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DOWNSTREAM PROCESS DEVELOPMENT PLATFORM FOR BIOSIMILAR MONOCLONAL ANTIBODY DRUG MOLECULE

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DECLARATION

I declare that this thesis has been composed by myself and it has not been submitted in any previous application for any other degree. Except collaborative contributions, the experimental work and the data analysis were entirely done by my own. References have been used for supporting literatures.

Dilara Baş

06.07.2020

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CAGR	Compound Annual Growth Rate
CCD	Central Composite Design
CD20	CD20 Marker
CDR	Complementarity-determining Region
CHO	Chinese Hamster Ovary
CQAs	Critical Quality Attributes
CV	Coefficient of Variation
DBC	Dynamic Binding Capacity
DNA	Deoxyribonucleic Acid
DoE	Design of Experiment
DS	Drug Substance
DSP	Downstream Processing
df	Degrees of Freedom
Fab	Fragment Antigen Binding
Fc	Fragment Crystallizable
H	Heavy
HER2	Human Epidermal Growth Receptor 2
HTP	High-throughput
Ig	Immunoglobulin
IL-12	Interleukin-12
IL-23	Interleukin-23
kLa	Volumetric Mass Transfer Coefficient

L	Light
mAbs	Monoclonal Antibodies
PA	Protein
PD-1	Programmed Cell Death Protein-1
pI	Isoelectric Point
PRESS	Predicted Residual Error Sum of Squares
SS	Sum of Squares
QbD	Quality by Design
rPA	Residual Protein A
TMAE	Trimethylaminoethyl
TNF	Tumor Necrosis Factor
USP	Upstream Processing
VEFG	Vascular Endothelial Growth Factor
VIF	Variance Inflation Factors

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SUMMARY

The importance and commercial value of drugs produced with biotechnology are increasing rapidly. One of the most important conditions for creating a competitive advantage in this expensive technology is the development of purification method with high quality, high efficiency and low cost in a short time. During the method development studies, due to the high number of parameters, a large number of experiments are needed to determine ideal working conditions. This issue causes significant losses in terms of time and cost in the industry. Robotic technologies are required with micro-volume column platforms for purification to eliminate long method development times and high cost. Manual high-throughput screening with micro-volume columns are of great importance in contributing to the development of the robotic systems. The information obtained from this study will be transferred to robot system within the Euripides (European Smart Electronic Systems) project. Monoclonal antibodies are complex proteins used in therapeutic applications. It is essential that monoclonal antibodies used for therapeutic purposes are of high purity. After bioreactor production, monoclonal antibodies need to be purified using various chromatographic techniques, using downstream process development platforms. In this study, a downstream process development platform was created to optimize monoclonal antibody purification. In this platform, Design of Experiments was used. The optimum conditions were determined by manual high-throughput screening with less sample volume and time. Then, confirmation was performed with laboratory scale columns.

Keywords: Biotechnology, Chromatography, Design of Experiments, Manual High-Throughput Screening, Monoclonal Antibody Purification

ÖZET

Biyobenzer Monoklonal Antikor İlaç Molekülü İçin Alt Akım Proses Geliştirme Platformu

Günümüzde biyoteknoloji ile üretilen ilaçların önemi ve ticari değeri hızla artarken bu pahalı teknolojide rekabet avantajı oluşturabilmenin en önemli koşullarından biri de kısa sürede, yüksek kalitede, yüksek verimde ve düşük maliyetle saflaştırma yönteminin geliştirilmesi biyoteknoloji alanında önem taşımaktadır. Biyoteknolojik üretim geliştirme çalışmalarında değişken sayısının fazla olması ve ideal üretim koşullarını belirlerken çok fazla sayıda deneme yapılması gerekliliği, bu denemelerde elde edilen örneklerin birçok nedenle değişkenlik göstermesi, endüstride gerek süre gerekse maliyet açısından önemli kayıplara neden olmaktadır. Bu kayıpların giderilmesi, üretim geliştirme sürelerinin kısaltılması, kalite ve verimliliğin artırılması ve böylece maliyetlerin düşürülmesi için mikro hacimli kolonlarla ile birlikte robot sistemlerine ihtiyaç vardır. Mikro hacimli kolonlarla manuel yüksek çıktılı tarama çalışmalarının yapılması robot sisteminin geliştirilmesi için katkı sağlamaktadır. Buradan elde edilen sonuçlar Euripides (European Smart Electronic Systems) projesi (Proje Numarası: 9170058) kapsamında geliştirilmekte olan robot sistemine aktarılacaktır. Monoklonal antikorlar tedavi amaçlı uygulamalarda kullanılan bir protein çeşitidir. Tedavi amaçlı olarak kullanılan monoklonal antikorların yüksek saflıkta olması esastır. Biyoreaktör üretiminden sonra monoklonal antikorların alt akım proses geliştirme teknikleri kullanılarak farklı kromatografik tekniklerle saflaştırılması gerekmektedir. Bu çalışmada, monoklonal antikor saflaştırma sürecini en kısa sürede optimize etmek için çok sayıda proses parametresinin denenebileceği bir platform oluşturulmuştur. Oluşturulan bu platformda Design of Experiments yöntemi kullanılmıştır. Kısa sürede daha az örnek hacmi ile daha çok parametre taranarak optimum koşullar belirlenmiştir ve ölçek büyütme işlemleri ile laboratuvar ölçeğine transferi gerçekleştirilmiştir.

Anahtar Kelimeler: Biyoteknoloji, Deney Tasarımı, Monoklonal Antikor Saflaştırma, Manuel Yüksek Çıktılı Tarama, Kromatografi

1 AIM OF THE STUDY AND INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) are target-specific protein used by suffering from cancer, autoimmune diseases and communicable. Therefore, the increasing importance of therapeutic mAbs is apparent and they are the fastest-growing sector in the biopharmaceutical industry.

Development of manufacturing process for mAbs requires the consideration of many different parameters including removal of impurities and ready availability of raw materials for large-scale production.

High-throughput (HTP) system is employed for process development for saving time, investment and cost. HTP systems allow running large number of experiments in a minimal scale.

In this study, a downstream process development platform for chromatographic technique was created with resin selection and parameter screening using the experimental design. Optimum parameters were determined by manual high-throughput screening. Then, confirmation runs were performed with laboratory scale columns.

2 BACKGROUND

2.1 Immunoglobulins

Immunoglobulins (Ig), also known as antibodies, are heterodimeric proteins with a Y shaped structure. There are two main purposes of them: Immunoglobulins permit cell signaling and cell activation on cell-surface receptors and they bind and neutralize antigens, such as bacteria or viruses, individually (1). In 1890, the first earlist to antibodies came from Emil von Behring and Shibasabura Kitasato (2). The side-chain theory, also called “lock and key”, was first to propose for model of binding to foreign material, known as antigen, by Paul Ehrlich, in 1900 (3). Astrid Fagraeus in 1948 showed that plasma B cells involved antibody generation specifically (4). Gerald Edelman and Rodney Porter published the molecular structure of immunoglobulins in 1959 (5).

Immunoglobulin is composed of two heavy chains (H) and two light chains (L) connected by disulfide bonds, called hinge region. An antibody is made of a variable region, which changes to various structures depending on antigens, and a constant region, not change on immunoglobulin.

Recognition of unique antigens is happened on fragment antigen-binding (Fab) via variable region. Fab is consists of one constant and one variable domain from each heavy and light chain of the antibody. The variable region domain can be subdivided into complementarity-determining region (CDR) which contact and bind antigen directly serve as a scaffold for CDR.

Large Y shape of an antibody plays a role in activating the immune system through interaction with cell surface receptors, called fragment crystallizable region (Fc). In Fc region, glycosylation plays critical role in mAb effector function. Depending on the subclasses of an antibody, this region has two heavy chains consisting of two or three constant domains.

Heavy chain domains have five subclasses. These are; IgM, IgG, IgA, IgD and IgE. These differences are associated with amino acid sequences in Fc region's heavy chains (1). The general concept of Ig is shown in Figure 1 (11).

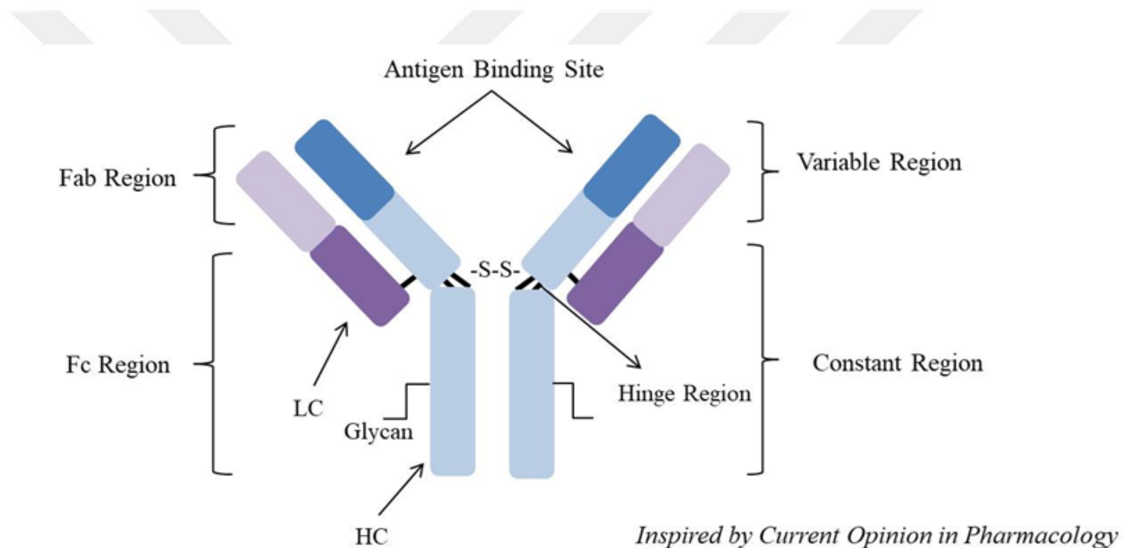

Figure 1. The view of Immunoglobulin

2.1.1. Immunoglobulin G

Immunoglobulin G (IgG) is predominant Ig class and is present in human serum (6). It is only type of Ig that can cross on the placenta in human. IgG is composed of 50 kDa H chains and two identical 25 kDa chains, connected together by interchain disulfide bonds (7). IgG can be divided in four subclasses; IgG1. IgG2. IgG3. IgG4.

These subclasses are differentiated on the size of the hinge region, position of interchain disulfide bonds and molecular weight that affect their binding to molecules, receptors and functionality (8).

2.2 Therapeutic Monoclonal Antibodies

Georges Köhler and Cesar Milstelin have initiated the modern era of antibody research and discovery in 1975. The first therapeutic monoclonal antibodies (mAbs) were made by using the hybridoma technology (9). This technology is very effective and of critical importance for raising mAbs against antigens of interest.

The conventional method for hybridoma technology is that mouse is immunized with the antigen of interest. Spleen cells of the immunized mouse are fused with B-lymphocytes, produce antibodies that bind to injected antigen, and myeloma cells. After a high antibody titer has been made, myeloma cells generate hybridomas to produce mAbs (10). Also, development of immunotherapy with hybridoma technology allowed for specific targeting of antigens both *in vitro* and *in vivo*.

There are four major antibody types have been developed. The order of immunogenicity with their levels is murine, chimeric, humanized and human. Hybridoma technology requires expertise to produce, time and money. Recombinant mAbs are executed *in vitro*, using recombinant DNA technology. This technique allows the production of mAbs with high specificity and reproducibility. The antibody genes are isolated and then joined into plasmid DNA vectors. As a result, plasmids are transfected into expression systems (bacteria. yeast or mammalian cell) (12).

Chinese hamster ovary (CHO) cells are most commonly used for production of recombinant mAbs. Therapeutic mAbs should have appropriate glycosylation and post-translational modification for performing their biological function.

Among the available expression systems, CHO cells are close to natural mAb. Also, exogenous (antibody) genes can be integrated steadily in CHO cells (13).

2.2.1 Market Research and Application Area of Biosimilar Therapeutic Monoclonal Antibodies

The first FDA approved therapeutic mAb, muromonab, has been published in 1986 (14). The global therapeutic mAbs market are increasing with developing new mAbs and supporting the growth in this market. According to Transparency Market Research, the global mAb therapeutics market will get through US$ 245.8 bn by the end of 2024 with the rate of 12.6% CAGR between 2016 and 2024.

Many global pharma companies as Novartis AG, AbbVie Inc., Pfizer Inc. Bayer AG and F.Merck&Co exert significant efforts to get their drugs approved to obtain advantage against their competitors (15). In 2018, the global top eight mAb drugs, shown in Table 1 with their application area, have combined shares of 55.6% in global market (16).

Table 1. The Top Eight Monoclonal Antibody Drug in the Global Market and Their Application

Name	Trade Name	Drug Class & Application Area*
Humira	Adalimumab	TNF Inhibitor Rheumatoid arthritis
Herceptin	Transtuzumab	Antineoplastic agent HER2 Positive breast cancer
Avastin	Bevacizumab	VEGF Inhibitor Colon cancer
Keytruda	Pembrolizumab	Target : PD-1 Melanoma
Rituxan	Rituximab	Target : CD20 Chronic lymphocytic leukemia
Remicade	Infliximab	TNF Inhibitor Crohn's disease
Stelara	Ustekinumab	Target: IL-12 and IL-23 Psoriasis
Enbrel	Etanercept	TNF Inhibitor Rheumatoid arthritis

**All information was taken from " drugbank.ca".*

2.3 Therapeutic Monoclonal Antibody Production

The conventional biopharmaceutical processing for mAbs is divided by Upstream Processing (USP) and Downstream Processing (DSP). USP refers to the first step of processing to grow biomolecules by mammalian or bacteria cell culture. Recovery and purification of mAbs from cell culture is called downstream processing (DSP). Development and optimization for both processes can be achieved by technological platform-based like high-throughput (HTP) system.

Large scale is only used to validate the suitable experimental data obtained from HTP systems. In addition, HTP systems can be combined with statistical approaches such as, Design of Experiments (DoE), to screen several factors in one set of experiments.

Furthermore, Quality by Design (QbD) with HTP is very helpful to understand manufacturing principle, risks with product quality. USP and DSP can be successful with platform-based development and optimization.

2.3.1 Upstream Processing

The development and optimization for USP consist of many stages; cell line development, cell clone selection, media and feed development, bioreactor design and scale-up (17). Product concentration, glycosylation and charge variant profiling have to be controlled and considered as quality attributes for USP. With the development of genetic engineering, CHO cells are currently preferred for the production of mAbs because CHO cell expression system can be adapted for the production of heterologous recombinant proteins yielding high quality mAbs (13). Media and feed development is a key factor for productivity and high titer. Effective media and feed are selected with feeding strategies, such as fed batch, batch or continuous. Fed- batch, operation of intermittent feeding with one or more substrate, is commonly used for mAb production using CHO cells due to high cell density and product quality. Fed-batch with stirred tank bioreactor platform is frequently used to reduce shear stress. pH, temperature, dissolved oxygen, glucose consumption, dissolved CO_2 removal, mixing rate are the essential parameters for development, optimization and manufacturing. Optimized parameters can be scaled-up mathematically using power input (related to mixing rate) and volumetric mass transfer coefficient (kLa) (oxygen transfer) (18).

2.3.2 Downstream Processing

DSP development and optimization is based on yield, purity and productivity. A mAb purification platform consists of many operations such as filtration or centrifugation and the sequence of different chromatography methods. The first step for mAb purification is cell harvesting to remove cell debris using filtration, centrifugation or depth filtration. After cell harvesting, chromatographic separations are used to reduce process-related impurities. Chromatography is the backbone of DSP to reduce the level of impurities down to the acceptable levels to ensure product safety and effectiveness. Host cell proteins (HCPs) are the main source of impurities. HCPs are different from each other in their molecular mass and structure (17). Residual DNA (rDNA) is another important process-related impurity.

The main purpose of DSP is to purify process-related impurities such as residual Protein A. Also, it is responsible for product-related impurities such as aggregation and HCP. These impurities are critical to influence of biochemical and biophysical properties of mAb (19). Development of liquid chromatography process for mAb production requires the selection of best separating resin and optimization of several operation parameters such as reduction of impurities. pH, buffer type, loading concentration and residence time of the column. Typically, three chromatography steps are used of mAb purification platform; the first being capture step – Protein A chromatography followed by two polishing steps that are selected based on the nature of the impurities that need to be removed.

2.3.2.1 Chromatography

Protein A (PA) chromatography refers to the capture step due to its high binding affinity and specificity toward the Fc region of IgG. Other ligands such as Protein G and Protein L are also suitable for mAb capture; but they have some limitations such as low capacity and hard elution condition (20). Protein A step yields a concentrated product (>10g/L). Generally, Protein A chromatography has four steps.

The clarified cell culture is loaded directly on the column at neutral pH and the product is eluted with at low pH. A wash step is used between loading and elution step to remove HCP and other contaminants. Finally, the column is stripped or sanitized according to properties of Protein A ligand (21). There are many Protein A ligands available in the market. Protein A is usually covalently bound to a natural (agarose or cellulose) or synthetic (pore glass. polyvinyl ether. polymethacrylate) base matrix (22). Protein A resins from different providers usually yield varying level of process impurities. Therefore, during chromatographic development of Protein A. the study parameters are screened and operating strategy is selected. The study Protein A dynamic binding capacity (DBC) is essential to design the chromatography process. DBC is described as the amount of a target protein bound to a Protein A ligand with determined flow rate at a breakthrough point of 10%. Other parameters to be considered are the life-time, stability under acidic or alkaline conditions and maximum allowable flow rate.

Protein A chromatography has several limitations. The main disadvantage is the high cost of the protein A resin, which makes it crucial to develop an operating strategy with smaller columns. Protein A resins also add another impurity, called residual Protein A (rPA), which is the Protein A ligand leached from the base matrix in elution step (22).

After Protein A chromatography, appropriate polishing steps are selected such as ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and mixed mode chromatography (MMC).

Selection of an appropriate IEX chromatography type depends on the isoelectric point of the protein to be purified. Isoelectric point (pI) is the pH at which the protein has no net charge and it shows the property of electric field of the protein. The pI value can be estimated using the primary sequence of the protein. A protein will be negatively charged at pH values higher than the pI of a protein; conversely. it will be positively charged at pH values lower than the pI (23).

IEX chromatography can be operated in two ways; flow-through mode and bind-elute mode. In flow-through mode, the target protein does not bind to the resin, whereas the impurities are captured. Then, weakly bound proteins are collected in wash step to increase yield. Higher column loading, which is called overloading, can be applied in flow-through mode (21). The impurities appear to bind stronger than the protein when flow-through with overload chromatography is applied. Also, it has been preferred for high yield and short process time with high flow rate (24).

CEX resin is made of negative charged particles, whereas AEX resin has positively charged particles. CEX is generally employed to reduce the level of HCP and mAb aggregation. AEX has been demonstrated to be useful in reducing HCP and viral clearance for mAbs (21). Cation and anion exchangers are divided into two types: strong and weak. These differences result from the functional groups of the resin (hydroxyl. methyl. carboxyl and sulfhydryl) and capacity of working pH range.

The principle of strong exchangers is to work independently of pH. On the other hand, weak exchangers are pH-dependent. Working range of pH is important for resin capacity of weak exchangers (23).

Design and optimization of IEX chromatography depends on; the concentration of protein and operating conditions such as working pH. flow rate and properties of the resin.

HIC and MMC chromatographies are also preferred in polishing steps for mAb purification. HIC is based on hydrophobic interactions between the protein surface and hydrophobic sorbent (phenyl. butyl. octyl) of the resin. The main mechanism is based on the ionic strength. When the salt concentration of the running buffer is reduced, the interaction is reversed. Therefore, the protein with the lowest hydrophobicity is first eluted from the column. (26).

MMC, also referred to as mixed-mode, is an alternative way to separate through at least one ligand with more than one interaction: charge, hydrophobicity and affinity (27).

2.3.3 High Throughput Screening in Downstream Processing

The large number of parameters have significant effect on chromatography performance, so the large number of experiments are performed at lab-scale to find optimum operation parameters. However, it requires large amount of material and long experiment times. High-throughput (HTP) screening with micro-scale formats is central to the development and optimization of mAb purification process. Also, HTP improves prediction robustness of the related parameters.

HTP for chromatography development consists of a pre-packed micropipette column with large number of experiments. Therefore, lab-scale experiments can then be run for confirmation or optimization studies only. Incorporation of HTP platform with Design of Experiment (DoE) is systematic integration for effective and rapid screening for optimum experimental conditions (28).

2.3.3.1 Design of Experiments

Design of experiments, also referred as DoE, is a systematic tool to understand influence of the experimental parameters on the responses and to find optimum process parameters. DoE offers screening, optimization and robust parameter design (29). It includes controllable and uncontrollable input factors, replication and responses. Controllable input factors means that input parameters can be modified in experiment or process, whereas uncontrollable input factors not.

Replication shows an estimation for the amount of random error. Responses or output, are process outcome for understanding the effect of input factors. There are five main model types; screening design, response surface design, full factorial design, mixture design and taguchi design. Choosing the best model depends on the objectives of the experiment and the number of factors (30).

DoE analysis is performed by Analysis of Variance (ANOVA), which is statistical based decision-making. It helps to determine the ideal factors for each of the responses using designed models (31).

ANOVA provides the following outputs; summary statistics, coefficients and equations.

Model variance is explained by the p-value which identify model terms and F-value shows the confirmation of model significance. Variance inflation factors (VIF) detects linearity in regression analysis. Summary statistics show predicted and adjusted R-squared to understand fitting the model and reliably with data. The equations of predicted (1) and adjusted (2) R-squared are shown below. Residual shows how much variation is unexplained.

Equation 1;

$$Adj.R^2 = 1 - \left[\left(\frac{SS(residual)}{df(residual}\right) / \left(\frac{SS(residual) + SS(model)}{df(residual) + df(model)}\right)\right]$$

$$= 1 - \left[\left(\frac{SS(residual)}{df(residual}\right) / \left(\frac{SS(total) - SS(curvature) + SS(block)}{df(total) - df(curvature) + df(block)}\right)\right]$$

Degrees of Freedom (df): It is the number of predicted parameters used to compute the source sum of squares.

Equation 2;

$$Pred.R^2 = 1 - \left[\frac{PRESS}{SS(residual) + SS(model)}\right]$$

$$= 1 - \left[\frac{PRESS}{SS(total) - SS(curvature) - SS(block)}\right]$$

PRESS: Predicted Residual Error Sum of Squares, it is a measurement of how the model fits each of point in the design. The related equation is shown below (3);

Equation 3;

$$PRESS = \sum_{i=1}^{n} (e - i)^2$$

$$e - i = y_i - y^{\wedge}{}_{-i} = \frac{e_i}{1 - h_{ii}}$$

$(e - i)^2$: It is a deletion residual by fitting the model.

e_i : It is the residual for each observation.

h_{ii} : It is the leverage of the run.

Adequate precision value is used for model optimization part and ratios greater than 4 to indicate model discrimination and coefficient of variation % (CV %) is used for the capability of the process. Coefficients display the confidence intervals with the estimated model and they are helpful to understand significance for all analyzed responses (32).

2.3.3.1.1 The Methodology of Design Modelling

Response surface is the collection of statistical techniques used for studying the relationship between factors and responses. It is evaluated with multiple sample points and the metal usually as linear or quadratic. There are several types of functions are found to generate response surface modelling; linear, cubic and quadratic. These approaches are related to be approximated at several sample points. Central composite design (CCD) and optimal (custom) design are type of the model design of the response surface.

CCD is based on 2-level factorial design with center and axial points to fit quadratic models. Also, it can be 3-level per factor with changing axial points. Numeric and categoric factors can be involved (33).

Optimal designs occur with a pseudo-random set of model points. It has a less experimental runs with the best groups of design points and criteria. During the choosing design type, optimality-criteria are chosen. For example, L-optimal algorithm is selected for minimizing the prediction variance across the factor space (34).

3 MATERIALS AND METHODS

3.1 Sample Description

Monoclonal antibody, IgG1 (pI: 8 – 8.5), was produced with recombinant CHO cells. CHO cells were grown in Mobius® 3L single-use bioreactors (Merck KGaA. Darmstadt. Germany) and the following set points; pH 7-7.1, 37°C, dissolved oxygen between 30-60% of air. Clarified CHO cell culture supernatant was provided with Millistak+® Pod Disposable Depth Filter System (Merck KGaA. Darmstadt. Germany) with a concentration of 2.55 mg/ml.

3.2 Manual High-Throughput Process Development of Chromatography Screening

All experiments were designed with Design Expert Software 12 (Stat-Ease. Inc., Minneapolis. MN). All column screens were based on a response surface modeling approach. 100µl (0.5 cm x 0.5 cm) OPUS® PipetColumns (Repligen Corporation) were used for screening with electronic Multipipette® E3x (Eppendorf). There are eight various types of PA and three different types of AEX and CEX resins were screened.

3.2.1 Protein A Chromatography

3.2.1.1 Dynamic Binding Capacity

DBC studies were performed by purified mAb at a concentration of 7.63 mg/ml. All columns were equilibrated with 30 mM citrate. 150 mM NaCl pH 7 at 5 CV. Flow rate was 4 µl/sn with a residence time 25 sec. During DBC studies, fraction was taken in the post-column every 5 steps and mAb concentration was measured. When the concentration of mAb was reached 0.1 mg/ml, the total fraction was determined as maximum volume bounded the column. Then, this value was multipled by stock mAb concentration to get DBC value. Unbound proteins were washed with 30 Mm citrate. 1M NaCl pH 7 and 10 mM citrate pH 7, respectively. Sanitization was performed and elution was not applied.

3.2.1.2 Protein A Resin Screening

Clarified CHO cell culture supernatant was used for PA screening with bind-elute mode. Build informations for PA are shown in Table 2. Factors were entered the software; elution pH between 0.1 M Acetic acid 3.20-3.60, loading percentage between 40%-80% and resin type. Responses were yield and HCPs.

Table 2. Build Informations of Protein A Screening

File Version	12.0.6.0			
Study Type	Response Surface		**Subtype**	Randomized
Design Type	I-optimal	Coordinate Exchange	**Runs**	37.00
Design Model	Quadratic		**Blocks**	No Blocks
Name	**Units**	**Type**	**Low**	**High**
Elution pH		Factor	3.2	3.6
Loading Percentage	%	Factor	40	80

During loading step, DBC data was used for each resin. Flow rate was 4µl/sn at a residence time 25 sec. Columns were washed with 20 mM sodium phosphate. 1 M NaCl pH 6 and 20 mM sodium phosphate pH 7 at 5 CV. respectively. The first 25 step at elution was not collected. Working template for Protein A screening is given in Table 3.

Table 3. Working Template for Protein A Screening

Step	**Buffer**	**CV**
Equilibration	20 mM Sodium Phosphate pH 7	5
Load	Clarified CHO Cell Culture Supernatant	Residence time 25 sec
Wash 1	20 mM Sodium Phosphate. 1 M NaCl pH 6	5
Wash 2	20 mM Sodium Phosphate pH 7	5
Elution	0.1 M Acetic Acid between pH 3.20-3.60	7
	Eluate was collected between 25 – 150 step	
Sanitization	Depending on resin type was changed between 0.3 M – 0.5 M NaOH or 125 mM Orthophosphoric Acid. 167 mM Acetic Acid. 2.2% Benzylic Alcohol	5
Rinsing	20 mM Sodium Phosphate pH 7	3
Storage	20% Ethanol	5

3.2.2 Anion Exchange Chromatography

AEX screening was applied with flow-through mode at a concentration of between 21-26 mg/ml due to pH differences. pH adjustment was applied with 2M Tris. Then, filtration was applied. Build informations are shown in Table 4. Factors were pH between 20 mM sodium phosphate 6-8, loading quantity between 400mg - 800mg and resin type. Responses were yield, HCPs and aggregation.

Table 4. Build Informations of AEX Screening

File Version	12.0.6.0		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Central Composite	**Runs**	39.00
Design Model	Reduced Cubic	**Blocks**	No Blocks

All columns were regenerated with 2 M NaCl at 5 CV and equilibrated with 20 mM sodium phosphate using design layout pH at 8 CV. Flow rate was 4 µl/sn at a residence time 25 sec. Columns were washed with equilibration buffer at 1 CV and wash step was collected. Working template for AEX screening is shown in Table 5.

Table 5. Working Template for AEX Screening

Step	**Buffer**	**CV**
Equilibration	20 mM Sodium Phosphate between pH 6-8	5
Regeneration	2M NaCl	5
Equilibration	20 mM Sodium Phosphate between pH 6-8	8
Load	Pooled PA Eluate between pH 6-8	Depends on Loading Quantity
Wash	Related Equilibration Buffer	1 CV
Sanitization	Depending on resin type was changed between 0.3 M – 0.5M NaOH	5
Rinsing	20 mM Sodium Phosphate pH 7	5
Storage	20% Ethanol	5

3.2.3 Cation Exchange Chromatography

Screening was done at flow-through mode at a concentration of between 13-17 mg/ml due to pH changes. pH adjustment was applied with 1 M Acetic Acid. Then, filtration was applied. Build informations for CEX are shown in Table 7. Factors were pH between 20 mM sodium phosphate 4 – 5.50 and resin type. Responses were yield, HCPs and aggregation.

Table 6. Build Informations of CEX Screening

File Version	12.0.6.0			
Study Type	Response Surface		**Subtype**	Randomized
Design Type	I-optimal	Coordinate Exchange	**Runs**	17.00
Design Model	Quadratic		**Blocks**	No Blocks

All columns were regenerated with 2 M NaCl at 5 CV and equilibrated with 20 mM sodium phosphate using design layout pH at 8 CV. Loading quantity was applied from determined confirmation run of AEX for all CEX screening columns. Flow rate and wash step process were the same with AEX screening.

3.3 Confirmation Runs with Scale-up Studies

After screening of each chromatography methods, determined operating parameters from DoE were done with ÄKTA Avant 25 (GE Healthcare) with 10 ml and 1 ml pre-packed columns. Schematic workflow from HTP Chromatography Screening to Confirmation Run with Scale-up is shown in Figure 2.

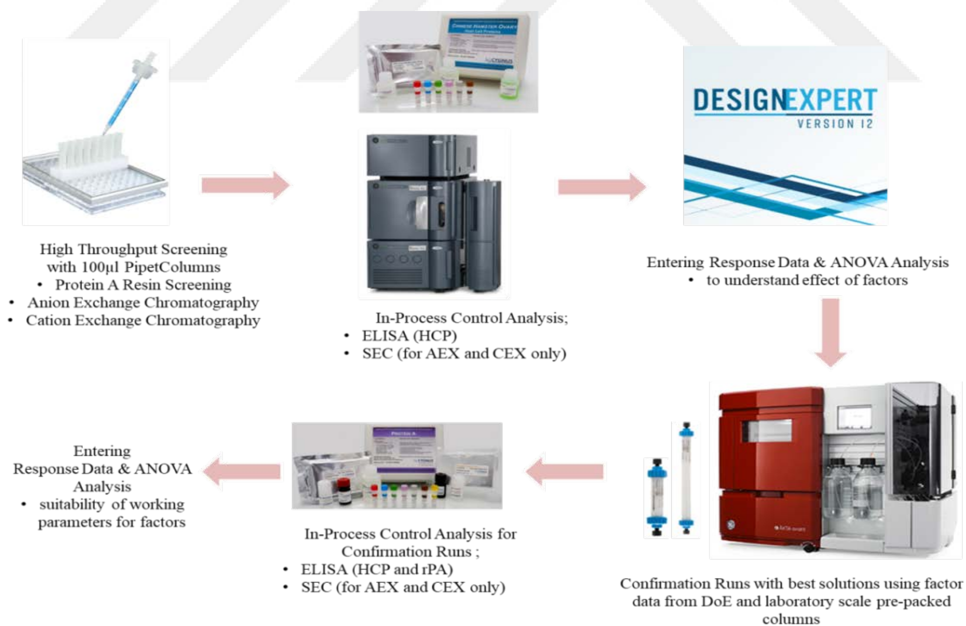

Figure 2. Schematic workflow from Downstream Process Development Platform from Screening to Confirmation Runs with Scale-up

3.3.1 Protein A Chromatography

Prediction of implementation from Protein A screening was done by performing (1.13 cm x 10 cm) with 10 ml pre-packed column. DBC was performed with related PA column at a residence time 6 minutes. DBC was calculated when the concentration of mAb in the column effluent was detected by 280 nm with 10% relative to mAb concentration (breakthrough point of 10%).

Loading quantity of Protein A was between 350-400 mg at 1.67 ml/ min flow rate a residence time 6 minutes. Working template which comes from screening was adapted on ÄKTA Avant 25, expect elution pH. Operating of process factors from DoE were performed at three times. Elution was taken once the UV increased above 20 mAU until decrease 20 mAU. Analytical characterization was performed for HCPs, rPA, aggregation and yield.

3.3.2 Anion Exchange Chromatography

Confirmation run was applied with provided process factors from DoE at three times with (0.5 cm x 5 cm) 1 ml pre-packed column. Flow rate was 0.33 ml/min and working template which comes from screening was adapted on ÄKTA Avant 25, expect loading pH and loading quantity. Analytical methods were performed for HCPs, rPA, aggregation and yield.

3.3.3 Cation Exchange Chromatography

Operating of process factors from DoE were conducted at three times with (0.5 cm x 5 cm) bed height 1 ml pre-packed column.

Flow rate was same with confirmation runs for AEX and working template which comes from screening was adapted on ÄKTA Avant 25, expect loading pH. Analytical methods were performed for HCPs, rPA, aggregation and yield.

3.4 Analytical Characterization

3.4.1 Protein Quantification

NanoDrop™ One (Thermo Fisher Scientific) was used to measure mAb concentration for all experiments using 280 nm and the extinction coefficient of 1.65 mL.mg−1. cm−1.

3.4.2 Size Exclusion Chromatography

SE-UPLC (Waters Corporation) was used to measure aggregates (HMW %) with BEH SEC200 (4.6 x 300 mm. 1.7 µm. 200 Å), (Waters Corporation) and 20 mM Phosphate Buffer, 188 mM NaCl, pH 7.4 mobile phase. Flow rate was 0.25 ml/min and qualified using reference mAb.

3.4.3 ELISA Quantification

CHO HCPs were measured using ELISA method from a Cygnus kit with sample diluent (Cat. #I028), (#F550, Cygnus Technologies, Soutport, NC). Dilution factor ranges of AEX screening CHO HCPs were applied between 1:400 - 1:600 for loading sample and 1:60 - 1:120 for FT.

Dilution factor ranges of CEX screening CHO HCPs were applied between 1:30 - 1:60 - 1:90 for loading sample and 1:10 - 1:20 - 1:40 for FT. Also, ntwith Cygnus kit (#F600, Cygnus Technologies, Soutport, NC). rPA samples were diluted between 1:100 - 1:800 for PA elution and AEX Load. 1:5 - 1:30 for CEX load and AEX/CEX FT.

4 RESULTS

4.1 Protein A Chromatography

4.1.1 Dynamic Binding Capacity

Working capacity for all mini screen columns is shown below in Table 7. The results showed that the highest DBC has PA 6, PA 7 and PA 1, respectively.

Table 7. DBC Results of Pipet Columns at 100 µl

Resin Name	DBC (mg) at 100 µl
PA 1	4.3
PA 2	2.3
PA 3	1.4
PA 4	2
PA 5	2.7
PA 6	5.2
PA 7	3.8
PA 8	2.5

4.1.2 Protein A Resin Screening

Analytical results with design layout of PA resin screening are shown in Table 8. The supernatant CHO HCP value was 4675243 ppm. Coefficients in terms of coded factors, final equation in terms of coded factors and actual factors for all responses of Protein A resin screening are shown in Appendix 1.

Table 8. Analytical Results with Design Layout for PA Resin Screening

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A:Elution pH	B:Loading Percentage (%)	C:Resin Type	Yield (%)	HCP (ppm)
1	3.44	40	PA 3	39.42	4191
2	3.51	49.2027	PA 7	82.00	3776
3	3.29	70.6	PA 7	88.00	3512
4	3.6	73.4	PA 2	79.00	866
5	3.294	49.4	PA 4	73.00	475
6	3.596	53	PA 3	77.00	589
7	3.51	49.2027	PA 7	87.00	3583
8	3.596	53.8	PA 5	86.00	876
9	3.32	79.6	PA 5	82.00	673
10	3.4	40	PA 8	82.00	1453
11	3.24	44	PA 3	84.00	1026
12	3.2	80	PA 4	79.00	320
13	3.6	60	PA 8	78.60	1327
14	3.208	68.2	PA 1	81.00	954
15	3.2	60	PA 8	77.38	349
16	3.5	41	PA 6	86.19	1007
17	3.20347	64	PA 6	83.08	1119
18	3.59319	52.6	PA 2	94.99	360
19	3.51	70.8	PA 4	76.91	634
20	3.294	49.4	PA 4	78.97	667
21	3.328	80	PA 3	76.19	595
22	3.568	80	PA 3	75.26	511
23	3.51	70.8	PA 4	74.58	1510
24	3.6	40	PA 4	74.38	1788
25	3.6	80	PA 7	63.54	2785
26	3.4	80	PA 8	59.23	405
27	3.328	79.4912	PA 2	69.92	509
28	3.24	43.2769	PA 5	98.88	223
29	3.46	40.2	PA 1	93.11	777
30	3.546	77.8	PA 6	78.77	1341
31	3.372	60	PA 3	73.50	1093
32	3.234	43.5845	PA 2	88.09	263
33	3.2	40	PA 7	84.85	2984
34	3.568	75.6	PA 1	80.80	1936
35	3.394	57	PA 5	77.27	660

Run	Factor 1 A:Elution pH	Factor 2 B:Loading Percentage (%)	Factor 3 C:Resin Type	Response 1 Yield (%)	Response 2 HCP (ppm)
36	3.29	70.6	PA 7	78.16	1906
37	3.20347	64	PA 6	95.31	1408

Evaluation of model terms is shown in Table 9. Standard error was below 1, which means that the design is balanced. Also, low VIF value and R_i^2 show that the model is significant.

Table 9. Model Terms for PA Resin Screening

Term	Standard Error*	VIF	R_i^2	Power
A	0.2403	1.14149	0.1240	96.9 %
B	0.2490	1.14736	0.1284	95.9 %
C[1]	0.4670			23.0 %
C[2]	0.3965			
C[3]	0.4400			
C[4]	0.4882			
C[5]	0.5351			
C[6]	0.4819			
C[7]	0.4769			
AB	0.3913	1.41807	0.2948	62.5 %
AC[1]	0.6578			11.3 %
AC[2]	0.5553			
AC[3]	0.6776			
AC[4]	0.6744			
AC[5]	0.7843			
AC[6]	0.7445			
AC[7]	0.7788			
BC[1]	0.6611			11.1 %
BC[2]	0.5638			
BC[3]	0.6768			
BC[4]	0.6955			
BC[5]	0.8159			
BC[6]	0.7584			
BC[7]	0.7620			
A^2	0.6150	1.88237	0.4688	82.4 %
B^2	0.6088	1.9648	0.4910	83.2 %

Fit statistics is shown is Table 10 for Yield (%). Predicted R^2 of 0.1631 is not as close to the Adjusted R^2 of 0.4088 as one might normally expect. This may indicate a large block effect or a possible problem with the model and/or data. Thanks to adequate precision, it is suitable to navigate the design space.

Table 10. Fit Statistics for Response 1: Yield

Std. Dev.	6.27		**R^2**	0.5608
Mean	80.50		**Adjusted R^2**	0.4088
C.V. %	7.79		**Predicted R^2**	0.1631
			Adeq Precision	6.9400

ANOVA analysis for Yield (%) is shown in Table 11. F-value shows the model is significant for Yield (%). Lack of fit is not significant relative to the pure error. P-values less than 0.0500 indicate model terms are significant. Also, linearity of the data is shown in Figure 3. In this case, B is a significant model term.

Table 11. ANOVA for Quadratic model for Response 1: Yield

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**
Model	1306.66	9	145.18	3.69	0.0043 **significant**
A-Elution pH	57.58	1	57.58	1.46	0.2373
B-Loading Percentage	584.22	1	584.22	14.84	0.0007
C-Resin Type	628.86	7	89.84	2.28	0.0593
Residual	1023.27	26	39.36		
Lack of Fit	866.95	21	41.28	1.32	0.4096 **not significant**
Pure Error	156.32	5	31.26		
Cor Total	2329.93	35			

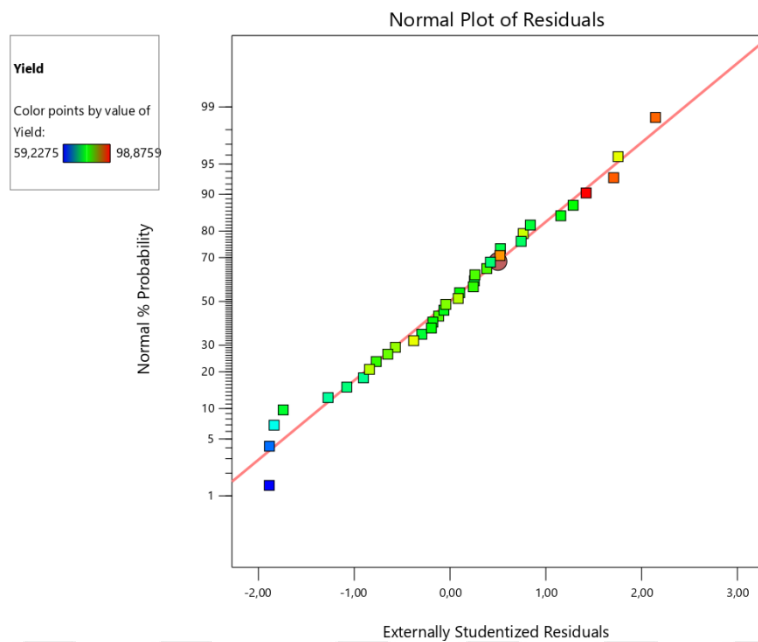

Figure 3. Data Points Linearity for Response 1: Yield

Fit statistics is shown is Table 12 for HCP (ppm). Differences between Adjusted R and Predicted R² are 0.0733 that the model is fit. Adequate precision and CV% is suitable for model discrimination and capability.

Table 12. Fit Statistics for Response 2: HCP

Std. Dev.	456.43		**R^2**	0.8386
Mean	1229.44		**Adjusted R^2**	0.7827
C.V. %	37.12		**Predicted R^2**	0.6920
			Adeq Precision	12.5919

ANOVA analysis for HCP (ppm) is shown in Table 13. F-value shows the model is significant for Yield (%). Lack of fit is not significant relative to the pure error. P-values less than 0.0500 indicate model terms are significant. Also, data point linearity is shown in Figure 4. In this case A and C are significant model terms.

Table 13. ANOVA for Quadratic model for Response 2: HCP

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	2.813E+07	9	3.126E+06	15.00	< 0.0001 **significant**
A-Elution pH	1.269E+06	1	1.269E+06	6.09	0.0205
B-Loading Percentage	2.244E+05	1	2.244E+05	1.08	0.3089
C-Resin Type	2.688E+07	7	3.839E+06	18.43	< 0.0001
Residual	5.417E+06	26	2.083E+05		
Lack of Fit	3.664E+06	21	1.745E+05	0.4980	0.8809 **not significant**
Pure Error	1.752E+06	5	3.504E+05		
Cor Total	3.355E+07	35			

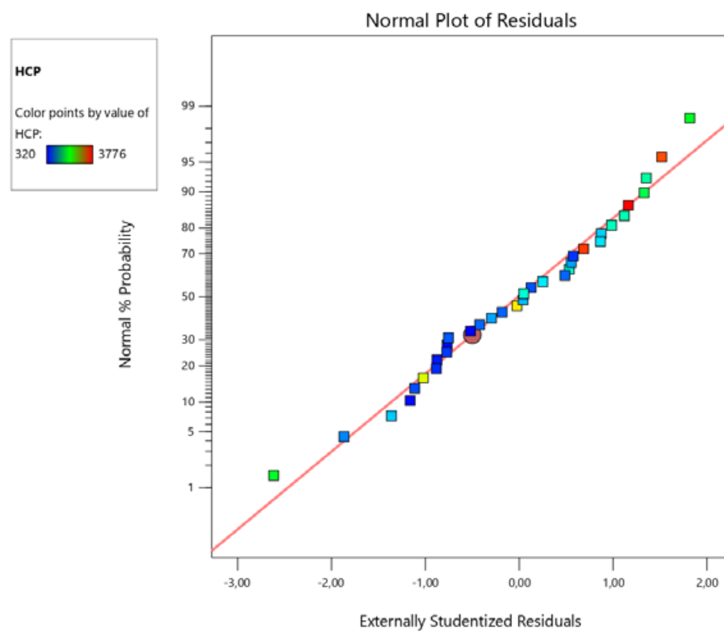

Figure 4. Data Points Linearity for Response 2: HCP

4.1.3 Confirmation Run for Protein A Screening with Scale-up Studies

After PA resin screening analysis, confirmation run with scale-up studies were done. The criteria were entered in DoE software. Working parameters for elution pH, loading percentage and resin type were kept in range. The goal for HCP was minimize to reduce impurities. Yield was maximize for achievement of process.

The criteria are shown in Table 14. The solution of DoE is shown in Table 15 with a desirability point of 0.851.

Table 14. Constraints for PA Resin Screening

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:Elution pH	is in range	3.2	3.6	1	1	3
B: Loading Percentage (%)	is in range	70	80	1	1	3
C:Resin Type	is in range	PA 8	PA 4	1	1	3
Yield (%)	maximize	59.2275	98.8759	1	1	5
HCP (ppm)	minimize	223	3776	1	1	5

After solution from DoE software, DBC was measured again at a residence time 6 minutes at a breakthrough 10%. DBC was found 60 mg/ml for PA 5. Then, confirmation run was applied with 10 ml pre-packed column with using factors in given Table 15.

Table 15. Solution for PA Resin Screening

Number	Elution pH	Loading Percentage (%)	Resin Type	Yield (%)	HCP (ppm)	Desirability	
1	3.200	70.000	PA 5	84.379	417.000	0.851	**Selected**

Confirmation run chromatogram is shown in Figure 5.Also, rPA was measured. Post-analysis with confirmation and coefficient table for Protein A resin screening are given in Appendix 2.

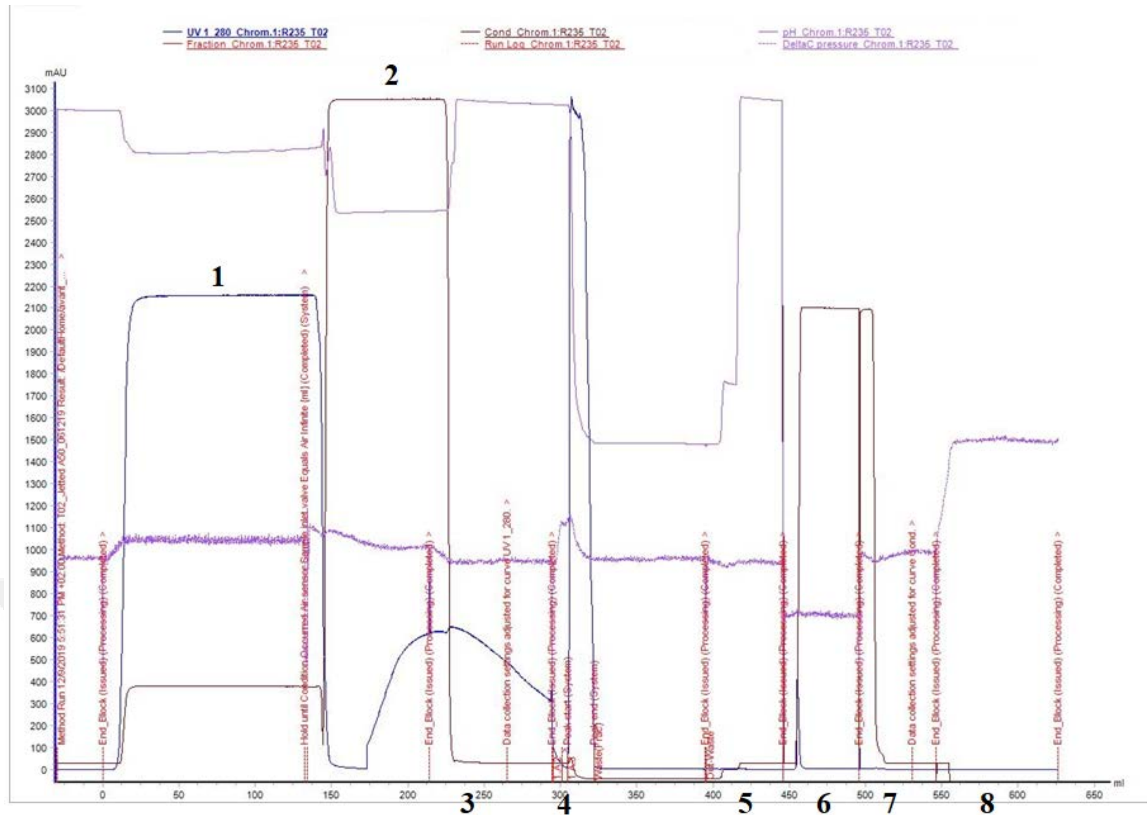

Figure 5. Confirmation Chromatogram for Protein A Screening

1. Loading: Product binding with bind-elute mode.
2. Wash 1: HCPs reduction.
3. Wash 2: Decreasing ionic strength of the column and get ready for elution.
4. Elution: Collection of product was applied.
5. Rinsing: Changing pH of the column.
6. Sanitization: Column cleaning.
7. Rinsing: Removing sanitization buffer.
8. Storage: Related column storage buffer was passed for protection of resin.

Table 16. Confirmation Run Results with 10 ml Pre-Packed Column

Run	HCP (ppm)	Yield (%)	rPA (ppm)
Confirmation Run	1378	87	7.79

4.2 Anion Exchange Chromatography

Analytical results with design layout of AEX screening is shown in Table 17. Pre-HCPs values were observed different from each other due to pH changes. Loading HCP value with different pH is shown in Appendix 3 and coefficients in terms of coded factors, final equation in terms of coded factors and actual factors for all responses of anion exchange chromatography are shown in Appendix 4.

Table 17. Analytical Results with Design Layout for AEX Screening

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Run	A:pH	B:Loading Quantity (mg)	C:Resin Type	HMW (%)	HCP (ppm)	Yield (%)
1	7	600	AEX 1	4.46	30.58	99.70
2	7	600	AEX 1	8.25	50.95	87.17
3	7	600	AEX 2	4.15	128.93	83.56
4	5.58579	600	AEX 1	0.83	99.29	90.18
5	7	882.843	AEX 3	4.37	12.79	97.51
6	7	882.843	AEX 2	5.67	136.79	81.24
7	8	400	AEX 2	4.70	173.83	82.65
8	6	800	AEX 1	1.65	66.75	96.37
9	7	600	AEX 3	4.16	11.91	100.65
10	8	800	AEX 1	5.48	29.22	90.70
11	7	600	AEX 2	4.01	5.73	78.74
12	7	600	AEX 2	4.24	47.41	76.44
13	7	317.157	AEX 1	5.11	24.29	89.40
14	7	600	AEX 1	5.04	58.55	90.33
15	7	317.157	AEX 3	4.03	111.9	94.98
16	7	600	AEX 3	3.62	10.48	95.04
17	6	400	AEX 2	1.61	20.11	90.36
18	5.58579	600	AEX 3	0.83	31.03	85.11
19	7	600	AEX 1	3.86	21.73	87.91
20	8	800	AEX 2	6.38	107.36	83.03
21	7	600	AEX 2	3.84	11.58	92.89
22	8.41421	600	AEX 2	3.56	74.11	89.89
23	8.41421	600	AEX 3	3.30	6.02	75.36
24	5.58579	600	AEX 2	0.81	29.08	79.95

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Run	A:pH	B:Loading Quantity (mg)	C:Resin Type	HMW (%)	HCP (ppm)	Yield (%)
25	6	800	AEX 3	1.84	16.18	93.16
26	7	600	AEX 1	4.19	21.27	79.60
27	6	800	AEX 1	1.07	18.99	85.56
28	7	600	AEX 2	5.23	47.85	88.80
29	6	400	AEX 1	1.76	41.01	96.33
30	7	600	AEX 2	3.73	6.88	92.41
31	8	800	AEX 2	5.28	5.12	90.40
32	7	317.157	AEX 1	5.15	6.42	88.29
33	8	400	AEX 3	4.79	3.72	82.65
34	7	600	AEX 3	4.21	5.7	93.74
35	8	400	AEX 2	5.45	7.55	92.03
36	7	600	AEX 3	4.18	4.81	93.38
37	6	400	AEX 3	1.68	11.86	93.73
38	8.41421	600	AEX 1	4.33	25.6	85.61
39	7	882.843	AEX 3	5.84	129.82	85.42

Evaluation of model terms is shown in Table 18. Standard error was below 1, which means that the design is balanced. Also, low VIF value and R_i^2 show that the model is significant.

Table 18. Model Terms for AEX Screening

Term	Standard Error*	VIF	R_i^2	Power
A	0.2041	1	0.0000	99.7 %
B	0.2041	1	0.0000	99.7 %
C[1]	0.3651			99.2 %
C[2]	0.3651			
AB	0.2887	1	0.0000	91.0 %
AC[1]	0.2887			92.4 %
AC[2]	0.2887			
BC[1]	0.2887			92.4 %
BC[2]	0.2887			
A^2	0.2189	1.01731	0.0170	99.9 %
B^2	0.2189	1.01731	0.0170	99.9 %
ABC[1]	0.4082			65.0 %
ABC[2]	0.4082			
A^2C[1]	0.3096			88.3 %
A^2C[2]	0.3096			
B^2C[1]	0.3096			88.3 %
B^2C[2]	0.3096			

Fit Statistics is shown is Table 19 for HMW (%). Differences between Adjusted R and Predicted R^2 are less than 0.2 that the model is fit. Adequate precision and CV% is suitable for model discrimination and capability.

Table 19. Fit Statistics for Response 1: HMW

Std. Dev.	0.9000		**R^2**	0.7986
Mean	3.92		**Adjusted R^2**	0.7166
C.V. %	22.99		**Predicted R^2**	0.6260
			Adeq Precision	11.8156

ANOVA analysis for aggregation is shown in Table 20. F-value shows that the model is significant for HMW (%) for quadratic model. Lack of fit is not significant relative to the pure error. P-values less than 0.0500 indicate model terms are significant. Also, data points linearity is shown in Figure 6. In this case, A, A^2 are significant model terms for HMW (%). ANOVA analysis showed that for pH condition has aggregation challenges.

Table 20. ANOVA for Quadratic Model for Response 1: HMW

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	86.73	11	7.88	9.73	< 0.0001 **significant**
A-pH	50.47	1	50.47	62.30	< 0.0001
B-Loading Quantity	0.6529	1	0.6529	0.8061	0.3772
C-Resin Type	4.05	2	2.03	2.50	0.1008
AB	0.6030	1	0.6030	0.7445	0.3958
AC	0.4569	2	0.2285	0.2820	0.7564
BC	0.0599	2	0.0300	0.0370	0.9637
A^2	27.37	1	27.37	33.79	< 0.0001
B^2	1.11	1	1.11	1.37	0.2521
Residual	21.87	27	0.8100		
Lack of Fit	7.68	15	0.5121	0.4332	0.9359 **not significant**
Pure Error	14.19	12	1.18		
Cor Total	108.60	38			

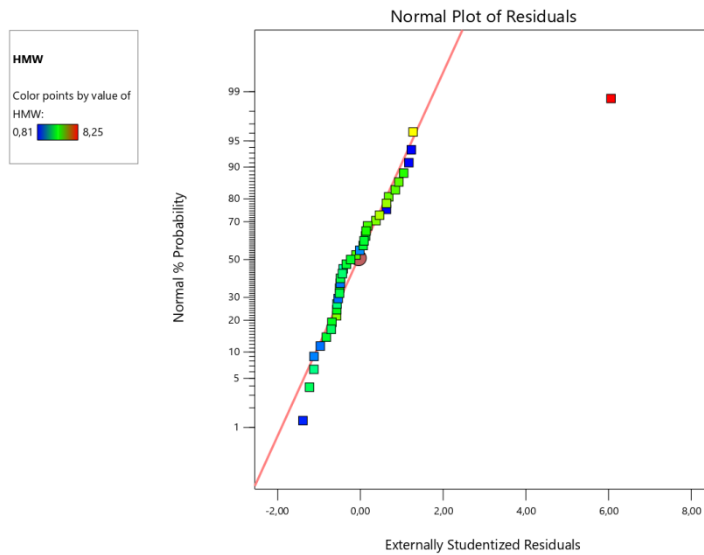

Figure 6. Data Points Linearity for Response 1: HMW

Fit Statistics analysis is shown is Table 21 for HCP (ppm). Although predicted R^2 did not match with adjusted R^2, the model was observed as significant due to adequate precision and CV (%).

Table 21. Fit Statistics for Response 2: HCP

Mean	42.39		**Adjusted R^2**	0.3905
C.V. %	82.48		**Predicted R^2**	-0.0644
			Adeq Precision	6.2015

ANOVA analysis for HCP is shown in Table 22. F-value shows the model is significant for HCP (ppm) for quadratic model with 3.21. P-values less than 0.0500 indicate model terms are significant. Also, data points linearity is shown in Figure 7. In this case, C, AC, B^2 are significant model terms for HCP (ppm), respectively. During AEX at FT mode, resin type is important for reduction of HCP.

Table 22. ANOVA for Quadratic Model for Response 2: HCP

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	43210.17	11	3928.20	3.21	0.0066 **significant**
A-pH	240.66	1	240.66	0.1969	0.6608
B-Loading Percentage	1334.90	1	1334.90	1.09	0.3053
C-Resin Type	12842.92	2	6421.46	5.25	0.0118
AB	436.09	1	436.09	0.3567	0.5553
AC	15655.66	2	7827.83	6.40	0.0053
BC	7463.18	2	3731.59	3.05	0.0638
A^2	158.07	1	158.07	0.1293	0.7220
B^2	5226.71	1	5226.71	4.28	0.0484
Residual	33007.52	27	1222.50		
Lack of Fit	22126.53	15	1475.10	1.63	0.2005 **not significant**
Pure Error	10880.99	12	906.75		
Cor Total	76217.69	38			

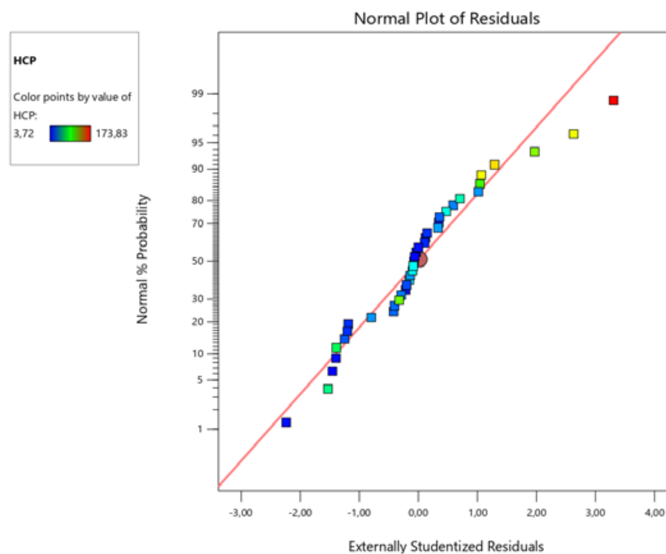

Figure 7. Data Points Linearity for Response 2: HCP

Fit Statistics is shown is Table 23 for HMW (%). Differences between Adjusted R and Predicted R^2 are less than 0.2 that the model is fit. Adequate precision and CV% is suitable for model discrimination and capability.

Table 23. Fit Statistics for Response 2: HMW

Std. Dev.	5.72		**R^2**	0.2623
Mean	88.73		**Adjusted R^2**	0.1755
C.V. %	6.45		**Predicted R^2**	0.0371
			Adeq Precision	5.5698

ANOVA analysis for Yield is shown in Table 24. F-value shows the model is significant for Yield (%) with 3.02. P-values less than 0.0500 indicate model terms are significant. Also, linearity of the data is shown in Figure 8. C is a significant model term.

Table 24. ANOVA for Quadratic Model for Response 3: Yield

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**
Model	396.07	4	99.02	3.02	0.0310 **significant**
A-pH	67.48	1	67.48	2.06	0.1604
B-Loading Quantity	4.64	1	4.64	0.1417	0.7089
C-Resin Type	323.94	2	161.97	4.94	0.0130
Residual	1113.83	34	32.76		
Lack of Fit	674.68	22	30.67	0.8380	0.6539 **not significant**
Pure Error	439.15	12	36.60		
Cor Total	1509.89	38			

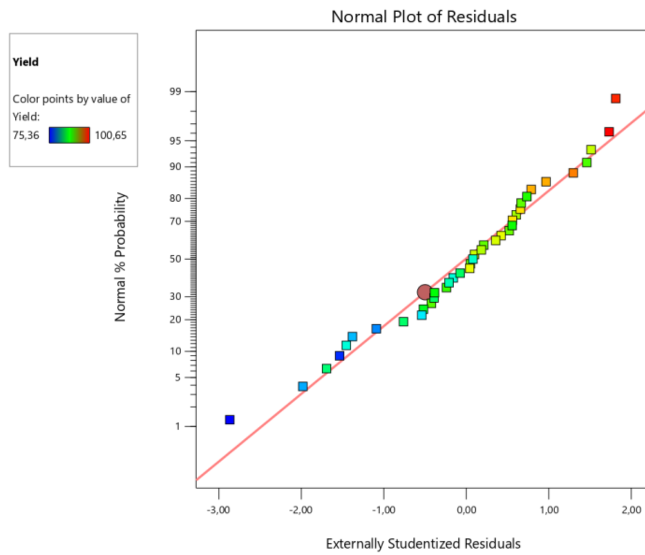

Figure 8. Data Points Linearity for Response 3: Yield

4.2.1 Confirmation Runs for Anion Exchange Chromatography with Scale-up Studies

After AEX screening analysis, confirmation run with scale-up studies were done. The criteria were entered in DoE software. Working parameters for pH, loading quantity and resin type were kept in range. The goal for HMW and HCP was minimize to reduce impurities. Yield was maximize for achievement of process. The criteria are shown in Table 25. The solution of DoE is shown in Table 26 with a desirability point of 0.831.

Table 25. Confirmation Run Criteria for AEX

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:pH	is in range	6	8	1	1	3
B:Loading Quantity (mg)	is in range	400	800	1	1	3
C:Resin Type	is in range	AEX 1	AEX 2	1	1	3
HMW (%)	minimize	0.81	8.25	1	1	5
HCP (ppm)	minimize	3.72	173.83	1	1	5
Yield (%)	maximize	75.3622	100.651	1	1	5

Table 26. Factors and Responses for Confirmation Run of AEX

Number	pH	Loading Quantity (mg)	Resin Type	HMW (%)	HCP (ppm)	Yield (%)	Desirability	
1	6.000	641.369	AEX 3	1.695	14.927	92.981	0.831	**Selected**

Confirmation run chromatograms are shown in Figure 9, Figure 10 and Figure 11. Comparability was done at three times. The results showed that responses are close to each other in Table 27. Therefore, AEX chromatography was confirmed 100µl to 1 ml pre-packed column. Also, rPA was measured. It is observed that there is no critical issue for rPA impurity. Post-analysis with confirmation and coefficient table are given in Appendix 5.

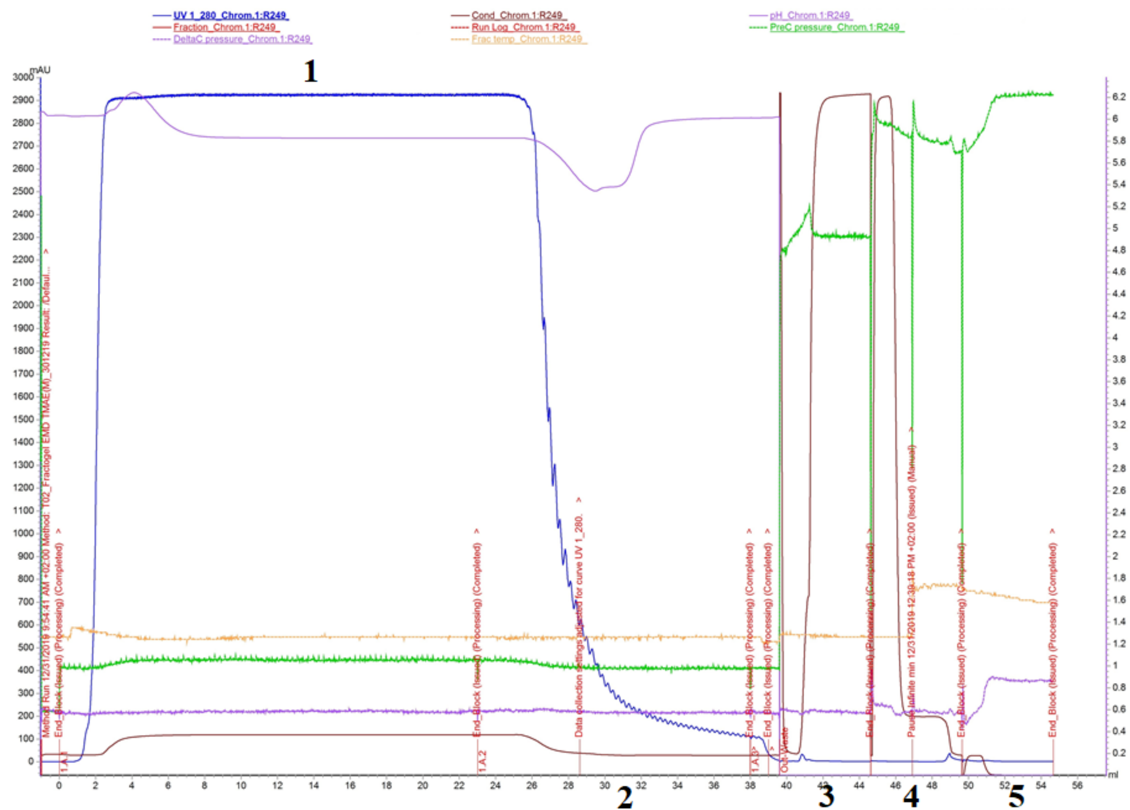

Figure 9. Chromatogram for AEX Confirmation Run 1

1. Loading: Product loading with flow-through mode.
2. Wash: Increasing the yield.
3. Sanitization: Column cleaning.
4. Rinsing: Removing sanitization buffer.
5. Storage: Related column storage buffer was passed for protection of resin.

   This chromatography process was applied for both cycle 2 and cycle 3 for confirmation runs for AEX chromatography.

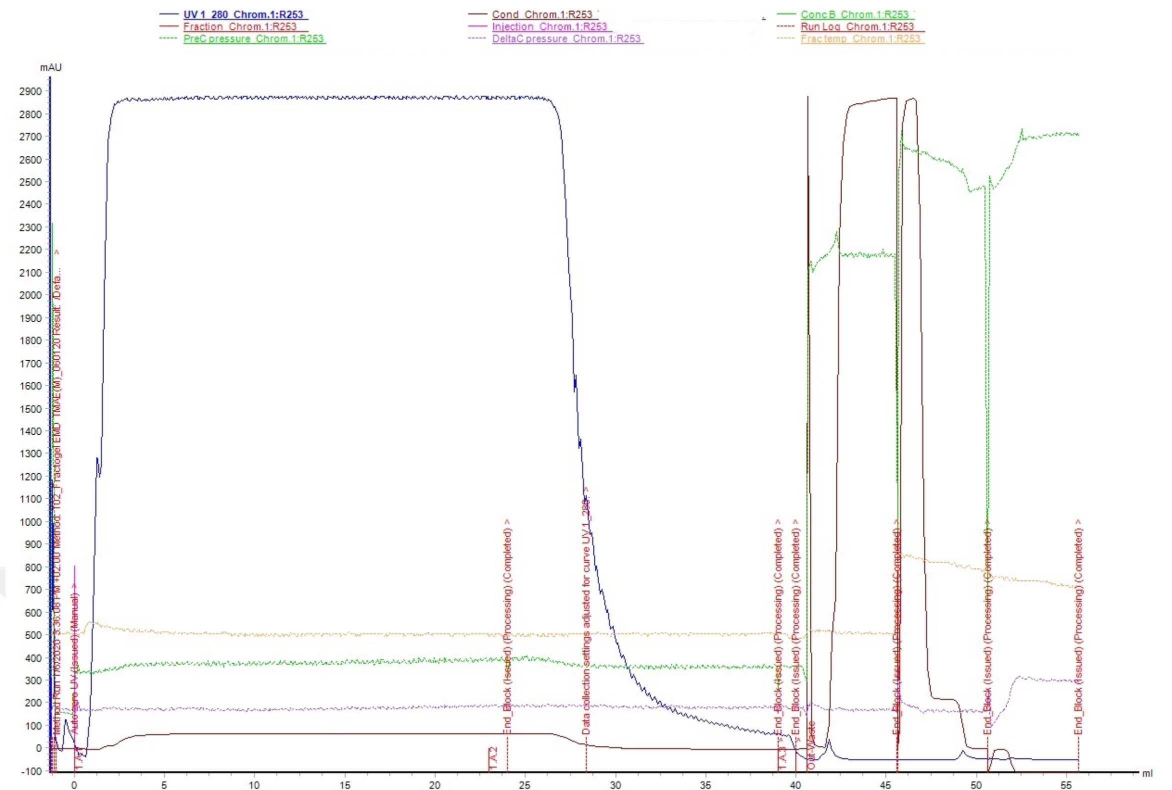

Figure 10. Chromatogram for AEX Confirmation Run 2

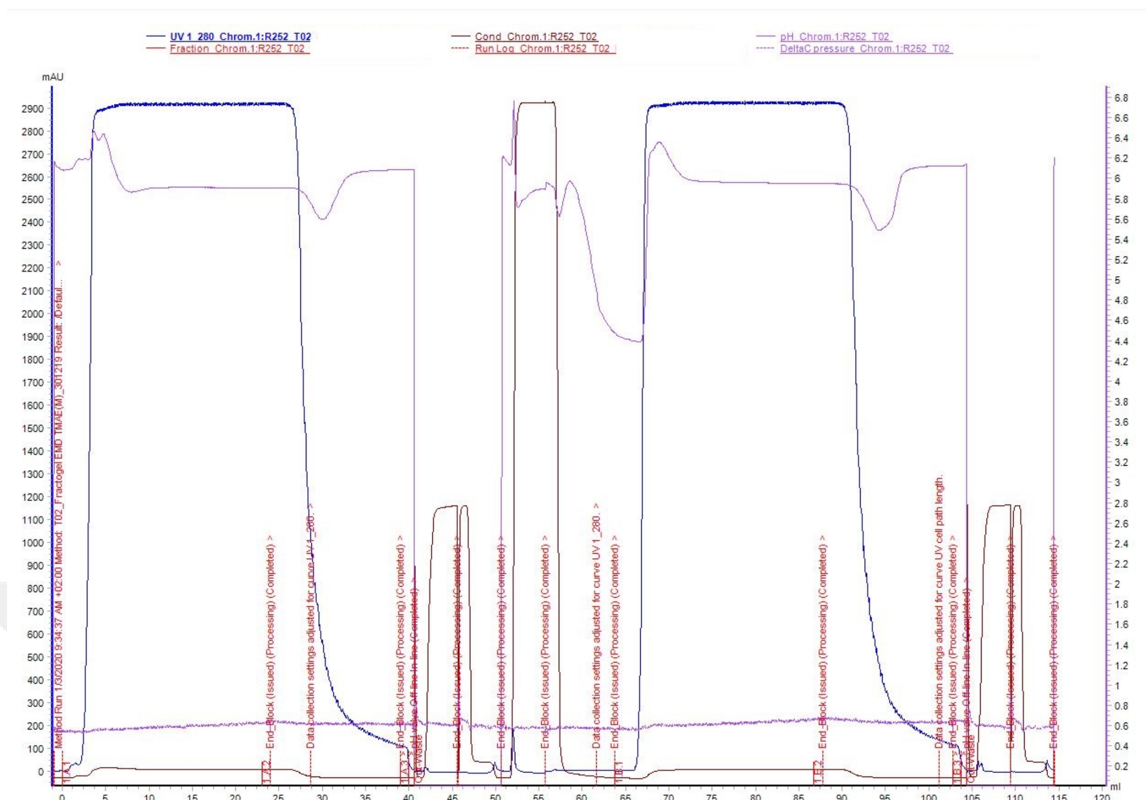

Figure 11. Chromatogram for AEX Confirmation Run 3

Table 27. Confirmation Run Results with 1 ml Pre-Packed Column

Run	**HMW (%)**	**HCP (ppm)**	**HCP Reduction %**	**Yield (%)**	**rPA (ppm)**
Confirmation Run 1	1.88	11.91	86 %	99	1.08
Confirmation Run 2	1.70	14.3	93 %	98	1.03
Confirmation Run 3	1.67	18.65	89 %	93	0.64

4.3 Cation Exchange Chromatography

Analytical results with design layout of CEX screening is shown in Table 28. Since the working range of pH values were close. Pre-HCP value is accepted as 17 ppm. Loading pH with HCP values are shown in Appendix 6 and coefficients in terms of coded factors, final equation in terms of coded factors and actual factors for all responses of cation exchange chromatography are shown in Appendix 7.

Table 28. Analytical Results with Design Layout for CEX Screening

	Factor 1	Factor 2	Response 1	Response 2	Response 3
Run	A:Loading pH	B:Resin Type	HMW (%)	Yield (%)	HCP (ppm)
1	4.375	CEX 1	0.55	78.53	3.87
2	4	CEX 1	0.67	85.22	3.52
3	4.75	CEX 3	0.39	88.03	9.20
4	4.2025	CEX 3	0.24	88.34	8.53
5	4.9225	CEX 2	0.57	83.98	3.25
6	5.305	CEX 3	0.66	86.24	8.42
7	5.5	CEX 1	0.96	68.24	4.90
8	5.125	CEX 1	0.67	74.59	4.35
9	5.305	CEX 3	0.80	80.92	7.53
10	4.75	CEX 1	0.60	80.61	3.88
11	5.29	CEX 2	0.78	81.45	4.44
12	5.29	CEX 2	0.71	80.16	4.28
13	4.195	CEX 2	0.49	84.14	1.10
14	4.5625	CEX 2	0.54	85.13	3.12
15	4.2025	CEX 3	0.25	78.97	5.79
16	4.75	CEX 1	0.58	82.06	3.17
17	4.195	CEX 2	0.51	84.63	1.56

Evaluation of model terms is shown in Table 29. Standard error, VIF and R_i^2 values showed that the design is balanced and significant.

Table 29. Model Terms for CEX Screening

Term	Standard Error*	VIF	R_i^2	Power
A	0.3812	1.00393	0.0039	66.0 %
B[1]	0.3389			71.5 %
B[2]	0.3554			
AB[1]	0.5279			34.7 %
AB[2]	0.5474			
A^2	0.7862	1.00692	0.0069	63.1 %

Statistics analysis is shown is Table 30 for HMW (%). Differences between Adjusted R and Predicted R^2 are 0.0733 that the model is fit. Adequate precision and CV % is suitable for model discrimination and capability.

Table 30. Fit Statistics for Response 1: HMW

Std. Dev.	0.0426		**R^2**	0.9669
Mean	0.5865		**Adjusted R^2**	0.9470
C.V. %	7.27		**Predicted R^2**	0.8737
			Adeq Precision	25.0755

ANOVA analysis for HMW (%) is shown in Table 31. F-value shows the model is significant for HMW (%) with 48.64. P-values less than 0.0001 indicate model terms are significant. Also, data points linearity is shown in Figure 12. A, B, AB and A^2 are significant model terms for HMW (%).

Table 31. ANOVA for Quadratic Model for Response 1: HMW

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.5300	6	0.0883	48.64	< 0.0001 **significant**
A-pH	0.2953	1	0.2953	162.61	< 0.0001
B-Resin Type	0.1221	2	0.0610	33.61	< 0.0001
AB	0.0464	2	0.0232	12.78	0.0018
A^2	0.0715	1	0.0715	39.35	< 0.0001
Residual	0.0182	10	0.0018		
Lack of Fit	0.0055	5	0.0011	0.4299	0.8122 **not significant**
Pure Error	0.0127	5	0.0025		
Cor Total	0.5482	16			

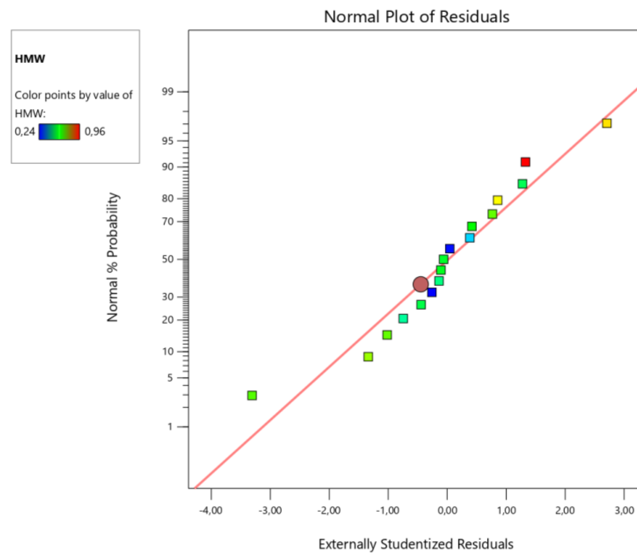

Figure 12. Data Points Linearity for Response 1: HMW

Fit Statistics is shown is Table 32 for Yield (%). Predicted R^2 of 0.1869 is not as close to the Adjusted R^2 of 0.6468 as one might normally expect. This may indicate a large block effect or a possible problem with the model and/or data. Thanks to adequate precision, it is suitable to navigate the design space.

Table 32. Fit Statistics for Response 2: Yield

Std. Dev.	2.98		**R^2**	0.7793
Mean	81.84		**Adjusted R^2**	0.6468
C.V. %	3.64		**Predicted R^2**	0.1869
			Adeq Precision	9.5335

ANOVA analysis for Yield (%) is shown in Table 33. F-value and vales show the model is significant for Yield (%). Also, data points linearity is shown in Figure 13. A and B are significant model terms.

Table 33. ANOVA for Quadratic Model for Response 2: Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	313.09	6	52.18	5.88	0.0073 **significant**
A-pH	87.44	1	87.44	9.86	0.0105
B-Resin Type	125.10	2	62.55	7.05	0.0123
AB	69.96	2	34.98	3.94	0.0546
A^2	29.67	1	29.67	3.35	0.0973
Residual	88.69	10	8.87		
Lack of Fit	28.64	5	5.73	0.4769	0.7822 **not significant**
Pure Error	60.05	5	12.01		
Cor Total	401.78	16			

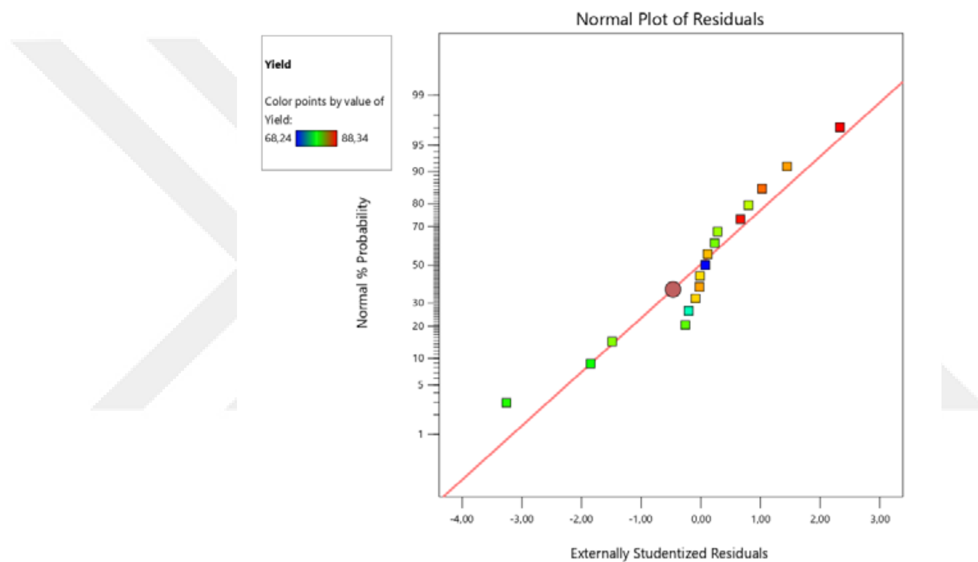

Figure 13. Data Points Linearity for Response 2: Yield

Fit Statistics is shown is Table 34 for HCP. Difference between Adjusted R and Predicted R^2 is less than 0.2 that the model is fit. Adequate precision and CV % is suitable for model discrimination and capability.

Table 34. Fit Statistics for Response 3: HCP

Std. Dev.	0.8770		**R^2**	0.9154
Mean	4.76		**Adjusted R^2**	0.8646
C.V. %	18.43		**Predicted R^2**	0.7021
			Adeq Precision	12.0841

ANOVA analysis for HCP is shown in Table 35. F-value and vales show the model is significant. Also, data points linearity is shown in Figure 14. A and B are significant model terms.

Table 35. ANOVA for Quadratic model for Response 3: HCP

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	83.18	6	13.86	18.03	< 0.0001 **significant**
A-pH	7.65	1	7.65	9.95	0.0103
B-Resin Type	72.24	2	36.12	46.97	< 0.0001
AB	2.86	2	1.43	1.86	0.2057
A^2	0.0622	1	0.0622	0.0808	0.7820
Residual	7.69	10	0.7691		
Lack of Fit	3.16	5	0.6324	0.6982	0.6485 **not significant**
Pure Error	4.53	5	0.9058		
Cor Total	90.87	16			

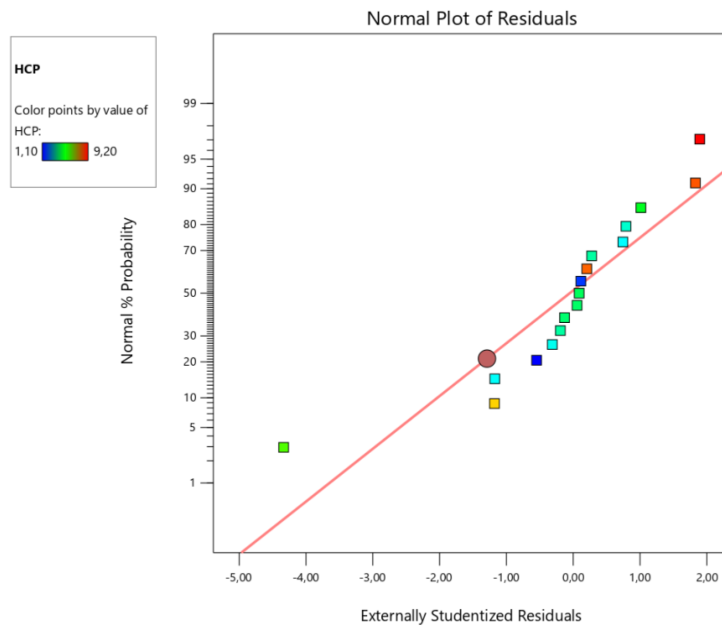

Figure 14. Data Points Linearity for Response 3: HCP

4.3.1 Confirmation Runs for Cation Exchange Chromatography with Scale-up Studies

After CEX screening analysis, confirmation run with scale-up studies were done. The criteria were entered in DoE software. Working parameters for pH and resin type were kept in range. The goal for HMW and HCP was minimize to reduce impurities. Yield was maximize for achievement of process.

The criteria are shown in Table 36. The solution of DoE is shown in Table 37 with a desirability point of 0.785.

Table 36. Confirmation Run Criteria for CEX

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:pH	is in range	4	5.5	1	1	3
B:Resin Type	is in range	CEX 1	CEX 2	1	1	3
HMW (%)	minimize	0.24	0.96	1	1	5
Yield (%)	maximize	68.2417	88.3351	1	1	5
HCP (ppm)	minimize	1.1	9.20241	1	1	5

Table 37. Factors and Responses for Confirmation Run of CEX

Number	pH	Resin Type	HMW (%)	Yield (%)	HCP (ppm)	Desirability	
1	4.322	CEX 2	0.496	84.875	1.858	0.785	**Selected**

Confirmation run chromatogram is shown in Figure 15. This chromatogram has 3 cycles. Comparability was done at three times. Loading quantity was determined 640 mg like AEX chromatography. The results showed that responses are close to each other in Table 38. HCP values are different from responses only in DoE software due to scale-up.

Confirmation run values for HCP can be accepted because of drug substance ranges shown in Table 39. Therefore, CEX chromatography was confirmed from 100µl to 1 ml pre-packed column. Also, rPA was measured. It is observed that there is no critical issue for rPA impurity. Post-analysis with confirmation and coefficient table are given in Appendix 8.

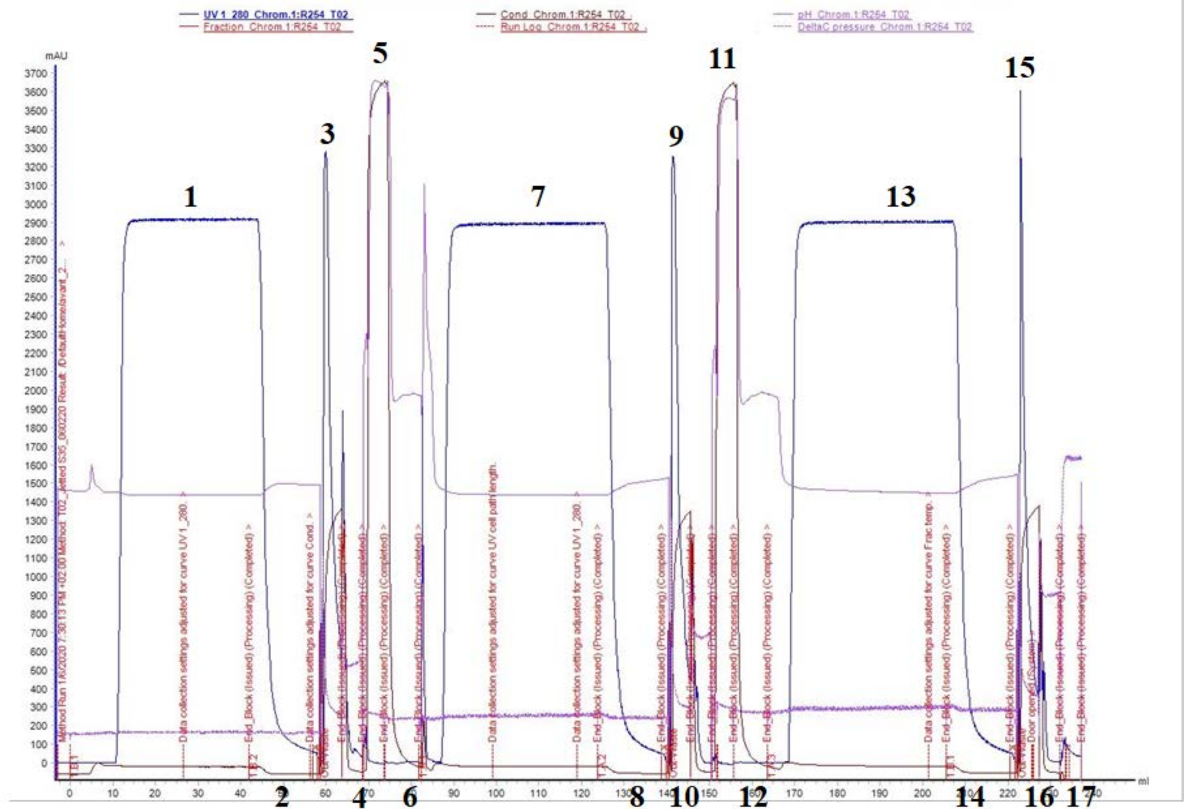

Figure 15. Chromatogram for CEX Confirmation 1,2 and 3

1-7-13 Loading: The product was passing through the column with flow-through mode.

2-8-14 Wash: This step was applied for increasing the yield.

3-9-15 Sanitization: Interaction between resin and impurities was broken and the column was cleaned.

4-10 Rinsing: This step was applied to remove sanitization buffer.

5-11 Regeneration: The aim of this step is to exhausted resin beads and removes ions which coming from last cycle.

6-12-16 Equilibration: It was applied for cycle 2 and cycle 3. Only 16.step was applied to remove sanitization buffer.

17 Storage: Related column storage buffer was passed for protection of resin.

Table 38. Confirmation Run Results with 1 ml Pre-Packed Column

Run	HMW (%)	HCP (ppm)	Yield (%)	rPA (ppm)
Confirmation Run 1	0.54	1.72	80	0.02
Confirmation Run 2	0.51	3.51	81	0.29
Confirmation Run 3	0.49	5.56	89	0.20

Table 39. Quality Attributes for Drug Substance compared to CQAs

Quality Attribute	Value Obtained	Process Target
HMW (%)	0.51 (average of CEX results)	1.48 - 3.85
HCP (ppm)	btw 1.72 - 5.56	< 100
rPA (ppm)	btw 0.02 - 0.29	< 4

5 DISCUSSION AND CONCLUSION

High-throughput strategy increases the amount of design space in development step for downstream process development platform using micro-volume columns. It helps to reduce the cost and development time with small sample volume. Decision-making tool, DoE, is very helpful for HTP within given experimental protocols. These protocols provide understanding the differences between parameters and limitations on the process development. Each mAb has a unique purification process, especially the type and the order of different chromatography steps.

Protein A chromatography is the best first capture step for mAb purification due to high selectivity and yielding high purity. For subtypes of IgG1, Protein A chromatography is preferable (20). Increased usage of Protein A resins has resulted in the emergence of variety of Protein A resins to the market. Therefore, evaluation of the performance of Protein A resins has become very important. The aim of Protein A resin screening was to find the best resin type amongst eight different Protein A resins using micro-volume columns. This study was helped to achieve selecting suitable resin type for our mAb. Base matrix and Protein A ligand type are very essential for Protein A chromatography. The results showed that the best resin type is PA 5 for the mAb used in this study. PA 5 is agarose-based base matrix. It gives high stability, binding capability and reduction of impurity (HCPs) during purification (35).

During Protein A resin screening, dynamic binding capacity was calculated. It depends on residence time, based on product loading time and flow-rate (22). Therefore, we had to know DBC value with using screening flow-rate.

In industry, product loading is applied at between 70-80% of their DBC due to safety factor and protection of life-time of the resin. Therefore, loading percentage was kept in a range of between 40-80% in DoE software for screening.

Elution of product from the protein A resin was carried out using a low pH buffer. Elution from the resin is essentially the reverse process of binding. pH elution affect the binding sites directly with reducing their affinity. This principle is the most common way to elute the mAb from the resin. Therefore, during the screening of elution conditions, pH value range was kept narrow to find the best elution condition that is suitable for the base matrix and Protein A ligand (36).

The feed composition on Protein A resin impacts host cell proteins. Also, the amount of HCPs is associated with the resin life-time performance. After loading of column, HCPs clearance are maximized with washing steps. Firstly, washing buffer with high ionic strength is applied for HCPs reduction. Then, second wash was applied with neutral pH and low ionic strength to maximize clearance without disrupting the interaction between product and Protein A ligand (22).

The results showed that resin type ($p<0.0001$) is significant in given Table 13. As discussed above, agarose-based Protein A resins achieve reduction of HCPs significantly.

During the confirmation run, residence time was selected at 6 minutes. Back pressure in the column may occur during loading of less than at a residence time 6 minutes. It may lead product loss at the industry. Therefore, DBC was calculated again at a residence time of 6 minutes before the confirmation run. Confirmation results showed that HCP value (1378ppm) is higher than expected value from DoE (417ppm).

At the same time, screening results showed that HCP value is about 1000 – 3776 ppm. It is not critical because HCPs reduction was achieved > 99 % with high process yield. Low elution pH has led to leakage of rPA from the base matrix. Laboratory scale results showed that rPA value is very low.

Anion Exchange chromatography was selected as the second polishing step in the flow through mode. Anion Exchange chromatography helps to reduce HCPs, viral clearance and process-related impurity, rPA (21). Also, the main purpose of AEX chromatography study is to find the optimum working conditions with three different types of micro-volume resins, for operating in flow through mode using AEX. pH value should be kept lower than the pI (8 - 8.5) of the mAb in order to make them positively charged. Therefore, the working pH range was selected between 6-8. The results showed that AEX 3 is best the resin type. It is a strong anion exchanger with trimethylaminoethyl group (TMAE). This resin type offered high binding capacity with working the full pH range (23). For AEX study, DBC value was not critical, because impurities are directly binding to resin whereas the product is passing through to the column.

AEX screening result showed that resin type is important for HCP reduction. At flow-through mode, HCPs bind to resin so, interaction between HCPs and resin is essential part of reduction. As discussed above, pH of the solution is about 6-8 to get positive charge on the mAb. This means that the isoelectric point of HCPs is lower than that of the mAb. As a result, HCPs has negative charge at the operating pH and HCPs bind to the resin strongly to get high reduction level. Therefore, HCPs reduction level was designated to be “high importance” in DoE for confirmation studies. Confirmation results showed that the main HCPs reduction (> 85%) is observed at AEX chromatography. Also, three confirmation run results were similar for responses, HCP, HMW% and yield. This means that designed model is suitable for process development platform.

Aggregation level is the major concern for the quality, safety and efficacy of the product. Aggregates have to removed or minimized by the chromatography steps for preparation of drug substance. AEX screening and confirmation results showed that the aggregation level (HMW %) was higher.

This issue is specific for this mAb and aggregate type for this study may be reversible non-covalent aggregation given in Mahler et al.

Non-covalent aggregates can be formed with weak non-protein interactions such as hydrophobic/hydrophilic interactions at short distance (36). A weak non-covalent reversible aggregation has been observed for the mAb molecule which is anti-VEGF given in Moore et al (37). The reversibility usually has equilibrium between the monomer and HMW%. This equilibrium can shift by changing pH or decreasing the mAb concentration. AEX screening result showed that pH ($p < 0.001$) is critical for HMW%. During confirmation runs for AEX, HMW% value was not suitable for quality attributes of therapeutic mAb drug. Therefore, cation exchange chromatography was used as third polishing step operated in flow-through mode for removing the aggregates.

During CEX study, overloading chromatography was applied with the differential binding between the product and impurities (24). Working pH range was 4-5.5 which means that mAb had posivitely charge. CEX resins have negatively charged particle, so they are supposed to bind positively charged molecules, because of the pI value of mAb, the product was also bound to the resin based on dynamic binding capacity. In this technique, impurities were bound to the resin as well as the mAb.

The results showed that aggregation reduction was successfully done for both screening and confirmation studies. Screening results presented that pH ($p > 0.001$) and resin type ($p > 0.001$) are important for aggregation. As discussed above, aggregate level can change with pH (37). This study showed that lower pH decreased the aggregation level (Table 37). Also, resin type has a critical role for both reduction of aggregation and HCPs. Because both impurities bind to the resin with overloading, like AEX, resin type was observed as significant due to binding of the resin.

For both AEX and CEX studies, residence time was selected at 3 minutes. Since impurities (HCP, HMW%, rPA) bind according to their pI value and functional groups, so faster operation flow rate provides an advantage for the industrial production plan. Also, there is no need to require elution step with flow-through mode. Therefore, buffer and material consumption are reduced. This is also another advantage for reducing cost of industrial production.

During the confirmation run, the optimum parameters were obtained from DoE to keep the impurities (HCPs and HMW %) at low levels. Three confirmation runs showed that responses are similar. The model was designed efficiently to understand the effect of the factors. Also, rPA reduction was done efficiently.

Selected resin has 90 mg dynamic binding capacity. Loading quantity was 640 mg. This means that yield should be > 75 %. The results showed that yield value was around 80-89%. Yield was increased with wash step to collect the product. With CEX confirmation run, aggregation level was also optimized, so the product became ready to be considered as a drug substance.

In this study, downstream process development platform was created for unique mAb purification with micro-volume column with less sample volume and time.

Qualitative and quantitative comparability was also seen with mini-column volume and laboratory scale column. Also, the behavior of the mAb at different conditions was observed. The information obtained from this study will be transferred to the robotic system. These are; development of conductivity, volume and pH sensors and determining system properties for sensors of the robotic system. Working range for all sensors will be provided by this study.

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7 LIST OF APPENDICES

Appendix 1: ANOVA Results for PA Resin Screening

Coefficients in Terms of Coded Factors (Sum Contrasts) for Yield

Term	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	81.29	1	1.07	79.08	83.49	
A-Elution pH	-1.72	1	1.43	-4.66	1.21	1.02
B-Loading Percentage	-5.66	1	1.47	-8.67	-2.64	1.01
C[1]	-6.98	1	2.92	-12.99	-0.9797	
C[2]	-0.7129	1	2.46	-5.78	4.35	
C[3]	-2.95	1	2.66	-8.43	2.52	
C[4]	2.69	1	2.93	-3.34	8.72	
C[5]	4.17	1	3.32	-2.65	10.98	
C[6]	4.71	1	2.93	-1.32	10.75	
C[7]	4.20	1	2.93	-1.82	10.21	

Final Equation in Terms of Coded Factors for Yield

Yield	=
+81.29	
-1.72	A
-5.66	B
-6.98	C[1]
-0.7129	C[2]
-2.95	C[3]
+2.69	C[4]
+4.17	C[5]
+4.71	C[6]
+4.20	C[7]

Final Equation in Terms of Actual Factors for Yield

Yield	=
Resin	PA 8
+120.59080	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 7
+126.86156	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 3
+124.62152	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 2
+130.26407	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 1
+131.74034	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 6
+132.28896	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 5
+131.77119	
-8.62324	Elution pH
-0.282832	Loading Percentage
Resin	PA 4
+122.45761	
-8.62324	Elution pH
-0.282832	Loading Percentage

Coefficients in Terms of Coded Factors (Sum Contrasts) for HCP

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1149.26	1	78.11	988.71	1309.81	
A-Elution pH	256.02	1	103.74	42.78	469.26	1.02
B-Loading Percentage	-110.86	1	106.82	-330.43	108.70	1.01
C[1]	-265.76	1	212.51	-702.59	171.07	
C[2]	1941.38	1	179.29	1572.84	2309.91	
C[3]	-394.24	1	193.85	-792.70	4.23	
C[4]	-686.85	1	213.36	-1125.42	-248.27	
C[5]	65.10	1	241.21	-430.72	560.93	
C[6]	125.98	1	213.52	-312.92	564.87	
C[7]	-534.02	1	212.99	-971.83	-96.21	

Final Equation in Terms of Coded Factors for HCP

HCP	=
+1149.26	
+256.02	A
-110.86	B
-265.76	C[1]
+1941.38	C[2]
-394.24	C[3]
-686.85	C[4]
+65.10	C[5]
+125.98	C[6]
-534.02	C[7]

Final Equation in Terms of Actual Factors for HCP

HCP	=
Resin	PA 8
-3136.21394	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 7
-929.07843	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 3
-3264.69319	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 2
-3557.30139	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 1
-2805.35085	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 6
-2744.47758	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 5
-3404.47521	
+1280.08848	Elution pH
-5.54312	Loading Percentage
Resin	PA 4
-3122.05118	
+1280.08848	Elution pH
-5.54312	Loading Percentage

Appendix 2: Post-analysis with confirmation and coefficient table for Protein A resin screening

Confirmation Table

Two-sided Confidence = 95%

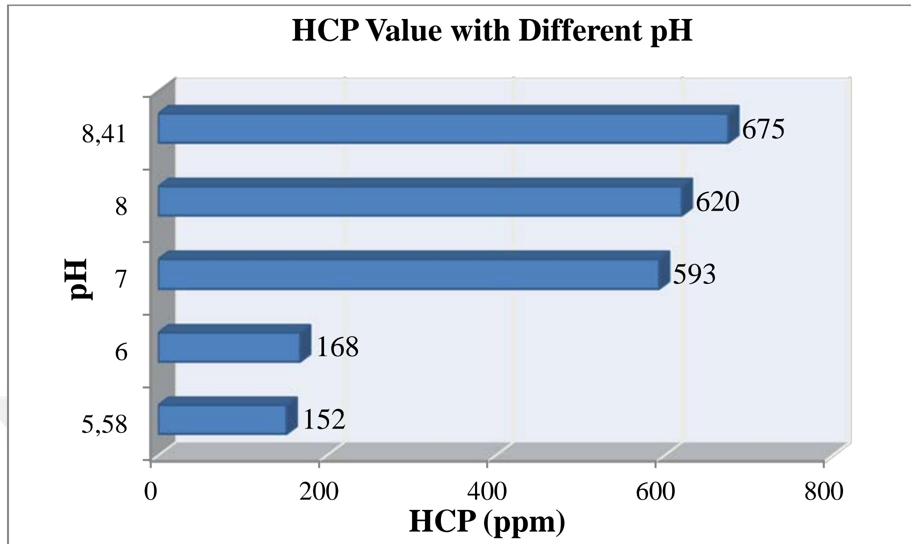
Response	Predicted Mean	Predicted Median	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Yield	82	82	6	1	7	66	95	97
HCP	349.223	349.223	469.282	1	564.954	-816.784	1378	1515.23

Coefficient Table

p-value shading: $p < 0.05$ $0.05 \le p < 0.1$ $p \ge 0.1$

	Intercept	A	B	C[1]	C[2]	C[3]	C[4]	C[5]	C[6]	C[7]
Yield	81	-1,725	-5,657	-6,984	-0.712949	-2,953	2,690	4,166	4,714	4,197
p-values		0.2373	0.0007	0.0593	0.0593	0.0593	0.0593	0.0593	0.0593	0.0593
HCP	1169.1	232.44	-135.553	-285.595	1921.46	-407.425	-657.601	48,329	103.906	-451.88
p-values		0.0509	0.2548	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Appendix 3: HCP values with Different pH for AEX Loading



Appendix 4: ANOVA Results for AEX Screening

Coefficients in Terms of Coded Factors (Sum Contrasts) for HMW

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	4.48	1	0.2324	4.00	4.95	
A-pH	1.45	1	0.1837	1.07	1.83	1.0000
B-Loading Quantity	0.1649	1	0.1837	-0.2120	0.5419	1.0000
C[1]	0.4118	1	0.2038	-0.0064	0.8300	
C[2]	-0.3751	1	0.2038	-0.7933	0.0430	
AB	0.2242	1	0.2598	-0.3089	0.7572	1.0000
AC[1]	0.1086	1	0.2598	-0.4244	0.6417	
AC[2]	-0.1947	1	0.2598	-0.7278	0.3384	
BC[1]	-0.0459	1	0.2598	-0.5790	0.4872	
BC[2]	-0.0236	1	0.2598	-0.5567	0.5095	
A^2	-1.15	1	0.1970	-1.55	-0.7410	1.02
B^2	0.2306	1	0.1970	-0.1736	0.6348	1.02

Final Equation in Terms of Coded Factors for HMW

HMW	=
+4.48	
+1.45	A
+0.1649	B
+0.4118	C[1]
-0.3751	C[2]
+0.2242	AB
+0.1086	AC[1]
-0.1947	AC[2]
-0.0459	BC[1]
-0.0236	BC[2]
-1.15	A^2
+0.2306	B^2

Final Equation in Terms of Actual Factors for HMW

HMW	=
Resin Type	AEX 1
-55.71288	
+16.91972	pH
-0.014168	Loading Quantity
+0.001121	pH * Loading Quantity
-1.14525	pH^2
+5.76458E-06	Loading Quantity ^{2}
Resin Type	AEX 2
-54.44341	
+16.61639	pH
-0.014057	Loading Quantity
+0.001121	pH * Loading Quantity
-1.14525	pH^2
+5.76458E-06	Loading Quantity ^{2}
Resin Type	AEX 3
-56.34939	
+16.89714	pH
-0.013591	Loading Quantity
+0.001121	pH * Loading Quantity
-1.14525	pH^2
+5.76458E-06	Loading Quantity ^{2}

Coefficients in Terms of Coded Factors (Sum Contrasts) for HCP

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	30.96	1	9.03	12.43	49.48	
A-pH	3.17	1	7.14	-11.48	17.81	1.0000
B-Loading Quantity	7.46	1	7.14	-7.19	22.10	1.0000
C[1]	4.27	1	7.92	-11.97	20.52	
C[2]	-24.05	1	7.92	-40.30	-7.81	
AB	-6.03	1	10.09	-26.74	14.68	1.0000
AC[1]	-25.07	1	10.09	-45.78	-4.36	
AC[2]	-9.99	1	10.09	-30.70	10.72	
BC[1]	17.12	1	10.09	-3.59	37.83	
BC[2]	-24.26	1	10.09	-44.97	-3.55	
A^2	2.75	1	7.65	-12.95	18.46	1.02
B^2	15.83	1	7.65	0.1215	31.53	1.02

Final Equation in Terms of Coded Factors for HCP

HCP	=
+30.96	
+3.17	A
+7.46	B
+4.27	C[1]
-24.05	C[2]
-6.03	AB
-25.07	AC[1]
-9.99	AC[2]
+17.12	BC[1]
-24.26	BC[2]
+2.75	A^2
+15.83	B^2

Final Equation in Terms of Actual Factors for HCP

HCP	=
Resin Type	AEX 1
+265.47905	
-42.34576	pH
-0.140866	Loading Quantity
-0.030142	pH * Loading Quantity
+2.75217	pH^2
+0.000396	Loading Quantity ^{2}
Resin Type	AEX 2
+255.76103	
-27.26652	pH
-0.347800	Loading Quantity
-0.030142	pH * Loading Quantity
+2.75217	pH^2
+0.000396	Loading Quantity ^{2}
Resin Type	AEX 3
-109.91670	
+17.77617	pH
-0.190785	Loading Quantity
-0.030142	pH * Loading Quantity
+2.75217	pH^2
+0.000396	Loading Quantity ^{2}

Final Equation in Terms of Actual Factors for Yield (D) and Final Equation in Terms of Coded Factors for Yield (E)

Yield	= (D)	= (E)	
Resin Type	AEX 1	+88.73	
+103.11464		-1.68	A
-1.67684	pH	-0.4398	B
-0.002199	Loading Quantity	+1.33	C[1]
Resin Type	AEX 2		
+104.45197			
-1.67684	pH		
-0.002199	Loading Quantity		
Resin Type	AEX 3		
+97.78023			
-1.67684	pH		
-0.002199	Loading Quantity		

Coefficients in Terms of Coded Factors (Sum Contrasts) for Yield

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	88.73	1	0.9165	86.86	90.59	
A-pH	-1.68	1	1.17	-4.05	0.6975	1.0000
B-Loading Quantity	-0.4398	1	1.17	-2.81	1.93	1.0000
C[1]	1.33	1	1.30	-1.30	3.97	
C[2]	2.67	1	1.30	0.0356	5.30	

Appendix 5: Post-analysis with confirmation and coefficient table for Anion Exchange Chromatography

Confirmation Table
Two-sided Confidence = 95%

Response	Predicted Mean	Predicted Median	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
HMW	1.69	1.69	0.90	3.00	0.68	0.31	1.75	3.08
HCP	14.93	14.93	34.96	3.00	26.26	-38.95	14.95	68.82
Yield	92.98	92.98	5.72	3.00	3.86	85.15	96.67	100.82

Coefficient Table

p-value shading: $p < 0.05$ $0.05 \leq p < 0.1$ $p \geq 0.1$

	Intercept	A	B	C[1]	C[2]	AB	AC[1]	AC[2]	BC[1]	BC[2]	A^2	B^2
HMW	4.478	1.4501	0.164942	-0.375128	0.224167	0.108638	-0.194692	-0.0458947	-0.0235876	-1.1453	0.230583	0.23058
p-values		< 0.0001	0.3772	0.1008	0.1008	0.3958	0.7564	0.7564	0.9637	0.9637	< 0.0001	0.2521
HCP	309.573	3.167	7.458	4.273	-24.051	-0,603	-25.067	-9.988	17.124	-24.263	2.752	15.826
p-values		0.6608	0.3053	0.0118	0.0118	0.5553	0.0053	0.0053	0.0638	0.0638	0.7220	0.0484
Yield	887.251	-1.677	-0.439777	1.332	2,670							
p-values	0.1604	0.7089	0.0130	0.0130								

Appendix 6: List of HCP value for CEX Loading Samples

Loading pH for CEX Screening	**HCP (ppm)**
4	21
4.19	22
4.2	21
4.37	18
4.56	17
4.75	17
4.92	16
5.12	17
5.29	14
5.3	15
5.5	14

Appendix 7: ANOVA Results for CEX Screening

Coefficients in Terms of Coded Factors (Sum Contrasts) for HMW

Term	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	0.4943	1	0.0172	0.4561	0.5325	
A-pH	0.2115	1	0.0162	0.1753	0.2477	1.00
B[1]	0.0898	1	0.0144	0.0576	0.1220	
B[2]	-0.1184	1	0.0151	-0.1522	-0.0847	
AB[1]	-0.0715	1	0.0225	-0.1216	-0.0213	
AB[2]	0.1163	1	0.0233	0.0643	0.1683	
A^2	0.2102	1	0.0335	0.1355	0.2848	1.01

Final Equation in Terms of Coded Factors for HMW

HMW	=
+0.4943	
+0.2115	A
+0.0898	B[1]
-0.1184	B[2]
-0.0715	AB[1]
+0.1163	AB[2]
+0.2102	A^2

Final Equation in Terms of Actual Factors for HMW

HMW	=
Resin Type	CEX 1
+8.12704	
-3.36264	pH
+0.373612	pH^2
Resin Type	CEX 3
+6.72942	
-3.11225	pH
+0.373612	pH^2
Resin Type	CEX 2
+7.89735	
-3.32716	pH
+0.373612	pH^2

Coefficients in Terms of Coded Factors (Sum Contrasts) for Yield

Term	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	83.72	1	1.20	81.05	86.39	
A-pH	-3.38	1	1.14	-5.90	-0.8455	1.00
B[1]	-3.73	1	1.01	-5.98	-1.48	
B[2]	2.63	1	1.06	0.2693	4.99	
AB[1]	-4.20	1	1.57	-7.71	-0.7013	
AB[2]	3.36	1	1.63	-0.2687	7.00	
A^2	-4.28	1	2.34	-9.50	0.9343	1.01

Final Equation in Terms of Coded Factors for Yield

Yield	=
+83.72	
-3.38	A
-3.73	B[1]
+2.63	B[2]
-4.20	AB[1]
+3.36	AB[2]
-4.28	A^2

Final Equation in Terms of Actual Factors for Yield

Yield	=
Resin Type	CEX 1
-43.77397	
+62.21723	pH
-7.61291	pH^2
Resin Type	CEX 3
-85.34659	
+72.30768	pH
-7.61291	pH^2
Resin Type	CEX 2
-70.88829	
+68.94269	pH
-7.61291	pH^2

Coefficients in Terms of Coded Factors (Sum Contrasts) for HCP

Term	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	5.02	1	0.3530	4.23	5.81	
A-pH	1.06	1	0.3343	0.3158	1.81	1.00
B[1]	-0.9886	1	0.2972	-1.65	-0.3263	
B[2]	2.96	1	0.3117	2.26	3.65	
AB[1]	-0.4099	1	0.4629	-1.44	0.6216	
AB[2]	-0.5068	1	0.4801	-1.58	0.5630	
A^2	-0.1960	1	0.6894	-1.73	1.34	1.01

Final Equation in Terms of Coded Factors for HCP (D) and Final Equation in Terms of Actual Factors for HCP (E)

HCP	= (D)		= (E)
+5.02		Resin Type	CEX 1
+1.06	A	-7.95369	
-0.9886	B[1]	+4.17853	pH
+2.96	B[2]	-0.348510	pH^2
-0.4099	AB[1]	Resin Type	CEX 3
-0.5068	AB[2]	-3.39397	
-0.1960	A^2	+4.04941	pH
		-0.348510	pH^2
		Resin Type	CEX 2
		-17.33615	
		+5.94732	pH
		-0.348510	pH^2

Appendix 8: Post-analysis with confirmation and coefficient table for Cation Exchange Chromatography

Confirmation Table

Two-sided Confidence = 95%

Response	Predicted Mean	Predicted Median	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
HMW	0.50	0.50	0.04	3.00	0.03	0.42	0.51	0.57
Yield	84.87	84.87	2.98	3.00	2.39	79.56	83.33	90.19
HCP	1.86	1.86	0.88	2.00	0.79	0.10	2.61	3.61

Coefficient Table

p-value shading: $p < 0.05$ $0.05 \le p < 0.1$ $p \ge 0.1$

	Intercept	A	B[1]	B[2]	AB[1]	AB[2]	A^2
HMW	0.494303	0.21147	0.0897984	-0.118444	-0.0714699	0.116328	0.210156
p-values		< 0.0001	< 0.0001	< 0.0001	0.0018	0.0018	< 0.0001
Yield	837.212	-337.505	-372.954	262.746	-420.398	336.386	-428.226
p-values		0.0105	0.0123	0.0123	0.0546	0.0546	0.0973
HCP	501.962	106.068	-0.988562	295.784	-0.409917	-0.506757	-0.196037
p-values		0.0103	< 0.0001	< 0.0001	0.2057	0.2057	0.7820

8 CURRICULUM VITAE

Personal Information

Name	Dilara	Surname	Baş
Place of Birth	İstanbul /Turkey	Date of Birth	06.06.1995
Nationality	TC	Telephone Number	05300419237
E-mail	dlrabs7@gmail.com		

Education

	Institution Name	Graduation Year
Master of Science	Acıbadem Mehmet Ali Aydınlar University – Medical Biotechnology	2020
Undergraduate	Yeditepe University – Genetics and Bioengineering	2018
High School	Başkent University Ayşe Abla High School	2013

Work Experience

Position	Corporation	Duration
Downstream Process Specialist	Turgut Pharmaceuticals	05.2020 - …
Downstream Process Development Junior Specialist	Turgut Pharmaceuticals	06.2018 – 04.2020

Foreign Languages

Language	Reading*	Speaking-	Writing*
English	Advanced	Advanced	Advanced
German	Intermediate	Intermediate	Intermediate

* Evaluated as advanced, good,intermediate,beginner

Foreign Language Exam Results#

KPDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE	DİĞER
									YÖKDİL 55

All successful exams should be enrolled.

KPDS: Kamu Personeli Yabancı Dil Sınavı; ÜDS: Üniversitelerarası Kurul Yabancı Dil Sınavı; IELTS: International English Language Testing System; TOEFL IBT: Test of English as a Foreign Language-Internet-Based Test TOEFL PBT: Test of English as a Foreign Language-Paper-Based Test; TOEFL CBT: Test of English as a Foreign Language-Computer- Based Test; FCE: First Certificate in English; CAE: Certificate in Advanced English; CPE: Certificate of Proficiency in English

Other Exams

Name of the Exam	Quantitative	Equally Weighted	Verbal
ALES**	57	58	58

** ALES: Akademik Personel ve Lisansüstü Eğitimi Giriş Sınavı

Computer Skills

Program	Ability to Use
Microsoft Office	Advanced
Design Expert	Advanced
Akta Avant UNICORN Software	Advanced