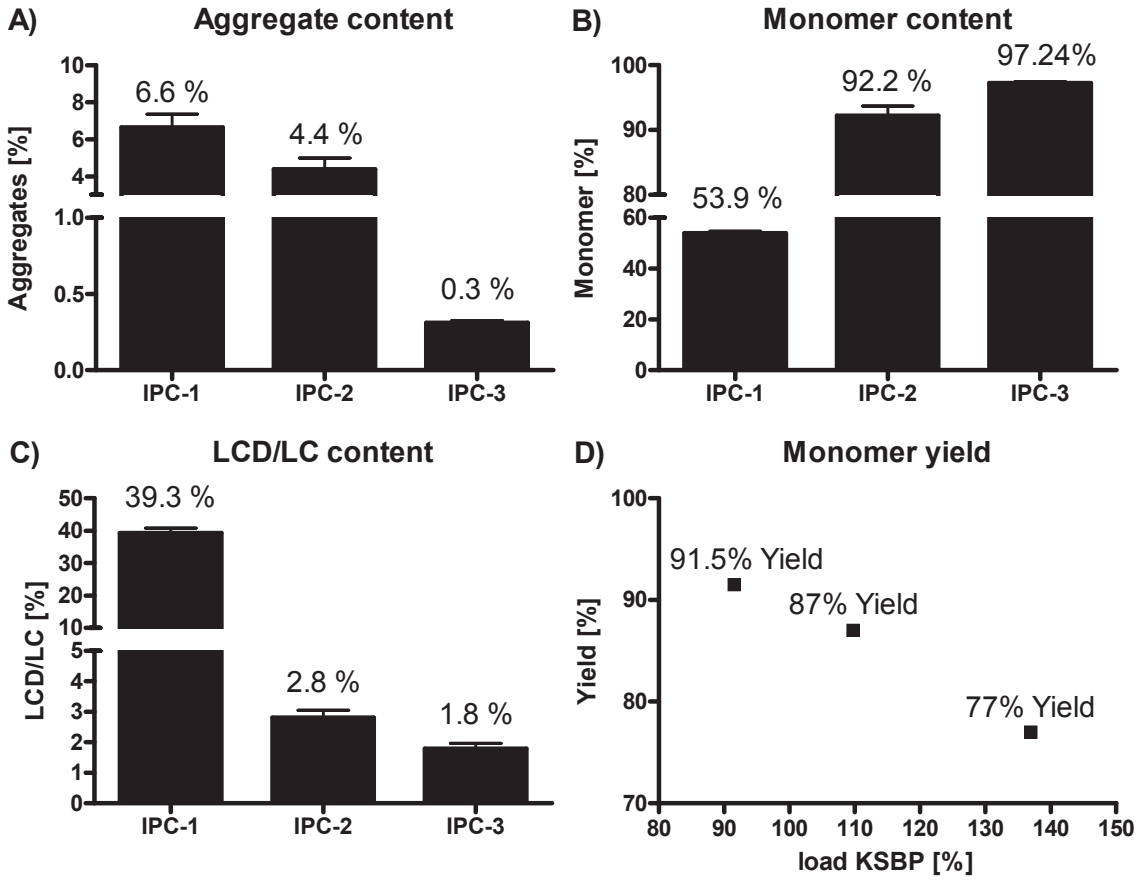


Figure 3.32: **Small-scale model - (SEC Superdex)**
A-C: Content of product-related impurities detected via SEC Superdex; D: calculation of monomer yield; 100 % KSBP load corresponds to 130 mg KSBP; verification batches AH1.1-3

3.3.3.2 SDS-PAGE

(see appendix figure 5.7 at page 146)
SDS-PAGE analysis exhibits analogue bands compared to SEC-analysis for all three verification batches. No additional or missing bands are visible (except for the fact that the aggregate fraction is missing on SDS-PAGE because of the heat-treatment in SDS-PAGE preparation).

3.3.3.3 Analytical CIEX / UHPLC

(see appendix figure 5.8 at page 146)
All three chromatograms of verification batches exhibits almost the same elution profile consisting of one main peak, two acidic variants, one acidic shoulder of the main peak and two basic variants.

3.3.3.4 Process related impurities

(see figure 3.33)
With small-scale process AH1, it was possible to reduce the HCP content from almost 6000 ppm via 52 ppm to 20.8 ppm and HCDNA content from 670 ppm to less than 1.5 ppm. The main HCP- and HCDNA-clearance post affinity chromatography was achieved via AIEX.
The removal of process-related impurity "leached KappaSelect" (LKS) could not be evaluated due to a very high background and a false-low signal of the ELISA. After incubation at 95 °C, LCD/LC are not denatured and still bind to the KappaSelect ligand resulting in a false low signal of the ELISA. In IPC-3, the LCD/LC content is still 1.8 % and might therefore still result in a false-low signal of the ELISA. Solely a theoretical consideration on removal efficiency of the process could be performed; an isoelectric focusing electrophoresis of the KappaSelect ligand (from ELISA kit) was performed to predict the property of the KappaSelect ligand on AIEX (see figure 3.34 at page 95). IEP of KappaSelect-ligand is lower than 5.85 and can therefore in theory be removed very effectively via AIEX.

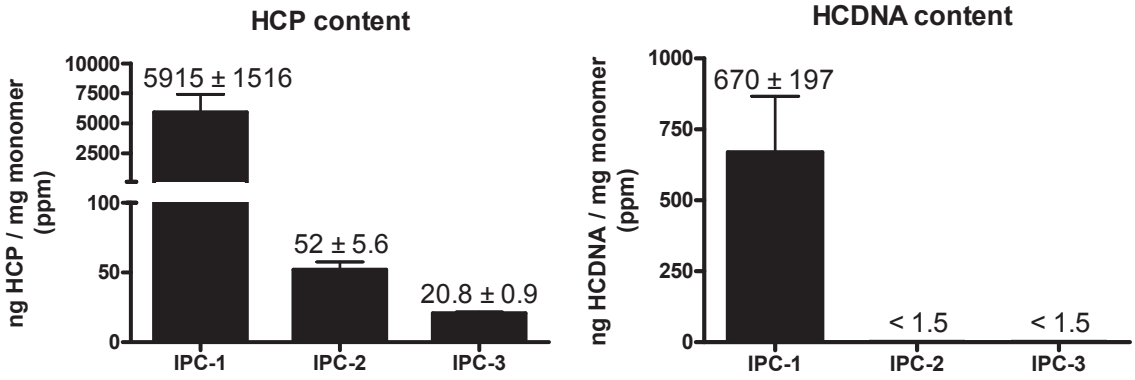


Figure 3.33: **Small-scale model - Removal of Process-related impurities**
Concentration of process-related impurities detected via ELISA (HCP) and QPCR (HCDNA); concentrations normalized to monomer; verification batches AH1.1-3

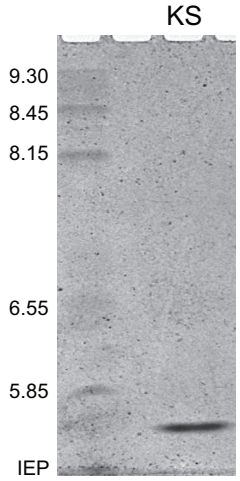


Figure 3.34: **Isoelectric focusing electrophoresis - KappaSelect ligand**
Ligand from KappaSelect ELISA kit; marker: "Isoelectric Focusing Calibration Kit High Range pI (pH 5 - 10.5)"

3.3.3.5 Flow cytometric analysis

(see appendix figure 5.9 at page 147 and 5.10 at page 148)
All titration curves of monomer-normalized KSBP of IPC-1 to 3 exhibit the same progression for all verification batches on NALM-16 ($FLT3^+$) and on Jurkat cells ($CD3^+$) and are comparable to reference standard (NF-CU_{N297Q} (SP2/0)).

3.3.3.6 T-cell proliferation assay

(see appendix figure 5.11 at page 149)
All titration curves of monomer-normalized KSBP of IPC-1 to 3 exhibit the same progression for all verification batches. The reference standard (NF-CU_{N297Q} (SP2/0)) exhibits a slightly weaker cell-proliferation inducing property. This may be due to the fact that the reference standard was already two years old and stored in DPBS instead of a suitable formulation buffer.

3.3.3.7 Potency Assay (Promega)

(see appendix figure 5.12 at page 150 and table 3.6 at page 96)
For this experiment, only IPC-3 of batches AH1.1 to AH1.3 were compared to reference NF-CU_{N297Q} (SP2/0). Overall EC_{50} of the three batches is 3.0 ± 0.2 ng/mL, which corresponds to only $33.3\,\% \pm 0.8\,\%$ relative potency compared to reference. NF-CU_{N297Q} seems to lose potency during purification via process AH1.

Table 3.6: **Small-scale model - Potency Assay (Promega) - Calculation of EC_{50}-values of IPC-3 for batches AH1.1-3**

	EC_{50} [ng/mL]	**% of Ref**
Experiment 1		
AH1.1	2.8	32
AH1.2	2.7	33
Ref	0.9	100
Mean AH1.1/2	**2.75 ± 0.05**	**32.5 ± 0.5**
Experiment 2		
AH1.1	3.2	34
AH1.3	3.2	34
Ref	1.1	100
Mean AH1.1/3	**3.2 ± 0**	**34 ± 0**
Experiment 1 and 2		
mean AH1.1/2/3	**3.0 ± 0.2**	**33.3 ± 0.8**

3.3.3.8 Summary and Conclusion

Aggregates and LCD/LC are very effectively removed via process AH1 resulting in a monomer yield of 90 %. For all three batches purified, SDS-PAGE, SEC (Superdex), analytical CIEX / UHPLC were almost identical. HCP and HCDNA are very effectively removed especially via AIEX. LKS is theoretically removed very efficiently via AIEX as well. Biological activity decreases during purification, this can only be observed in the potency assay (Promega).
Despite some loss of potency and incomplete analytical evaluation, the small-scale model AH1 exhibits excellent results and is suitable for up-scaling.

3.3.4 Nanofiltration

The purpose of this test was to find a suitable filter for the reduction of potential viral contamination for a production process. Because of previous experience with "Asahi Kasei"-Nanofilters in the production process of the first product of Synimmune GmbH, 4G8SDIEM, filtration performance was solely evaluated on "Asahi Kasei"-filters. For performance of a process realistic small-scale-experiment, the technical objectives for the production process must first be defined. As discussed in 4.2.2 at page 117, maximum monomer content per batch is 6050 mg with a concentration of 1 mg/mL. With addition of a safety factor of 5 %, 6300 mg antibody monomer with a concentration of 1 mg/mL are to be filtrated. The formulation buffer for this experiment was DPBS.
The following filters were tested: Planova™ 15 N, Planova™ 20 N and Planova™ BioEx.
Process development scientists of "Asahi Kasei" proposed a 1:120 small-scale model for Planova™ 15 N and Planova™ 20 N and a 1:100 small-scale model for Planova™ BioEx. Parameters for filtration are listed in table 3.7

Experimental procedure:
As first step, each filter was flushed with 20 mL water for injection. A leakage test was performed and was negative for all three filters. Subsequently, the filters were flushed with 20 mL DPBS and filtration was performed according to the parameters in table 3.7 and 3.8. A post-use-leakage test was performed and was negative for all three filters.

Table 3.7: **Nanofiltration - Parameters for filtration**

Filtration volume in process	6.3 L
Filter area process filters	0.120 m^2 (15 N,20 N) 0.100 m^2 (BioEx)
Filtration volume downscale model	52.5 mL (15 N, 20 N), 63 mL (BioEx)
Filter area downscale filters	0.001 m^2
Maximal TMP	98 kPa (15 N, 20 N), 330 kPa (BioEx)
Mode of filtration	isobar
Concentration of antibody solution	1.03 mg/mL

Table 3.8: **Nanofiltration - Filtration procedure**

Filter Planova™	**15 N**	**20 N**	**BioEX**
Filtrated volume - void volume [mL]	45	49.4	49.8
Filtrated Monomer [mg]	46.35	50.88	51.29
% of required filtration volume according to setpoint	**88.3 %**	**97 %**	**81.4 %**

Table 3.9: **Nanofiltration - Results of filtration**

Filter Planova™	**15 N**	**20 N**	**BioEX**
Filtration time [h]	**1.25**	**0.75**	**0.42**
Average flux [L/h/m²]	44	73.33	132
Product recovery [mg]	44	51.15	45.1
Product recovery (rel)	**95 %**	**100 %**	**88 %**

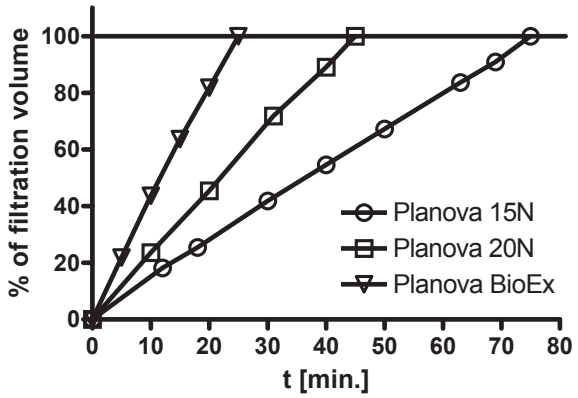


Figure 3.35: **Nanofiltration**

Results:
Filtration can be performed in less than 1.5 h with all three filters. Thus, all filters would be suitable for nanofiltration in production process. However, product recovery is an issue for Planova BioEx with only 88 %. Recovery of Planova 20 N is slightly higher than in Planova 15 N (100 % instead of 95 %) and the filtration time is much shorter (0.75 h instead of 1.25 h). In conclusion, the use of the following nanofilter will be recommended for production process:

Planova™ 20 N; area: 0.120 m²

3.4 Formulation development

A suitable protein formulation buffer increases thermodynamic and kinetic stability as well as shelf-life of the active pharmaceutical ingredient (API). The following parameters were predetermined for formulation development:

- Development of a liquid dosage form
- Antibody concentration of 1 mg/mL

In 2011, Warne et al. stated that the effort for the development of protein formulations for Phase-I-biopharmaceuticals can be reduced drastically by using a platform approach (Warne, 2011). Based on formulations of approved drugs, those investigators tried narrowing down reasonable combinations of buffer compositions and properties.

When analyzing pH of formulations of approved antibody therapeutics (see examples in table 3.10), it can be seen that a mild acidic pH is common and the main buffer ions are Histidine or Phosphate. Histidine was used as buffer component for NF-CU_{N297Q} due to its pKa-value of 5.97 (instead of 7.2 for Phosphate). Thus, Histidine exhibits a higher buffer capacity in mild acidic environment; it is also recommended as starting buffer by Warne et al. in buffers from pH 5.60 to 6.80.
The addition of a protectant against the influence of several stress factors such as ultra-/diafiltration or freeze-thaw-cycles is recommended. Saccharose is quite common in lyophilized dosage forms. When using an acidic pH in liquid dosage forms, Saccharose is an unfavorable choice due to glycolysis. This degradation reaction can especially be observed in the presence of Imidazole (Farr and Heitz, 1974). A reasonable alternative is Trehalose because of a more stable α-α-1,1-glycosidic bond between the monosaccharides. Concentrations up to 100 g/L Trehalose are common in formulations for monoclonal antibodies.
In order to maintain a physiological osmolality of roughly 300 mOsm/kg, sodium chloride is added to each test buffer if required. Usually, surfactants are added to the protein formulation to reduce the risk of aggregate formation. The addition of Tween® 20 or Tween® 80 is common in concentrations ranging from 0.01 to 0.2 %.

The formulation development experiments were performed using a statistical strategy termed "Design of experiments" (DoE) (Fisher, 1990). With this approach, multiple factors (parameters) of an experiment are altered simultaneously and a correlation to multiple responses (results) can be deducted and verified. This is an established approach for process- and formulation development of biopharmaceuticals.
Differential scanning fluorimetry (DSF) was used for determination of thermodynamic stability of the API in a certain buffer. The half-maximal denaturation temperature (melting temperature) of the monomer was determined with this method. The thermal stress test was used for determination of kinetic stability of the API. After incubation of the antibody solution at 40 °C for 1 or 4 weeks, SEC (Superdex) / UHPLC analysis of the antibody in the respective buffer was performed. The amount of aggregates and the amount of monomer normalized to

the whole peak area were determined.

Table 3.10: **Formulation composition of selected monoclonal antibodies in liquid dosage form (FDA-Accessdata, 2015)**

Drug	Approval	Conc.	pH	Buffer	Isotonisation	Sugar	Surfactant
Rituximab	1997	10 mg/ml	6.5	25 mM Citrate	150 mM NaCl	none	0.07 % Tween® 80
Daclizumab	1997	5 mg/mL	6.9	70 mM Phosphate	80 mM NaCl	none	0.02 % Tween® 80
Palivizumab	1999	100 mg/mL	6.0	25 mM Histidine 1.5 mM Glycine	10 mM NaCl	none	none
Adalimumab	2002	50 mg/mL	5.2	15 mM Phosphate 10 mM Citrate	100 mM NaCl	12 g/L Mannitol	0.1 % Tween® 80
Tositumomab	2003	2.5 mg/mL	7.2	10 mM Phosphate 18 mM KOH	150 mM NaCl	100 g/L Maltose	none
Cetuximab	2003	2 mg/mL	7-7.4	15 mM Phosphate	150 mM NaCl	none	none
Natalizumab	2004	20 mg/mL	6.1	15 mM Phosphate	150 mM NaCl	none	0.02 % Tween® 80
Bevacizumab	2005	25 mg/mL	6.2	50 mM Phosphate	none	60 g/L Treh.*2 H_2O	0.04 % Tween® 20
Ranibizumab	2006	10 mg/ml	5.5	10 mM Histidine	none	100 g/L Treh.*2 H_2O	0.01 % Tween® 20
Panitumumab	2006	20 mg/mL	5.6-6.0	80 mM NaOAc	100 mM NaCl	none	none
Eculizumab	2007	10 mg/mL	7.0	15 mM Posphate	150 mM NaCl	none	0.022 % Tween® 80
Golimumab	2009	100 mg/mL	5.5	5.6 mM Histidine	none	41 g/L Sorbitol	0.015 % Tween® 80
Ipilimumab	2011	5 mg/mL	7.0	100 µM DTPA 20 mM Tris	100 mM NaCl	10 g/L Mannitol	0.01 % Tween® 20
Tocilizumab	2011	20 mg/mL	6.5	15 mM Phosphate	none	50 g/L Saccharose	0.05 % Tween® 80
Nivolumab	2014	10 mg/mL	6.0	20 µM DTPA	50 mM NaCl	30 g/L Mannitol	0.02 % Tween® 80

3.4.1 Formulation screening

In the formulation screening experiment, the screening buffers comprised the following constant properties:

- 20 mM Histidine
- Osmolality 300 mOsm/kg
- Antibody concentration 1 mg/mL

The experiment was performed with the following factors (parameters or variables) as full factorial analysis (see figure 3.36):

- pH 5.60 to 6.80
- Tween® 20 or Tween® 80 from 0 % to 0.15 %
- Trehalose from 0 to 100 g/L[1]

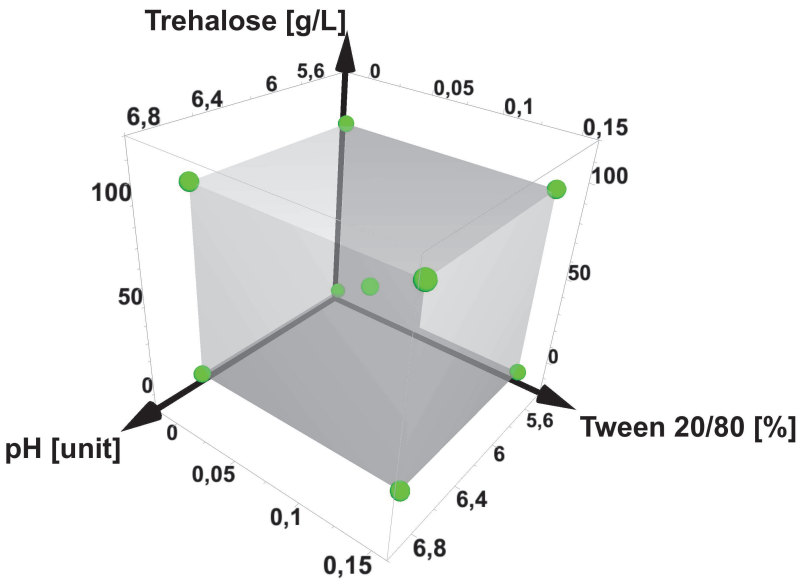


Figure 3.36: **Formulation screening: Design region**
Performed experiments are marked in the corners and in the center of design region

The following responses (results) were investigated:

- Melting temperature determined via differential scanning fluorimetry (DSF) T_{m1}
- Aggregate content after 1 week at 40 °C determined via SEC (Superdex) on UHPLC
- Monomer content after 1 week at 40 °C determined via SEC (Superdex) on UHPLC

[1]NaCl was added to maintain osmolality of 300 mOsm/kg ranging from 150 mM NaCl for 0 g/L Trehalose to 0 mM NaCl for 100 g/L Trehalose

For evaluation of connections between factors and responses, the fitting method "partial least squares" (PLS) was performed by software "Modde 10.0". Results are presented in heat maps, green is a favorable condition, red is an unfavorable condition. The R^2, Q^2 and residual standard deviation (RSD) of each plot are noted in the caption of the figures.

To evaluate the informative value of a model, the following statistical parameters were considered:

- **R^2** (Coefficient of correlation) shows the model fit; 0.5 is a model with rather low significance.

$$R^2 = \frac{\text{the sum of squares of Y corrected for the mean, explained by the model}}{\text{the total sum of squares of Y corrected for the mean}} \tag{3.2}$$

- **Q^2** shows an estimate of the future prediction precision. Q^2 should be greater than 0.1 for a significant model and greater than 0.5 for a good model.

$$Q^2 = 1 - \frac{\text{the prediction residual sum of squares}}{\text{the total sum of squares of Y corrected for the mean}} \tag{3.3}$$

- **RSD** (residual standard deviation)

3.4.1.1 Results for Tween® 20 buffers

The results are presented in table 3.11 and figures 3.37, 3.38 and 3.39. For response T_{m1}, R^2 and Q^2 are both higher than 0.5. RSD is with 0.32 relatively low.
For response "Aggregate content [%] at 40 °C for 1 week" R^2 is higher than 0.5. Q^2 is lower than 0.5. RSD is with 1.58 relatively low.
For response "Monomer content [%] at 40 °C for 1 week" R^2 is higher than 0.5. Q^2 is lower than 0.5. RSD is with 2.30 relatively low.
The calculated models are not fitted perfectly. Due to the fact that this experiment is only the screening for a preliminary formulation, the results are evaluated anyway.

For response T_{m1}, the most favorable conditions are found at pH 5.6, Tween® 20-concentration of 0.15 % and 100 g/L Trehalose with a melting temperature of T_{m1} = 73 °C. The worst conditions are pH 5.6, no Tween® 20 and no Trehalose with T_{m1} = 67 °C.
For responses "Aggregate content [%] at 40 °C for 1 week" and "Monomer content [%] at 40 °C for 1 week" the most favorable conditions are found at pH 6.8, no Tween® 20 and 100 g/L Trehalose with down to 0 % aggregation and 98 % monomer. The worst conditions are found at high concentrations of Tween® 20 from pH 5.6 to 6.8. Trehalose has a positive effect on aggregation property, especially at lower pH-values. The highest determined amount of aggregation was 5 % and the lowest amount of monomer was 93 %.

Table 3.11: **Formulation screening - Buffer composition and responses for Tween® 20 buffers**

Parameters			Responses		
pH	**Tween® 20 [%]**	**Trehalose [g/L]**	**T_{m1} [°C]**	**Aggregate content [%] 40 °C 1 week**	**Monomer content [%] 40 °C 1 week**
5.6	0	0	66.8	4.04	93.53
6.8	0	0	67.2	0.21	98.06
5.6	0.150	0	69.0	5.15	91.92
6.8	0.150	0	68.0	6.32	91.75
5.6	0	100	71.8	1.01	95.17
6.8	0	100	70.4	0.20	97.32
5.6	0.150	100	73.2	3.45	91.43
6.8	0.150	100	71.0	5.33	92.00
6.2	0.075	50	70.0	0.61	97.47
6.2	0.075	50	70.1	1.38	96.77
6.2	0.075	50	70.1	1.65	96.38

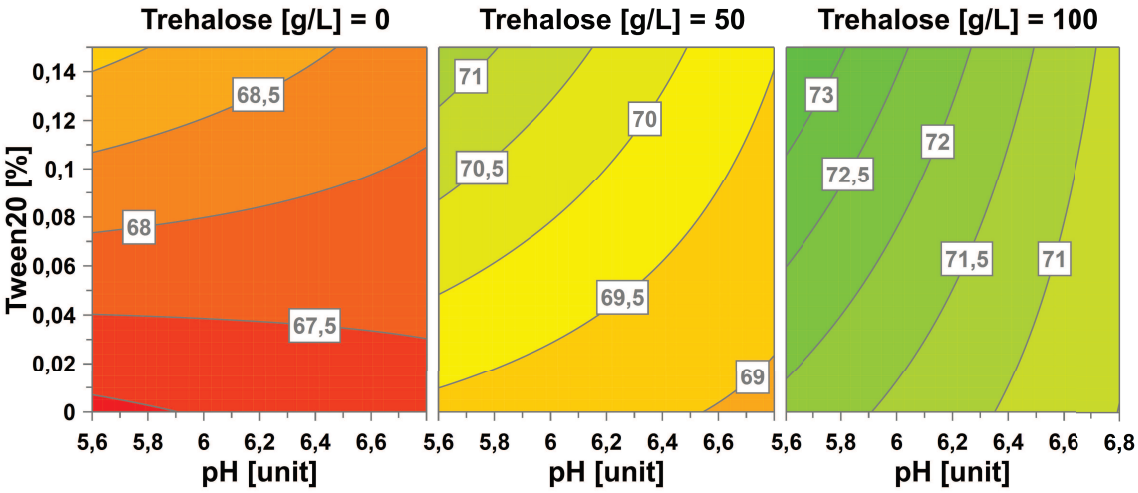


Figure 3.37: **Formulation screening - Tween® 20 - Melting temperature T_{m1}**
Numbers and heat lines in the plot represent T_{m1}; $R^2 = 0.99$; $Q^2 = 0.62$; RSD = 0.32

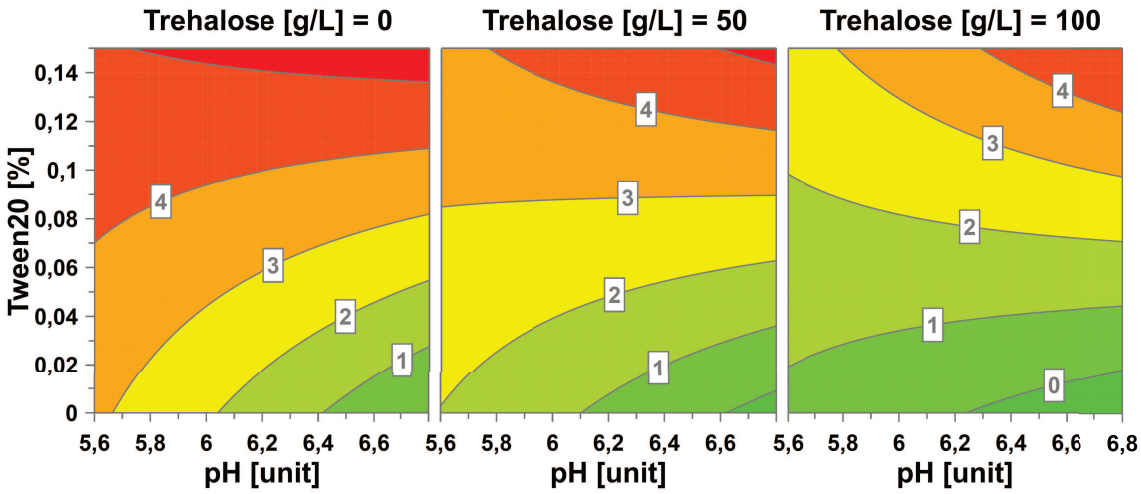


Figure 3.38: **Formulation screening - Tween® 20 - Aggregate content** [%] **after 1 week at 40 °C** Numbers and heat lines in the plot represent the aggregate content in % of protein; $R^2 = 0.80$; $Q^2 = 0.23$; RSD = 1.58

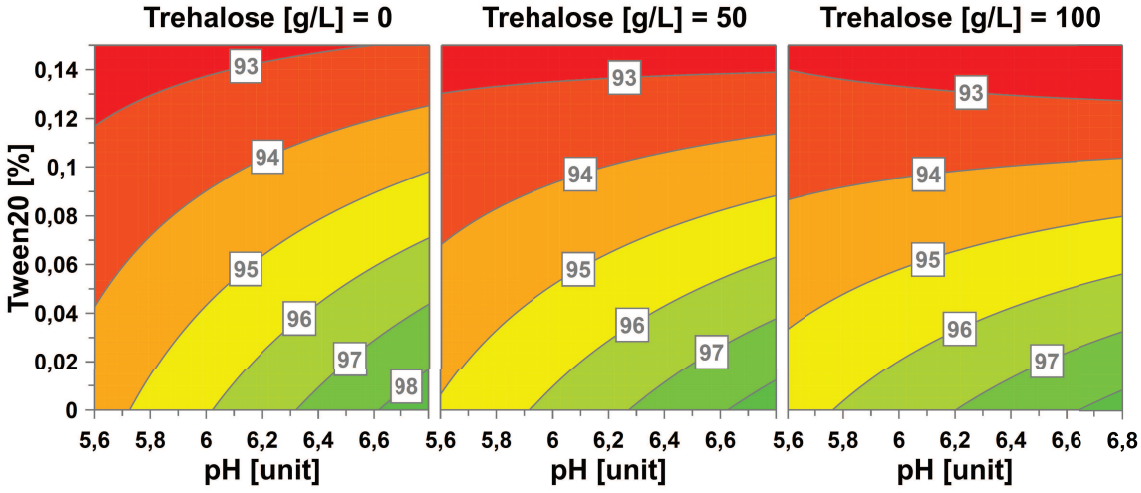


Figure 3.39: **Formulation screening - Tween® 20 - Monomer content** [%] **after 1 week at 40 °C** Numbers and heat lines in the plot represent the monomer content in % of protein; $R^2 = 0.69$; $Q^2 = 0.12$; RSD = 2.30

3.4.1.2 Results for Tween® 80 buffers

The results are presented in table 3.12 and figure 3.40. For response "Aggregate content [%] at 40 °C for 1 week", R^2 and Q^2 are both higher than 0.5. RSD is with 1.20 relatively low. The statistical significance of this model is very high and is evaluated.

A direct significant correlation of Tween® 80-concentration and the aggregation property can be observed. As a consequence, all further experiments with buffers containing Tween® 80 were omitted because this compound seems to induce aggregation.

Table 3.12: **Formulation screening - Buffer composition and responses for Tween® 80 buffers**

Parameters			Responses		
pH	**Tween® 80 [%]**	**Trehalose [g/L]**	**T_{m1} [°C]**	**Aggregate content [%] 40 °C 1 week**	**Monomer content [%] 40 °C 1 week**
5.6	0	0	66.8	4.04	93.53
6.8	0	0	67.2	0.21	98.06
5.6	0.150	0	69.2	23.51	71.70
6.8	0.150	0	68.8	21.86	76.54
5.6	0	100	71.8	1.01	95.17
6.8	0	100	70.4	0.20	97.32
5.6	0.150	100	74.4	22.82	73.72
6.8	0.150	100	71.8	23.34	74.53
6.2	0.075	50	70.3	10.99	87.45
6.2	0.075	50	70.4	10.10	88.32
6.2	0.075	50	70.6	11.84	86.53

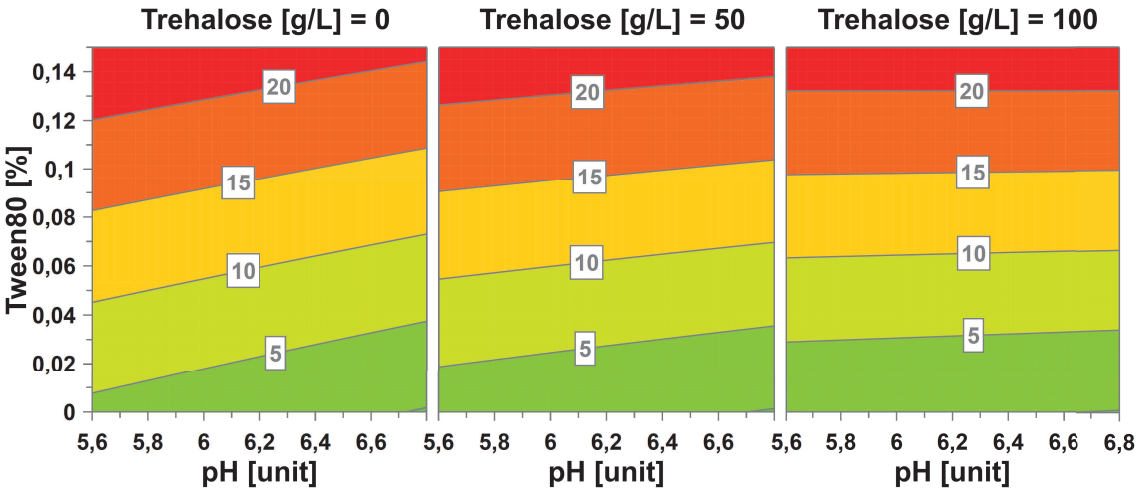


Figure 3.40: **Formulation screening - Tween® 80 - Aggregate content [%] after 1 week at 40 °C** Numbers and heat lines in the plot represent the aggregate content in % of protein; $R^2 = 0.99$; $Q^2 = 0.73$; RSD = 1.20

3.4.1.3 Sweet spot analysis

The sweet spot analysis tool allows the combination and visual presentation of acceptance criteria for various responses. In order to determine a sweet spot area for the Tween® 20 experiment, the following specifications were set for each response:

- T_{m1}: >70°C
- Aggregate content after 1 week at 40 °C: 0-1 % of whole peak area
- Monomer content after 1 week at 40 °C: >95 % of whole peak area

The sweet spot area is located in the area of pH 5.6 to 6.8 and Tween® 20-concentration of 0 to 0.075 % with 100 g/L Trehalose (see figure 3.41). The so-called sweet spot is marked in green, two criteria met is marked in pale blue, one criterion met is marked in dark blue and no criteria met is marked in white.

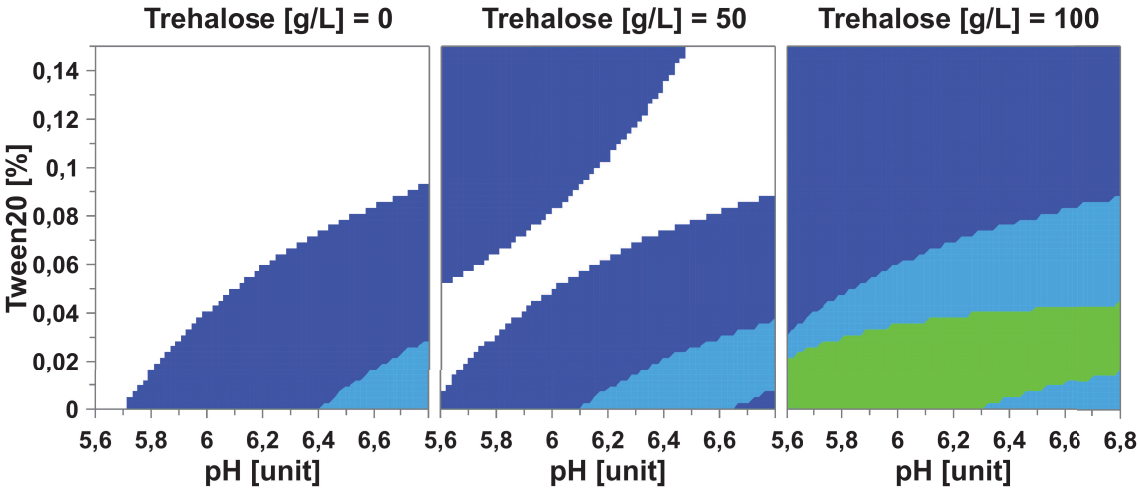


Figure 3.41: **Formulation screening - Sweet spot analysis**
The so-called sweet spot is marked in green, two criteria met is marked in pale blue, one criterion met is marked in dark blue and no criteria met is marked in white

3.4.2 Formulation optimization

Due to the fact that the sweet spot area of the formulation screening experiment is still very large, an optimization experiment was performed with the following constant properties for each optimization buffer:

- 20 mM Histidine
- Osmolality 300 mOsm/kg
- Antibody concentration 1 mg/mL
- 100 g/L Trehalose

The experiment was performed with the following factors (parameters) as full factorial analysis including star points in the central composite design face-centered (CCF-design):

- pH 5.60 to 6.80
- Tween® 20 from 0 % to 0.075 %

The buffers investigated are marked on the sweet spot plot of the formulation screening experiment (see figure 3.42).

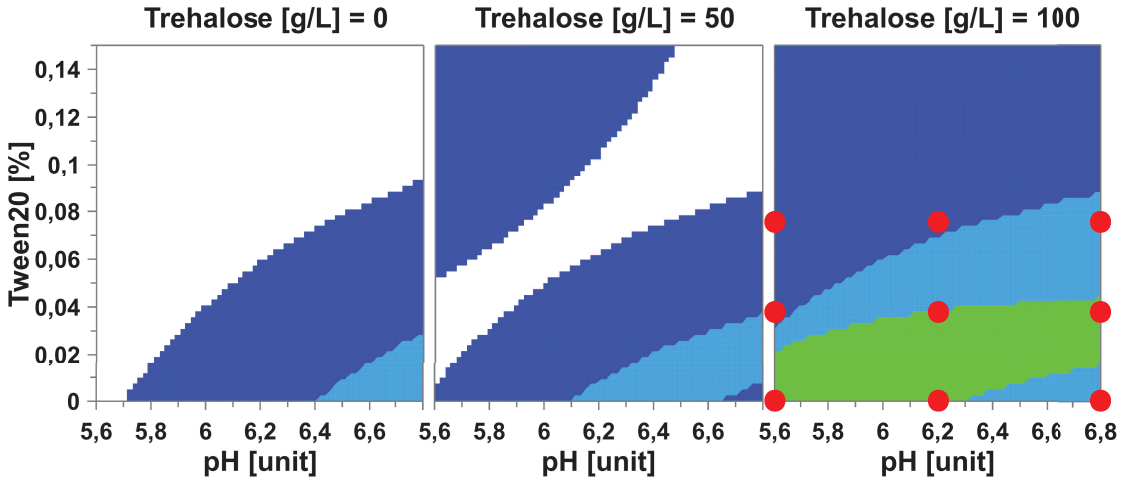


Figure 3.42: **Formulation optimization - Design region** deducted from the sweet spot analysis of the formulation screening experiment

The following responses were investigated:

- Melting temperature determined via DSF (T_{m1})
- Aggregate content after four weeks at 40 °C determined via SEC (Superdex) on UHPLC[1]

[1]The assessment of aggregate and monomer content was performed after 4 weeks instead of 1 week because no difference in SEC-profiles was detectable.

- Monomer content after four weeks at 40 °C determined via SEC (Superdex) on UHPLC

For evaluation of connections between factors and responses, the fitting method "partial least squares" (PLS) was performed by software "Modde 10.0". Results are depicted in heat maps. The R^2, Q^2 and residual standard deviation (RSD) of each plot are noted in the caption of figure 3.43.

3.4.2.1 Results for Tween® 20 buffers

For calculation of the models, all relationships that were not statistically significant were omitted from model fitting. This was done to increase the informative value of the models.
For response T_{m1} the omitted relationships were T20*T20 and T20*pH. For responses "Aggregate content [%] at 40 °C for 1 week" and "Monomer content [%] at 40 °C for 1 week" the omitted relationships included T20, T20*T20 and T20*pH.
For all responses the statistical parameters R^2, Q^2 meet their specifications and RSD is relatively low. Despite of some weaknesses in the model fitting, the statistical significance of these models is high enough for preliminary formulation studies.

For response T_{m1}, the best conditions were found at pH 5.8 and Tween® 20-concentration of 0.075 %. The melting temperature at these conditions was T_{m1} = 73.5 °C (see table 3.13 and figure 3.43). The worst condition was pH 6.8 / no Tween® 20; the melting temperature was less than 71 °C.
For responses Aggregates [%] and Monomer [%] the best conditions are found at pH-values higher than 6.2. The Tween® 20-concentration is irrelevant. Best values for aggregation were less than 0.5 % and more than 95 % monomer. The worst conditions are found at pH 5.6 with an aggregate content of more than 1.5 % and less than 90 % monomer.

Table 3.13: **Formulation optimization - Buffer composition and responses**

Parameters		**Responses**		
pH	**Trehalose [g/L]**	**T_{m1} [°C]**	**Aggregate content [%] 40 °C 4 weeks**	**Monomer content [%] 40 °C 4 weeks**
5.6	0	72.2	0.97	90.70
6.8	0	70.2	0.32	96.29
5.6	0.0750	73.6	1.66	89.85
6.8	0.0750	71.4	0.39	95.71
5.6	0.0375	73.3	2.68	85.80
6.8	0.0375	71.4	0.38	95.76
6.2	0	72.8	0.28	94.69
6.2	0.0750	73.0	0.24	94.57
6.2	0.0375	72.8	0.34	94.36
6.2	0.0375	72.7	0.31	94.24
6.2	0.0375	72.9	0.24	94.52

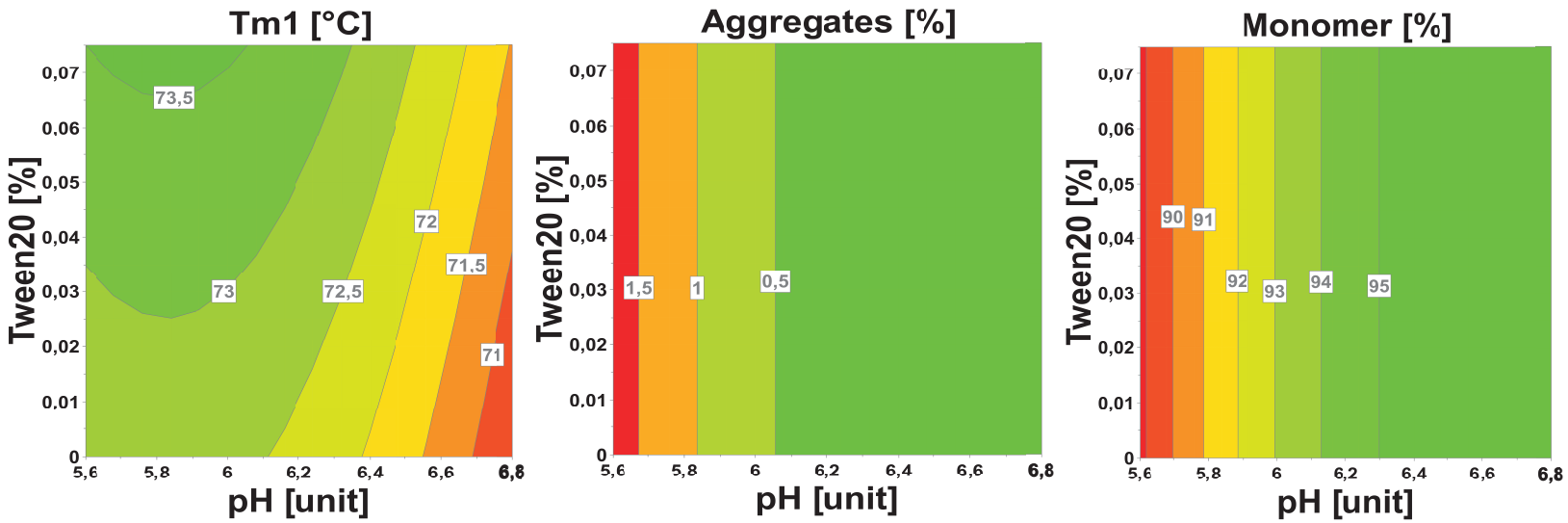


Figure 3.43: **Formulation optimization - Melting temperature T_{m1}, Aggregate and monomer content [%] after 4 weeks at 40 °C**
Tm1: $R^2 = 0.92$; $Q^2 = 0.77$; RSD = 0.34
Aggregates [%]: $R^2 = 0.76$; $Q^2 = 0.57$; RSD = 0.43
Monomer [%]: $R^2 = 0.86$; $Q^2 = 0.76$; RSD = 1.33

3.4.2.2 Sweet spot analysis

In order to determine a sweet spot area for the formulation optimization experiment, the following specifications were set for each response:

- T_{m1}: >72°C
- Aggregate content after 4 weeks at 40 °C: 0-1 % of whole peak area
- Monomer content after 4 weeks at 40 °C: >95 % of whole peak area

The sweet spot for these conditions is a narrow area in the range of pH 6.3 to 6.6 from 0 to 0.075 % Tween® 20 (see figure 3.44).

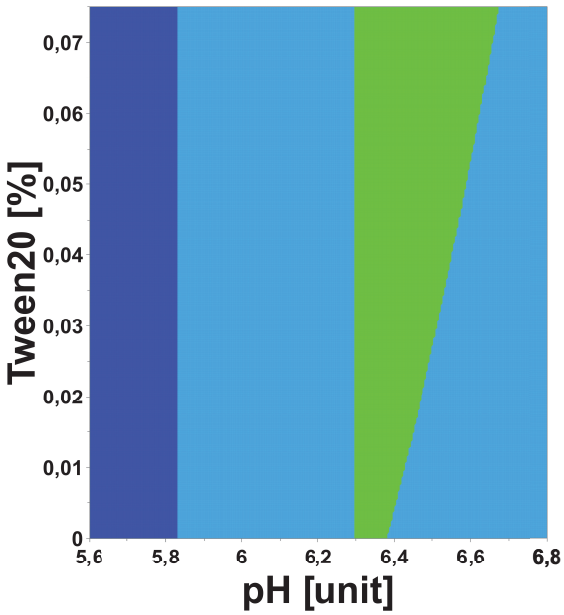


Figure 3.44: **Formulation optimization - Sweet spot analysis**
The so-called sweet spot is marked in green, two criteria met is marked in pale blue, one criterion met is marked in dark blue and no criteria met is marked in white

The analysis of the sweet spot plot results in a preliminary formulation buffer with the following properties:

- 20 mM Histidine pH 6.4
- 100 g/L Trehalose
- 0.05 % Tween® 20

Chapter 4

Discussion

Contents

The purpose of this thesis was to develop a production process and a preliminary protein formulation buffer for the bispecific antibody NF-CU$_{N297Q}$. The GMP-grade active pharmaceutical ingredient (API) obtained from the production process is indented for application in a clinical phase I/IIa trial for the treatment of AML.
A main issue of biopharmaceutical process development is to develope a process, which is capable of producing an API with the required purity. In most cases, regulatory authorities do not demand specified quality objectives but rather high quality and scientific and risk-based justification of specifications for critical quality attributes (CQA) (ICH-Q6B, 1999). Another fact to consider is the required amount of the API for a clinical phase I/IIa trial and for validation of all necessary production steps and assays. The outcome of these quality and quantity objectives is the prerequisite for process development.
The achievements of process development for bispecific antibody NF-CU$_{N297Q}$ will be evaluated in terms of these objectives.

4.1 Determination of quality objectives

ICH-Guideline Q11 subdivides critical quality attributes (CQAs) in properties or characteristics that affect identity, purity, biological activity and stability of the active pharmaceutical ingredient (API) (ICH-Q11, 2012).
In the following part, the determined specifications for identity, biological activity and stability of the API are discussed. Subsequently, specifications for product- and process-related impurities as well as for viral- and TSE-safety are discussed.
Specifications for CQAs were adopted from mandatory authoritative requirements, if existing. If no specific values are demanded, commonly applied specifications were adopted. If commonly applied specifications were not feasible (e.g. for product-related impurities), the determination of specifications was based on characterization experiments. All determined specifications for the process- and formulation development are summarized in table 5.5 at page 151.

4.1.1 Active pharmaceutical ingredient (API)

The monomeric bispecific antibody NF-CU$_{N297Q}$ can be identified via SDS-PAGE, isoelectric focusing electrophoresis and flow cytometric titration. The results must accord with theoretical values.
The purification process may influence the biological activity of the API. Thus, the specifications for biological activity were set as follows.
Flow cytometric analysis: a comparable binding property to cells presenting the antigens of interest compared to an appropriate reference and no loss of binding activity during purification.
T-cell proliferation assay: a comparable proliferation-inducing property of T-cells compared to an appropriate reference and no loss of activation properties during purification.
Potency assay (Promega): a comparable activity of the purified API of three batches.

The unspecific activation of T-cells in the absence of target cells was already observed in initial characterization experiments at the Department of Immunology (Durben, 2012; Durben et al., 2015). The assumption that this activation could be assigned to aggregates was not correct. The unspecific activation is triggered by the monomer which may bind to FLT3 on monocytes and thereby activates T-cells (Gabbianelli et al., 1995; Durben et al., 2015). This unintended biological activity is an immanent property of the monomeric bispecific antibody and can therefore not be reduced or removed.
Stability of an API must be ensured in all phases of clinical development and shelf-life of the product should be at least one year. No real-time/real-temperature studies in an appropriate formulation buffer were performed so far. However, surrogate specifications for thermodynamic and kinetic stability can be deducted from performed experiments and can be applied for formulation development: Melting temperature $T_{m1} > 72\,°C$, aggregate content $< 1\,\%$ after four weeks at 40 °C and monomer content $> 95\,\%$ after four weeks at 40 °C. If and to what extent these specifications correlate to a high stability and long shelf-life must be verified in real-time/real-temperature studies. Those studies are not part of this thesis. If a preliminary formulation buffer indicates sufficient stability, it should be suitable for application in clinical phase I/IIa trials. A profound formulation development and stability study should be performed for a clinical phase III trial.

4.1.2 Impurities

Specifications for the critical concentration of protein-based impurities such as aggregates, light chain dimers (LCD), light chains (LC), Host cell proteins (HCP) and Leached affinity ligands (LKS) were defined. These impurities are immunogenic and might therefore lead to the generation of α-API antibodies in patients (de Zafra et al., 2015; Romer et al., 2007). This might have a significant impact on efficacy. Thus, the specifications for these protein-based impurities should be very low in all phases of clinical development as well as for approved drugs (Chon and Zarbis-Papastoitsis, 2011).
Because of their API-specific properties, the biological activity of the product-related impurities aggregate, LCD and LC was characterized in order to develop scientific and risk-based purity specifications. These impurities exhibited only minimal biological activity. However, in order to increase safety and to minimize unintended immunological or renal reactions, the acceptable aggregate and LCD level was lowered to $< 1\,\%$ and to $< 2\,\%$ for LC (determined via SEC Superdex).
Specifications of process-related impurities were adopted from similar processes with monoclonal antibodies, because the CHO-based production process followed by biospecific interaction chromatography can be regarded as well-known. The specification for HCP was set to $< 100\,$ppm, for Host cell DNA (HCDNA)[1] to $< 10\,$ppm and for leached affinity ligand to $< 10\,$ppm. Those threshold values are generally regarded as safe (Chon and Zarbis-Papastoitsis, 2011; WHO-Biologicals,

[1] a commonly accepted specification for HCDNA content is 10 ng/dose; assuming a maximum dose of 1 mg/day, which is already overestimated (see 4.2 at page 116), a specification of 10 ng/mg (10 ppm) was set. Because of the dose-overestimation, this is a safe specification.

2010). The specification for the critical concentration of HCDNA was set very low because of the possibility to transmit oncogenes or proviruses along with the DNA. Despite the fact that a clinical confirmation of this mechanism has not been shown so far (11/2015), the specifications for this impurity should be low in all phases of clinical development because of the potential severe effects of a gene transmission. In contrast to most other impurities, the influence of endotoxins on patients is very well described. Thus, regulatory authorities issued mandatory specifications for this impurity: 5 IU/h/kg (Ph.Eur. 7.0, 2015).

4.1.3 Viral and TSE safety

Another critical aspect is to ensure viral safety. Specifications for viral safety were adopted from the European medicines agency (EMA): This regulatory authority states that a conversion of the demanded sterility assurance level (SAL) of 10^{-6} for sterile products to viral safety is justifiable; the assurance level is the likelihood of contamination risk per vial (EMA-CPMP/BWP/268/95, 1996). However, the confirmation of this assurance level can only be obtained in viral clearance studies with a small-scale model of the production process. That study is not part of this thesis.
The risk of transmissible spongiform encephalopathy (TSE) proteins is still not completely understood and therefore the specification from EMA is: "the absence of any TSE-proteins" (EMA-2011/C-73/01, 2011). The presence of these proteins should be completely avoided in all phases of clinical development as well as in drug approval. No animal-derived raw material will be used in the process (except for the use of the CHO-based production cell line). Thus, this specification will be fulfilled.

4.2 Determination of quantity objectives

4.2.1 Estimated dose, final product concentration and batch size requirements

A realistic dose estimation for an active pharmaceutical ingredient (API) is important for reliable process development. The batch size requirements for a planned clinical trial can only be deducted from this estimation. Because of the fact that no safety study with animals was conducted so far, the estimated dose can only crucially be derived from similar drugs in clinical development. The most recently FDA-approved bispecific antibody is Blinatumomab (FDA-Accessdata, 2015). In a clinical phase II trial, patients received 15 µg/m²/day for 28 days, which is about 30 µg per day and 840 µg in 28 days (Clinicaltrials.gov, 2015).
The minimal reliable filling volume for the intended liquid dosage form is 0.5 mL per vial and concentration is to be set to 1 mg/mL because previous observations indicated that the protein is stable at this concentration. In conclusion, 500 µg of NF-CU_{N297Q} is available per vial.
Assuming a medication cycle of at most 28 days, one patient needs 28 vials and

therefore 14 mg API. Assuming a maximum of 50 patients per trial, 700 mg API will be required for a clinical phase I/IIa trial.
However, 94 % of the vial content will be discarded with this liquid dosage form. The development of a lyophilized dosage form would be more economic; Blinatumomab contains 35 µg of lyophilized API per vial (BlinCyto, 2015). The development of a lyophilized dosage form will not be performed for the clinical phase I/IIa trial because of the higher development effort compared to a liquid dosage form. That development could be pursued for a clinical phase III trial or for drug approval.
Calculation of the required API for additional regulatory requirements such as safety studies, stability studies, a viral clearance study and validation of parts of production and quality control is difficult to predict at this stage of process development.

4.2.2 Technical limitations of the production process

KappaSelect binding proteins (KSBP) exhibit a $DBC_{10\,\%}$ of 18.5 mg/mL towards the KappaSelect affinity resin. A range of 30 to 70 % of this capacity will be applied in production process in order to avoid a shift of the proportion of monomer to LCD/LC; the latter residues exhibit a higher affinity towards the KappaSelect resin due to their smaller molecular weight and same (LC) or twice as many binding sites (LCD) compared to the monomer.
The smallest KappaSelect column connectable to an ÄKTA ready purification system is a 1 L column (GE-Healthcare, 2015) which can bind 5550 mg to 12950 mg KSBP at 30 - 70 % of $DBC_{10\,\%}$.
In a test fermentation run, productivity in a 10 L-wave-reactor was 110 mg/L KSBP consisting of approximately 55 % NF-CU_{N297Q}-monomer, 40 % LCD/LC and 5 % aggregates. A 100 L wave-reactor will be used in the production process. Therefore, approximately 11,000 mg KSBP comprising 6050 mg NF-CU_{N297Q}-monomer will be produced. Thus, a purification process with a polishing recovery of at least 80 % produces 5000 mg of polished NF-CU_{N297Q}-monomer. In conclusion, two to three batches should be sufficient for a clinical phase I/IIa trial including all regulatory requirements.

4.3 Achievement of quality objectives

4.3.1 Molecular biology and Cell banking

A stably transfected Chinese hamster ovary (CHO)-based clone was generated for permanent and reliable production of bispecific antibody NF-CU_{N297Q} under serum-free conditions. This primary seed bank (PSB)-clone is suitable for the generation of a Master cell bank (MCB) and a Working cell bank (WCB) under GMP-conditions. The MCB and WCB must be generated and tested according to ICH-Guidelines Q5D and Q5A (ICH-Q5D, 1997) (ICH-Q5A (R1), 1999). This task is not part of this thesis.
Quality objectives for the absence of microbial contamination, viral safety and

Transmissible spongiform encephalopathy (TSE)-proteins were met. Separate reports were generated for the plasmid construction (by Dr. Ludger Große-Hovest) and for the generation of a PSB (by Gregor Neumann).

4.3.2 Fermentation

Fermentation in a university-related setting was only possible because disposable fermentation equipment was available for lab- and verification scale of the fermentation. Verification scale was a 10 L-wave reactor. Development with stainless steel equipment would not have been feasible.
As already discussed in 4.2.2 at page 117, the achieved production titer of 110 mg/L KappaSelect binding protein (KSBP) is sufficient for the intended production process. Quality objectives for the absence of microbial contaminants and viral safety will be fulfilled when the production process is performed under GMP-conditions in a clean room. The fermentation will be performed with animal-component free fermentation media and equipment. Therefore, the quality objectives for TSE-safety will be fulfilled as well.

4.3.3 Purification

4.3.3.1 Affinity chromatography

KappaSelect binding proteins (KSBP) exhibit a $DBC_{10\,\%}$ of 18.5 mg/mL towards KappaSelect affinity resin. This is an average capacity compared to manufacturers data sheet (GE-Healthcare, 2012). GE Healthcare states that the $DBC_{10\,\%}$ for Fab-fragments is 15 mg/mL. NF-CU_{N297Q} has 1.75-times the weight of one Fab-fragment. Thus, KappaSelect should theoretically bind 26.25 mg NF-CU_{N297Q} per mL KappaSelect resin. This capacity is propably reduced due to sterical reasons and therefore the value of 18.5 mg/mL is an average capacity. This $DBC_{10\,\%}$ is sufficient as already discussed in 4.2.2 at page 117.
The affinity chromatography was not further optimized in terms of removal of product- and process-related impurities. A modification of washing conditions or flow rates was not evaluated because the polishing process in small-scale model AH1 was sufficiently effective to allow removal of product- and process-related impurities.

4.3.3.2 Polishing applications

Usually, the purity achieved after initial purification is not sufficient for application in a clinical phase I/IIa trial. Thus, additional chromatographic techniques were evaluated for their suitability to purify the active pharmaceutical ingredient (API) without affecting its biological activity. Those methods are termed “polishing applications”. This discussion part refers to the buffer verification models (see results part 3.3.2.2 at page 61 ff.).

Anion exchange chromatography (AIEX):
When comparing the isoelectric points (IEPs) of the monomer and LCD/LC to the IEPs of process-related impurities, it can be seen that a high pH (but lower

than the IEP of the monomer) is favorable. In that way, all components with IEPs below the applied pH bind to the positively charged resin, whereas the components with higher IEPs such as the monomer and the aggregate flow through the column (see figure 4.1). A pH-value of 8.00 and conductivity of 3.0 mS/cm is therefore very suitable for removal of product- and process-related impurities.
The reduction of the aggregate-, LCD and LC content is efficient with this method. However, a second, at best orthogonal chromatography step such as Hydrophobic interaction chromatography (HIC) or Multimodal chromatography (MMC) would be necessary for a more efficient removal of these impurities. Yield is average at 89 %. The method effort is low due to the application of a flow-through mode (FT mode). The theoretical properties for removing process-related impurities and viruses are very promising. The removal of product- and process-related impurities was verified in the small-scale model (see chapter 4.3.3.3 at page 121).

Cation exchange chromatography (CIEX):
In figure 4.1 can be seen that the application of a high pH (but lower than the IEP of the monomer) is favorable. In that way, only the monomer and the aggregate bind to the negatively charged resin, whereas all the other impurities flow through the column. A pH-value of 7.30 and conductivity of 3.5 mS/cm is therefore a suitable application buffer for the removal of impurities.
Unfortunately, aggregation is induced by this method. This may be caused by a fast conductivity shift from 3 to 15 mS/cm (binding to elution), which might induce aggregate formation. The method effort is very high due to the performance of a bind-elute (BE) mode and due to the small bead size of 40 µm, which results in a low flow rate.

Comparison of AIEX and CIEX for purification of NF-CU$_{N297Q}$
The most significant disadvantage of CIEX compared to AIEX is the aggregate-inducing property. A significant advantage of AIEX compared to CIEX is, that the DBC$_{10\,\%}$ of impurities towards the AIEX resin will not be reached in practice due to their low concentration compared to the monomer. In contrast, the DBC$_{10\,\%}$ of the monomer on a CIEX resin can very easily be reached and must be evaluated. Another advantage of AIEX is the applicability of a higher pH compared to CIEX resulting in better impurity removal (see figure 4.1). AIEX ensures a faster process step, because the FT mode is faster than the BE mode. Furthermore, a FT mode requires less development effort, because no elution buffer must be developed. The elution buffer of the BE mode has a higher conductivity than the FT-buffer and therefore co-elutes more impurities than the FT-buffer.
One advantage of CIEX is the enrichment of the monomer, whereas AIEX dilutes the monomer.
In conclusion, AIEX exhibits more advantages than CIEX for purification of NF-CU$_{N297Q}$ and should be applied. In addition, CIEX is not suitable, especially because of the aggregate-enriching properties.

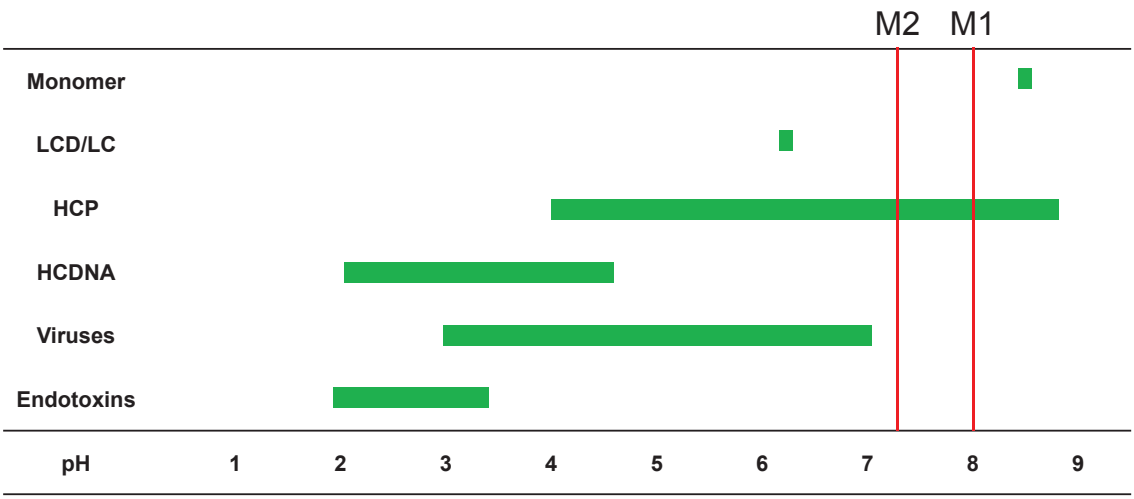


Figure 4.1: **Isoelectric points/areas (IEP) of process- and product-related impurities**
Marker M1: pH-conditions for Anion exchange chromatography
Marker M1: pH-conditions for Cation exchange chromatography
LCD = Light chain dimer; LC = Light chain; HCP = Host cell proteins; HCDNA = Host cell DNA

Hydrophobic interaction chromatography (HIC):
Due to the fact that the performance of a protein on a HIC chromatography medium is difficult to predict, seven different media were evaluated for their suitablity to separate the monomer from LCD/LC with the same buffer conditions. Because of the relatively low aggregate content, this KSBP could not be evaluated in the purification chromatograms. LCD/LC on the other hand, represent almost 50 % of KSBP and can therefore be used as "reporter impurities". The resin with the best peak separation was chosen for further investigation; Phenyl HP. LCD and LC elute at a lower conductivity than the monomer and are therefore more hydrophobic than the monomer. This was an expected behavior, because in mAbs, heavy and light chain heterodimerize not only via disulfide bonds, but also through hydrophobic interaction in the molecule parts directed to the complementary chain. If one of these chains is not paired, it exposes a huge hydrophobic flank resulting in increased hydrophobicity compared to a mAb-monomer. The aggregate is also more hydrophobic than the monomer; when the monomer is exposed to stressful conditions such as low pH, the tertiary structure partially unfolds and exposes hydrophobic flanks resulting in aggregation. Some of these flanks are still exposed on the surface of the aggregate and result in higher hydrophobicity compared to the monomer.
In conclusion, the method is suitable for the removal of aggregates and LCD/LC. Yield is average at 90 %. An advantage of this HIC-process is the possibility to perform the process in FT mode (see AIEX). The conductivity, which is required for separation is not very high (18 mS/cm) and did not show any influence on biological activity.
In contrast to that, it is not possible to predict the properties of process-related impurities on HIC. Those properties were verified in the small-scale model (see chapter 4.3.3.3 at page 121).

Multimodal chromatography (MMC):
The properties of KSBP and process-related impurities on a MMC chromatography medium are almost impossible to predict. Because of the HIC-properties of MMC chromatography media, processes are much more salt tolerant than classical CIEX applications. Required load and elution conductivities are usually much higher and were determined with 20 and 63 mS/cm respectively. The high load conductivity resulted in reduced binding capacity for the monomer. The even higher elution conductivity might have an influence on biological activity of the monomer and might lead to co-elution of previously bound impurities. The application of a BE mode is a disadvantage in later production process.
Removal of aggregates and LCD/LC is very efficient with this MMC method. Unfortunately, the yield is too low with only 75 %. This might be the consequence of the high binding conductivity as already assumed earlier (see above). The MMC process would be suitable for further optimization. However, the process was not developed further because the results of AIEX and HIC were more promising and were verified with a small-scale model.

Summary and conclusion:
Biological activity does not decrease after application of the polishing methods; no difference in binding properties and T-cell proliferation induced by the API was observed.
In conclusion, a very reasonable arrangement for polishing is the use of AIEX and HIC. The methods utilize different chemical properties of the API and the impurities and therefore ensure high purity. Despite the fact that no theoretical predictions about removal properties for process-related impurities are feasible for HIC, those predictions are very promising for AIEX and were verified with a small-scale model. The intended application of AIEX would therefore be the removal of LCD/LC, some aggregates, process-related impurities and viruses, whereas HIC removes LCD/LC and especially aggregates.
When a polishing process is performed with the AIEX method first and then the HIC method, no buffer exchange would be required. Subsequently after performance of AIEX in FT mode, $(NH_4)_2SO_4$ is added to increase conductivity and a second FT-mode is performed with HIC. Thus, because of two FT modes, the process can be performed very fast and no elution buffers must be developed.

4.3.3.3 Process verification

Process verification refers to the small-scale model AH1 (see results part 3.3.3 at page 92 ff. and appendix chapter 5.3 at page 139).

Process AH1 combines the initial purification using KappaSelect and the polishing applications anion exchange and hydrophobic interaction chromatography. The process exhibits very effective removal properties for aggregates and LCD/LC. This correlates to previous results (see above).
Monomer yield decreases with increasing load of KSBP onto the process columns. Process AH1.2 with 131 % KSBP load exhibits a higher concentration than process AH1.3 with 90 % KSBP load. This might result in more unintended retention of

the monomer to the AIEX or HIC resins. Thus, 100 % load, which equals 130 mg KSBP for the small-scale model should be pursued.
For all three produced batches, SDS-PAGE, SEC (Superdex), CIEX / UHPLC were nearly identical. CIEX / UHPLC shows three acidic variants, two basic variants and one main peak of the API in all three batches with similar peak areas. Thus, this pattern indicates a stable, robust and reproducible process. Usually, glycosylation is responsible for this phenomenon. NF-CU_{N297Q} is not N-glycosylated. The acidic variants may be the result of posttranslational modifications such as asparagine deamidation. Basic variants may be the result of pyroglutamate formation from N-terminal glutamine (Brorson and Jia, 2014; Rehder et al., 2006).
HCP and HCDNA are efficiently removed especially during AIEX purification and meet their specifications. This correlates to earlier predictions (see above). The removal of leached KappaSelect (LKS) could not be verified because of an influence of LCD/LC on the ELISA readout. LCD and especially LC did not completely denature at 95 °C for 15 min. and seemed to bind to KappaSelect ligand with their binding sites resulting in a false-low signal. The theoretical properties of AIEX for removal of LKS-Ligand are very promising due to its low IEP of 5.
Determination of endotoxin-level has no informative value, since the research-equipment might be contaminated. The endotoxin concentration will be determined when process AH1 is performed in process scale under GMP-conditions. The risk of contamination will be very low, because no gram-negative bacteria such as *Escherichia coli* will be used for production. However, removal properties of AIEX for endotoxins are very promising because of the low IEP of Lipopolysaccharides.
All titration curves of flow cytometric analysis of IPCs are comparable, no loss of affinity is observed during purification. All titration curves of the T-cell proliferation assay of the IPCs are comparable, no loss in proliferation-inducing properties during purification can be observed. Potency assay (Promega) is a very sensitive assay. A minor reduction of potency compared to the reference can be observed in all three batches. Perhaps the polishing resins alter the secondary and / or tertiary structure of the monomer to a small extent, which cannot be observed in flow cytometric analysis or T-cell proliferation assays. This loss of potency is not critical as long as it is reproducible. All three batches show almost the same extent of potency loss and the $EC_{50} = 3.0$ ng/mL still indicates a high potency. Thus, this phenomenon can be considered as not critical.
In conclusion, the developed small-scale model AH1 is suitable for up-scaling to a production process. The API produced with a process based on small-scale model AH1 is suitable for application in a clinical phase I/IIa trial. A summary of the achievement of quality objectives can be found in table 5.5 at page 151.

4.3.4 Viral and TSE safety

The three orthogonal virus reduction steps pH-inactivation, anion exchange chromatography and nanofiltration combined with the profound testing of the parental cell line and production under GMP-conditions reduce the risk of viral contamination of the final product tremendously. The effects of the virus reduction steps can only be discussed theoretically because the required viral clearance study was not performed so far.

pH-inactivation is a commonly applied method to reduce viral load and usually exhibits a logarithmic10 reduction value (LRV) of 4 for viruses. The isoelectric points of viruses are mostly acidic (see figure 4.1) and therefore they bind to the positively charged anion exchange resin at pH 8.00. In the applied flow-through mode, the viral load gets reduced as previously discussed for product- und process-related impurities. This methods usually exhibits a LRV of 5 for viruses.
Nanofiltration can be performed in less than 1.5 h with all three selected filters Planova™ 15 N, 20 N and BioEx. Thus, all filters would be suitable for nanofiltration in the production process. Nanofiltration usually exhibits LRVs of 4 to 6 for viruses. Nevertheless, product recovery is an issue for Planova™ BioEx with only 88 %. This may be due to the fact that the filtration fibers consist of hydrophilic modified polyvinylidene fluoride instead of cuprammonium regenerated cellulose in Planova™ 15 N and 20 N. This might lead to enhanced adsorption of the API. Monomer recovery is slightly better in Planova™ 20 N and filtration time is much shorter than in Planova™ 15 N. The suffix 15 N does not mean that the actual pore diameter of the filter is smaller and therefore holds back more viruses. Process development scientists of "Asahi Kasei" asserted that a statement about the comparability of virus removal between 15 N and 20 N can only be made after removal validation of spiked viruses from the API (viral clearance study). These results and the fact that Synimmune GmbH has the most experience with Planova™ 20 N resulted in the decision to apply this filter.
The confirmation of the viral safety assurance level of 10^{-6} can only be obtained in viral clearance studies with a small-scale model of the process. This study is not part of this thesis. However, the theoretical LRVs for viruses are very promising for this process.
TSE safety is achieved by completely avoiding the use of animal-derived raw material.

4.3.5 Preliminary formulation development

The development of the antibody formulation is regarded as preliminary. The tested buffers were suggestions by Warne et al. for antibody formulations for a clinical phase I trial (Warne, 2011). Despite a lack of fit for some mathematical models, the results of the formulation development were evaluated. However, formulation buffer development for a clinical phase III trial should include evaluation of more potential buffer components.
The suitability of a test formulation was assessed in thermodynamic and kinetic stability studies of the bispecific antibody in the respective buffer.

In the formulation screening experiment, three factors (parameters) which might influence each other were altered simultaneously. The three factors were pH-value, concentration of Trehalose and concentration of Tween® 20 or 80. The responses were the melting temperature T_{m1}, the aggregate- and the monomer content after 1 week at 40 °C of thermal stress. Evaluation and illustration of the connection of factors and responses was performed with an DoE approach.
For formulation screening of Tween® 20-buffers, the responses have their optimum in different parts of the evaluated buffer composition area (design region). However,

a high Trehalose concentration and a certain amount of Tween® 20 is necessary for optimal thermodynamic and kinetic stability of the protein. The effects of these two responses are pH-dependent.
The relevance of the responses for evaluation can be balanced with the sweet-spot analysis tool. Cutoff for T_{m1} was set not as strict as for responses aggregate and monomer content after 1 week at 40 °C. The reason for this is that the latter responses represent kinetic stability, which correlates more to the shelf-life of a protein. In addition, T_{m1} is already very high even in unfavorable buffers compared to other proteins (Menzen and Friess, 2013). The sweet-spot area was further investigated in the formulation optimization experiment.
In the formulation screening experiment of Tween® 80-buffers, only response "Aggregate content [%] at 40 °C after 1 week" was evaluated. A direct correlation of Tween® 80-concentration and aggregation can be seen. This observation was completely surprising; Tween® 80 is a common surfactant to prevent protein aggregation, not to induce it. All subsequent experiments with Tween® 80-buffers were omitted.

For formulation optimization, the sweet spot area of the Tween® 20-buffers from the formulation screening experiment was further investigated.
Trehalose concentration was kept constant at 100 g/L and therefore only two factors were altered during the experimental setting; pH-value and Tween® 20-concentration. A standard optimization design includes so-called star-points. The central composite design face centered (CFF)-design was chosen. The assessment of aggregate and monomer content was performed after 4 weeks instead of 1 week because no difference in SEC-profiles was detectable after 1 week. This indicates a high kinetic stability of the protein. Due to the fact that more experiments were performed in the design region, the optimization design resulted in a better mathematical model fitting than for the screening experiments. All non-significant connections between factors and responses were omitted from the model fitting, this is an accepted convention.
For evaluation of the formulation optimization experiments, T_{m1} was rated more important. The sweet spot analysis results in a relatively large area of suitable buffer compositions. The following buffer was chosen as preliminary formulation buffer:

- 20 mM Histidine pH 6.4
- 100 g/L Trehalose
- 0.05 % Tween® 20

ICH-Guideline Q5C states that the determination of the expiration date should be based on real-time/real-temperature studies (ICH-Q5C, 1995). Nevertheless, the use of accelerated stability studies for determination of thermodynamic and kinetic stability of a protein in certain formulation buffers is common (Weiss et al., 2009). However, it has been shown that promising results in accelerated stability studies do not always correlate to a longer shelf-life (Wang et al., 2007). Thus, these results must be verified with real-time/real-temperature studies.

In conclusion, API formulated in the developed preliminary formulation buffer should be suitable for application in a clinical phase I/IIa trial. A summary of the achievement of quality objectives can be found in table 5.5 at page 151.

4.4 Review of the development process

The special aspect of this thesis was the realization of good manufacturing practice (GMP)-compliant late-stage development of a protein-based pharmaceutical drug at an university-related setting. This section evaluates the technical feasibility to achieve previously defined quality and quantity objectives at a small spin-off company operating at an university campus.

The profound characterization of biological activity of product-related impurities was performed with standard equipment of the Department of Immunology. Process development is possible with qualified personal and minimal resources. However, the development process requires a profound overview over various technical and legal requirements. Nevertheless, a viral clearance study must be entrusted to a specialized company. In addition, the success of the preliminary formulation development can only be assessed in real time/real temperature experiments. However, the formulation development approach performed in this thesis should be applicable for a wide range of antibody therapeutics.
The development process was performed in a period of 1.5 years. The investment costs were about 150,000 € and include a UHPLC, an ÄKTA-FPLC, a Q-PCR-device, an orbital shaker and a 10 L wave reactor. These expenses do not include standard university equipment such as scales, centrifuges and gel chambers. Personnel costs were 55,000 € and the expenses for chemicals, reagents, chromatography columns, cell culture media, kits and plastic supply were about 24,000 €. This calculation of costs does not include expenses for the transfection plasmid, the cell line or the viral clearance study. Those development expenses are very low compared to the development costs at a contract manufacturing organization.
In conclusion, a small spin-off company operating at an university campus is capable of developing a production process and formulation buffer for a biopharmaceutical drug for application in a clinical phase I/IIa trial. This fundamental work lowers the expenses for GMP-development and -production at a contract manufacturing organization. The consecutive process development for a clinical phase III trial should be performed by a pharmaceutical company with development experience.

4.5 Outlook

The next step towards a clinical phase I/IIa trial is to up-scale process AH1 and to perform a verification run under GMP-conditions. The process data should be utilized to verify the up-scaling and to verify or adapt specifications for critical quality attributes (CQAs). The department of quality control must develop an ELISA for leached KappaSelect and validate all required assays. The formulated API can be used for safety studies (ICH-Guidelines S1-S11). The master and working cell banks must be developed and characterized.

Subsequently, a viral clearance study can be performed. The virus removal of the following process steps should be validated: viral inactivation via pH, AIEX-polishing and nanofiltration. In addition, a validation of the final sterile filter and of the filling of drug product is requested. After validating the safety assays of the quality control department, the production and quality parts of an investigator medicinal product dossier (IMPD) can be written. The IMPD is a direct prerequisite for a clinical trial.

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Chapter 5

Appendix

Contents

5.1 List of abbreviations

°C	Degree Celsius
AC	Affinity chromatography
ACF	Animal component-free
ADC	antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
AIEX	Anion exchange chromatography
AH1	Polishing process combination AIEX, HIC number 1
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
API	Active pharmaceutical ingredient
Appl.	Application
AU	Absorption units
BE mode	Bind-elute mode
CCF	central composite design face-centered
CDC	Complement dependent cytolysis
CDR	Complementary determining region
CHO	Chinese hamster ovary
CIEX	Cation exchange chromatography
CD	Cluster of differentiation
Conc.	Concentration
Cond.	Conductivity
CQA	Critical quality attribute
Cs	Caesium
CV	Column volume
Da	Dalton
$DBC_{10\,\%}$	Dynamic binding capacity at 10 % flow through
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate buffered saline
DSF	Differential scanning fluorimetry
EC_{50}	Effective concentration for 50 %
ELISA	Enzyme-linked immunosorbent Assay
E:T	Effector : target ratio
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	Food and drug administration
FF	Fast flow
FFF	Field flow fractionation
FPLC	Fast protein liquid chromatography
FT mode	Flow-through mode
g	Gram
GMP	Good manufacturing practice
Gy	Gray
h	Hour
HCDNA	Host cell deoxyribonucleic acid
HCP	Host cell protein
HIC	Hydrophobic interaction chromatography
HP	High performance

ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IEP	Isoelectric point
IEX	Ion exchange chromatography
IMDM	Iscove's modified Dulbecco's medium
IMPD	Investigator medicinal product dossier
IPC	In process control
IU	International units
KS	KappaSelect
KSBP	KappaSelect binding protein = Aggregates, Monomer, LCD and LC of NF-CU$_{N297Q}$
L	Litre
LC	Light chain
LCD	Light chain dimer
LKS	Leached KappaSelect
LPS	Lipopolysaccharides
LRV	Logarithmic10 reduction value
m	Meter
M	Molar
mAb	monoclonal antibody
MEM-NEAA	Minimum essential Medium - Non-essential Amino Acid Solution
MCB/WCB	Master cell bank / Working cell bank
MDS	Myelodysplastic syndrome
MFI	Mean fluorescence intensity
min.	Minute
MMC	Multimodal chromatograpy
Mon	Monomer
MRD	Minimal residual disease
MW	molecular weight
NFAT	Nuclear factor of activated T-cells
OD_{280}	Optical density at $\lambda = 280\,\text{nm}$
Osm	Osmol
Pa	Pascal
PBMC	Peripheral blood mononuclear cell
pH	pondus Hydrogenii
Ph.Eur.	Pharmacopoeia europea = european pharmacopoeia
PHA	Phythemaglutinine
pKa	Negative log of acid dissociation constant
PLS	Partial least squares
ppm	Parts per million
PSB	Primary seed bank
QPCR	Quantitative polymerase chain reaction
R&D	Research and development
Ref	Reference
RLU	Relative luminescence units
RPMI	Roswell Park Memorial Institute medium
RSD	Residual standard deviation
S	Siemens
scFv	Single chain Fragment variable (antibody fragment)
SDS-PAGE	Sodiumdodecylsulfate - Polyacrylamide gel electrophoresis

SEC	Size exclusion chromatography
TFF	Tangential flow filtration
TMP	Trans membrane pressure
TSE	Transmissible spongiform encephalopathy
(U)HPLC	(Ultra) high performance liquid chromatography
V	Volume

5.2 Amino acid sequence of NF-CU$_{N297Q}$

Heavy chain:
QVQLQQPGAELVKPGASLKLSCKSSGYTFTSYWMHWVRQRPGHGLEWIGE
IDPSDSYKDYNQKFKDKATLTVDRSSNTAYMHLSSLTSDDSAVYYCARAITT
TPFDFWGQGTTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPE
PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKKVEPKSCDKTHTSPPSPAPPVAGPSVFLFPPKPKDTLMISRT
PEVTCVVVGVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKQLPSPIEKTISKAKGQPSGDIQMTQSPSSLS
ASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFSG
SGSGTDYTLTISSLQPEDFATYYCQQGNTLPWTFGQGTKVEIKGGGGSGGG
GSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGK
GLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAV
YYCARSGYYGDSDWYFDVWGQGTLVTVSS

Light chain:
DIVLTQSPATLSVTPGDSVSLSCRASQSISNNLHWYQQKSHESPRLLIKYASQ
SISGIPSRFSGSGSGTDFTLSINSVETEDFGVYFCQQSNTWPYTFGGGTKLEI
KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG
NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF
NRGEC

5.3 Small-scale model

5.3.1 Purification

Table 5.2: **Small-scale model - Process Parameters for AH1.1-3**; IPC = In process control

		AH1.1	AH1.2	AH1.3
KappaSelect V = 10 mL; h = 20 cm; v = 400 cm/h				
Application	Name Supernatant	Spin150128-A	Spin150512-E	
	Volume Supernatant [mL]	1064	1060	790
	Amount KSBP [mg]	143 (110 %)	178 (135 %)	119 (90 %)
	Wash with PBS until OD_{280}	< 20 mAU	< 20 mAU	< 20 mAU
Elution	Frac from to	100 mAU to 100 mAU		
	Volume Elution [mL]	12.8	13.9	12.3
	pH	2.86	2.86	2.78
Preparation for AIEX (Capto Q)				
	Add. 150 mM Tris pH 9.60 [mL]	5.40	5.45	5.45
	pH	7.994	7.999	7.997
	Cond. [mS/cm]	4.65	5.02	4.32
	Add. water [mL]	10.5	14	9
	Cond. [mS/cm]	2.98	3.02	3.01
	Volume (sum) [mL]	28.7	33.35	26.75
	⟹IPC-1			
AIEX (Capto Q): V = 9.4 mL; h = 20 cm; v = 150 cm/h				
Application	Volume [mL]	26	30	21,7
	Amount Monomer [mg]	71.70	85.85	50.71
Elution	Frac from to	50 mAU to 100 mAU		
	Volume Elution [mL]	33.5	37.6	28.8
	⟹IPC-2			
Preparation for HIC (Phenyl HP)				
	Add. 1M $(NH_4)_2SO_4$ [mL]	2.8	3.4	2.2
	Cond. [mS/cm]	18,6	18.5	18.75
	Conc. [mg/mL]	2.05	2	1.72
HIC (Phenyl HP) V = 9.4 mL; h = 20 cm; v = 120 cm/h				
Application	Volume [mL]	31.6	37	27
	Amount Monomer [mg]	60.39	66.96	43.15
Elution	Frac from to	50 mAU to 100 mAU		
	Elution volume	37	42	32
	⟹IPC-3			

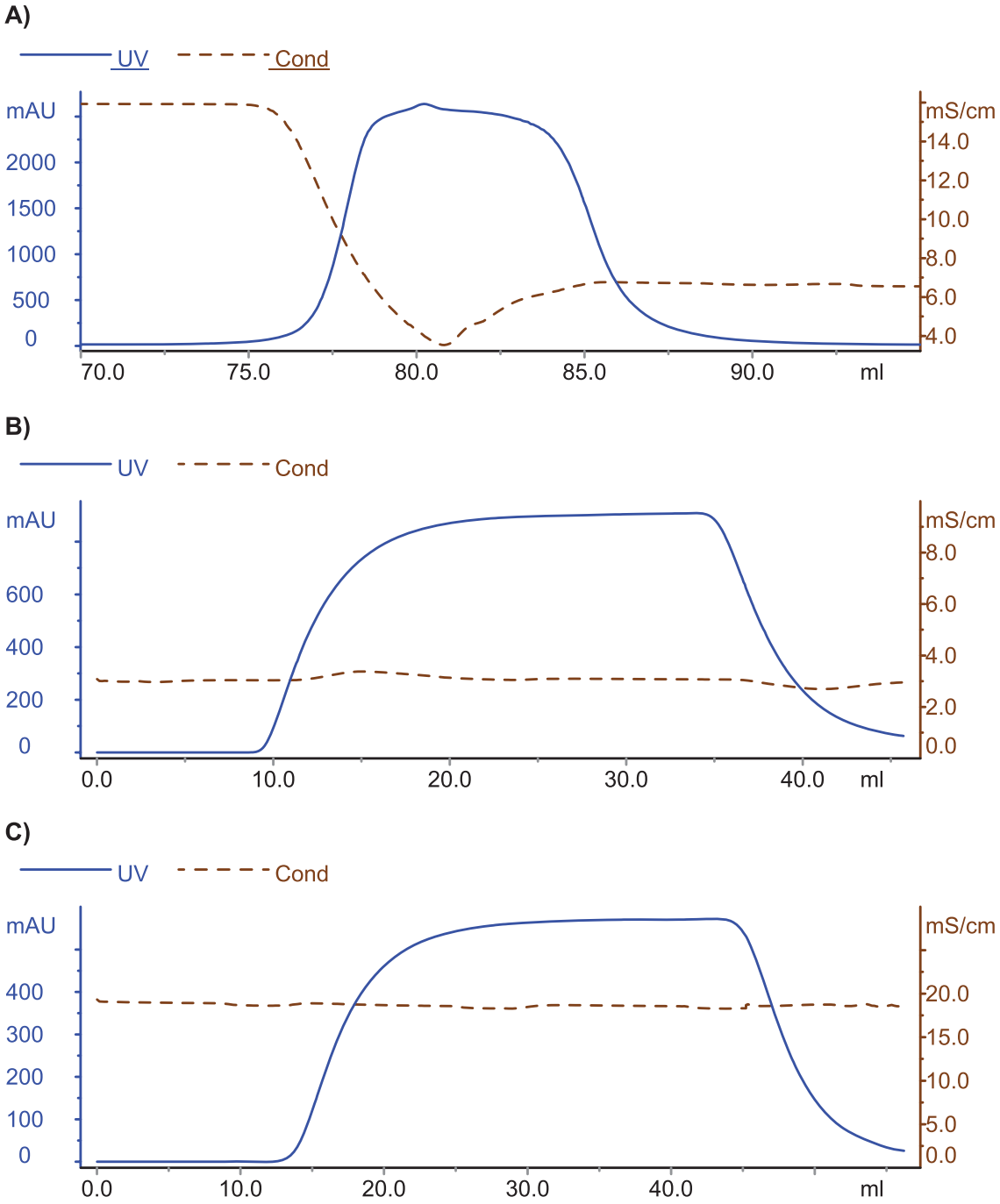

Figure 5.1: **Small-scale model for production process - Process AH1.1**
Column dimensions: 2 x Hiscreen™ (V = 9.4 mL; h = 20 cm) for each purification step
Amount KSBP: 142.5 mg (110 %)
A: KappaSelect; v = 400 cm/h; BE mode DPBS and 0.1 M Glycine pH 2.5
B: AIEX; Capto Q; v = 150 cm/h; FT mode 50 mM Tris pH 8.00 / Cond. 3 mS/cm
C: HIC; Phenyl HP; v = 120 cm/h; FT mode 50 mM Tris pH 8.00 / Cond. 18.5 mS/cm

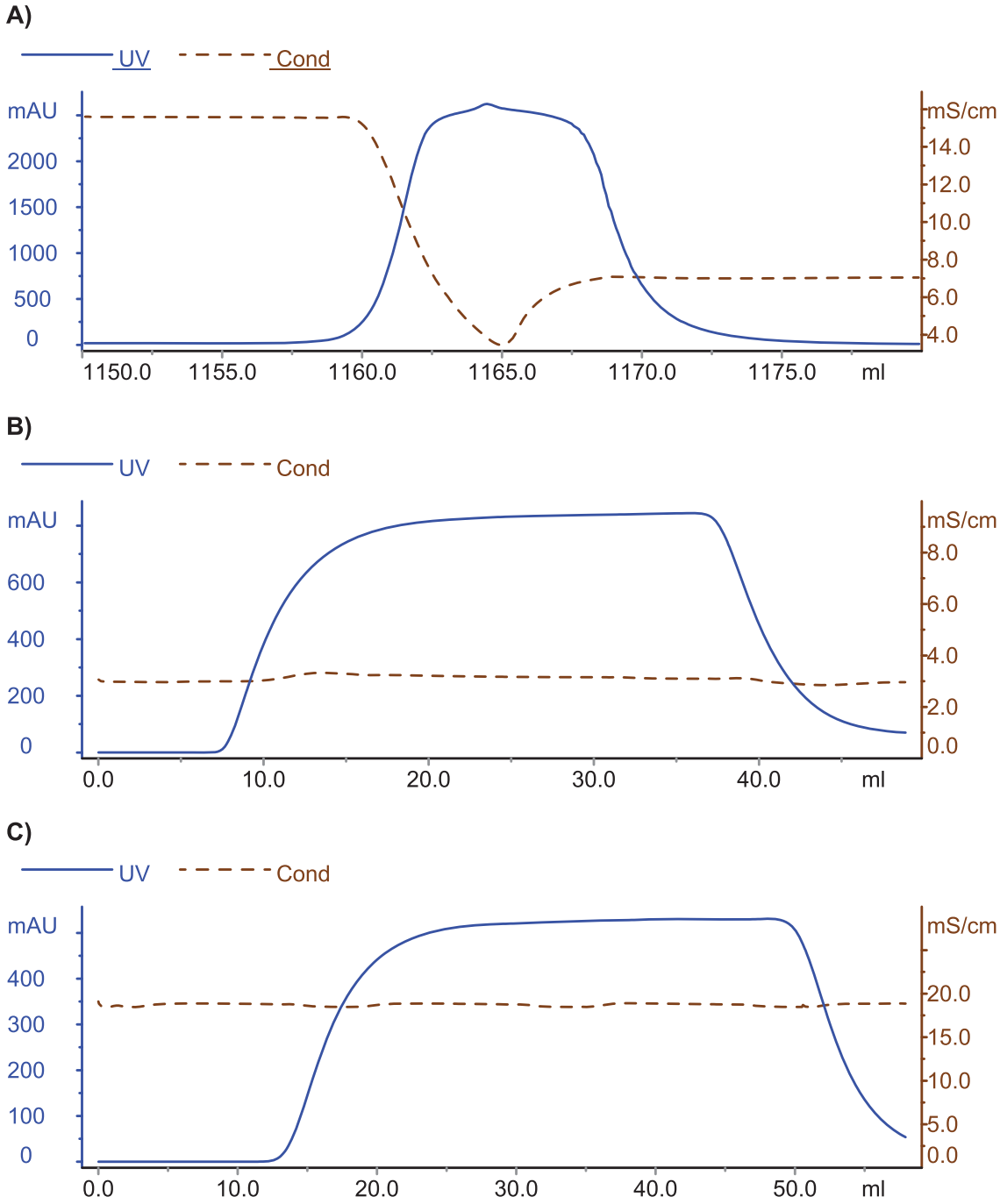

Figure 5.2: **Small-scale model for production process - Process AH1.2**
Column dimensions: 2 x Hiscreen™ (V = 9.4 mL; h = 20 cm) for each purification step
Amount KSBP: 178 mg (135 %)
A: KappaSelect; v = 400 cm/h; BE mode DPBS and 0.1 M Glycine pH 2.5
B: AIEX; Capto Q; v = 150 cm/h; FT mode 50 mM Tris pH 8.00 / Cond. 3 mS/cm
C: HIC; Phenyl HP; v = 120 cm/h; FT mode 50 mM Tris pH 8.00 / Cond. 18.5 mS/cm

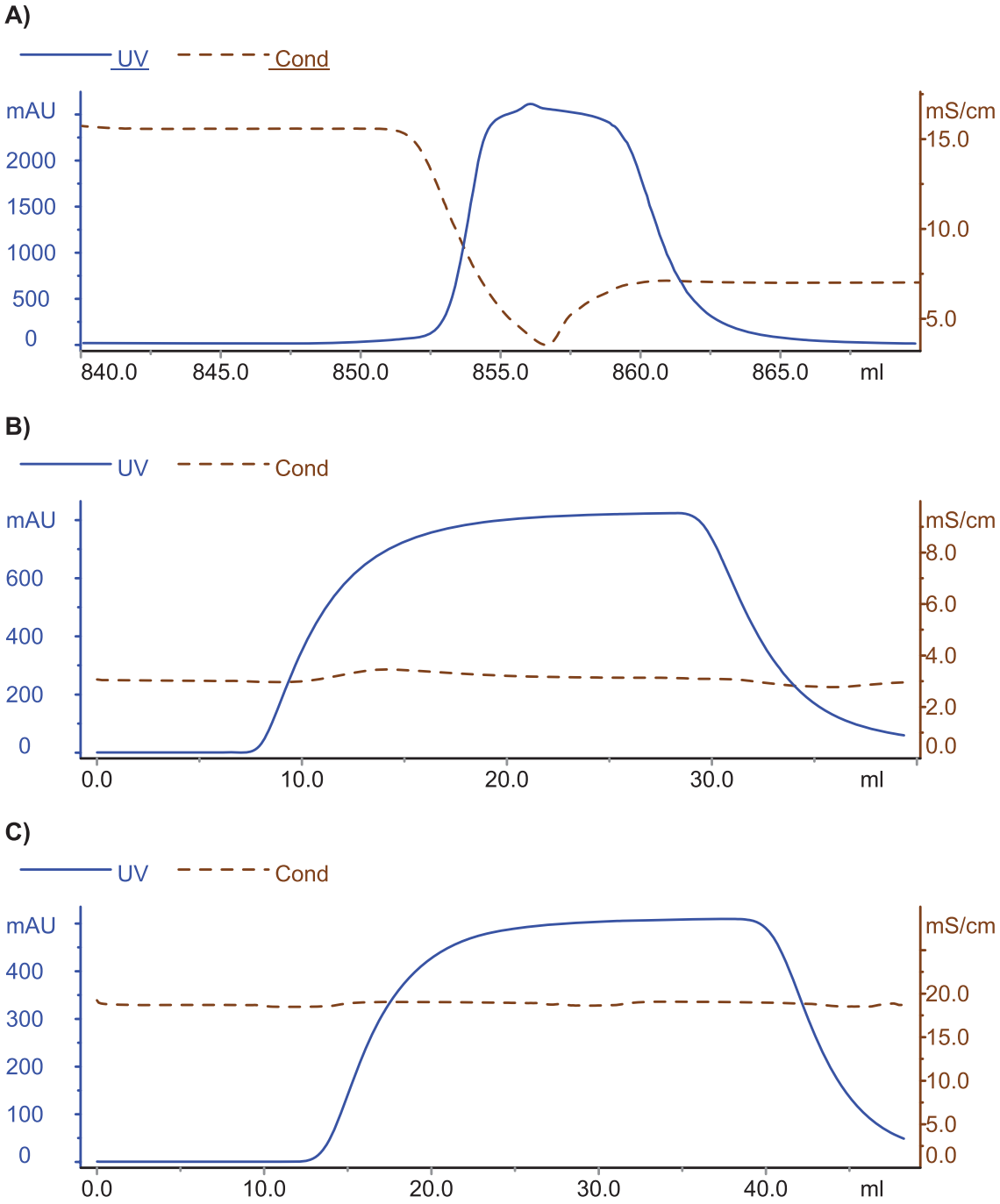

Figure 5.3: **Small-scale model for production process - Process AH1.3**
Column dimensions: 2 x Hiscreen™ (V = 9.4 mL; h = 20 cm) for each purification step
Amount KSBP: 119 mg (90 %)
A: KappaSelect; v = 400 cm/h; BE mode DPBS and 0.1 M Glycine pH 2.5
B: AIEX; Capto Q; v = 150 cm/h; FT mode 50 mM Tris pH 8.00 / Cond. 3 mS/cm
C: HIC; Phenyl HP; v = 120 cm/h; FT mode 50 mM Tris pH 8.00 / Cond. 18.5 mS/cm

5.3.2 Analysis

5.3.2.1 Compendium of Analysis

Table 5.3: **Small-scale model - Compendium - Results for IPC**
Concentration was determined via OD_{280}-measurement; Aggregate-, monomer- and LCD/LC-content was determined via SEC Superdex; HCP-content was determined via ELISA; HCDNA-content was determined via QPCR; Note: results for SDS-PAGE, Flow cytometric analysis, T-cell proliferation assay and potency assay (Promega) are not included in this compendium; IPC = In process control; KSBP = KappaSelect binding protein; HCP = Host cell proteins; HCDNA = Host cell DNA, QPCR = Quantitative PCR; LCD = Light chain dimer; LC = Light chain

		AH1.1	**AH1.2**	**AH1.3**
IPC-1	conc. [mg/mL]	4.97	5.34	4.44
	Amount KSBP [mg]	142.64	178	119
	Aggregates [%]	6.88	5.84	7.2
	Monomer [%]	55.49	53.59	52.63
	LCD/LC [%]	37.63	40.56	39.58
	Amount Monomer [mg]	79.15	95.39	62.51
	HCP [ppm]	5584	4591	7569
	HCDNA [ppm]	629	497	884
IPC-2	conc. [mg/mL]	2.1	2.2	1.87
	Amount KSBP [mg]	70.35	82.72	53.86
	Aggregates [%]	3.61	5.56	3.96
	Monomer [%]	93.22	90.48	92.91
	LCD/LC [%]	2.63	3.29	2.51
	Amount Monomer [mg]	65.58	74.85	50.04
	HCP [ppm]	46.0	52.8	57.2
	HCDNA [ppm]	< 1.5	< 1.5	< 1.5
IPC-3	conc. [mg/mL]	1.59	1.45	1.29
	Amount KSBP [mg]	58.83	60.9	41.28
	Aggregates [%]	0.28	0.32	0.33
	Monomer [%]	97.69	97.15	96.88
	LCD/LC [%]	1.47	1.87	2.05
	Amount Monomer [mg]	57.47	59.16	39.99
	HCP [ppm]	20.6	20.0	21.8
	HCDNA [ppm]	< 1.5	< 1.5	< 1.5

5.3.2.2 SEC analysis via Superdex™ 200 Increase 10/300 GL

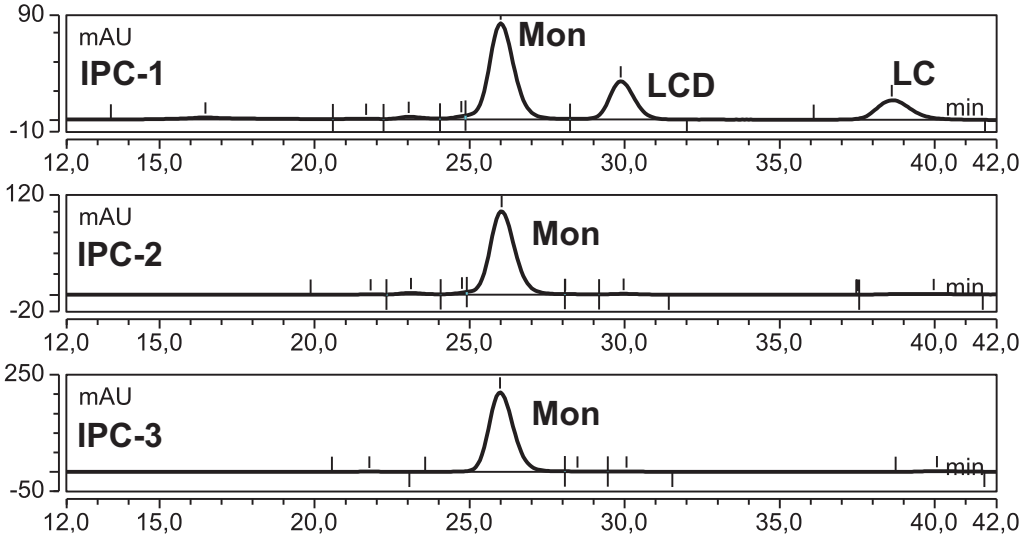

Figure 5.4: **Small-scale model - SEC (Superdex) - Process AH1.1**
UHPLC BioRS 3000
Column: Superdex™ 200 Increase 10/300 GL
Analysis of In-process controls (IPC1-3); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

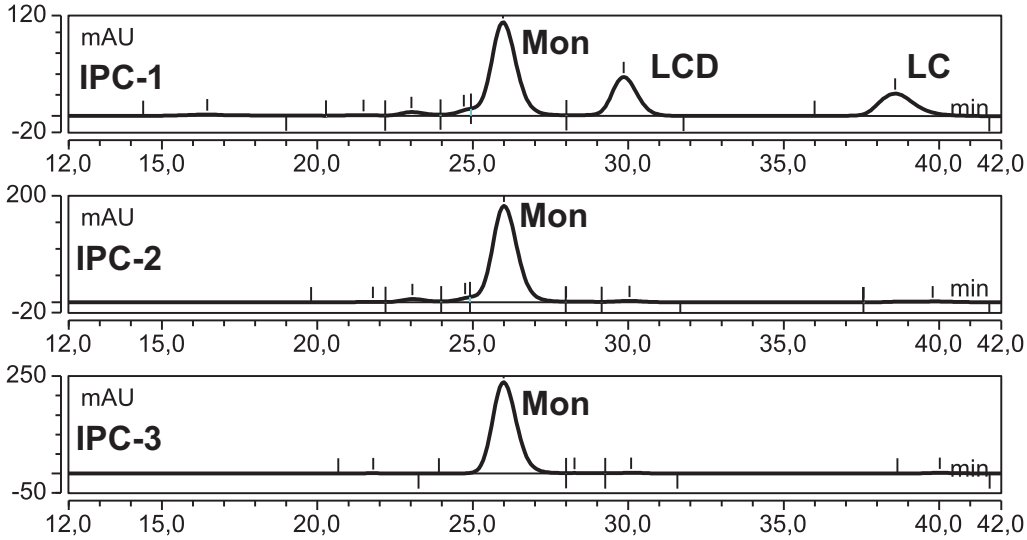

Figure 5.5: **Small-scale model - SEC (Superdex) - Process AH1.2**
UHPLC BioRS 3000
Column: Superdex™ 200 Increase 10/300 GL
Analysis of In-process controls (IPC1-3); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

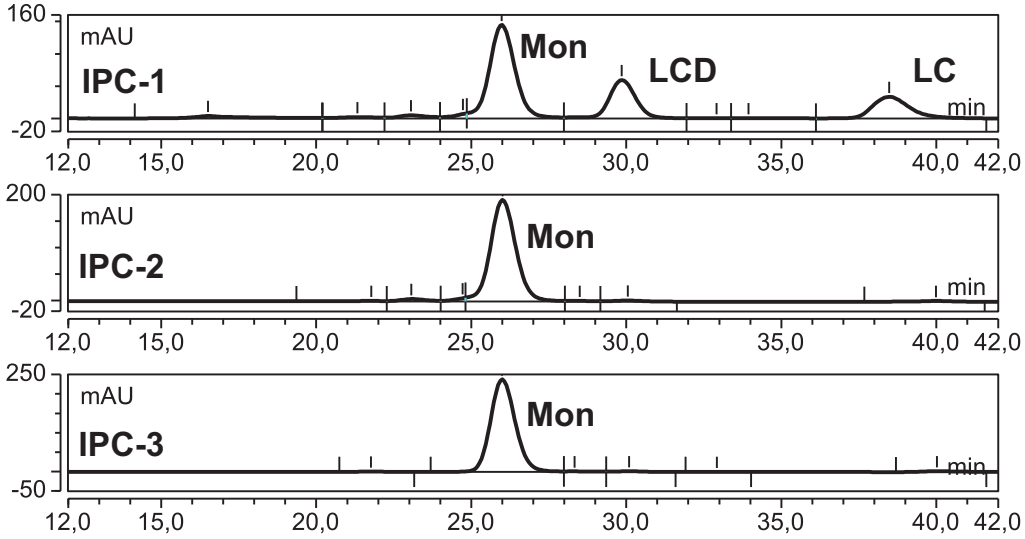

Figure 5.6: **Small-scale model - SEC (Superdex) - Process AH1.3**
UHPLC BioRS 3000
Column: Superdex™ 200 Increase 10/300 GL
Analysis of In-process controls (IPC1-3); Mon = Monomer, LCD = Light chain dimer, LC = Light chain

Table 5.4: **Small-scale model - SEC (Superdex)**
Peak detection and quantification via software "Chromeleon 7.2"; Analysis of In-process controls (IPC1-3) of process AH1

		Aggregate [%]	Monomer [%]	LCD [%]	LC [%]
AH1.1	IPC-1	6.9	55.5	22.1	15.6
	IPC-2	3.6	93.2	1.1	1.6
	IPC-3	0.3	97.7	0.4	1.1
AH1.2	IPC-1	5.8	53.6	22.4	18.2
	IPC-2	5.5	90.5	1.5	1.8
	IPC-3	0.3	97.2	0.9	0.9
AH1.3	IPC-1	7.2	52.6	22.2	17.4
	IPC-2	4.0	92.9	1.1	1.4
	IPC-3	0.3	96.9	0.9	1.2

5.3.2.3 SDS-PAGE

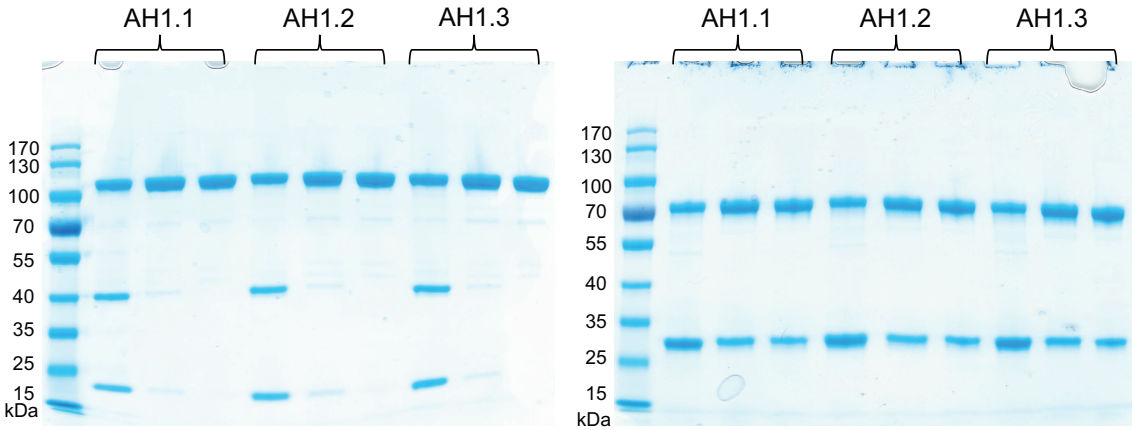

Figure 5.7: **Small-scale model - SDS-PAGE**
IPC-1-3 for verification batches AH1.1 to AH1.3; A = unreduced condition; B = reduced condition; marker: "PageRuler™ Prestained Protein Ladder"

5.3.2.4 CIEX / UHPLC

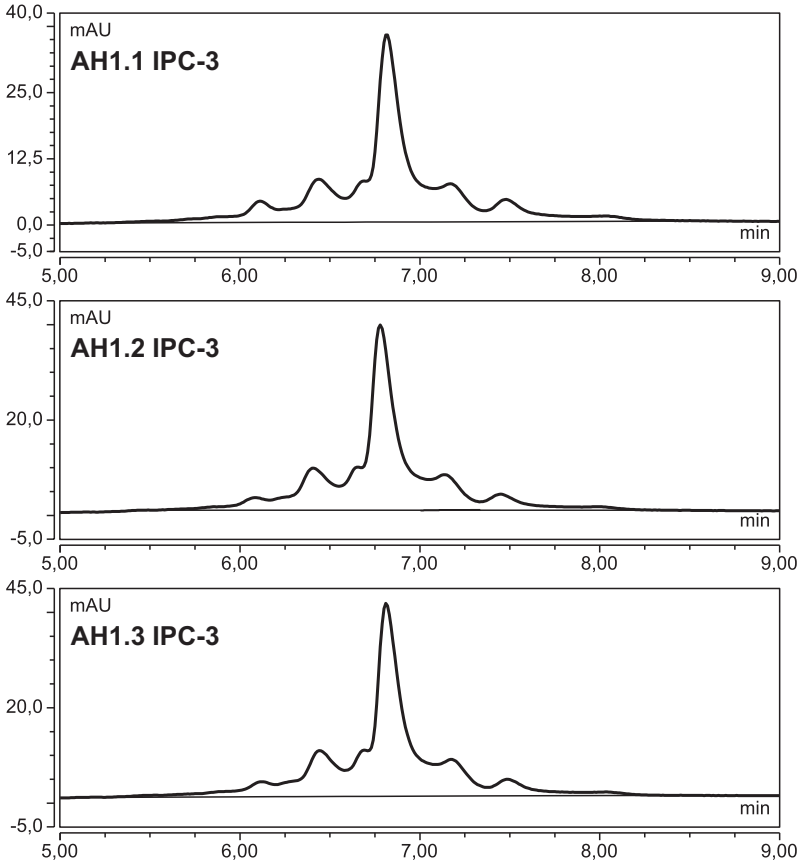

Figure 5.8: **Small-scale model - CIEX / UHPLC**
UHPLC BioRS 3000
Column: MAbPac™ SCX-10 RS, 5 µm BioLC™ 2.1x50 mm
Analysis of In-process control 3 (IPC-3) of process AH1

5.3.2.5 Flow cytometric analysis

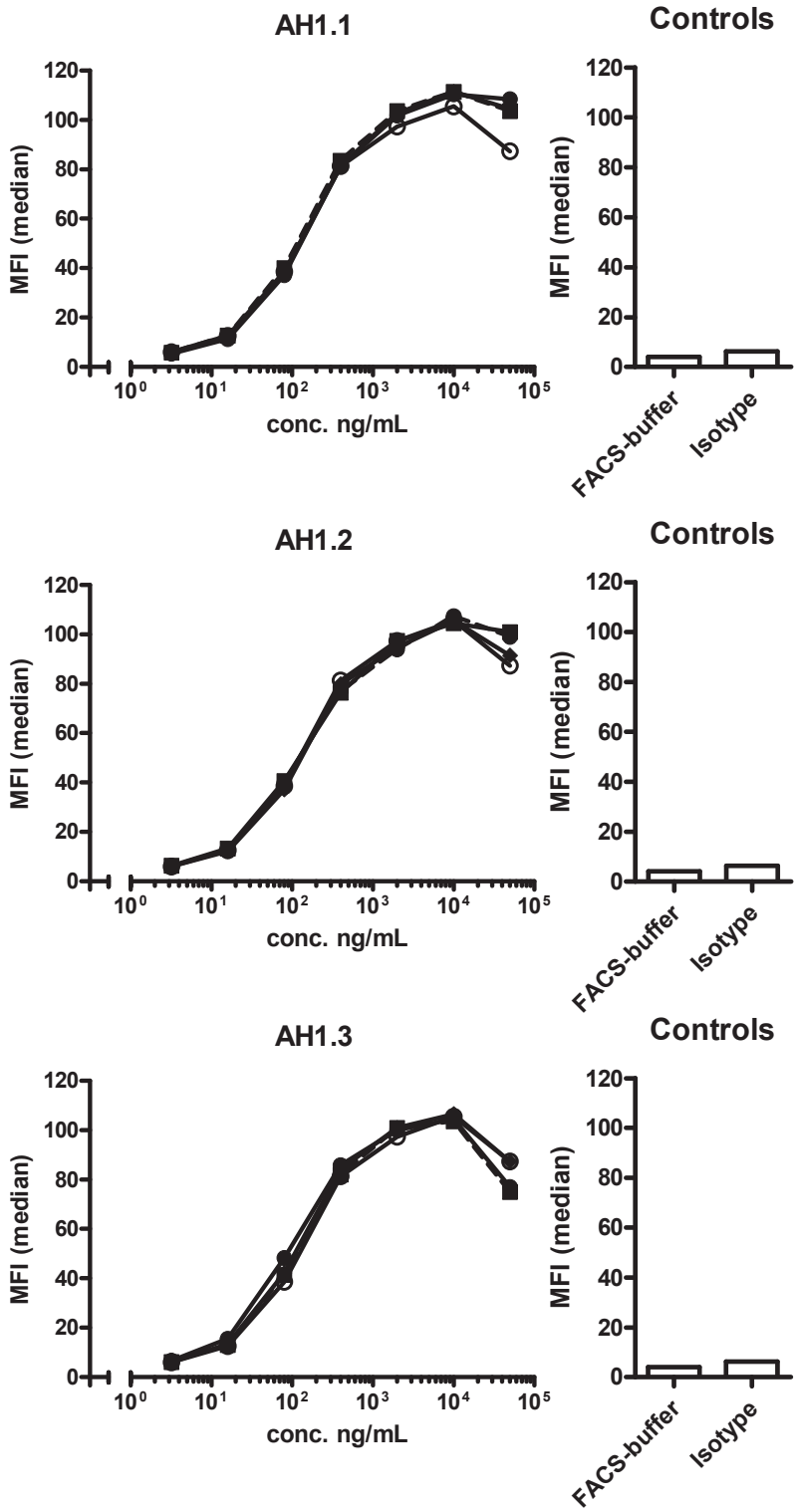

Figure 5.9: **Small-scale model - Flow cytometric analysis - NALM-16 cells**
Titration of monomer-normalized IPC-1 (●), IPC-2 (■) and IPC-3 (♦) of process AH1 on NALM-16 cells; Isotype = NP-C16$_{N297Q}$ (SP2/0) 50 µg/mL; Reference (○) = NF-CU$_{N297Q}$ (SP2/0); Detection: GaH-Fc-PE

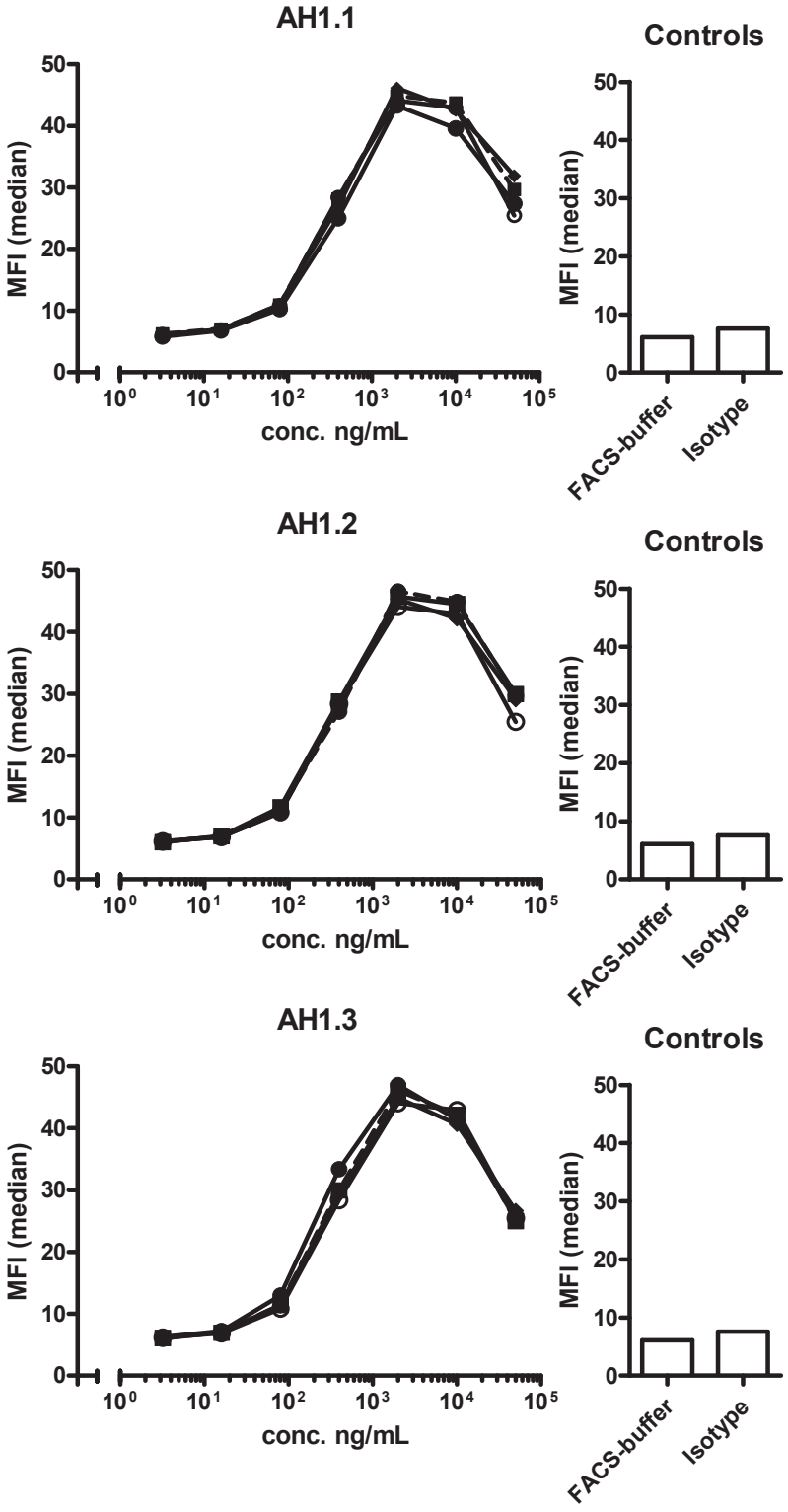

Figure 5.10: **Small-scale model - Flow cytometric analysis - Jurkat cells**
Titration of monomer-normalized IPC-1 (●), IPC-2 (■) and IPC-3 (⧫) of process AH1 on Jurkat cells; Isotype = NP-C16$_{N297Q}$ (SP2/0) 50 µg/mL; Reference (○) = NF-CU$_{N297Q}$ (SP2/0); Detection: GaH-Fc-PE

5.3.2.6 T-Cell proliferation assay

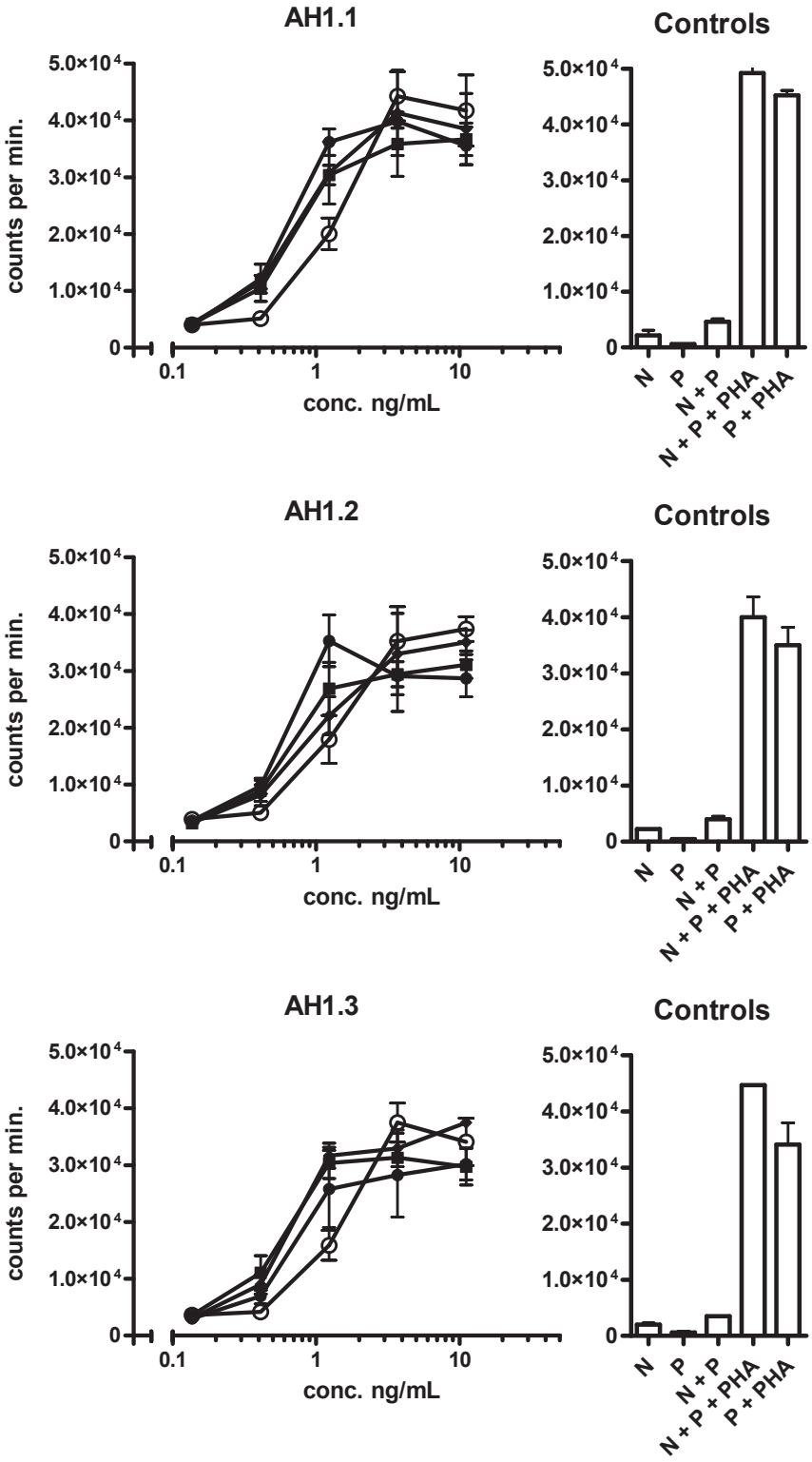

Figure 5.11: **Small-scale model - T-cell proliferation assay**
Titration of monomer-normalized IPC-1 (●), IPC-2 (■) and IPC-3 (⧫) of process AH1; Reference (○) = NF-CU$_{N297Q}$ (SP2/0); E:T = 1:1; N = NALM-16; P = PBMC; PHA = 10 µg/mL phytohemagglutinin

5.3.2.7 Potency assay (Promega)

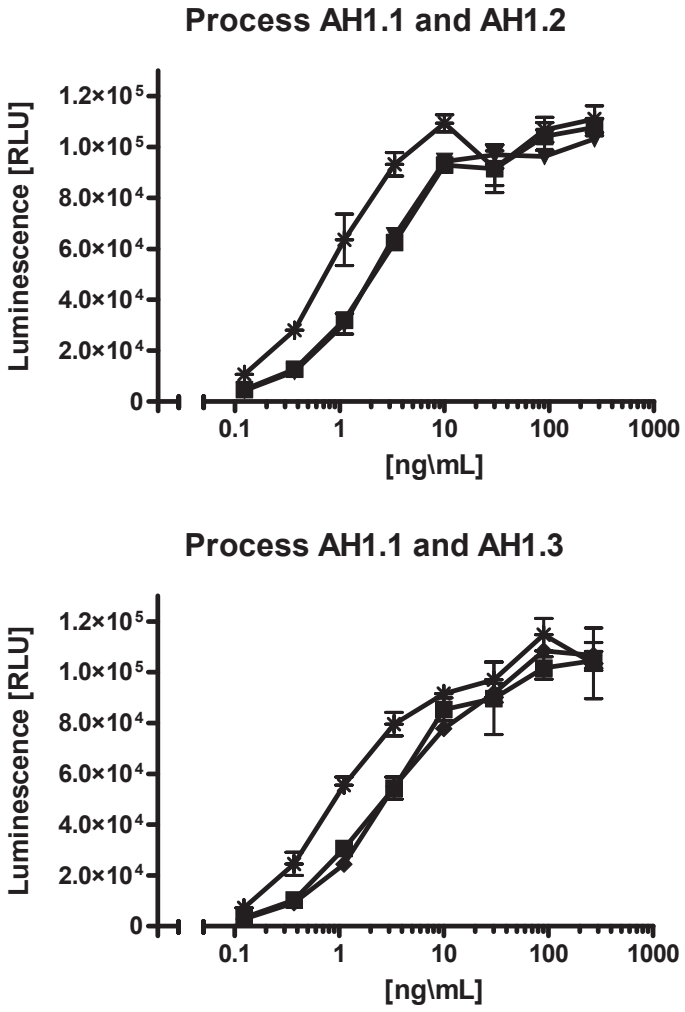

Figure 5.12: **Small-scale model - Potency assay (Promega)**
Titration of monomer-normalized IPC-3 of batches AH1.1 (■), AH1.2 (▼) and AH1.3 (⧫); Overall EC_{50} of the three batches is 3.0 ± 0.2 ng/mL, which corresponds to 33.3 % ± 0.8 % relative potency compared to reference.
Reference (✻) = NF-CU_{N297Q} (SP2/0); RLU = relative luminescence units; E:T = 2:1; E = modif. Jurkat; T = NALM-16

5.3.3 Specifications and Achievements for the Small-Scale model

Table 5.5: **Definition and achievement of quality objectives - Specifications and results for critical quality attributes**

CQA	Specification	Achievement
Identity		
	Distinctive Identification of Aggregates, Monomer, LCD and LC	identified
Purity		
Aggregates	$< 1\,\%$ (SEC Superdex)	$0.3\,\%$
LCD	$< 1\,\%$ (SEC Superdex)	$0.7\,\%$
LC	$< 2\,\%$ (SEC Superdex)	$1.1\,\%$
HCP	$< 100\,\text{ppm}$ (ELISA)	$20.8\,\text{ppm}$
HCDNA	$< 10\,\text{ppm}$ (QPCR)	$< 1.5\,\text{ppm}$
LKS	$< 10\,\text{ppm}$ (ELISA)	**theoretical**
Endotoxins	$5\,\text{IU/h/kg}$ (Ph.Eur. 7.0 part 2.6.14)	**theoretical**
Viral safety	Assurance level of 10^{-6}	**theoretical**
TSE safety	Absence of TSE proteins	achieved
Biological activity		
FACS	comparable binding properties to cells presenting the antigens of interest compared to an appropriate reference and no loss of binding activity during purification	verified
T-Cell proliferation	comparable proliferation-inducing properties of T-cells compared to an appropriate reference and no loss of activation properties during purification	verified
Potency Assay (Promega)	comparable activity of the purified API of three batches	verified
Stability		
Melting temperature	$T_{m1} > 72\,^{\circ}\text{C}$ (DSF)	verified
$40\,^{\circ}\text{C}$ 4 weeks	$< 1\,\%$ Aggregate (SEC Superdex)	verified
	$> 95\,\%$ Monomer (SEC Superdex)	verified

5.4 Contributions

Figure 1.2 at page 6	Synimmune GmbH, Tübingen
Figure 2.1 at page 32	Synimmune GmbH, Tübingen
Figure 3.16 at page 71	GE Healthcare, München

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