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DEVELOPMENT AND OPTIMIZATION OF THE PRODUCTION PROCESS OF A RECOMBINANT HUMAN PROTEIN BY HIGH-THROUGHPUT TECHNIQUES

SVILUPPO E OTTIMIZZAZIONE DEL PROCESSO DI PRODUZIONE DI UNA PROTEINA UMANA RICOMBINANTE ATTRAVERSO L'UTILIZZO DI TECNICHE HIGH-THROUGHPUT

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Contents

List of abbreviations

AEX	Anion Exchange Chromatography
ANOVA	Analysis Of Variance
API	Active Pharmaceutical Ingredient
AS	Ammonium Sulfate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumine
BSE	Bovine Spongiform Encephalopathy
CCC	Central Composite Circumscribed
CCD	Central Composite Design
CCF	Central Composite Face centered
CCI	Central Composite Inscribed
CEX	Cation Exchange Chromatography
CFRs	Code of Federal Regulations
CQAs	Critical Quality Attributes
CSS	Continuous Sterilizing System
CV	Column Volume
DEAE	Diethyl Amino Ethyl
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DoE	Design of Experiment
DP	Drug Product
DPBS	Dulbecco Phosphate Buffer Saline
DS	Drug Substance
DTT	DL-Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
EU	European Union
FDA	Food and Drug Administration
FF	Fast Flow
FT	Flow through
GDP	Good Distribution Practices
GLP	Good Laboratory Practices
GMPs	Good Manufacturing Practices
HA	Hydroxyapatite
HCD	Host Cell DNA
HCPs	Host Cell Proteins
HIC	Hydrophobic Interaction
HRP	Horseradish Peroxidase
HT	High throughput
HTPD	High-Throughput Process Development
ICH	International Council on Harmonization
IEX	Ion Exchange Chromatography
IL-1β	Interleukin 1β
IPC	In Process Control
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
ISO	International Organization for Standardization
MCB	Master Cell Bank
MMC	Mixed Mode Chromatography
MQ	Mustang Q
MW	Molecular Weight
NFF	Normal Flow Filtration
OD	Optical density
PEM	Protein Expression Medium

PES	PolyEtherSulfone
POI	Protein Of Interest
PPA	Polymeric Primary Amine
QbD	Quality by Design
qPCR	quantitative Polimerase Chain Reaction
R&D	Research & Development
R.T.	Room temperature
rDNA	residual DNA
RPC	Reverse Phase Chromatography
RSM	Response Surface Methodology
RV	Resin Volume
SEC	Size Exclusion Chromatography
SIP	Sterilizing In Place
TBS	Tris Buffer Saline
TFF	Tangential Flow Filtration
TLR	Toll Like Receptor
TNFα	Tumor Necrosis Factor α
TSE	Transmissible Spongiform Encephalopathy
UPE	Ultra-high molecular weight PolyEthylene
US	United States
VCC	Viable Cell Count
WB	Western Blot
WCB	Working Cell Bank
WFI	Water For Injections
WHO	World Health Organization

1. Introduction

1.1 Healthcare biotechnology

Biotechnology has revolutionized the way diseases are treated. Recombinant DNA technology has allowed the development of bio-pharmaceuticals, which can mimic the complex body proteins. These unique agents have helped the treatment of diseases in entirely new ways. An important of modern pharmacotherapy is based on “biologics” which are the biotechnology-derived drugs. The ability to produce biologics has resulted in the development of innovative drugs and vaccines for clinical needs, including cancers, rheumatoid arthritis, Crohn's disease, multiple sclerosis, macular degeneration, retinal vein occlusions and psoriatic skin diseases (1).

Biological products include a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins and can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances, or may be living entities such as cells and tissues. Biologics are originated from human, animal, or microorganism and may be produced by biotechnology methods and other cutting-edge technologies. Gene-based and cellular biologics, for example, often are at the forefront of biomedical research, and may be used to treat a variety of medical conditions for which no other treatments are available. While many drugs have a known structure and are chemically synthesized, most biologics are complex mixtures that are not easily identified or characterized. Biological products, including those manufactured by biotechnology, tend to be heat sensitive and susceptible to microbial contamination. Therefore, it is necessary to use aseptic principles from initial manufacturing steps, which is also in contrast to most conventional drugs.

Biological products often represent the cutting-edge of biomedical research and, in time, may offer the most effective means to treat a variety of medical illnesses and conditions that presently have no other treatments available (2).

The academic research groups require recombinant mammalian proteins for functional analysis (e.g. cellular signaling pathways) and high resolution structure determination and the production of these proteins in recombinant systems had a profound impact in many areas such as the biotech sector. As a matter of fact, the biotech sector has heavily invested in the production of protein therapeutics (i.e. biologics), as a relatively new and transformative approach to treating human diseases (3).

1.2 Biotech drug market steadily expands

Today several biologics such as monoclonal antibodies, insulins, peptide hormones and analogues, haematopoietic and non-haematopoietic growth factors, interferons, interleukins, erythropoietins, fusion proteins, "recombinantly produced antigens" (vaccines) and other innovative products that account for a substantial portion of all human medicines have entered into the global market of pharmaceuticals. Globally the sale of biologics was approximately $150 billion in 2015. Approximately, 30% of the pharmaceutical and biotech industries pipeline is composed of biologics, and by 2020 ten of 20 top selling drugs will be biologics. Presently biologics, including Humira (Adalimumab), Enbrel (Etanercept), Remicade (Infliximab), Avastin (Bevacizumab), Lantus (Insulin Glargine), Rituxan (Rituximab), Herceptin (Trastuzumab), Prolia (Denosumab) and Lucentis (Ranibizumab), are among the top selling pharmaceuticals worldwide. They are often prescribed long-term for chronic medical conditions, although they are expensive yet. For this reason there is a demand for generic cheaper versions of these drugs. The generic version of biologics, called "biosimilars" are the most required on the market although they cannot be exactly the same as innovators owing to structural and manufacturing complexities of biopharmaceuticals, making them similar but not exactly same as in the case of less complex small molecule pharma drugs (1).

1.3 Manufacturing of a biotechnological product: an overview

Large molecules (e.g., therapeutic proteins) are manufactured by a number of methods, including extraction from natural sources (as done in the past to extract erythropoietin from urine), modification of naturally occurring protein, mammalian cell culture *in vivo*, production by microorganisms, and chemical synthesis.

Generally, manufacturing recombinant therapeutic proteins involves:

• Cloning of a specific gene in the laboratory, or the construction of a synthetic gene;

• Insertion into a host cell and subcloning in a microorganism or cell culture;

• Process development on a pilot scale to optimize yield and quality;

• Large-scale fermentation or cell culture processes;

• Purification of the macromolecular proteins;

• Animal testing, clinical testing, regulatory approval, and marketing.

This applies to both recombinant deoxyribonucleic acid-derived products as well as recombinant proteins.

The manufacturing processes follow similar basic requirements for process validation, environmental control, aseptic manufacturing, and quality control/quality assurance systems as required for pharmaceutical products, though with a great deal more complexity, as the processes of cell propagation, purification methods, and analytical controls are significantly different and more detailed (4).

Overall, the first part of manufacturing process is the upstream that refers to cell culture, leading to fermentation. Manufacturing starts with cell cultivation where an aliquot (a vial containing cell material) is taken from the cell bank and incubated on a small scale (shake flask). This is followed by a sequence of scale-up steps, which are typically different in volume by a factor 10, to generate the inoculation culture for the production fermenter. Cells are propagated and the target protein generated in the fermentation step. The nutrients for cell metabolism are supplied through the medium and aeration. In

upstream the major strategic issue is whether the cell culture should be run in the batch, fed-batch, or in continuous mode, the latter being very attractive at low expression levels because of higher yields in continuous processing. After harvesting the cells from the cultivation broth, the supernatant (the aqueous water phase) is separated from the cell mass and this can be done by centrifugation or filtration. If the product is expressed intracellularly in inclusion bodies, the cells have to be disrupted, the cell debris separated and the target proteins dissolved in a suitable aqueous solvent. If the target protein is denatured, a refolding step may become necessary to restore the three-dimensional structure and therefore its therapeutic functionality. After refolding, the solution contains the correctly folded protein and impurities from the preceding process steps. This is comparable to the situation of extracellularly expressed proteins after elimination of the biomass. In this latter case the product does not reside in the cells, but in the watery supernatant (5).

The downstream process begins with the "harvest" where the cells are separated from the supernatant. The target proteins are separated from host and process-related impurities by several purification unit operations. These are divided into capture, intermediary purification, and polishing, resulting in the purified bulk material (drug substance). The aim of this section is to remove impurities similar to the product, such as HCPs, denatured forms of the protein, residual DNA or other byproducts. For isolation and concentration of the protein of interest (POI) a chromatographic step (direct-capture chromatography) and ultrafiltration are used; furthermore, the aqueous buffer can be exchanged by diafiltration in order to prepare the solution for the subsequent purification steps. Chromatography requires a solid matrix as stationary phase. After isolation/concentration, the protein is obtained in solution, free of crude impurities. Ultimate purification is done in "polishing" phase, where a whole range of chromatographic processes can be applied. The end of the process chain is represented by the formulation step for preparing a dosage form ready for administration to humans by converting Drug Substance (DS) into a Drug Product (DP). The aim of purification is to obtain the

dissolved protein as pure as possible and with optimal stability to make long-term storage at moderate conditions possible (5).

The entire manufacturing process must be tightly connected at each unit of operation of upstream and downstream processing. Yield variation, impurity diversity, and potency achieved are the factors that can significantly affect all steps (4-5). A scheme of a hypothetical drug substance production process is shown in Figure 1. It is interesting to note that the process is designed in order to achieve a fully disposable set-up.

1.4 Key factors for the process development of a therapeutic recombinant protein

The key factors for the process development evaluation are related with the costs, simplicity, robustness and reproducibility of the manufacturing operations. Starting from the upstream phase of a process, a crucial key factor is the fermentation titer that is the achievable product concentration in grams per liter fermentation volume at the end of the fermentation. The duration of the process depends on the cell growth rate, the achievable cell density and the productivity of the individual cell. Another key factor in the downstream process is related with the overall yield which represents the final product content referred to initial volume of the production campaign.

Type, capacity and time of process steps are the main factors for the evaluation and optimization of plant usage. The type and capacity of the process steps determine the type of the plant and the process time.

The capacity of an individual step indicates how often such a step has to be performed for the manufacturing of a desired product quantity.

Robustness of the process is very important because delivers a consistent product quality within a wide range of process parameter variations. High robustness simplifies process validation and technical process control, decreasing the risks of rejects. The specification ranges of the input parameters (e.g. stirrer speed, gas flow rate, temperature range, volume, linear flow rate,

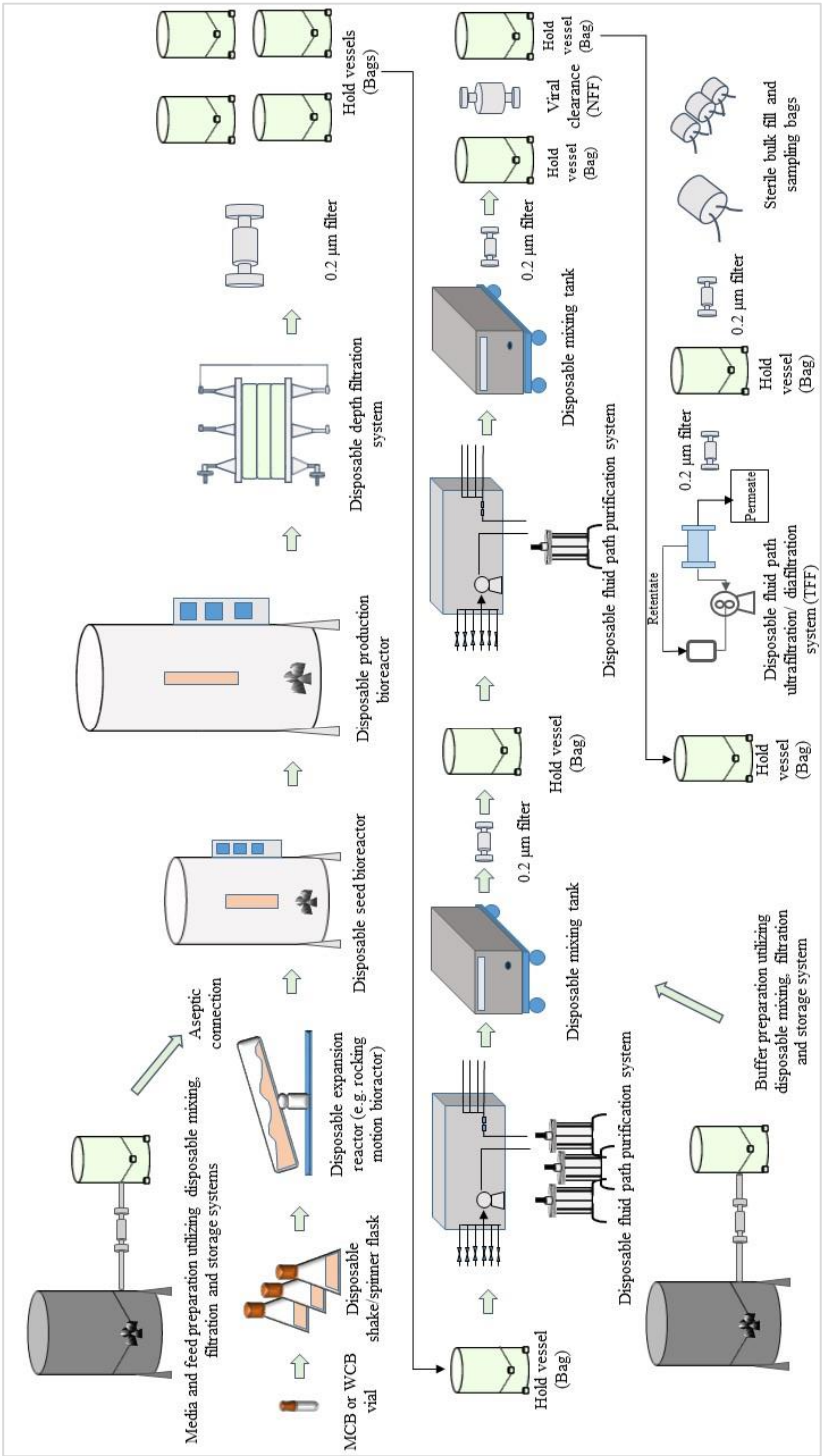


Figure 1: A biopharmaceutical drug substance production process (6)

etc.) are normally established in development studies and should be broad and accomplishable with technical equipment.

All the steps of the process are monitored by analytical approaches because it should be clear which and how many tests have to be performed for validation, In-Process Control (IPC), release, stability testing and environmental monitoring.

Raw materials are media, buffer preparations, chromatography gels, pharmaceutical water and packing material. Raw materials such as media and excipients of animal origin are very important in the evaluation and in the risk assessment of the manufacturing process but they are difficult to procure due to the limited offer of certified Transmissible Spongiform Encephalopathy (TSE)/Bovine Spongiform Encephalopathy (BSE)-free material; moreover, there is a remaining risk for contamination and high regulatory hurdles for approval of processes using these materials. Synthetic media can be used although they are too much expensive and may not support the process as well as their natural equivalents. Considering chromatography gels, the operation of some particular preparative chromatographic columns requires facilities suited to handle organic solvents. Finally, the transfer of processes is significantly simplified if standard packaging materials are used instead of custom-made vials or syringes.

Product stability means the ability of a product to retain its properties over a long period of time under defined environmental conditions. Usually, biopharmaceuticals tend to degrade with time and they are sensitive to extreme environmental conditions. Product stability has a significant impact on process design as well as transport and storage conditions. Poor stability impose a quick turnover after the upstream phase and downstream processing and the use of cold temperatures of 2–8 °C could be necessary. It is also important to consider that virus or protease inactivation steps, that need to apply high temperature or pH shifts, can go along with high product losses. Intermediates have to be correctly stored and tested for stability. At the end of the supply chain, the distribution to the client is associated with risks, since the sensitive

drug leaves the area controlled by the manufacturer. This can cause restrictions to the marketing profile and consequentially disadvantages compared to competitive products (5). The European Commission has published EU (European Union) Guidelines on Good Distribution Practice (GDP) in 1994. Revised guidelines were published in March 2013 in order to take into account recent advances in practices for appropriate storage and distribution of medicinal products in the European Union, according to new requirements introduced by Directive 2011/62/EU. Moreover a "question and answer" document (March 2014) respond to frequently asked questions in relation to the guidelines on GDP of medicinal products for human use (7).

1.5 The Good Manufacturing Practices (GMPs)

The GMPs are defined as "that part of quality assurance which ensure that products are consistently produced and controlled to the quality standards appropriate to their intended use". GMPs are guidelines issued and elaborated by international organizations and institutions, in collaboration with Pharmaceutical Industry and several national regulatory authorities in different regions and countries. GMPs are guidelines which govern the production, distribution and supply of a drug and they are a necessary condition for marketing authorization. They guarantee the highest standards of efficacy, quality and safety in any process that involves the manufacture of health products (8).

The European Medicines Agency (EMA) is a decentralized agency of the EU. The Agency is responsible for the scientific evaluation of medicinal products developed by pharmaceutical companies in the EU. EMA is responsible for emphasizing the development of guidelines, setting standards and contribution to international cooperation activities with authorities outside the EU. Directive 2001/83/EC provides the Community codes for medicinal products for human use. On 2004, the Council of the EU and the European Parliament decided some amendments (Regulation [EEC] No 2309/93 by Regulation [EC] No 726/2004) (9-10).

Volume 4 of "The rules governing medicinal products in the European Union" contains guidance for the interpretation of the principles and guidelines of good manufacturing practices for medicinal products for human and veterinary use laid down in Commission Directives 91/356/EEC, as amended by Directive 2003/94/EC, and 91/412/EEC respectively. All medicinal products are assessed by a competent authority to ensure compliance with contemporary requirements of safety, quality and efficacy (11).

The EU, including the European Commission and the EMA, has confidentiality arrangements with the Food and Drug Administration (FDA), the agency of the US Department of Health and Human Services, responsible for protecting the Public Health by assuring the appropriate regulation of medicinal products for human use, and through the encouragement of product innovations. The FDA GMPs regulations can be found in Title 21 of the Code of Federal Regulations (CFRs) (12). The arrangements allow the exchange of confidential information between the EU and the FDA as part of their regulatory and scientific processes. This includes information on advance drafts of legislation and regulatory guidance documents, as well as non-public information related to ensuring the quality, safety and efficacy of medicinal products for human and veterinary use.

A close cooperation between the several national and international entities is necessary in order to achieve a regulatory harmonization of GMP for medicinal products for human use by the competent authorities. For this reason in 1990 the International Conference on Harmonization (ICH) was established, with the propose of making recommendations, implementing standards of the International Organization for Standardization (ISO) and employing harmonization in the guidelines and technical requirements for registration of pharmaceutical products. This organization is very important because brings together the drug regulatory authorities and the pharmaceutical industry in Europe, Japan and the United States (13).

1.5.1 The process development of a biotechnological product meets the Good Manufacturing Practices (GMPs)

1.5.1.1 Cell lines and expression systems

The starting material for manufacturing therapeutic proteins are the bacterial, yeast, insect, or mammalian cell culture that expresses the protein product or monoclonal antibody of interest. The cell seed lot, that consists of aliquots of a single culture, are used by manufacturers to ensure identity and purity of the starting raw material. The Master Cell Bank (MCB) is derived from a single colony (bacteria, yeast) or a single eukaryotic cell, stored cryogenically to ensure genetic stability. The Working Cell Bank (WCB) is the quantity of cells derived from one or more ampoules of the MCB used to initiate the production batch (Figure 2) (4). The MCB and the WCB should be tested and properly characterized in accordance with the prescribed International Conference on Harmonization (ICH) Q5D protocol (14).

The tests that are generally performed for rigorously test the identity of the MCB are genotypic characterization by DNA fingerprinting, phenotypic characterization by nutrient requirements, isoenzyme analysis, growth and morphological characteristics, reproducible production of desired product, molecular characterization of vector/cloned fragment by restriction enzyme mapping, sequence analysis, assays to detect viral contamination, reverse transcriptase assay to detect retroviruses, sterility test and mycoplasma test to detect other microbial contaminants (4).

For WCB, a reduced level of characterization is required. The tests that are generally performed are the phenotypic characterization, restriction enzyme mapping, sterility and mycoplasma testing and the reproducible production of the desired product (4). Seed lots and cell banks should be stored and used in such a way as to minimize the risks of contamination, (e.g. stored in the vapor phase of liquid nitrogen in sealed containers) or alteration and control measures should be implemented to prevent mix-up and cross contaminations (15).

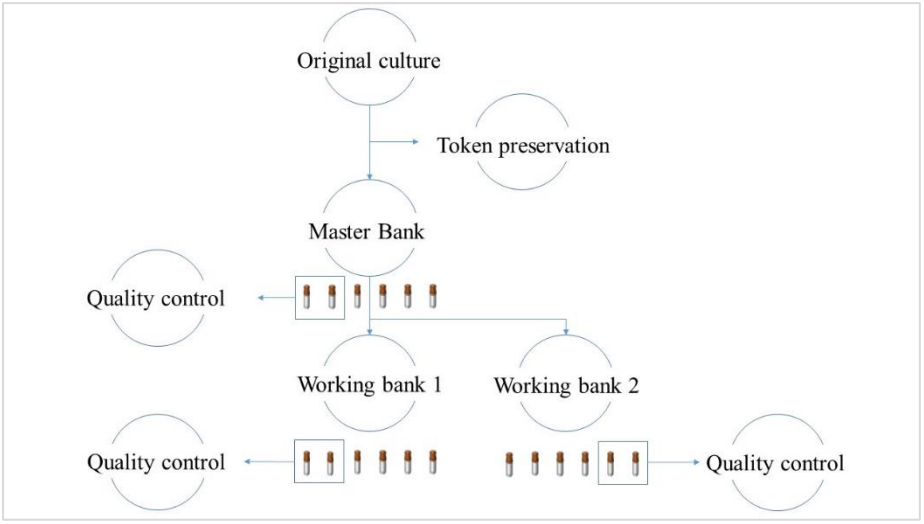


Figure 2: When a cell line is to be used over many manufacturing cycles, a cell banking system consisting of a master cell bank (MCB) and a working cell bank (WCB) is recommended. Cells from the MCB are expanded to form the WCB, which is characterized for cell viability prior to use in the manufacturing process (16)

The choice of expression system depends on factors such as type of target protein, post-translational modifications, expression level, intellectual property rights, and economy of manufacture. Information about the construction of the expression vector, the fragment containing the genetic material that encodes the desired product, and the relevant genotype and phenotype of the host cell(s) are submitted as part of a product application (4).

1.5.1.2 Media and buffers

In fermentation the cells are incubated in the nutrient medium, an aqueous solution that contains the nutrients important for cell growth and metabolism. In a process development the medium should support the vital functions and the cellular reactivity of the target cell and should be composed in such a way that purification is not unnecessarily complicated. It should not interfere with the analytical methods used in the process and should not interact with the segregated target protein or other segregated proteins (5).

Most mammalian cell cultures require animal fetal serum for growth. Serum should be sterile, although sometimes could be a source of contamination by adventitious organisms like mycoplasma. Serum could also be contaminated with BSE agent, therefore it's important to know the source of the serum and require certification that the serum does not come from areas where BSE is endemic as matter of fact that there is no sensitive *in vitro* assay to detect the presence of this agent. Other potential sources of BSE may be proteases and other enzymes derived from bovine sources (4). The risk of contamination of starting and raw materials during their passage along the supply chain must be assessed, with particular emphasis on TSE (14-15).

Biological product manufacturers have been requested to determine the origin of these materials used in manufacturing. The media used must be sterilized generally by Sterilizing In Place (SIP) or by using a Continuous Sterilizing System (CSS) process. Any nutrients or chemicals added beyond this point must be sterile (4).

Annex 2 of the volume 4 of EU guidelines regulates the characteristics of medium for the production of a biotechnology product. In particular, since media and reagents are designed to promote the growth of cells or microbial organisms, typically in an axenic state, it is necessary to control and prevent the unwanted bioburden and associated metabolites and endotoxins. The source, origin and suitability of biological starting and raw materials (e.g. cryoprotectants, feeder cells, reagents, culture media, buffers, serum, enzymes, cytokines and growth factors) should be clearly defined. Where the necessary tests take a long time, it may be permissible to process starting materials before the results of the tests are available, the risk of using a potentially failed material and its potential impact on other batches should be clearly understood and assessed. In such cases, release of a finished product is conditional on satisfactory results of these tests. The identification of all starting materials should be in compliance with the requirements appropriate to its stage of manufacture.

The growth promoting properties of culture media should be demonstrated to be suitable for its intended use. If possible, media should be sterilized in situ. In-line sterilizing filters, for routine addition of gases, media, acids or alkalis, anti-foaming agents etc. to fermenters, should be used where possible. The level and type of micro-organism present in raw materials, media, biological substances, buffers, intermediates or products must be measured by bioburden analysis.

Starting and raw materials may need additional documentation on the source, origin, distribution chain, method of manufacture, and controls applied, to assure an appropriate level of control including their microbiological quality (14-15).

1.5.1.3 Culture growth (upstream manufacturing)

Upstream manufacturing include operations for cell expansion and culture growth, starting with a single vial of frozen cells and reaching the large-scale terminal reactor where the targeted protein is expressed. These operations require highly skilled specialists trained in microbiological processes, GMPs, fermenter and bioreactor systems, automation systems, and in-process analysis instruments (17).

Cell cultures are run in batch, fed-batch, or continuous mode depending on expression system used and process development results. Bioreactor inoculation, transfer, and harvesting operations must be done using validated aseptic techniques and additions or withdrawals from industrial bioreactors are generally done through steam sterilized lines, steam-lock assemblies or disposable sterile ports. It is important for a bioreactor system to be closely monitored and tightly controlled to achieve the proper and efficient expression of the desired product. Growth rate, pH, waste by-product level, viscosity, addition of chemicals, density, mixing, aeration, and foaming are the parameters for the fermentation process and these must be specified and monitored. Other factors that can affect the finished product are shear forces, process-generated heat, and effectiveness of seals and gaskets (4).

There are two main types of bioreactors: multiple-use (stainless steel) or single-use bioreactors (disposable). The first one is the most important and the predominant version of bioreactor. It generally requires a large capital investment for purchase and installation and also validated processes for cleaning, and sterilization. For this reason single-use bioreactors are being used increasingly.

Disposable bioreactors utilize a disposable sterilized cell chamber in which the cell culture is maintained and where the risk of cross-contamination is minimized. The use of disposable bioreactors decreases the amount of validation, cleaning, sterilization, and maintenance needed per bioreactor run. For this reason, disposable bioreactors runs are able to be scheduled closer together allowing for an increase in plant production (Figure 3).

a) b)

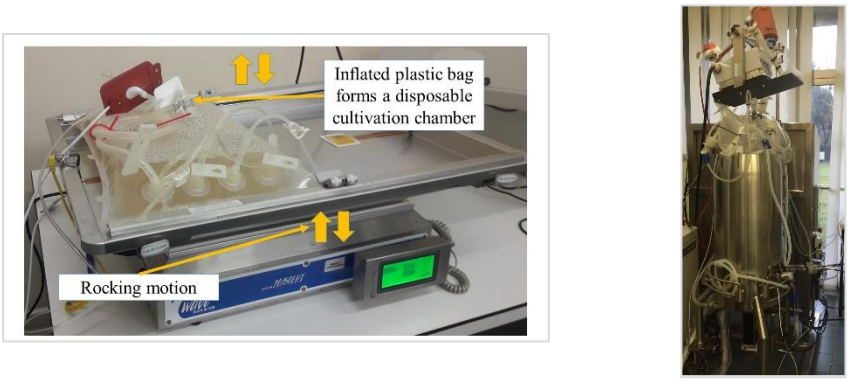


Figure 3: Disposable bioreactors: a) Rocking motion bioreactors are mechanically-driven reactor systems able to produce a "wave" inside a disposable plastic bag in order to provide mixing and gas transfer for cell growth. b) Disposable stirred tank bioreactor. These bioreactors have single-use mixing systems, and disposable bag assemblies for GMP biomanufacturing (18)

The addition of materials or cultures to fermenters and other vessels and sampling should be carried out with closed systems or under carefully controlled conditions to prevent contamination as described in ICH Q5D guidelines and in the operating principles chapter of the annex 2 of the volume 4 EU GMP guidelines. Moreover these documents underline the importance of continuous monitoring some production processes (e.g. in fermentation pH,

temperature, agitation rates, pressure cell growth, viability and productivity) and these data should form part of the documentation used to control the production process (batch record). All the containers, tubes and cell culture equipment, if not disposable, must be cleaned, sanitized and sterilized. The directive underline the necessity of appropriate in place procedures to detect contamination and determine the course of action to be taken. This should include procedures to determine the impact of the contamination on the product and those to decontaminate the equipment. Foreign organisms observed during fermentation processes should be identified as appropriate and the effect of their presence on product quality should be assessed, if necessary and the results of such assessments should be taken into consideration in the disposition of the material produced. Computer programs used to control the course of fermentation, data logging, and data reduction and analysis should be validated in accordance with 21 CFR part 11 (14-15).

1.5.1.4 Downstream bioprocessing

Downstream bioprocessing refers to the separation, purification, and modification of macromolecules from complex biological feedstocks. The feedstock is a cell suspension containing host cells that synthesizes the macromolecule of interest. A downstream unit operation, a single step in the downstream process, can be categorized into a mechanical separation, chemical separation, or dual mechanical/chemical separation step. The molecule of interest is separated from the remaining impurities mechanically, by its dimensional (size, shape) characteristics, or chemically, by its biochemical (electrical charge, interaction with other macromolecules, oiliness) properties. Several downstream processing techniques apply both mechanical and chemical separations simultaneously, and can be highly selective for the molecule of interest. Product separation and purification is accomplished through a series of process steps including filtration, chromatography, precipitation, and centrifugation. In addition to the separation

and purification of the target drug molecule, downstream processes modify the drug molecule and its environment.

1.5.1.4.1 Clarification

Downstream processing begins with the separation of large insoluble contaminants from the feedstock or "harvest" solution, usually whole cells and cell debris. This mechanical separation process is called clarification. For expression systems in which the molecule of interest is secreted outside the cell, into the surrounding solution, and a relatively low density of cell debris is present, depth filtration is a common clarification technique. Depth filtration is a key purification unit operation and often the final step in particle conditioning processes involving precipitation and/or flocculation (17).

The 3D matrix of depth filters is commonly employed for clarification of cellular debris, HCPs and DNA, or for capture of solid product and are widely used because of low equipment cost and easy scalability from bench scale to production phase (Figure 4). Although often used for steric retention, the adsorptive properties of certain filters offer the potential for targeted removal of impurities at any location in a purification process and have utility as polishing steps (19). Several varieties of depth filters are readily available with some acting as both mechanical and chemical separators that bind charged contaminants from the host cell such as DNA and proteins.

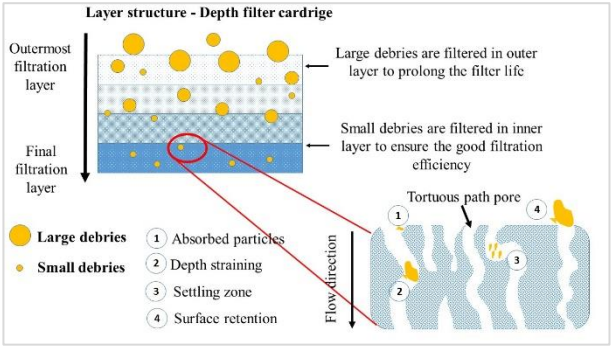


Figure 4: Internal structure of a depth filter. Due to the channel-like nature of the filtration medium, the particles are retained throughout the medium within its structure (20)

Another way to remove large particulate from the feedstock is centrifugation. Continuous flow centrifuges are better than traditional laboratory centrifuges because they allow the separation of the product from the feedstock in a single batch, taking advantage of density differences between liquids and solids. After this step the harvest solution is passed through different fine filters, in order to protect further downstream processing steps from unwanted contaminants and debris (19). It is important that harvesting steps are performed using equipment and areas designed to minimize the risk of contamination according to rules governing medicinal products in the EU and ICH guidelines. Harvest is defined as the procedure to remove or inactivate the producing organism, cellular debris and media components and must not degrade, contaminate or reduce the quality of the molecule of interest. Moreover, the clarification step should be adequate to ensure that the intermediate or Active Pharmaceutical Ingredient (API) is recovered with consistent quality (21-22).

1.5.1.4.2 Chromatography

Chromatography is the primary tool used in downstream processing and it refers to the separation of molecules that exist together in a solution. Column chromatography is the most common form of chromatography in biomanufacturing, in which a liquid mobile phase containing the molecule of interest passes through a solid stationary phase. The stationary phase, commonly referred to as chromatography resin or media, contains immobilized chemicals called ligands and can operate in different ways depending on the downstream process operation. The different affinity mechanisms have lead to the development of different chromatography principles described in Table I.

The ligands can bind to the product molecule, allowing other unwanted molecules to pass through the column and be discarded. This strategy is referred to as “bind and elute mode” chromatography. The opposite occurs in “flow through” chromatography, in which the molecule of interest passes through the column while impurities bind to the ligands.

Table I: Chromatographic techniques (5)

CHROMATOGRAPHIC TECHNIQUE	*DESCRIPTION*
Ion exchange chromatography (IEX)	IEX works on basis of the different electrostatically charged molecular moieties in a protein. They are capable of binding to oppositely charged molecules that are immobilized on the solid gel particles in IEX. CEX (negatively charged immobilized ions) and AEX (positively charged immobilized ions) depend on the polarity of the bound proteins.
Affinity chromatography	In affinity chromatography, binding to the molecules is achieved by a very specific complementary structure between ligand and protein.
Size exclusion chromatography (SEC)	In SEC, the separation principle is based on the pores in the particles. When passing the particles, small molecules diffuse easier and deeper into the pores than big ones. Consequentially the small molecules lose speed relative to the large ones and arrive at the column outlet later.
Hydrophobic interaction chromatography (HIC)	Sample molecules containing hydrophobic and hydrophilic regions are applied to an HIC column in a high-salt buffer. The salt in the buffer reduces the solvation of sample solutes. As solvation decreases, hydrophobic regions that become exposed are adsorbed by the hydrophobic media. The more hydrophobic the molecule, the less salt is needed to promote binding. Usually a decreasing salt gradient is used to elute samples from the column in order of increasing hydrophobicity. Sample elution may also be assisted by the addition of mild organic modifiers or detergents to the elution buffer.
(Reverse phase chromatography) RPC	RPC separates molecules according to differences in their hydrophobicity. In theory, HIC and RPC are closely related techniques since both are based upon interactions between hydrophobic patches on the surface of biomolecules and the hydrophobic surfaces of a chromatography medium. The surface of an RPC medium is usually more hydrophobic than that of a HIC medium. This leads to stronger interactions that, for successful elution, must be reversed using non-polar, organic solvents such as acetonitrile or methanol.
Mixed mode chromatography (MMC)	Mixed-mode chromatography materials contain ligands of multimodal functionality that allow protein adsorption by a combination of ionic interactions, hydrogen bonds, and/or hydrophobic interactions.

Many commercially resins, sharing the same ligands, differ from each other in polymer that holds the ligands that, normally, does not interact with macromolecules in solution. The polymer backbone is a highly porous spherical bead in column chromatography, or in a 3D matrix in the case of membrane chromatography. The capture chromatography step is the first chromatography step of a downstream process and is necessary in order to bind the molecule of interest. Capture chromatography usually involves the use of an affinity ligand, a molecule that strongly attracts the product macromolecule. A common affinity ligand is Protein A, a 42 kDa surface protein found in the cell wall of *Staphylococcus aureus*, that binds human antibodies. Protein A-based resins are widely used in biomanufacturing to separate monoclonal antibody products from mammalian host-cell impurities. For recombinant proteins most chromatography resins can be used as the initial capture step such as cationic, anionic and mixed mode exchangers. Another chromatographic step is the polishing that is able to increase the purity of the target macromolecule reducing the amount of contaminant such as HCPs and DNA for delivery of the drug to the patient (17).

Membrane chromatography is an alternative to traditional column chromatography, as ligands are attached to a 3D matrix rather than a spherical bead. Membrane adsorbers technology is available for some time and recently it was used into industrial processes, although only in the Anion Exchange Chromatography (AEX), mostly quaternary amine "Q" chemistry. There have been publications on the use of Hydrophobic Interaction Chromatography (HIC) membrane chromatography to mediate a precipitation purification, which has the added innovation of combining the particulate-retaining capability of the filter. Another possibility is the use of Cation Exchange Chromatography (CEX) membranes. This is predominantly used as a column chromatography step in binding and elution mode for the removal of trace impurities, predominantly aggregates. The use of membrane chromatography (Figure 5) is advantageous for some downstream processes since higher flow rates through the chromatography matrix can be achieved as compared to most

traditional resin types, and capital costs are lower without the need for expensive column hardware. However, membrane chromatography can be not convenient to use at commercial production scales, where the material costs of the membranes can exceed the cost of column chromatography. A small subset of membrane chromatography systems combines the flow rate advantages of membrane chromatography with the reusability of traditional column chromatography (23).

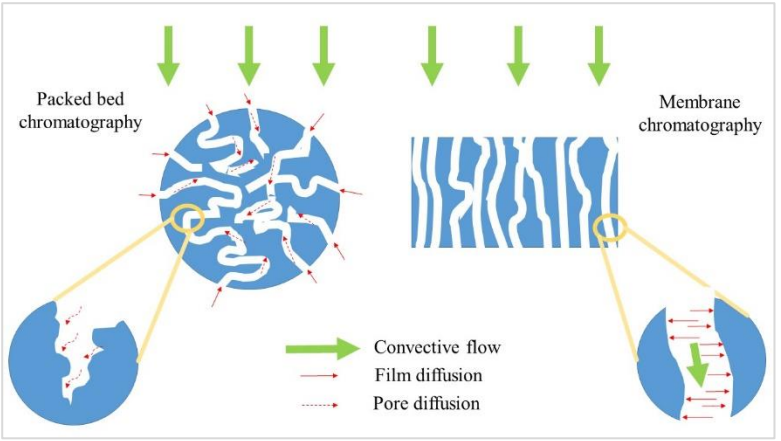


Figure 5: A significant functional advantage of membranes over resins is that the transport of molecules to their binding sites takes place mainly by convection with minimal pore diffusion, which results in a binding capacity more or less independent of the flow rate (24)

In a downstream processing operation, selecting the right mobile phase is equally important as choosing the proper stationary phase. The salt solutions that pass through chromatography media that establish the proper mobile phase conditions are called buffers. A great deal of attention is directed at buffer preparation to ensure that the chemical components meet rigorous regulatory standards for pharmaceutical use and the buffers are prepared correctly. Advances in disposable technology are particularly applicable to the buffer preparation process. A cost to store large volumes of buffer solutions in stainless steel tanks is not feasible for most biomanufacturers, so disposable plastic bags are preferred (17).

The purification procedures that remove contaminants should reduce degradation, contamination, and loss of quality of the product of interest. In a GMP facility all equipment should be properly cleaned and, as appropriate, sanitized after use. Multiple successive batching without cleaning can be used if intermediate or API quality is not compromised. When open systems are used, purification should be performed under environmental conditions appropriate for the preservation of product quality. Additional controls, such as the use of dedicated chromatography resins or additional testing, may be appropriate if equipment is to be used for multiple products (21-22).

1.5.1.4.3 Tangential Flow Filtration (TFF)

TFF is a rapid and efficient method for separation and purification of biomolecules. It can be applied to a wide range of biological fields such as immunology, protein chemistry, molecular biology, biochemistry, and microbiology. TFF can be used to concentrate and desalt sample solutions ranging in volume from 10 mL to thousands of liters. It can be used to fractionate large from small biomolecules (diafiltration), harvest cell suspensions, and clarify fermentation broths and cell lysates.

The pores in a tangential flow filter are small enough that the drug product does not pass through and continues flow parallel to the filter surface. Sample solution flows through the feed channel and tangent to the surface of the membrane as well as through the membrane. The crossflow prevents build up of molecules at the surface that can cause fouling. Impurities, salts, and water pass through the filter and are discarded. TFF can be utilized as a preparative step between chromatography steps or to formulate the product of interest with the optimal salts and excipients (25).

1.5.1.4.4 Viral clearance

Several mammalian expression systems contain viruses that are intentionally present to manufacture the drug macromolecule. Foreign viruses can also contaminate the cell culture and can be difficult to detect. To protect

patients from harmful viral agents, downstream processes have a shield to eliminate viral contamination, known as “viral clearance”. The primary viral clearance operation in most downstream processes is known as viral filtration. Many types of specialized viral filters are available which allow the product molecule to pass through but trap viruses. The challenge with viral filtration is that some viruses, especially parvoviruses, are very small and similar in size to product molecules therefore the precision to which these filters are made is critical for patient safety. To ensure the filter performs properly, an air test is performed to detect microscopic leaks that could have allowed a virus through. Viruses are often susceptible to extreme pH, temperature and detergents, while many biologic drugs are not as sensitive. Acid treatment is a common strategy for viral reduction in a monoclonal antibody downstream process, while detergent treatment is sometimes used to reduce viral contamination for enzyme products (15).

The volume 4 of GMP guidelines refers to ICH Guideline Q5A for specific information on viral safety evaluation of biotechnology products derived from cell lines. In particular, in the first part, this document describes the potential sources of viruses contamination that could occur in MCB or adventitious viruses that could be introduced during production. The second part describes the testing for viruses for cell line qualification and unprocessed bulk while the last part is dedicated to the evaluation and characterization of viral clearance procedure. Viral removal and viral inactivation steps are defined as critical steps for some processes and should be performed within their validated parameters. Appropriate precautions should be taken to prevent potential viral contamination from pre-viral to post-viral removal/inactivation steps. Therefore, open processing should be performed in areas that are separate from other processing activities and have separate air handling units. The same equipment is not normally used for different purification steps. However, if the same equipment is to be used, it should be appropriately cleaned and sanitized before reuse. Appropriate precautions should be taken to prevent potential virus carry-over (e.g. through equipment or environment)

from previous steps. In order to validate the viral clearance step a scale down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should be shown to be representative of commercial-scale manufacturing. Deviations which cannot be avoided should be discussed with regard to their influence on the results (26).

1.6 Quality by Design (QbD) and the design space

QbD is receiving a lot of attention in both the traditional pharmaceutical and biopharmaceutical industries subsequent to the FDA published "Guidance for Industry: Q8 Pharmaceutical Development" in May 2006 (27). Primary challenges in successfully implementing QbD are requirements of a complete understanding of the product and the process. This knowledge must include understanding the variability in raw materials, the relationship between the process and the Critical Quality Attributes (CQAs) of the product, and finally relationship between the CQA and the clinical properties of the product (28).

A good definition of QbD can be found in ICH Q8 (R2): "Quality by Design (QbD) is a systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management". It means designing and developing formulations and manufacturing processes to ensure a predefined quality. QbD requires an understanding how formulation and process variables influence product quality (29-30).

A systematic approach to pharmaceutical development should start with the desired clinical performance and then move to product design. The desired product attributes should then drive the process design, and the process design should drive the strategies to ensure process performance. This systematic

approach may be iterative and thus the circular design as shown in Figure 6. The inner circle interacts with many other specific measures of pharmaceutical manufacturing, such as specifications and critical process parameters. This QbD circle can be divided into two major semicircles, product knowledge and process understanding. A critical tool for enabling QbD manufacturing is a defined way of linking these two semicircles. These can be connected using the concept of a design space described in ICH Q8. A design space is the multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality (28).

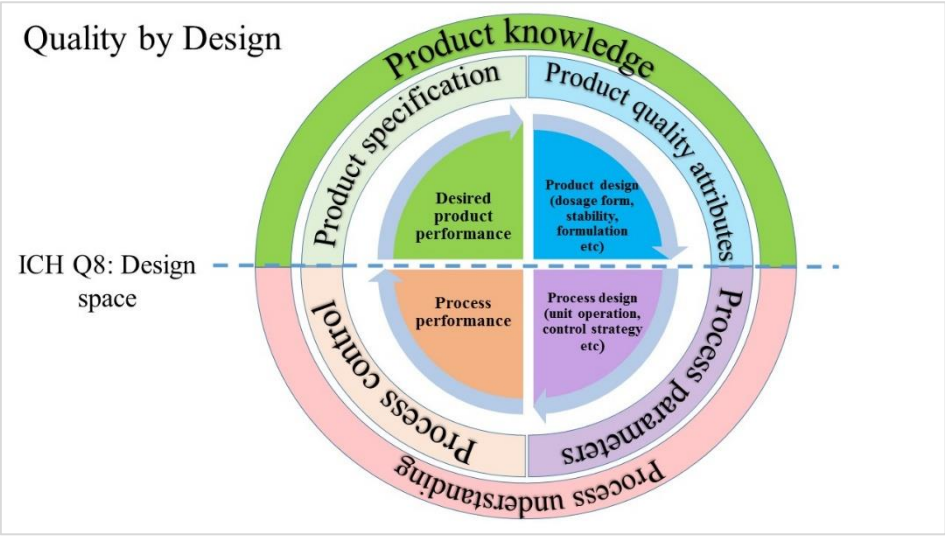


Figure 6: QbD systematic approach (28)

1.7 The Design of Experiments (DoE) in the downstream process development

Downstream bioprocessing for biotechnology products may afford more immediate opportunities for generation of large design spaces. The use of a design space approach is compared to more traditional process limits. In the simplest traditional approach to optimize experiments, one parameter is varied while all others are fixed. It can wrongly be assumed that the optimum levels for the factors analyzed can simply be found by using the optimum levels of

the factors obtained in the two series of experiments. Further, the traditional set-up does not take into account the parameters interactions where experimental factors can be dependent of each other. As follows, with the one-factor-at-a-time experimental set-up, there is a great risk that the true optimum for the studied process is not identified (31).

DoE is a technique for planning experiments and analyzing the information obtained. The technique allows to use a minimum number of experiments, in which several experimental parameters are varied simultaneously to obtain sufficient information on the effect of each individually parameter as well as combined. Based on the obtained data, a mathematical model of the studied process is created. The model can be used to understand the influence of the experimental parameters on the outcome and to find an optimum for the process. The design can be visualized by a cube that represents the experimental space to be explored and where factors are represented by the axes of the cube (x1, x2, and x3 represent three different factors, e.g., pH, conductivity, and temperature). Using DoE, multiple factors handled in a single series of experiments can be viewed in arrangements called hypercubes as the set-up becomes multidimensional and, depending on the study to be performed, different types of designs are available (32).

1.8 How to set-up a DoE

In order to start a DoE set-up, some basic information regarding the process to be studied must be available, for example which factors could possibly impact the process. Hence is necessary to define the overall project goals and the study objective, define process requirements (measurable) or issues that are not strictly part of the DoE, define the size of the study, identify all parameters that have an effect on the end result and exclude irrelevant ones, pool all available information about the factors and responses, define factors and their levels.

The starting point for any DoE work is to state the objective, define the questions about the process to be answered, and choose the relevant factors

and ranges. For example in a screening study, the objective could be to identify key parameters that impact purity and yield in an affinity chromatography capture step. Another objective could be to identify the most suitable chromatography medium for achieving high target protein homogeneity in a capture or polishing step. These screening studies could subsequently be followed by optimization studies, again using DoE, with objectives such as maximizing purity and yield.

Screening DoE explores the effects of a large number of factors in order to identify the ones that have significant effect on the response of a process or system and to determine which factors need to be further characterized or optimized. DoE is typically used for screening studies, optimization studies, and robustness testing. Screening explores the effects of a large number of factors in order to identify the ones that have significant effect on the response of a process or system and to determine which factors need to be further characterized or optimized. All critically important variables are considered before reducing number of variables while optimization is used for determination of optimal factor settings for a process or a system. Robustness testing is used for determination of process robustness through the identification of the responses that do not vary significantly when the factor levels are changed.

Factors are the input parameters or conditions that have to be controlled and varied for a process and should have an impact on the response to measure. Quantitative factors are characterized by being on a continuous scale, for example, pH, flow rate, and conductivity. Qualitative factors are discrete (discontinuous), for example, column type, type of chromatography medium, and buffer substance. Uncontrollable factors might affect the response but are difficult to manage, for example, ambient temperature or target protein amount in the cell culture.

The second step of DoE set-up define what to measure as responses from the process, set the specification limits and define a reliable measurement methodology and perform a measurement system analysis.

In screening studies, the range should be large enough to increase the possibility of covering the optimum and to obtain effects above the noise. In optimization DoE, the range should, and can usually be reduced as there is more information available at this stage.

The third step of the DoE approach is the creation of an experimental design. This is a process performed by defining factors, factor ranges, and the objective. The experimental design is completed by a model, a mathematical description of the process, which depends on the complexity of the selected design. Terms used in modeling, are the main (linear), interaction (two-factor), and quadratic terms.

Screening designs are useful when the main effects must be determined or when we wish to disregard parameter interactions or nonlinear relationships. In optimization designs the experiment is set-up in order to quantitate nonlinear cause-and-effect relationships and allow to increase the complexity of the mathematical modeling by adding square terms, and hence, to spot a minimum or maximum for our process.

The DoE ends performing the experiments generated by the design approach and creating a mathematical model able to fit the results obtained by the experiments. Analysis of variance (ANOVA) can be used to assign significance to the effect of variables and interactions (32).

1.8.1 Types of design

For studying the effect of two factors (e.g., conductivity and pH) on process outputs (response variables), and including all combinations of high and low settings for both of these factors, a full factorial design can be used.

A visualization of a design space for a full factorial design using two variables is a square with four corner experiments. The corners represent all combinations of the two factors at a high and a low level. A full factorial design also includes replicated center points between the high and the low level ([high + low]/2) for both factors. The center point experiments are repeated at least three times and their main function is to measure variability. Center points will

also detect curvature but cannot assign a specific factor level as the cause of the curvature.

A visualization of the design space for three factors would be a cube with each corner representing an experiment as represented in Figure 7. The number of experiments can be calculated by the formula N = 2k where k is the number of factors and N is the number of experiments. Full factorial designs support linear effects and all interactions so that each factor can be evaluated separately. For a two-level full factorial design the number of experiments is: N = 2k + 3 where the number 3 represent the center points. The center point allows detection of curvature, and is usually run in triplicate to estimate the noise.

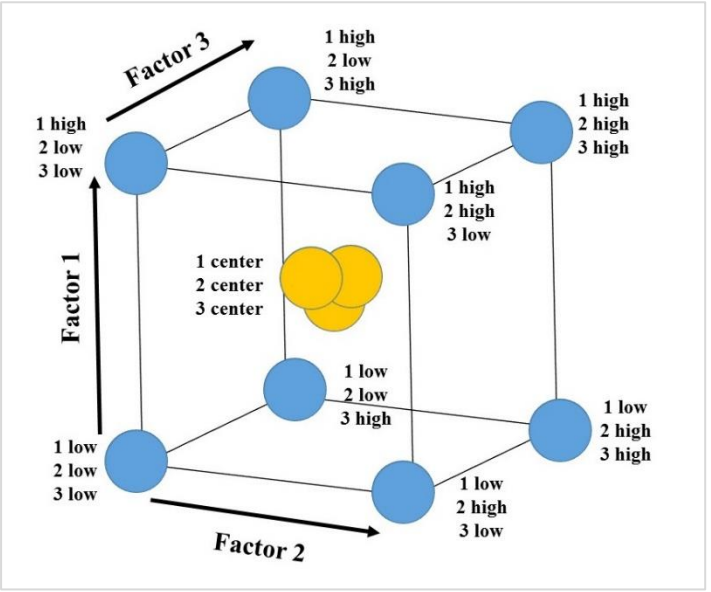


Figure 7: Three-factor full factorial design with three identical center-point experiments (32)

For studies where four or more factors are of interest, such as in a robustness test or a screening study, it is quite common to employ fractional factorial designs. A fractional factorial design is constructed in a way that it will still be possible to identify main effects without acquiring the detailed

information that a full factorial design provides. The experiments are selected by using a symmetrical selection of corners, diagonals, and opposite diagonals. In general, a fractional factorial design can be designated as $N = 2k–p$, where N is the number of experiments, k is the number of factors to be investigated, and p the size of the fraction (1 = ½, 2 = ¼, 3 = ⅛, etc.). Plackett-Burman is one of the most common screening design. This designs using $N = 4 \times k$ number of runs to investigate up to (N-1) factors, can only be used to fit linear models. However, these models are in general heavily confounded by interaction effects but, if these interactions are negligible, the Plackett-Burman design can be used for efficient detection of large main effects.

The Central Composite Design (CCD) contains a factorial or fractional factorial design with center points that is augmented of a value α, with a group of "star points" that allow estimation of curvature. If the distance from the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center of the design space to a star point is $|\alpha| > 1$. The specific value of α depends on certain properties desired for the design and on the number of factors involved. A central composite design always contains twice as many star points as there are factors in the design. The star points represent new extreme values (low and high) for each factor in the design (Figure 8). Central Composite Circumscribed (CCC) designs are the original form of the central composite design. The star points are at some distance α from the center based on the properties desired for the design and the number of factors in the design. The star points establish new extremes for the low and high settings for all factors. These designs have circular, spherical, or hyperspherical symmetry and require 5 levels for each factor.

For those situations in which the limits specified for factor settings are truly limits, the Central Composite Inscribed (CCI) design uses the factor settings as the star points and creates a factorial or fractional factorial design within those limits (in other words, a CCI design is a scaled down CCC design with each factor level of the CCC design divided by α to generate the CCI design).

Central Composite Face Centered (CCF) requires 5 levels of each factor. In this design the star points are at the center of each face of the factorial space, so $\alpha = \pm 1$. This variety requires 3 levels of each factor.

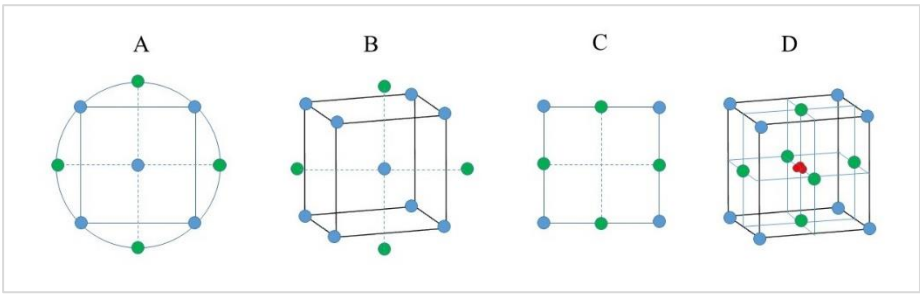


Figure 8: Central composite designs. (A) Two-factor CCC, (B) three-factor CCC, and (C) two-factor CCF design, and (D) three-factor CCF design (32)

Figure 9 represents an example of a CCD design where a three-factor CFF design was used to optimize the elution conditions of a human immunoglobulin (IgG) bound to protein A. The three factors considered in the design are pH (3 to 4), arginine concentration (0 to 1 M) and NaCl concentration (0 to 750 mM). The number of experiments to perform are seventeen including three replicates at the center point.

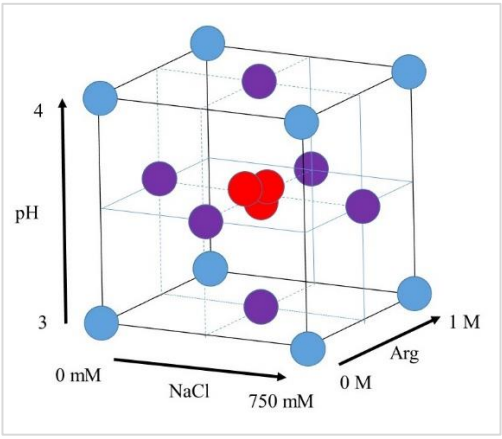


Figure 9: Example of a CCF design proposed to optimize the elution condition of an IgG. Each sphere represent a single experiment to perform (32)

In the Box-Behnken design, experiments are performed on the edges instead of in the corners. This design avoids the corner settings with all factors simultaneously at high/low. Instead, the Box-Behnken design supports linear, interaction and quadratic effects for all model terms. The Box-Behnken design is suitable for three to seven factors and is especially useful for investigations of many (five to seven) parameters. This design is also suitable to use when some corner-point settings are not feasible because of process limitations (32-33).

1.9 High-Throughput Process Development (HTPD)

Efficient development of the manufacturing process is a requirement in the biopharmaceutical industry as well as in other industries. A steadily increasing demand from regulatory authorities for a better understanding and control of manufacturing processes puts even more pressure on the development work. In HTPD, the initial evaluation of chromatographic conditions is performed in parallel, often using a 96-well plate format. Further verification and fine-tuning is typically performed using small columns before moving up to pilot and production scale. This approach to process development is performed using DoE.

Designing purification processes for biopharmaceutical proteins at industrial scale is challenging, as there are numerous possible combinations of chromatography media and conditions. Fast process development has lately come in focus. In order to facilitate a "short time to market" the development of these processes should be performed as soon as possible even though only small amounts of pure API may be available.

Chromatographic methods in packed bed format are widely used in Research & Development (R&D), process development and quality control for resin characterization. Chromatographic experiments are often time-consuming and require large sample and test substance amounts. HTPD has emerged as a tool of interest in bioprocessing. The primary motivation for creating such a platform is that it allows to examine the effects and interactions

amongst the numerous process parameters that can impact step by performing larger number of experiments with relatively limited costs and time (34).

The use of high-throughput methods such as parallel batch uptake experiments in microtiter plate format has the potential to substantially reduce both analysis time and material costs. Batch uptake assays have long been used in screening studies, but this study shows that analytical batch uptake methods can be used for determination of dynamic and total binding capacity for chromatographic resins (35-36-37).

2. Aim of the work

Most biotechnology unit operations are complex in nature with numerous process variables, feed material attributes, and raw material attributes that can have significant impact on the performance of the process. DoE-based approach offers a solution to this problem allowing for an efficient estimation of the main effects and the interactions with minimal number of experiments (38).

This PhD work can be divided in two main parts. In the first part DoE and the HTPD approaches were adopted in order to optimize a production process of a therapeutic recombinant protein expressed in PER.C6 cell line (39).

The macromolecule studied is a 340 kDa, glycosylated octameric human protein expressed in response to pro-inflammatory signals like Toll Like Receptor (TLR), Tumor Necrosis Factor α (TNFα) e Interleukin 1β (IL-1β). In particular, it is produced by many cell lines like mononuclear phagocytes, dendritic cells, fibroblasts endothelial cells. This proteins binds the complement protein C1q and specific microorganisms such as *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. The interest for this protein is related to its function as an inflammatory marker in many diseases and to its use for the development of antifungals and antibacterials.

In this PhD work the protein production process was further optimized in order to introduce a fully compliant GMP process. The optimization of the purification process can be driven by increasing the yield and by reducing the HCPs, residual DNA, protein aggregates and viral contamination. First, a new pilot scale disposable depth filtration technology as alternative to centrifugation for the harvest of the supernatant containing the protein of interest was evaluated. Second, the three chromatographic steps of the downstream process were optimized in terms of HCPs and rDNA reduction by screening and DoE approaches. In particular in the capture and polishing steps, these studies were used in order to introduce washing steps able to decrease impurities content. The same DoE approaches, were also used in the

intermediate step on hydroxyapatite to reduce the concentration of phosphate in elution buffer preventing the co-elution of the POI and the rDNA.

Further strategy followed to reduce contaminants was the introduction of a membrane chromatographic step by flow-through applications. An evaluation of three chromatographic membranes was performed in order to adapt this technology for trace contaminant removal and virus clearance applications. The last aim was the introduction of viral reduction steps achieved by thermal inactivation and filtration, in order to satisfy ICH Q5A guidelines. In particular, three different virus removal filter were tested and sized in order to identify the best in terms of performances for the implementation in the full scale process.

In the second part of the work, the DoE and HTPD technology were used as a systematic approach for the optimization of purification process for other recombinant proteins. These two innovative techniques, tested and developed during the optimization of the 340 kDa protein purification process, were used as a standardized "tool box" for the purification steps of other recombinant proteins or macromolecules such as plasmid DNA produced in microorganisms such as *Escherichia coli (E. coli).*

The intention was to propose a comprehensive approach based on a step by step "decision tree" in order to lead the experimenter in a well defined path with the purpose of purifying and formulate any protein of interest. The protein used to test the procedure was a 13 kDa protein expressed in *E. coli* as inclusion bodies and used in rescue of the retinal function in glaucoma. This hydrophobic and basic protein is challenging to purify and to formulate in a stable and high concentrate formulation; therefore this model represents a good case study to test the efficiency of the systematic approach.

2.1 Confidentiality statement

This PhD project was performed at Areta International S.r.l., an Italian biotech company specialized in contract development and GMP manufacturing of biodrugs and advanced therapy medicinal products. Areta is a multi-purpose service company that performs also contract research and is also co-developing a pipeline of products with other partners. For these reasons, some information related to the projects that have been performed as a service for the clients of the company are confidential and will not be disclosed in this work, such as the name of the drugs and their therapeutic indication. Anyway, the non-disclosure of such information does not represent a limitation to the aim of this work, which is strictly focused at the set-up of the methods that remain valid among similar variants of the same biological therapeutic class.

3. Materials and methods

3.1 DoE and HTPD approaches

3.1.1 Design of experiment using the statistical software MODDE pro 11.0

The experimental cycle of DoE using MODDE (Umetrics – MKS) consists of three phases. In the first phase a design of experiment is proposed where variables and their ranges, the responses and the objective of the design are defined.

The second step corresponds to the analysis phase where data are explored, the raw data and the fit are reviewed and finally a model to explain the data is refined. The last phase correspond to the prediction phase where the model is used to predict the optimum area for operability.

The statistical analysis, performed in order to evaluate the model fitting and to interpret the data obtained, are composed by the summary of fit plot, the coefficient plot, the interaction plot and the contour response surface plot.

In the summary of fit plot four model statistics are considered. The R^2 value gives a measure of how much of the overall data variance the model can explain. R^2 describes how well the model fits the current data. It can vary between 0 and 1, where 1 equals a perfect model and 0 corresponds to no model at all. A high R^2 value is necessary for a good model but not sufficient on its own. A value of 0.75 indicates a rough but stable and useful model and an R^2 of 0.5 is a model with rather low significance.

The Q^2 value is a measure of how well the model will work for future predictions and it can usually vary between 0 and 1. The higher Q^2 value, the better indicator of how well the model will predict new data. Q^2 should be greater than 0.1 for a significant model and greater than 0.5 for a good model. Q^2 is a better indicator of the usefulness of the model than R^2 that should not exceed Q^2 by more than 0.2–0.3 for a good model.

The model validity is a value representing the lack of fit (a low value indicates that the model suffers from lack of fit). Model validity tests a variety

of problems and it is only available if replicated experiments have been performed. A model validity > 0.25 indicates a good model while a model validity < 0.25 indicates statistically significant model problems, such as the presence of outliers, an incorrect model, or a transformation problem. A low value may also indicates the missing of interaction or square term. When the pure error is very small (replicates almost identical), the model validity can be low even though the model is good and complete.

The reproducibility compares the repeatability variation (replicates) with the overall variation (rest of the data). A reproducibility < 0.5 indicates that there is a large pure error and poor control of the experimental set-up (high noise level).

In the coefficient plot, we can view the effect and importance of each model term indicated by the height (positive or negative) of the response change as the factor changes from its low to high level. The coefficient plot is also useful for model refinement. Thus, nonsignificant terms are identified by checking the confidence intervals (the noise contained in the confidence intervals). If the confidence interval covers zero the term is not significant.

The interaction plot shows if there is no interaction between two factors. The factors are represented as two lines, if they are parallel no interaction can be observed, if not an interaction is evident. If the two lines are crossing, there is a strong interaction between the two factors.

A response-surface plot is generated to get a graphical representation of the experimental region. From this plot, the most interesting area can be used to plan new experiments, verifying experiments, and to get a better understanding of the impact of large factor interactions. The response-surface plot is a tool for visualizing interaction and curvature effects (40).

3.1.2 HTPD approaches

The chromatographic HTPD approaches were performed using the vacuum manifold apparatus (Pall). First, a 50% (v/v) resin slurries were prepared and the desired volume of media were added to the 96 well acrowell

filter plate. The slurries were mixed frequently to avoid settling and inconsistent volume delivery to the plate. Subsequently a 1 mL collection plate was placed underneath the filter plate and the excess liquid from the well was removed by applying vacuum for ten seconds (0.34-0.69 bar). Afterwards, about five Resin Volumes (RV) of binding buffers were added to the well and then vacuum was applied. After the equilibration of the resins, the volumes of desired protein samples were loaded and the filter plate was mixed thoroughly using a plate mixer for 1 hour at room temperature to facilitate binding. After the incubation, Flow-Throughs (FT) fractions, containing unbound proteins, were collected. The resins were washed twice with a total of 10 RV of equilibration buffer. Each addition of buffer was mixed for 5 minutes before evacuating. Finally, retained proteins were eluted with 3 RV of elution buffers (Figure 10).

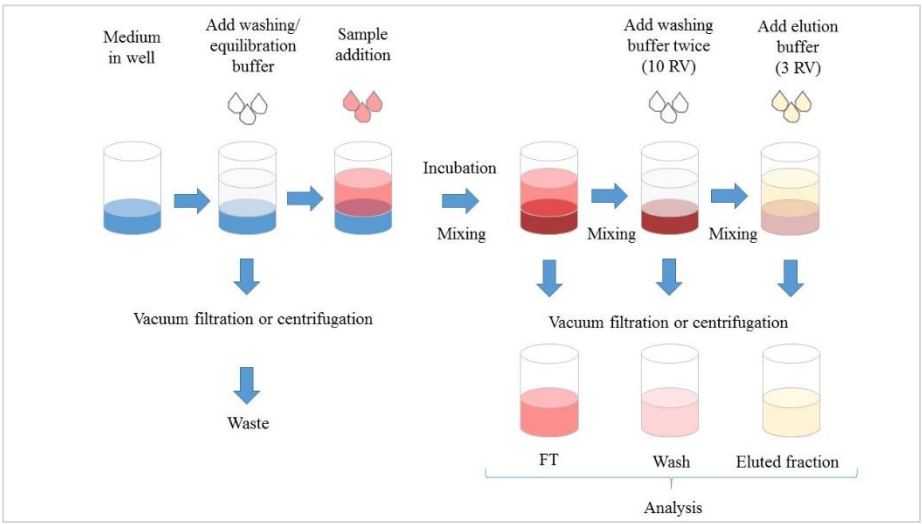


Figure 10: Illustration of the workflow a HTPD approach. The process starts with the equilibration of the resin, the sample addition, the mixing and, after another washing step, the elution of the bound proteins (41)

3.2 Development and optimization of the production process of a 340 kDa human protein by High-Throughput (HT) techniques

3.2.1 Upstream phase

The PER.C6 cells were cultured in HyClone CDM4PerMAb™ medium (GE Healthcare) supplemented with 4 mM glutamine (Lonza) and 0.05% (v/v) of Poloxamer 188 (Sigma-Aldrich). The cells were thawed and seeded in the complete medium on a shaking flask at 37 °C in a humidified atmosphere containing 5% CO_2. During the expansion phase, cells were monitored for cell count, viability and glucose concentration every two days and expanded in flasks until the total viable count (VCC) was at least 2×10^9 cells. The cells were further expanded on a 5 L working volume Cultibag RM (Sartorius) at a Viable Cell Count (VCC) of $0.5x10^6$ cells/mL and grown on a Biostat RM (Startorius) using a rocking speed of 20 rpm, an angle of 6°, an airflow of 0.5 L/min and a temperature of 37 °C. The cell culture was monitored daily for VCC, viability and glucose concentration. Viable cell count was performed with the NucleoCounter system (Sartorius) whereas the glucose concentration was determined with the Accu-Check Aviva device (Roche). The culture was supplemented with 0.5 L of complete Feed solution (75 g/L Amino Acid Powder Mark2 (AA Mark2) (Gibco), 31.2 g/L of Protein Expression Medium (PEM) (Thermo Scientific), 28.7 g CDM4PERMAb Feed Supplement (Gibco), 1.76 g tyrosine (Sigma-Aldrich), 1.2 g/L cysteine (Sigma Aldrich) and 0.1% Poloxamer 188 at days 3 and 5 and if necessary at day 7 starting from the inoculum. A total of 36×10^9 cells obtained from the 5 L Cultibag were used to inoculate four 25 L working volume Cultibag RM (Sartorius) containing 18 L of complete medium supplemented with 2 g/L of glucose with an initial viable cell density of $0.5x10^6$ cells/mL. The culture was monitored on a daily basis for glucose concentration, viability and pH. The culture was supplemented daily, starting from day 3, with 1 L of the feed medium. The culture was harvested when the viability dropped below 70%. The duration of the culture was about 10-14 days defined by the viability.

3.2.2 New disposable pilot scale depth filtration technologies

A volume of 10 L of PER.C6 fermentation was loaded on Millistak+ POD (D0HC) (Merck-Millipore) and on Supracap 100 (Pall) connected in series with a 0.022 m^2 0.22 µm Mini Kleenpak supor EAV membrane (Pall) disposable filter. Both depth filters have an area of 0.05 m^2 and the same construction materials, the retention range of the Millistak+ filter is 9-0.6 µm while for the Supracap it is 0.8-0.4 µm. The material of the 0.22 µm filter is PolyEtherSulfone (PES) with an area of 0.026 m^2. After the laboratory-scale screening, 100 L of the same high cell density fed batch cultivations was processed using two Stax disposable depth filter system (Medium-single layer, P series, superficial area 1 m^2, retention range 0.8-0.4 µm) (Pall) connected in series with a 0.22 µm Kleenpak Nova capsule filter PES supor EAV membrane (T style, with 2,16 m^2 of superficial area) (Pall). Both systems were connected to a peristaltic pump and a manometer. Before loading, the system was washed with Water For Injection (WFI). The broth was loaded and processed with a flux of 100 $L/m^2/h$. Pressure and the filter capacity were monitored throughout the process.

3.2.3 Determination of the Q Sepharose Fast Flow (FF), Hydroxyapatite (HA) and Phenyl Sepharose FF binding capacity

Three Tricorn columns 10/50 mm (GE) were used to pack 1 mL of Q Sepharose FF (GE), CHT ceramic HA type I 40 µm (Bio-Rad) and Phenyl Sepharose FF High sub (GE). The buffer used for the equilibration of Q Sepharose was 50 mM Tris-HCl, 400 mM NaCl pH 7, 10 mM sodium phosphate buffer pH 7 for the HA and 50 mM Tris-HCl, 2 M NaCl pH 7 for the Phenyl Sepharose. The purification was performed using the ÄKTA purifier system (GE) applying a linear flow rate of 80 cm/h. The Q Sepharose was loaded with 12 mL of PER.C6 supernatant, the HA with 12 mL of the eluate from Q Sepharose and the phenyl Sepharose with 12 mL of eluate from HA. All the samples were preventively diluted with equilibration buffers in order to have 0.25 mg/mL as concentration of protein of interest. NaCl was

added from a 5 M stock to HA eluate in order to reach the concentration of 2 M of NaCl. The FT was collected in twelve 1 mL fractions. The WB of the fractions was analyzed with Image J software in order to determine the concentration of the protein of interest.

3.2.4 Optimization of HA purification step with an HTPD approach

In order to improve the elution conditions on the HA, 5 mL of a 50% slurry composed of CHT ceramic HA 40 µm type I and 0.1 M NaOH were centrifuged at 1000 × g. Twenty-two wells were prepared as described in paragraph 3.1.2 with 200 µL of 50 % slurry (100 µL of resin). The wells were washed with 50 mM Tris-HCl, 400 mM NaCl pH 7 and then 200 µg of Q Sepharose eluate, containing the protein of interest at the concentration of 1 mg/mL, was added to each well. After 1 h of incubation the vacuum was applied and the dry resins were resuspended with three RV of 10 mM sodium phosphate buffer pH 7 repeating this step three times. An onion D-optimal design of experiment was planned in order to set-up an elution buffer matrix using as variables pH and sodium phosphate buffer concentrations. Both factors are quantitative with a pH range from 6.5 to 7.5 and a phosphate buffer concentration range from 10 to 350 mM. Figure 11 resumes the worksheet generated by MODDE planning the concentrations and pH of the elution buffer in the DoE. Three RV (300 µL) of the 28 different elution buffer were used to resuspend the dry resins and then the vacuum was applied in order to collect the FTs.

The responses considered in this DoE were HCPs, residual DNA and the protein of interest content.

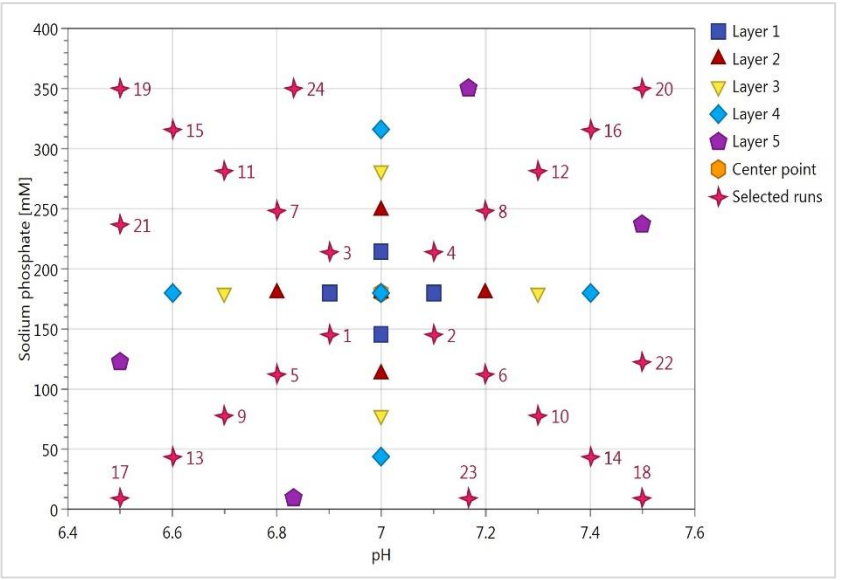


Figure 11: Onion design of the DoE performed for the optimization of HA purification step. The onion design represent the experimental space as comprising a number of sub-spaces called "layers" or "shells". This design allows to select a diverse range of experiments with uniform coverage across the experimental domain

3.2.5 Optimization of Q Sepharose FF and Phenyl Sepharose FF (high sub) chromatographic steps

In order to reduce HCPs in the Q Sepharose FF eluted fraction, a screening of different washing buffers containing different components (arginine (Sigma), $MgCl_2$ (Sigma), ethanol (Sigma), glycerol (Carlo Erba) and polysorbate 20 (Sigma)) at different concentrations was performed. The screening was performed with the vacuum manifold apparatus. An 1 mL receiver plate was placed at the bottom of the apparatus and upside a 96 well acrowell filter plates. A volume of 200 µL of Q Sepharose FF was added to every well of the plate and then was washed with equilibration buffer composed by 50 mM Tris-HCl, 400 mM NaCl pH 7. A volume of 200 µL of PER.C6 supernatant was loaded and, after a washing of five RV with equilibration buffer, the resins were washed with one volume of 50 mM Tris-HCl pH 7 with the addition of different excipients. Table II resumes the excipients concentrations used in the work.

Table II: Excipients concentrations used in washing buffer during capture step on Q Sepharose FF

(mM) Excipients	**0.9**	**1.8**	**4.5**	**5**	**10**	**50**	**100**	**200**	**342**	**428**	**500**	**684**	**856**	**1370**	**1710**
Arginine				✓	✓	✓	✓				✓				
$MgCl_2$				✓	✓	✓	✓	✓							
Glycerol									✓			✓		✓	
Ethanol										✓			✓		✓
Polysorbate 20	✓	✓	✓												

After the washing step, the protein of interest was eluted with two RV of 50 mM Tris-HCl, 650 mM NaCl pH 7. The eluates were analyzed by ELISA test to determine the yields of the protein of interest and HCPs.

A similar approach was performed to reduce HCPs in Phenyl Sepharose FF eluate and to increase the protein yield. The apparatus was prepared as the Q Sepharose FF experiment. A matrix of arginine and NaCl concentration in loading buffer (50 mM Tris-HCl pH 7) and in sample fraction was designed to identify the best combination able to reduce HCPs and increase yield.

A CCF design of experiment was planned as for the optimization of HA elution described in the previous paragraph. The range of the two factors considered was 0.5 to 2 M for NaCl concentration and 25 to 500 mM for arginine concentration. Table III resumes the worksheet generated by the software. The numbers of run are 22, composed by three center points and eight experiments with one replication for each test.

The resins were washed with two RV of loading buffer, then, samples were loaded (concentration of the protein of interest 1 mg/mL) and after two RV of re-equilibration the protein was eluted with WFI.

The eluates were analyzed by ELISA test to determine the yields of the protein of interest and HCPs.

Table III: DoE of the Phenyl Sepharose FF elution optimization

Exp No	Run Order	[arginine] (mM)	[NaCl] (M)
1	8	25	0.5
2	3	500	0.5
3	18	25	2
4	7	500	2
5	1	25	1.25
6	11	500	1.25
7	17	262.5	0.5
8	22	262.5	2
9	2	262.5	1.25
10	5	262.5	1.25
11	21	262.5	1.25
12	6	25	0.5
13	15	500	0.5
14	16	25	2
15	19	500	2
16	10	25	1.25
17	12	500	1.25
18	14	262.5	0.5
19	20	262.5	2
20	4	262.5	1.25
21	13	262.5	1.25
22	9	262.5	1.25

3.2.6 Reduction of HCPs and DNA: introduction of a membrane chromatographic step

A single-use, membrane-based ion exchanger chromatographic step was proposed in order to reduce the contaminants after Q Sepharose FF elution. The table IV refers to two different membrane anion exchangers and one cation exchanger that were screened in this work.

Pall acrodiscs were conditioned with 4 mL of NaOH, 4 mL of NaCl and 10 mL of equilibration buffer 50 mM Tris-HCl, 650 mM NaCl pH 7 while Chromasorb was washed with equilibration buffer.

Table IV: Single-use membrane-based ion exchanger proposed in the work. * Polymeric Primary Amine (PPA) **Ultra-high molecular weight PolyEthylene (UPE)

Membrane	***Manufacturer***	***Membrane***	***Pore size***	***Bed volume***	***Ion exchanger***
Acrodisc Mustang Q	**Pall**	PES	0.8 µm	0.18 ml	Q (anion exchanger)
Chromasorb	**Merck**	UPE**	0.65 µm	0.08 ml	PPA*
Acrodisc Mustang S	**Pall**	PES	0.8 µm	0.18 ml	S (cation exchanger)

A volume of 2 mL of Q Sepharose FF eluate (concentration of protein of interest 1 mg/mL) was loaded on each filter at a 1 mL/min flux using the ÄKTA Purifier system (GE). Resin FT was analyzed for yield, HCPs and DNA content.

3.2.7 Feasibility study of viral clearance by thermal inactivation and Normal Flow Filtration (NFF)

The fraction from the HIC step at the concentration of 0.4 mg/mL was processed for viral thermal inactivation at 60±2 °C for 4.0±0.1 hours under continuous stirring. Protein yield, aggregation (SEC) and reduction of HCPs evaluation were assessed. Virus clearance was evaluated by a NFF through the screening of three different prefiltration 0.1 µm filter and three 20 nm filters. The prefilter used for the work was Viresolve pro V shield 3.1 cm^2 (Merck-Millipore), Millipore express SHR optiscale 3.1 cm^2 (Merck-Millipore) and the prototype V shield H31 3.1 cm^2 (Merck-Millipore). The 20 nm filters used for the screening were: Viresolve Pro 3.1 cm^2 (Merck-Millipore), Ultipor VF grade DV20 9.6 cm^2 (Pall) and Pegasus SV4 virus removal 9.6 cm^2 (Pall). The combinations of filters and prefilters are resumed in table V.

Table V: Different combination of prefilters and filters used in the screening

		20 nm Filter		
		Viresolve V Pro	DV 20	SV 4
0.1 µm Filter	V SHIELD	✓	×	×
	SHR	✓	×	×
	V SHIELD H 31	✓	×	×
	NONE	✓	✓	✓

The screening was performed using a peristaltic pump at a constant pressure of 2 bar. All the conditions were compared for yield and by plotting the flux decay (J actual/J initial) vs loading (L/m^2) and simple capacity evaluation was carried out by measuring the volume filtered vs the time at constant operating pressure. The equation for calculating Vmax (total volume that can be filtered per unit membrane area) is:

$$\frac{t}{V} = \frac{t}{Vmax} + \frac{1}{Qi}$$

(t is the time, V is cumulative volume filtered and Qi is the initial flux).

The minimum area for 50 L scale-up batch size, to process in about four hour, was determined by the formula:

$$Amin = \frac{Batch\ Volume}{V75} + \frac{Batch\ Volume}{Qi \times process\ time}$$

where V75 is 0.5×Vmax and it is also defined as the volumetric throughput where Q=Qi×0.25. The first term of the equation is related to filter capacity, while the second is linked to productivity (process speed). If the relative Vmax is low and Qi is high, the second term in the equation is negligible compared to the first one, the filters are sized based on capacity (42).

3.2.8 Performance test of the downstream process

The complete downstream process was performed at a 25 L scale. Table VI resumes all the investigated downstream process steps. In order to assess the process reproducibility all the steps were analyzed in terms of yield, HCPs and rDNA content.

Table VI: Complete downstream process performed on a 25 L scale

STEP	**Apparatus/ Column**	**Flux/ Pressure**	**Procedure**	**Volume/ dimension**
1.Deph filtration	Stax (PALL)	100 L/m^2/h	75 mM NaCl washing buffer	50 L/m^2
2.Q Sepharose FF (Q)	ÄKTA Purifier-Pilot BPG Column (200/500)	1: 34 cm/h 2: 8.5 cm/h 3: 34 cm/h 4: 34 cm/h 5: 34 cm/h 6: 34 cm/h	1: 3 CV AQ (50 mM Tris-HCl, 400 mM NaCl pH 7) 2: Load sample 3: 1 CV AQ 4: 1 CV WQ (Buffer A + 50 mM Arg) 5: 2 CV AQ 6: Elution with BQ (50 mM Tris-HCl, 650 mM NaCl pH 7)	5 L
3.Mustang Q (MQ)	Mustang Q (Pall) - peristaltic pump	100 mL/min	1: 100 mL NaOH 1 M 2: 100 mL NaCl 1M 3: 300 mL BQ 4: Loading Q eluated fraction	60 mL
4.HA	ÄKTA Purifier-Pilot - BPG Column (100/500)	76 cm/h	1: 3 CV AHA (50 mM Tris-HCl, 50 mM sodium phosphate buffer ph 7.5) 2: Loading MQ FT after addition of 50 mM sodium phosphate buffer (final concentration) 3: Wash with 2 CV AHA and collect the FT 4: Elute DNA with 3 CV 500 mM sodium phosphate buffer ph 7.5	1.25 L
5.Phenyl Sepharose FF (HIC)	ÄKTA Purifier-Pilot BPG Column (100/500 25L)	76 cm/h	1: 3 CV AHIC (50 mM Tris-HCl, 2 M NaCl, 50 mM arginine ph 7) 2: Load HA FT + 2 M NaCl and 50 mM arginine (final concentrations) 3: 2 CV AHIC 4: Eluate with water for injection (WFI)	1.25 L
6.Viral inactivation and viral clearance	Thermal inactivation + 0.1 µm opticap millistax (Merck-millipore)+20 nm Filtration DV20 (Pall)	Operating pressure= 2 bar	Thermal inactivation: 4 h @60° C Viral clearance: NFF @ 2 bar	0.1 µm: 35 cm^2 (2x 47mm capsule); 0.07 m^2 DV20

STEP	Apparatus/ Column	Flux/ Pressure	Procedure	Volume/ dimension
7. TFF+ 0.2µm filtration	Novasep Cassette TangenX (100 kDa)	ΔP=0.7 bar	7 volume exchange and concentration to 2,5 mg/L PBS 10 mM sodium phosphate buffer, 150 mM NaCl pH 7	0.1 m^2

3.2.9 Scale-up of the fully GMP recombinant protein downstream process

The 100 L cell culture from four 25 L culture was harvested by depth filtration on a 2 m^2 STAX filter (Pall) followed by sterile filtration using a 2.16 m^2 0.22 µm Kleenpak Nova capsule filter (Pall). The non-purified bulk was stored at 2-8 °C in single use containers until further processed and no longer than 4 days.

The 100 L cell culture harvest was loaded on 20 L Q-Sepharose FF (GE Healthcare) previously packed on a BPG 300/500 column (GE Healthcare). The column equilibrated with 3 CV buffer AQ (50 mM Tris/HCl, 400 mM NaCl pH 7.0) at a linear flow rate of 34 cm/h. The non-purified bulk was loaded at 8.5 cm/h followed by a washing step of 1 CV of buffer AQ, 1 CV of buffer WQ (50 mM Tris/HCl, 50 mM arginine pH 7.0) and 2 CV of buffer AQ at 34 cm/h. The protein of interest was eluted in a single peak with a step gradient of buffer BQ (50 mM Tris/HCl, 650 mM NaCl pH 7.0) at the same linear flow rate. The eluted fraction was online sterile filtered with 0.22 µm filter (Pall) and stored at 2-8 °C in single use containers. The column was subsequently regenerated with 1 CV of buffer CQ (50 mM Tris/HCl, 1 M NaCl pH 7.0) and 2 CV of 20% v/v ethanol.

The eluted fraction from Q Sepharose FF was processed on a 140 mL Mustang Q XT membrane filter (Pall). The membrane was previously equilibrated 2 L of buffer BQ. The flow through (FT) was collected and filtered at 0.22 µm (Pall) and stored in single use containers at 2-8 °C.

The sample obtained from Mustang Q step was prepared for loading on the HA by adding 50 mM sodium phosphate, final concentration. A volume of 5 L of Macroprep Hydroxyapatite CHT-1, 40 µm (Bio-Rad) was packed on a

BPG 200/500 column (GE Healthcare), equilibrated with buffer AHA (50 mM sodium phosphate, 50 mM Tris/HCl pH 7.5). The sample was loaded at a linear flow rate of 76 cm/h and the protein of interest was eluted with 2 CV of buffer AHA. The eluted fraction containing the POI was diluted 1:2 with buffer AHA and then filtered through a 0.22 µm filter (Pall) and stored in single use containers at 2-8 °C.

The sample obtained from the HA step was further processed on 5 L Phenyl Sepharose FF high substitution column (GE Healthcare) packed on a BPG 200/500 column (GE Healthcare). In order to achieve the protein binding, the NaCl concentration was adjusted to 2 M and the arginine concentration to 50 mM. The sample was loaded on the column previously equilibrated with buffer AHIC (50 mM sodium phosphate, 2 M NaCl and 50 mM arginine pH 7.0) at a linear flow rate of 76 cm/h, followed by washing with 2 CV of buffer AHIC. Elution of protein of interest was performed with WFI in approximately 1.7 CV. The eluted sample was sterile filtered (Pall) and stored at 2-8 °C until further processed.

The fraction from the HIC step was processed for viral thermal inactivation at 60±2 °C for 4.0±0.1 hours under continuous stirring. The solution was filtered through a 0.22 µm filter (Pall) and subsequently on a millipak-20 100 cm^2 0.1 µm filter (Merck Millipore). The viral removal by filtration was performed on two 0.07 m^2 20 nm Ultipor® VF Grade DV20 filter (Pall) at a constant pressure of 2 bar with a flux of 24 LMH. The sample was filtered and stored at 2-8 °C in single-use containers until further processed.

The fraction obtained from the viral removal filtration was formulated at 2.5 mg/ml by tangential flow filtration on a 0.1 m^2 single use cassette with a cut-off of 100 kDa (TangenX) in 10 mM sodium phosphate, 150 mM NaCl pH 7.0. The final product was recovered from the system and sterile filtered at 0.22 µm and stored at 2-8 °C.

3.3 Development of a systematic approach for the purification of a 13 kDa human protein expressed in *E. coli*

3.3.1 *E. coli* cultivation, inclusion bodies preparation and refolding of the protein of interest

E. coli strain expressing the 13 kDa protein was cultivated in 2XYT medium (16 g/L Soytone (Difco), 10 g/L yeast extract (Difco) and 5 g/L NaCl (Sigma)) with the addition of 50 mg/L of kanamycin. A preinoculum of 50 mL (5% v/v final culture volume) was prepared and used to inoculate 1 L of the broth. The expression of the protein was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma) and 20 mM $MgCl_2$ (Sigma) when the cultivation reached an optical density $OD_{600\ nm}$ of 0.5. After 15 hours of incubation at 37° C with a shaking of 250 rpm, the culture was harvested by centrifugation.

The pellet was resuspended with 5 mL/g of lysis buffer (0.1 M Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM $MgCl_2$ pH 7) using an homogeneizator. The homogenate was subsequently sonicated with ten pulse of one minute. Between every pulse, the sample was equilibrated in ice for one minute. After the lysis step, half volume of Triton buffer (6% (v/v) of Triton X100 (Sigma), 1.5 M NaCl, 60 mM EDTA) was added to the lysate and the mixture was incubated at room temperature (R.T.) for 30 minutes under continuous stirring. At the end of incubation the mixture was centrifuged at 15000 × g for 20 minutes at 4 °C. In order to prepare the inclusion bodies the procedure described was performed two times. Subsequently, the inclusion bodies pellets were resuspended with inclusion body wash buffer (0.1 M Tris-HCl, 20 mM EDTA, pH 7) and centrifuged as previously described. The washing step was performed three times and, consequently, 5 mL/g of solubilization buffer (6 M guanidine-HCl (Sigma), 0.1 M Tris-HCl, 1 mM EDTA, 0.1 M dithiothreitol (DTT) (Sigma), pH 8) were used to dissolve the pellets. After three hours of incubation on a rotating wheel, the mixture was brought at pH 3 with 37% (v/v) HCl and then centrifuged for 10 minutes at 15000 × g. The supernatant was dialyzed against 6 M guanidine-HCl pH 3 at

4 °C replacing the buffer four times every 12 hours. After dialysis, total proteins were quantified using the bicinchoninic acid (BCA) assay. In order to obtain a final concentration of guanidine-HCl of 200 mM the refolding buffer (0.1 M Tris-HCl, 1 M arginine, 5 mM EDTA, 0.61 g/L oxidized glutathione, 1.53 g/L reduced glutathione pH 9.3) was prepared. Every hour 50 μg/mL of resolubilized protein was added to refolding buffer under vigorous stirring.

3.3.2 Arginine removal step: screening of suitable buffers

Arginine was used as chemical chaperone in order to help the refolding process, but, due to its high concentration it could interfere with the following purification steps. In order to reduce the amount of this amino acid, four alkaline dialysis buffers were evaluated (0.1 M Tris-HCl pH 9.5, 0.1 M carbonate buffer pH 9.5, 0.1 M glycine-NaOH pH 9.5, 0.1 M phosphate buffer pH 8). The dialysis were performed with a 7 kDa membrane (SnakeSkin dialysis tubing – Thermo Scientific) replacing the buffer three times every twelve hours. Protein stability was tested measuring the total protein content with BCA analysis, the protein of interest with ELISA test and performing turbidity assay measuring the absorbance at 410 nm.

3.3.3 Platform set-up for protein stability testing: sample preparation for chromatography (platform n°1)

Some variables such as pH, NaCl concentration, temperature, time and the starting total protein concentration could affect protein stability. In order to test how these factors can alter protein composition in 0.1 M Tris-HCl buffer, used during the chromatographic steps, a DoE based on a fractional factorial V+ resolution was performed. Table VII resumes the DoE performed. pH range of the samples was forced outside the buffer capacity of Tris-HCl (7-9.5), choosing 4 and 10.5 as limits of the test, NaCl concentration range was set between 0 to 1 M, incubation time between 0 to 2 hours and total protein concentration between 0.1 to 0.75 mg/mL. The temperature was set as multilevel quantitative variable at 4 and 20 °C.

Table VII: DoE for the protein stability evaluation

Exp No	Run Order	pH	[NaCl] (M)	Time (h)	[POI] (mg/mL)	Temp (°C)
1	35	4	0	0	0.1	20
2	37	10.5	0	0	0.1	4
3	18	4	1	0	0.1	4
4	24	10.5	1	0	0.1	20
5	16	4	0	2	0.1	4
6	5	10.5	0	2	0.1	20
7	38	4	1	2	0.1	20
8	1	10.5	1	2	0.1	4
9	34	4	0	0	0.75	4
10	33	10.5	0	0	0.75	20
11	30	4	1	0	0.75	20
12	36	10.5	1	0	0.75	4
13	13	4	0	2	0.75	20
14	21	10.5	0	2	0.75	4
15	8	4	1	2	0.75	4
16	12	10.5	1	2	0.75	20
17	32	7.25	0.5	1	0.425	4
18	20	7.25	0.5	1	0.425	4
19	7	7.25	0.5	1	0.425	4
20	3	4	0	0	0.1	20
21	31	10.5	0	0	0.1	4
22	9	4	1	0	0.1	4
23	22	10.5	1	0	0.1	20
24	11	4	0	2	0.1	4
25	17	10.5	0	2	0.1	20
26	28	4	1	2	0.1	20
27	27	10.5	1	2	0.1	4
28	29	4	0	0	0.75	4
29	26	10.5	0	0	0.75	20
30	2	4	1	0	0.75	20
31	10	10.5	1	0	0.75	4
32	25	4	0	2	0.75	20
33	4	10.5	0	2	0.75	4
34	19	4	1	2	0.75	4
35	23	10.5	1	2	0.75	20
36	6	7.25	0.5	1	0.425	4
37	14	7.25	0.5	1	0.425	4
38	15	7.25	0.5	1	0.425	4

One hundred µg of total proteins content were used for each point and conductivity was corrected adding a stock solution of 5 M NaCl in order to reach the desired concentration and pH was reduced or increased using 4 M HCl or 4 M NaOH respectively. The responses considered in this work were the total protein amount analyzed by BCA assay.

3.3.4 Chromatographic capture step: HTPD platform development (platform n°2)

An HTPD panel comprising cationic, anionic and mixed mode chromatographic media was proposed in order to build up a fast method to identify a resin able to significatively bind the protein of interest. Table VIII resumes the media and their properties.

Table VIII: Chromatographic media screened in the capture step

	Exchanger	Bead diameter	Matrix materials	pH stability
SP Sepharose FF HP (GE) SP	Sulfopropyl (strong cationic)	34 µm	Agarose	3-14
Eshmuno S (Merck) ES	Sulfo (strong cationic)	75-95 µm	Hydrophilic polyvinyl ether	2-12
Poros XS (Applied biosystems) PS	Sulfopropyl (strong cationic)	50 µm	Polystyrene-divinylbenzene	1-14
Nuvia S Bio-Rad) NS	Sulfo (strong cationic)	85 µm	Unosphere (cross liked polymer)	2-14
CM Sepharose FF (GE) CM	Carboxy methyl (weak cationic)	90 µm	Agarose	3-14
Fractogel SO3- (Merck) FS	Sulfo (Strong Cationic)	40-90 µm	Methacrylate polymer	2-12
Eshmuno HCX (Merck) EX	Sulfo, carboxy and phenyl (mixed mode)	75-95 µm	Hydrophilic polyvinyl ether	2-12

	Exchanger	Bead diameter	Matrix materials	pH stability
HA (Bio-Rad) HA	Ca^{2+}, PO^{4-}, OH^{-} (mixed mode)	40 µm	Hydroxyapatite	6.5-14
Nuvia Q (Bio-Rad) NQ	Quaternary amine (strong cationic)	µm	Unosphere (cross liked polymer)	2-14
Q Sepharose FF (GE) Q	Quaternary amine (strong cationic)	90 µm	Agarose	3-14
Poros HQ (Applied biosystems) PQ	Quaternary amine (strong cationic)	50 µm	Polystyrene-divinylbenzene	1-14

The screening approach was performed as described in paragraph 3.1.2 using 50 µL of resins with two levels full factorial DoE. The variables considered in this work were pH, conductivity and total protein loading. Table IX resumes the entire screening and sample preparation. The samples pH range was set from 9.5 to 7 for the cationic and mixed mode resins while 10.5 to 7 for anionic. NaCl concentration was set from 0 to 1 M and total protein loaded set from 50 to 360 µg. As described in the previous paragraph a concentrated solution of 5 M NaCl was used to modify the salt concentrations and 4 M HCl and 4 M NaOH to adjust pH. The FTs were analyzed by dot blot analysis, ELISA test and BCA assay.

Table IX: DoE matrix performed in capture step HTPD approach. Each box contains information about pH and NaCl concentration (M) of the sample and equilibration buffer (pH/[NaCl])

	50 µg load				360 µg load				205 µg load		
SP	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
ES	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
PS	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
NS	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
CM	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
FS	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
EX	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
HA	7/0	9.4/0	7/1	9.4/1	7/0	9.4/0	7/1	9.4/1	8.25/0.5	8.25/0.5	8.25/0.5
Q	7/0	10.5/0	7/1	10.5-1	7/0	10.5/0	7/1	10.5/1	8.75/0.5	8.75/0.5	8.75/0.5
NQ	7/0	10.5/0	7/1	10.5-1	7/0	10.5/0	7/1	10.5/1	8.75/0.5	8.75/0.5	8.75/0.5
PQ	7/0	10.5/0	7/1	10.5-1	7/0	10.5/0	7/1	10.5/1	8.75/0.5	8.75/0.5	8.75/0.5

3.3.5 Elution conditions optimization on anionic exchangers (platform n°3)

A similar DoE approach, as those mentioned in paragraphs 3.3.3 and 3.3.4, was proposed in order to optimize elution conditions of the three anionic resins (Nuvia Q, Q Sepharose FF and Poros HQ). The variables considered for elution buffers were the pH (range 10.5-6), the NaCl concentration (range 0-1 M) and the buffer nature as qualitative variable (100 mM of Tris or sodium phosphate) in order to plan a two level full factorial design. Starting sample represented

refolded protein dialyzed against 0.1 M Tris-HCl pH 9.5. First, 50 µL of resins were equilibrated with 0.1 M Tris-HCl pH 10.5 and subsequently 50 µg of total proteins were loaded. The buffers described in Table X were used for the elution step. Eluted fractions were quantified in terms of POI and HCPs content.

Table X: Elution buffers composition in the capture step

Exp No	Run Order	pH	[NaCl]	Buffer
1	9	6	0	Phosphate
2	6	10.5	0	Tris
3	5	6	1	Phosphate
4	2	10.5	1	Tris
5	10	6	0	Phosphate
6	7	10.5	0	Tris
7	8	6	1	Phosphate
8	11	10.5	1	Phosphate
9	3	8.25	0.5	Phosphate
10	4	8.25	0.5	Phosphate
11	1	8.25	0.5	Phosphate

3.3.6 Scale-up of the anionic capture step

The binding capacity of the three media considered in paragraph 3.3.5 was determined adopting the same approach described in paragraph 3.2.3. Subsequently, one milliliter of the three anionic resins, was packed in a Tricorn 10/50 column (GE) and equilibrated with ten volumes of 0.1 M Tris-HCl pH 10.5 at the linear flow rate of 76 cm/h. Refolded protein, dialyzed against 0.1 M Tris-HCl pH 9.5, was loaded at the same flow and then eluted using a linear gradient of ten CV from 0 to 100% of elution buffer (100 mM sodium phosphate buffer, 1 M NaCl pH 6). During the elution, fractions of 0.5 mL were collected and subsequently analyzed by dot blot in order to detect the peak containing the protein of interest. The linear gradient allowed the identification of the percentage of elution buffer in order to optimize a stepwise elution performed on 5 mL resin packed on a XK 16/20 column (GE). In particular, part of the *E. coli* HCPs and rDNA were eluted at 15% of elution buffer while the POI was eluted at the 37%. Others contaminants were

removed after the regeneration of the column with the 100 mM phosphate buffer, 1 M NaCl pH 6. The HCPs content, total protein and POI content were quantified in the fraction containing the 13 kDa protein.

3.3.7 Chromatographic intermediate/polishing step: HTPD platform development (platform n°4)

In order to introduce a second purification step able to reduce HCPs and rDNA an iterative approach was followed. First, all the cationic resins FTs samples, obtained in platform n° 2, were quantified for HCPs and rDNA content. The aim was to select a media for the introduction of a flow-through chromatographic step to identify conditions where contaminants were eliminated. Then, since HA resulted as a media able to highly bind the protein of interest, a three variable DoE was prepared to select the best elution condition. The first variable considered was the sodium phosphate concentration (range from 10 to 500 mM), second $CaCl_2$ concentration (from 0 to 20 ppm) and third the NaCl concentration (from 0 to 1 M). Also in this case, 50 µL of resin and 50 µg of POI eluted from Nuvia Q were used in the HTPD approach. Initially the resins were washed with 10 mM sodium phosphate buffer pH 7 subsequently the sample was loaded and finally the resins were washed with buffers described in Table XI used for the elution steps.

Table XI: CCF design for the optimization of the elutions in polishing/intermediate step

Exp No	**Run Order**	**[Sodium phosphate]**	**[NaCl]**	**[Ca^{2+}]**
1	17	10	0	0
2	16	500	0	0
3	8	10	1	0
4	12	500	1	0
5	11	10	0	20
6	14	500	0	20
7	3	10	1	20
8	6	500	1	20
9	4	10	0.5	10
10	10	500	0.5	10

Exp No	Run Order	[Sodium phosphate]	[NaCl]	[Ca^{2+}]
11	2	255	0	10
12	5	255	1	10
13	9	255	0.5	0
14	15	255	0.5	20
15	1	255	0.5	10
16	13	255	0.5	10
17	7	255	0.5	10

Another resin considered for the polishing step was the CIM multus Diethyl Amino Ethyl (DEAE)-1 monolithic column (BIA Separation). This resin is a weak anion exchanger with a similar binding characteristics of strong cation exchanger. Since information on interaction between the protein of interest and anion exchangers was available from platform n° 2, the peak eluted from Nuvia Q, containing the 13 kDa protein, was diluted twenty-fold with 0.1 M Tris-HCl pH 10.5 and loaded on the column. The same elution gradient described in paragraph 3.3.6 was performed. Another alternative for the polishing step was the hydrophobic Phenyl Sepharose HP resin (GE). Before loading the sample on the column a stability test was performed as described in paragraph 3.3.3. In this case the variables considered were the type of salt used (NaCl or ammonium sulfate) for the salting out and its concentrations (range 0 to 2 M). NaCl and ammonium sulfate were screened with a linear model L18 with three levels. The three salt concentrations tested by the model were 0, 1 and 2 M with three replicates.

The fraction containing the POI eluted from Nuvia Q was loaded on the Phenyl Sepharose HP after the addition of 1 M of ammonium sulfate. The resin was equilibrated with 50 mM phosphate buffer pH 8, 1 M ammonium sulfate and the elution buffer was the WFI. Initially, the elution was performed by a gradient of 10 CV from 1 M to 0 M of ammonium sulfate, subsequently, after the SDS-PAGE and WB analysis, the elution was optimized by a three step elution at the conductivity of 36, 20 and 0.2 mS/cm The peaks eluted were analyzed in SDS-PAGE, dot blot analysis, HCPs, rDNA, total and POI content.

3.3.8 Analytical methods

3.3.8.1 SDS-PAGE and Semi-quantitative Western blot (WB)

The protein samples were diluted with 5× of Laemmli buffer containing 2-mercaptoethanol and incubated at 95 °C for 5 minutes. The samples were loaded in a 4-15% (for the 340 kDa protein study) or 4-20% (for the 13 kDa protein study) polyacrylamide gel (TGX Bio-Rad) and the electrophoresis was performed applying 200 V. For SDS-PAGE staining biosafe coomassie blue brilliant G-250 (Bio-Rad) and silverQuest Silver staining kit (Life technologies) were used. For WB the proteins were then transferred to a nitrocellulose membrane with Transblot Turbo instrument (Bio-Rad) applying 2.5 A, 25 V for three minutes. The membrane was blocked for 30 minutes with 5% (w/v) of milk in Tris Buffer Saline (TBS)-Tween 20 and incubated for 2 hour with the primary antibody against 340 kDa protein (diluted 1/6000 in milk 1% (w/v)) or against 13 kDa protein protein (diluted 1/200 in BSA 1% (w/v)) and finally incubated for 1 hour with an anti-rabbit secondary polyclonal antibody Horseradish Peroxidase conjugated (HRP) diluted 1/4000 in milk 1% (w/v). The membrane was developed with 4-chloro-1-naphthol and then analyzed with the software Image J. This software, used for the semi-quantitative WB, allows to plot the bidimensional profile of the proteins area selected on the developed membrane. These areas can be compared and eventually quantified with the areas of a standard curve.

3.3.8.2 Dot blot analysis

A volume of 3 µL of the samples were spotted on a nitrocellulose membrane (GE) that was subsequently blocked for 30 minutes with 5% (w/v) of milk in TBS-tween 20 and incubated for 2 hour with the primary antibody and finally incubated for 1 hour with an anti-rabbit secondary polyclonal antibody HRP conjugated diluted 1/4000 in milk 1% (w/v). The membrane was developed with 4-chloro-1-naphthol.

3.3.8.3 Quantification of the protein of interest with Enzyme-Linked ImmunoSorbent Assay (ELISA) sandwich test

This assay consist in a commercially available quantitative sandwich enzyme immunoassay. A monoclonal antibody, used as “capture antibody” specific for the protein of interest, is coated onto a microplate. Plates are washed, and standards and samples are added to the wells: the protein of interest is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked HRP conjugated specific for the protein of interest is added to the wells. Following a washing step to remove any unbound conjugate, a TetraMethyl Benzidine (TMB) substrate solution is added to the wells and color develops in proportion to the amount of the protein of interest. The color development is stopped and the intensity of the color is measured at 450 nm.

3.3.8.4 Total proteins quantification

For the optimization of the 340 kDa and 13 kDa production process, the total protein content was determined by Bradford protein assay (Bio-Rad) and BCA kit (Sigma) respectively. The quantifications were performed following the manufacturer’s instructions and using an affinity purified 340 kDa protein as standard and lysozyme for the 13 kDa protein. In the case of pure samples, the determination of the 340 kDa protein content was determined by absorbance readings at 280 nm using an $Abs_{0.1\%}$ coefficient of 1.5 or 1.6 for the 350 or the 13 kDa protein (43).

3.3.8.5 HCPs quantification

The quantification of the HCPs was performed with the HCP ELISA kit (Cygnus) which allows the quantification of host proteins in samples. The kits used in this work were PER.C6 and *E. coli* HCP ELISA kit. Samples containing cell HCPs are incubated simultaneously with a HRP enzyme labeled anti-PER.C6 or *E. coli* cell antibody (goat polyclonal) in microtiter strips coated with an affinity purified capture anti-PER.C6 or *E. coli* cell antibody. The immunological reaction results in the formation of a sandwich

complex of solid phase antibody-HCP-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactant. The substrate, TMB is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of cell line HCPs present.

3.3.8.6 Size exclusion chromatography (SEC)

The SEC analysis was used to evaluate the composition of the HA eluates containing the 340 kDa protein. The analysis was performed on a Superose 6 10/300 GL Tricorn column (GE) equilibrated on the ÄKTA purifier with degassed Dulbecco Phosphate Buffer (DPBS) (Lonza). 100 µl of samples were loaded and eluted using a linear flow rate of 40 cm/h. The chromatographic profile was monitored at the wavelengths of 280 and 260 nm in order to detect the profile of the protein of interest, aggregates and the content of DNA.

3.3.8.7 Quantitative Polymerase Chain Reaction (qPCR)

The extraction of residual cell DNA from the samples was performed using the PrepSEQ® rDNA Sample Preparation Kit (Applied Biosystems) according to the manufacturer's instructions. The residual DNA quantification was performed by real-time PCR using the commercially available kit resDNASEQ® Human Residual DNA Quantitation Kit for the protein produced in PER.C6 cell line and resDNASEQ® *E. coli* Residual DNA Quantitation Kit (Applied Biosystems). The assay was performed according to the instructions provided by the manufacturer and all the data were obtained using the 7500 fast real time PCR system instrument (Applied Biosystems).

3.3.8.8 Bioactivity test of the 13 kDa protein: Rat C6 proliferation assay

Before the assay, Rat C6 cells were cultured for 1 week in DMEM (Dulbecco's Modified Eagle Medium) containing 2% (v/v) glutamine (Sigma) 0.2 % v/v cholesterol (Gibco), 0.6 % (v/v) 2-mercaptoethanol (Sigma), 0.2 %

(v/v) insulin-transferrin (Gibco) and 1% or 10% (v/v) Fetal Bovine Serum (FBS) (SAFC) in humidified atmosphere with 5% CO_2. Subsequently 2000 cells cultured in 1% FBS at the concentration of 15×10^3 cells/mL were plated in each well of a 96 well plate. The cells were exposed to the 13 kDa recombinant protein (concentration range: 0.1 to 30 µg/mL) and incubated for 72 hours. As positive control a commercial 13 kDa protein was used in the same concentration range of the purified protein and, as negative control, 1% FBS cells without the addition of the POI was used.

After the incubation time, cells amount was quantified using the ViaLight Plus cell proliferation cytoxicity bioassay (Lonza) following the manufacturer's instruction.

4. Results

4.1 Development and optimization of the production process of a 340 kDa human protein by HTPD techniques

4.1.1 Background of the process development: protein ID

The aim of the first part of this work was the optimization of the production process of a 340 kDa human protein expressed in response to pro-inflammatory signals. This molecule is a multimeric protein composed by eight identical subunits stabilized by interchain disulfide bonds able to stabilize four protein subunit in tetrameric arrangement and to link the tetramers into octamers. Each monomer is made of a specific sequence of 381 amino acids with an overall molecular mass of 40 kDa. In particular, the symmetric disulfide bonds are located among five cysteine residues located in position 47, 49, 103, 317 and 318 of the primary sequence. The octamer is organized in two opposite oriented tetramer link to each other by the interchain bonds 317-317 and 318-318 between the C-terminal of opposite oriented subunits.

SDS-PAGE analysis under reducing conditions shows how the monomer deviate from the predicted Molecular Weight (MW) of about 3-4 kDa and this discrepancy is due to glycosylation (Figure 12). Using the server NetNGlyc 1.0 (44), one glycosylation site can be predicted on asparagine 220 of the protein monomer, hence, the fully structured protein has eight glycosylation sites with fucosylated and sialyted complex-type sugars. These complex post-translactional modifications require the expression of this protein in a host cell able to ensure a glycosylation pattern coherent to the native one. For this reason the expression of the protein was performed in the PER.C6 cell line derived from primary culture of human fetal retinoblasts immortalized upon transfection with an E1 minigene of adenovirus type 5.

The generation of PER.C6 was performed in compliance with Good Laboratory Practice (GLP) and has been extensively documented and the cell banks meet all pertinent US and EEC regulatory requirements. Moreover,

PER.C6 cells can be grown in suspension to high cell densities (up to 10^7 cells/mL) in serum-free medium and without the aid of any solid support (45).

Figure 12: SDS-PAGE and WB of the 340 kDa protein under reducing conditions

Using the software CLC Main workbench (Qiagen) and the bioinformatics tool Protparam (Expasy), biochemical properties and information can be identified starting from the amino acid sequence. Table XII resumes the parameters of the 340 kDa protein monomer described in this part of the work.

Table XII: Biochemical characteristics of the 340 kDa protein monomer

Length	381 aa		
Weight	40.121 kDa		
Isoelectric point	5.11		
Aliphatic index	85		
Half-life: aa N-terminal glutamic acid	1 hour Mammals	30 min yeast	>10 hours *E. coli*
Extinction coefficient at 280 nm	Non reduced cysteines 60710 $mM^{-1}cm^{-1}$		Reduced cysteines 60170 $mM^{-1}cm^{-1}$
Extinction coefficient absorption at 280 nm 0.1% (=1g/L)	Non reduced cysteines 1.513		Reduced cysteines 1.500
Secondary structure number	Alpha helix: 8		Beta strand: 18

Figure 13a shows the plot of charge as a function of pH. The protein in its zwitterionic form has a neutral charge: its isoelectric point corresponds to 5.1.

A hydrophilicity/hydrophobicity plot is shown in Figure 13b: the plot has amino acids sequence of a protein on its x-axis, and degree of hydrophobicity and hydrophilicity on its y-axis. It is useful to characterize or identify possible structure or domains of a protein. There is a number of methods to measure the degree of interaction of polar solvents such as water with specific amino acids. For instance, the Kyte-Doolittle scale gives information about the protein structure. For instance, if a stretch of about 20 amino acids shows positive for hydrophobicity, these amino acids may be part of alpha-helix spanning across a lipid bilayer; on the converse, amino acids with high hydrophilicity indicate that these residues are in contact with solvent, and that they are therefore likely to reside on the outer surface of the protein (46). The 340 kDa-protein monomer shows both hydrophobic and hydrophilic regions. This information was taken into consideration for the process development design.

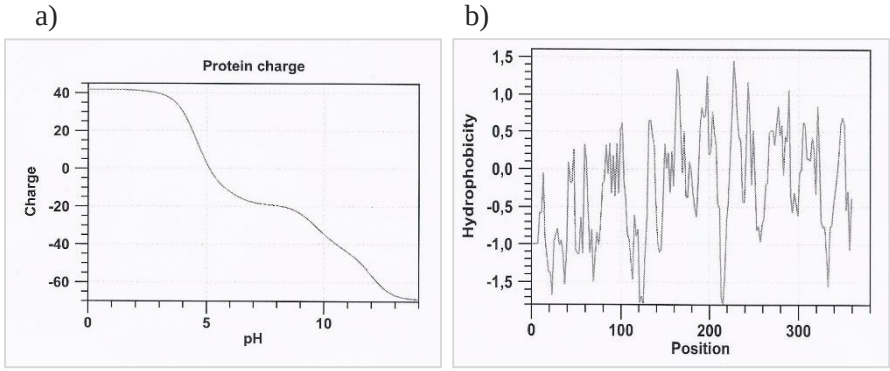


Figure 13: a) plot of charge as a function of pH, b) Kyte-Doolittle plot

4.1.2 Production of the recombinant protein

The production process of the protein of interest was performed starting from the cell bank expansion in flasks until the cell concentration reached 10-20×10^6 cell/mL in order to inoculate a 5 L working volume bag. The cell bank expansion lasted about 7-8 days. The viable cell count during the flask

expansion was always maintained above 90%. The 5 L bag was inoculated at a VCC of 0.5×10^6 cells/mL and, during the growth, feed medium was added depending on the glucose concentration in order to avoid levels below 3 g/L. The duration of this phase was of 6-7 days with a final VCC between 8.5 and 13.7×10^6 cells/mL whereas the final culture viability was within the 82-95% range. These results are shown in Figure 14a where the viability and VCC of growth curves obtained from three different 5 L cultures are shown.

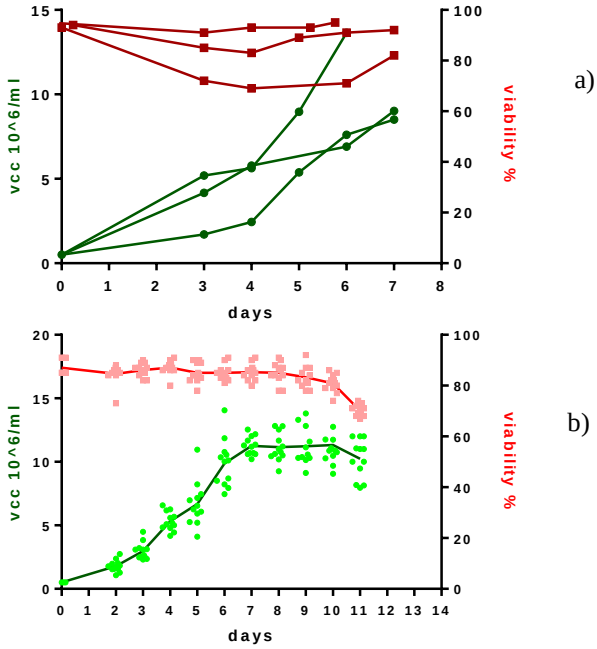


Figure 14: a) Growth curves of three cultures at the 5 L scale. b) Growth curves of the 25 L scale. VCC of each time point is represented in green whereas the viability is represented in red. The green and red lines correspond to the mean values

The 100 L production campaign necessary for the production of about 5 g of the 340 kDa protein from PER.C6 cells, was performed with four parallel bioreactors of 25 L. Cells were inoculated at a VCC of 0.5×10^6 cells/mL and grown with daily monitoring for glucose consumption, pH (Figure 15a-c) and VCC (Figure 14b). A clear pH drift could be observed along the duration of the culture with a final value at harvest of 6.3 (Figure 15c). The initial glucose consumption was 0.6-0.5 mg/10^6 cells/day starting from the second day to the

fourth day of culture and remained constant at 0.2 mg/10^6 cells/day for the remaining period (Figure 15b). The cultures were stopped when viability was below 70%. As shown in Figure 15d the average of the maximum VCC obtained for the four 25 L fermentations was 14.8×10^6 cells/mL and 11.7×10^6 at harvest. The average protein of interest production in PER.C6 cell line was 104 mg/L (Figure 15e).

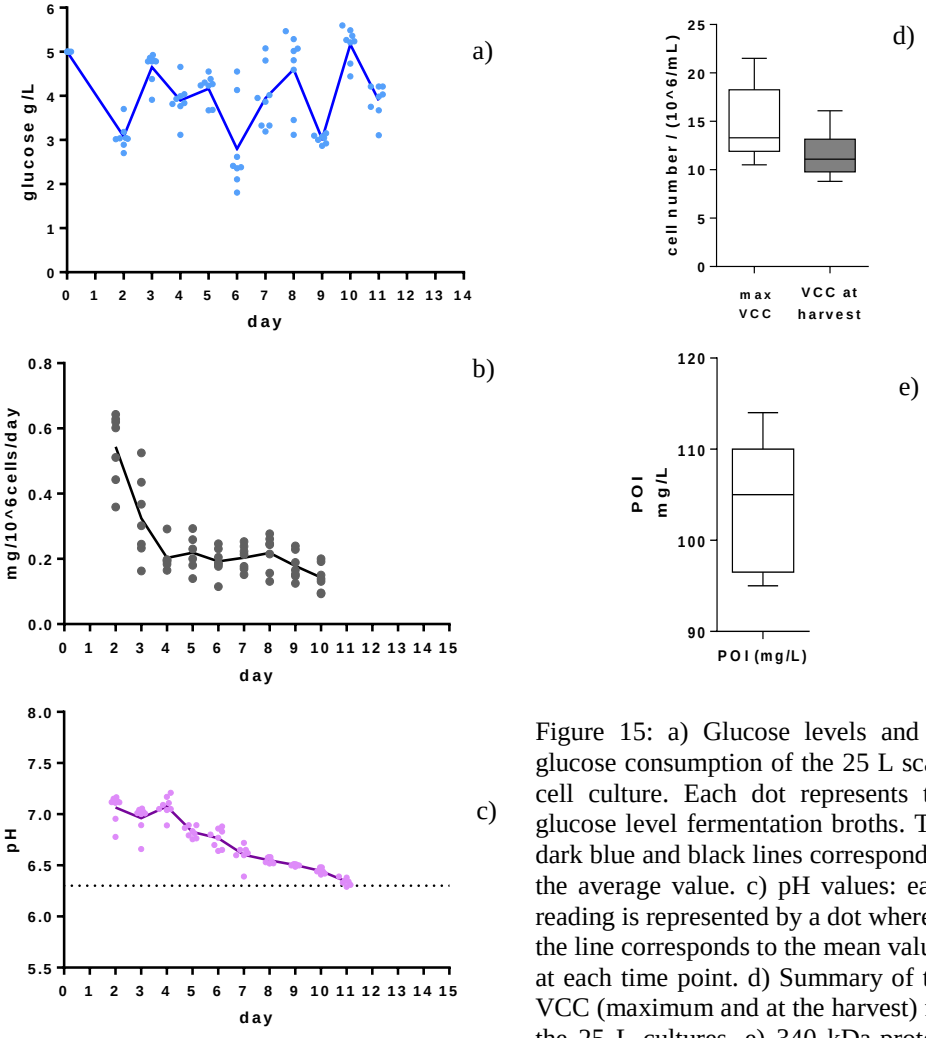


Figure 15: a) Glucose levels and b) glucose consumption of the 25 L scale cell culture. Each dot represents the glucose level fermentation broths. The dark blue and black lines correspond to the average value. c) pH values: each reading is represented by a dot whereas the line corresponds to the mean values at each time point. d) Summary of the VCC (maximum and at the harvest) for the 25 L cultures. e) 340 kDa-protein production level obtained from the 4 × 25 L production campaign

4.1.3 Limits of the non-optimized downstream process and proposals for new improvements

After the upstream phase, the feedstock of the PER.C6 culture needed to be processed in order to purify the protein of interest. In the non-optimized process the harvest of a 25 L cell supernatant was achieved by high-speed centrifugation but the scale-up of this step was a limit. Continuous centrifugation could be the key of this bottleneck but the choice is mainly a matter of costs: actually, this approach is the preferred clarification method for large scale process. The choice of a clarification method depends on equipment availability, the cell culture process (including the cell line and the cell viability at time of harvest), process economics and scale of the process. For relatively medium-high scale processes, up to 4000 L, depth filtration may be a more efficient method of clarification. Depth filtration use is also essential in a full-scale industrial process after an eventual continuous centrifugation. In this work a depth filtration step and its sizing was studied and tested in order to introduce this technology in the process.

The core of the downstream process is chromatography. The standard process was characterized by three chromatographic steps corresponding to capture, intermediate and polishing step (Figure 16). The capture step was performed by loading the feedstock on the Q Sepharose FF equilibrated with 50 mM Tris-HCl, 400 mM NaCl pH 7 and eluting the fraction containing the protein of interest with 50 mM Tris-HCl, 650 mM NaCl pH 7. The latter fraction was loaded on the hydroxyapatite conditioned with 10 mM sodium phosphate buffer pH 7 and the protein of interest was eluted with 350 mM sodium phosphate buffer pH 7. The last purification step was achieved by HIC using the Phenyl Sepharose FF resin. After the addition of NaCl, in order to reach the concentration of 2 M, the sample was loaded on column and eluted with WFI. The last step of the downstream phase was the concentration and diafiltration of the protein carried out by TFF on a 100 kDa MWCO membrane.

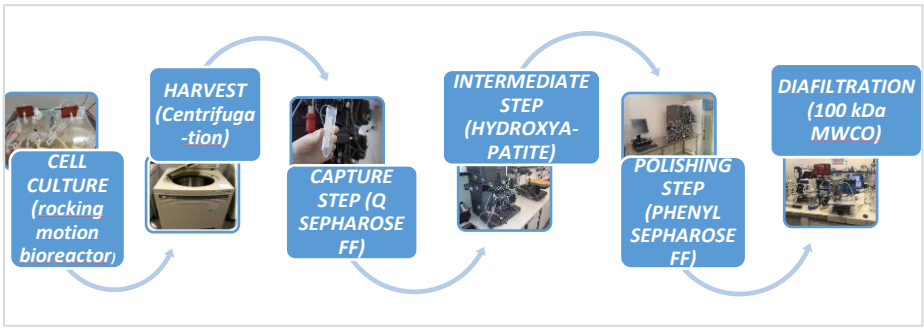


Figure 16: Non-optimized downstream process scheme of the 340 kDa-protein

A challenging aim of the purification steps is the reduction of contaminants such as HCPs, rDNA, aggregates and endotoxins. Table XIII resumes all the downstream steps and the contaminant content (where available) of the non-optimized process, pointing up the impact of the single steps on impurities reduction expressed by Log reduction.

Table XIII: Downstream steps and contaminants contents of the non-optimized process

STEP	**STEP YIELD**	**HCPs Log red**	**HCPs ppm**	**DNA Log red**	**DNA ppm**
AEX	100 %	2.6±0.05	8000	-	-
HA	70%	0.3±0.05	4800	-	-
HIC	55%	0.4±0.10	2600	-	-
TFF	95%	0.3±0.05	900	-	-

This purification approach fulfilled the endotoxin requirement of 10 EU/mg because all the process was performed in an endotoxin-free mode using disposable and sanitized equipment, but the others impurities levels were outside specifications. In particular HCPs were not in the range of 1-100 ppm and the content of rDNA was above the limit of 10 ng per dose of biotechnological drug as described by guidelines on the quality, safety, and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (World Health Organization (WHO)) (47).

In this PhD work the process was further optimized in order to introduce a fully compliant GMP process. First, the three chromatographic steps were

optimized in terms of HCPs and rDNA reduction by screening and DoE approaches. In particular in the capture and polishing steps, these studies were used in order to introduce washing steps able to decrease impurities content. The QbD, achieved by the DoE approaches, was also used in the intermediate step on hydroxyapatite to reduce the concentration of phosphate in elution buffer preventing the co-elution of the POI and the rDNA.

Further strategy followed to reduce contaminants was the introduction of a membrane chromatographic step. Membrane chromatography has demonstrated unparalleled performance for flow-through applications. For this reason, a screening of three chromatographic membranes was performed in order to adapt this technology for trace contaminant removal and virus clearance applications. Anion exchange chromatography is most widely used for this unit operation and has been validated for use since 2001 (48). Finally, further aim was the introduction of viral reduction steps achieved by thermal inactivation and filtration, in order to satisfy ICH Q5A guidelines. In particular, three different virus removal filter were tested and sized in order to identify the best in terms of performances for the implementation in the full scale process.

4.1.4 New disposable pilot scale depth filtration technologies

The protein of interest, expressed by PER.C6 cells, is secreted in the culture medium. At small to medium scales, single-use technology offers significant advantages over traditional reusable (e.g., stainless steel) manufacturing technology with regard to flexibility, cost of goods, implementation timelines, and maintenance. However, process design based on disposables does create new challenges. For processes based entirely on disposables, the disc-stack centrifuge needs to be replaced by filtration alone. In the first step, the cellulose Millistak+ POD D0HC and Supracap 100 filter, connected in series with a PES 0.22 µm filter for the final clarification, were tested. For the laboratory-scale screening 10 L of high density fed batch PER.C6 cultivation broth were used. Harvest was performed after 14 days

with a viability of 94%. During the filtration, a constant flux of 100 L/m^2/h was applied. The filter capacity, turbidity, yield and the pressure were monitored during the process. Figure 17 shows Millistak+ POD and Supracap 100 capacity (L/m^2) plotted against pressure (bar). The maximum filter capacity was determined at the pressure of 1 bar. At low differential pressure, the filters show exponential curve typical of a filtration process with constant flow. After processing 6 L of broth the MilliStak+ POD filtrate throughput showed turbidity and the 0.22 µm filter was clogged. This filter processed about 6 L reaching a maximum capacity of about 110 L/m^2 at 0.7 bar. The capacity of Supracap 100 was about 100 L/m^2 at 1 bar without turbidity.

The scale-up was performed processing 100 L of the same PER.C6 culture using two 1 m^2 Stax filters considering a capacity of 50 L/ m^2 (safety factor = 2). As showed in Figure 17, the Supracap results were confirmed with Stax system, in fact, with 2 m^2 of superficial area, the maximum capacity at 1 bar was not reached. The results obtained show that the Millipore Millistak+ POD filter was able to process about 110 L/m^2 but turbidity breakthrough at pressure below 1 bar was observed and consequently this filter was not considered for the scale-up. 1 m^2 Stax filter was necessary to process 50 L of PER.C6 culture avoiding turbidity and clogging of 0.22 µm filter.

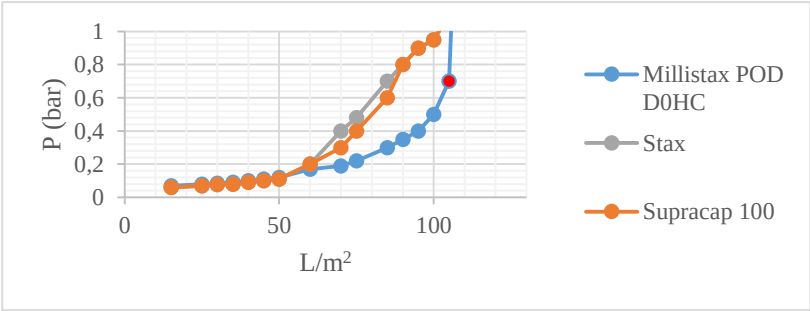


Figure 17: Comparison of the pressure/capacity relation for Millistak+ POD D0HC Supracap 100 and Stax filter. The red dot represents turbidity threshold of POD system

4.1.5 Determination of the Q Sepharose, HA and Phenyl Sepharose binding capacity

The binding capacity corresponds to the quantity of a specified molecule such as protein, DNA and virus that can be bound by a resin. It is common practice in chromatography to express the binding capacity in milligrams of proteins per milliliters of resin. The dynamic binding capacity may be determined from breakthrough curves. These are obtained by measuring the outlet protein concentration during continuous feeding of the equilibrated chromatographic medium. Total breakthrough is obtained when the outlet protein concentration equals the feed concentration. The dynamic binding capacity represent the quantity of protein bound to the resin under the operated flow conditions. It is commonly measured as the quantity of protein that has been retained by resin at 5% breakthrough.

As described in paragraph 3.2.3, 12 fractions of 1 mL were collected for each binding test and then analyzed by semi-quantitative WB in order to determine the concentration of the protein.

Appreciable intensity signals of the protein were identified after loading 1 mg for Q Sepharose and Phenyl Sepharose or 2 mg for HA (Figure 18). The binding capacity determined at 5% breakthrough at the flow rate of 80 cm/h were 1 mg/mL for Q Sepharose and Phenyl Sepharose while 2 mg/mL for HA.

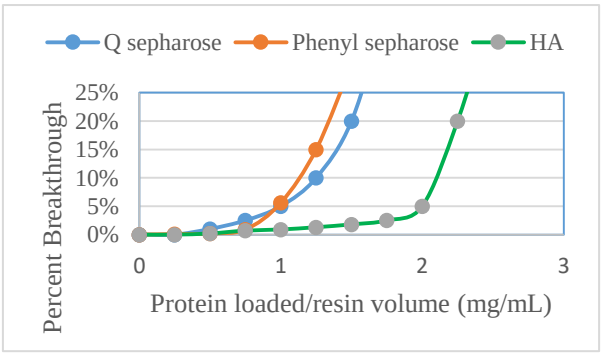


Figure 18: Relationship between percent breakthrough determined by semi-quantitative WB and protein loaded on the three resins. The binding capacity were 1 mg/mL for Q Sepharose and Phenyl Sepharose while 2 mg/mL for HA

4.1.6 Set-up of the HA purification step with HTPD

In the non-optimized process the fraction eluted from the Q Sepharose resin was loaded on HA. Subsequently the resin was washed with 10 mM sodium phosphate buffer pH 7 and the protein of interest was eluted with 350 mM of sodium phosphate buffer pH 7 while most of HCPs are eluted at 500 mM of the same buffer. Figure 19 represent the SEC chromatographic profile of the fraction eluted at 350 mM of sodium phosphate buffer. With this approach, the protein of interest co-elutes with contaminant host's DNA. The first peak contains the protein of interest while the peaks at higher retention times contains low molecular weight DNA fragments. The absorbance ratio 260/280 nm indicates the presence of residual DNA in the late eluting fractions (> 16 mL).

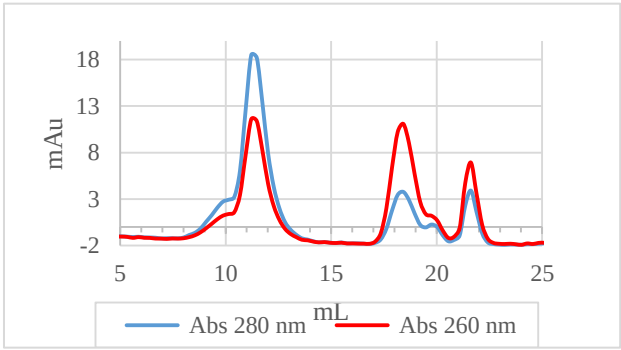


Figure 19: SEC of non-optimized hydroxyapatite eluate. The analysis was performed on a Superose 6 column and monitored following the absorbance at 260 nm and 280 nm

In order to reduce the contaminant DNA an HTPD approach was used as described in paragraph 3.2.4. The 28 elution conditions were analyzed by ELISA, HCPs test and quantified for rDNA. The results obtained allowed MODDE to propose a model to explain the protein elution behavior. The predicted contour graph is shown in Figure 20. The model had a R^2 of 0.795 and a Q^2 of 0.72, hence, was able to fit the data and was used for elution prediction. The results showed how there were not significant differences

between the three different elution buffers pH, in fact, this coefficient was not significant in the model. Furthermore, washing the resin with 50 mM phosphate buffer the yield of the protein of interest was about 70-80% while at lower concentrations the yield decreased up to 20%.

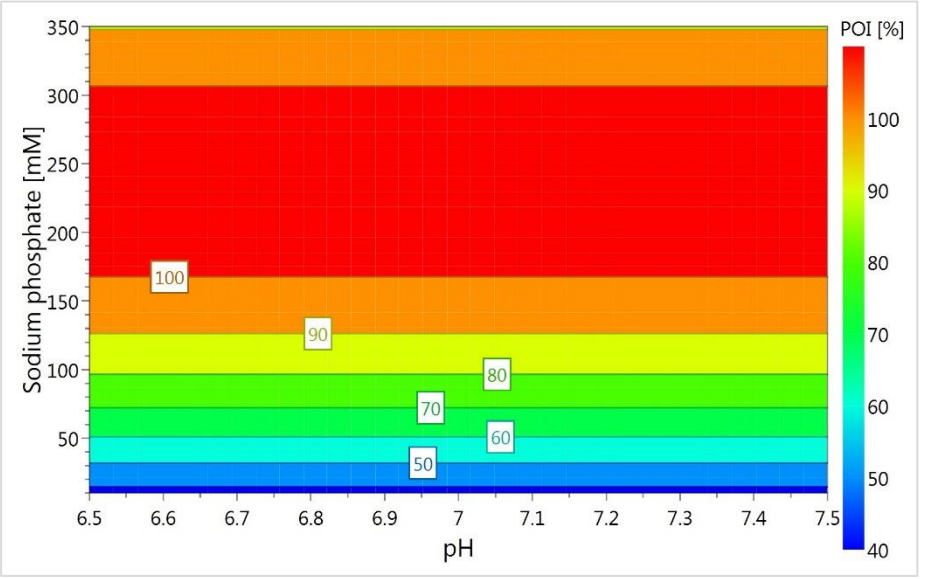


Figure 20: The contour graph resumes the yields of HTPD work. As shown in the figure there are not differences in terms of yield varying the pH and the maximum of recovery (about 70%) is reached at 50 mM of sodium phosphate

High and low molecular weight DNA strongly bound to HA at low phosphate buffer concentrations and approximatively a 100 mM solution was necessary for the elution. This observation and the results of the HTPD approach allowed to redefine the chromatographic step on HA. Sodium phosphate buffer at pH 7 was added from a 500 mM stock to Q Sepharose eluate in order to reach the concentration of 50 mM. The eluate was loaded on the resin and the protein of interest was collected in the FT. DNA and HCPs were eluted with 500 mM of sodium phosphate buffer pH 7. Figure 21 shows the SEC profile of the redefined chromatographic step. The profile shows highly reduced DNA peaks compared to the SEC of the non-optimized process. The yield of the protein of interest was about 70%, the HCPs Log

reduction step was about 0.6 and the Log reduction step of DNA by qPCR was 3.6.

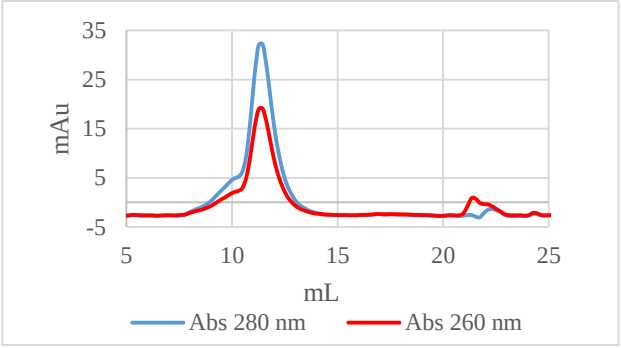


Figure 21: SEC analysis of the FT fraction of optimized HA step

4.1.7 HCPs and rDNA reduction approaches introduced in the downstream process

Process-related impurities are cell components such as HCPs or DNA, chemical additives, residual media components or leachables. HCPs are a complex mixture of proteins significantly differing from each other in their molecular mass, isoelectric point, hydrophobicity and structure. These properties present a challenge for product purification due to the modification in level, composition and property distribution during a single fermentation process. In the non-optimized downstream process the capture step on Q Sepharose FF allowed to reach an amount of 8000 ppm and a Log reduction of 2.6±0.05 of HCPs. As described in paragraph 3.2.5 some different components were tested in Q Sepharose washing buffer in order to evaluate their capability to reduce HCPs-anion exchanger or HCPs-protein of interest interactions. Figure 22 resumes the results obtained from this screening.

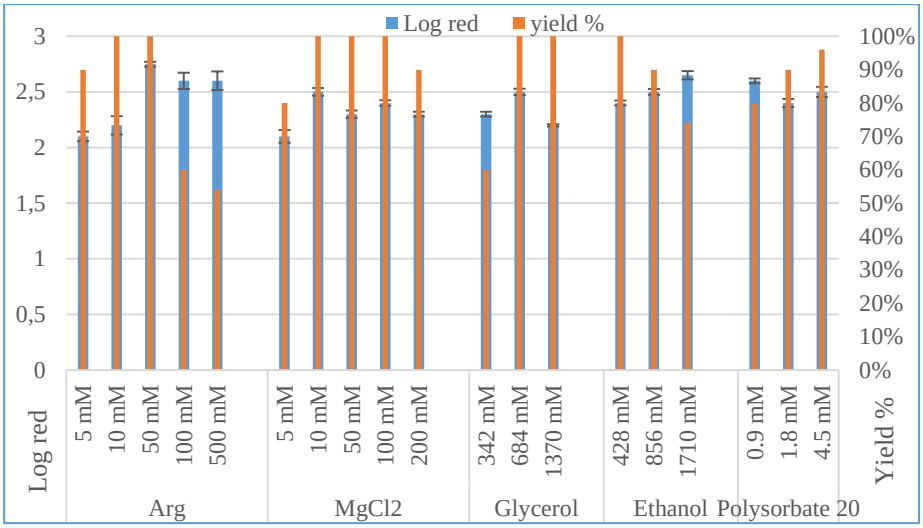


Figure 22: Log reductions and yields obtained from the use of different concentrations of components applied in washing steps

The results show that the best condition in terms of yield and Log reduction was obtained with the introduction of a washing step with 50 mM arginine achieving a Log reduction of 2.75±0.02 and about a full recovery of the protein of interest. Higher arginine concentration was able to reduce contaminant but yield was dramatically reduced because of the capability of arginine to weaken interaction between ion exchangers and proteins. On the other hand, no significant reductions of HCPs using different concentrations of $MgCl_2$, glycerol, ethanol or polysorbate 20 were observed.

Many studies demonstrated that arginine weakens hydrophobic interactions and facilitates elution of bound proteins from Phenyl Sepharose during decrease of salt concentration. In addition, inclusion of arginine in the loading sample increased the recovery of the total protein and decreased the aggregation during ion exchange chromatography (IEX) (49). In this work a screening of different concentrations of arginine and NaCl included in sample and loading buffer of HIC was performed in order to evaluate the possibility to reduce NaCl increasing arginine and to identify the best arginine

concentration able to eventually reduce HCPs content. Contours graphs in Figure 23 resumes the results obtained.

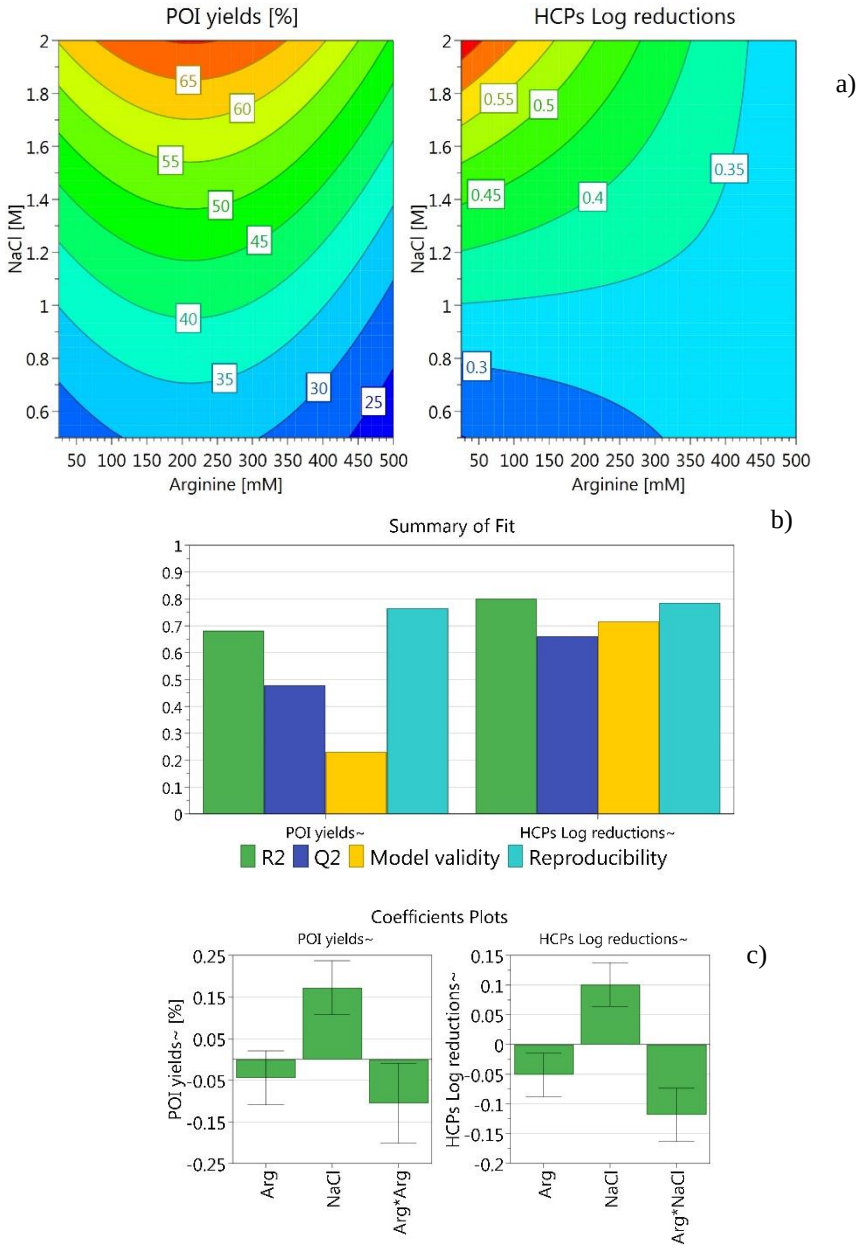


Figure 23: a) Contour graphs for Log reductions and yields obtained from the use of different concentrations of excipients applied in HIC screening. b) Summary of fit of the two response model. c) Coefficients plots of the two models

In the non-optimized downstream process the chromatographic step on Phenyl Sepharose FF allowed to obtain a yield of 55% and a Log reduction of 0.4±0.1 of HCPs. Figure 23 shows that below the concentration of 1 M NaCl the recovery of the protein of interest ranged from 35 to 20%, hence, these values were too low to allow the protein binding on the resin. Similarly, the recovery yields were about 20-30% increasing the amount of arginine at these NaCl concentrations. Best conditions were obtained at 2 M NaCl with the addition of 50 mM arginine where the yield reached values of 70% and a Log reduction of 0.6 was the best. The yields and Log reductions slowly decreased with increasing arginine concentration in the samples containing 2 M NaCl. This could be probably due to the altered interactions between the protein of interest or HCPs with the resin in presence of high concentrations of the two salts. Both the models proposed to describe the cause-and-effect relationship for POI yields and HCPs Log reduction were statistically relevant, hence, they were considered valid; moreover, the models had a good fit and a good capacity to predict future experiments as shown by the values obtained for R^2 and Q^2 (Figure 23b). Figure 23c resumes the coefficients plots for POI yields and HCPs Log reduction. In both the responses considered, increasing NaCl concentration significantly favored the protein of interest binding and reduction in HCPs. Arginine concentration was able to explain the POI yield only when considered as a quadratic effect term turning the linear regression into a curve. In the HCPs Log reduction model the increase of this amino acid boosted the HCPs interaction with the resin, moreover, the interaction between NaCl and arginine was significative: this interaction was evident only when one of the two salts increased and the other decreased (or vice versa).

A relatively new development in membrane technology is the membrane chromatography. These are symmetric microfiltration membranes functionalized with specific ligands attached directly to the convective membrane pores. Diffusive pores are eliminated, mass transfer of biomolecules depends on convection and the binding capacity is largely independent on flow rates. Significant advances have recently been made in

developing high permeability and high capacity sterile filters by application of composite membranes. Membrane adsorbers are used for polishing applications aimed to remove contaminants. Viruses, endotoxins, DNA, HCPs and leaches bind to the membrane at neutral to slightly basic pH and low conductivity values. Additional developments investigate the possibility to apply membrane adsorbers in capture and purification of large biomolecules and focus on new designs of structures for bind-and-eluate processes. In this work three different chromatographic membrane were tested in order to introduce a polishing step in the downstream process. The Q Sepharose FF eluate had a concentration of 650 mM NaCl and, at this salt concentration, the tests were performed in negative mode where the protein of interest is collected in the FT and all the undesired contaminants are trapped and bound to the membrane. Table XIV resumes the results obtained in terms of yields, HCPs and DNA reduction.

Table XIV: Yield and reduction of contaminants obtained in membrane chromatography screening tests

	DNA Log red	**HCP Log red**	**Yield**
MUSTANG Q	0.6±0.1	0.30±0.05	97%
CHROMASORB	0.1±0.1	0.17±0.05	69%
MUSTANG S	0	0.01±0.05	84%

Mustang Q had the best performance in terms of DNA reduction (0.6±0.1) compared with other membranes, coupled to a very high recovery. On the other hand, the HCPs reduction was limited with the three systems used. Nevertheless, Mustang Q performed better than Chromasorb and Mustang S. Based on these results, Mustang Q was introduced in the scale-up of downstream process.

4.1.8 Feasibility study of viral clearance by thermal inactivation and NFF

Biopharmaceutical products, such as monoclonal antibodies, recombinant proteins, vaccines, blood derivatives and animal products carry an inherent risk of transmitting infectious viruses due to the source material used, manufacturing processes, and routes of administration. All of the regulatory guidelines emphasize that each viral validation study should be reviewed on a case-by-case basis and that Log reduction factors obtained should be viewed under experimental limitations and product-specific risk factors. A manufacturing process for the production of biopharmaceuticals should incorporate at least two distinct robust virus clearance steps, with at least one step effective on non-enveloped viruses. Robust steps are those able to clear a wide range of viruses and are not influenced by process variables (pH, protein concentrations, buffers, temperatures etc.). Scaling-down the process steps to be evaluated is a prerequisite to performing the actual spiking experiments, as it would be impractical to use the actual production scale for the viral clearance study due to the volumes of virus needed. In this work, two steps for virus clearance were evaluated. First, the fraction from the HIC step at the concentration of 0.4 mg/mL was processed for thermal viral inactivation at 60±2 °C for 4.0±0.1 hours under continuous stirring. The protein yield after the treatment was 97% and the step HCPs Log reduction was 0.2. The SEC profile before and after the treatment was assessed and the profiles are reported in Figure 24.

a) b)

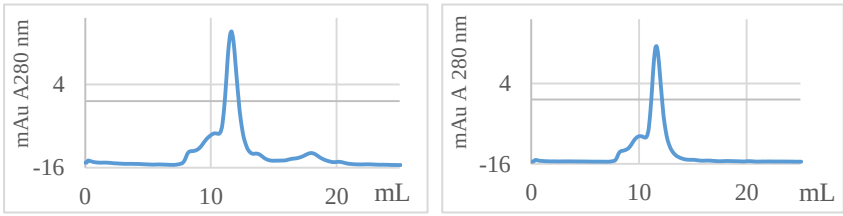


Figure 24: SEC profile of HIC eluates before (a) and after (b) thermal inactivation

The SEC profiles show no significant changes in dimer, trimer or aggregates before the main octameric protein peak. These results show that the heat-treatment can be easily introduced in the viral clearance step because it has no significant effect on protein structure and yield. As described in paragraph 3.2.7, after the thermal inactivation step the feasibility of virus clearance by a normal flow filtration through the screening of different 20 nm filters was evaluated.

In order to save the highest amount of product all trials were run with 50 mL of product. Collected data were placed in a graph by plotting the loading versus the flow decay (Figure 25).

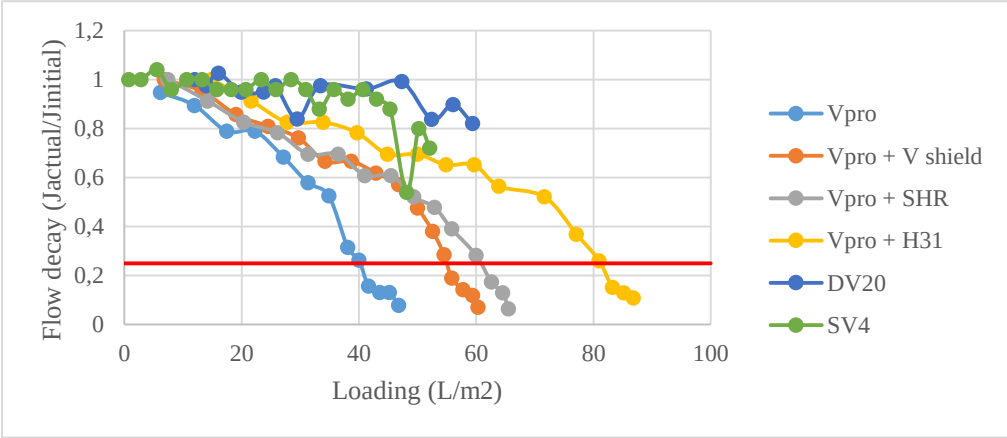


Figure 25: Viral clearance by 20 nm filtration. Flow decay vs loading are plotted for the six experiments

The red line represents the limit of the flow decay used for sizing calculation (flow at 25% of the initial means 75% of flow decay). The product loading (filter capacity) with dedicated prefilters is meaningfully higher than Virus filter (Vpro) alone. The performance given by the SHR filter is able to increase the Vpro capacity more than 50% but the Vshield H31 prefilter showed the best performance. DV20 and SV4 without any 0.1 µm filter were stopped at about 60 L/m^2 of loading and their flow decay trends were less pronounced that other filters. In particular this effect is more evident for DV20

filter. The results obtained are summarized in table (Table XV), also including the sizing for 50 L process.

Table XV: Summary table of the filtrations viral step clearance results

Device	Prefilter	Vmax (L/m^2)	V75 (L/m^2)	Pressure (bar)	Trial Loading (L/m^2)	Initial flux (LMH)	Average flux (LMH)	% yield	Sizing 50 L (m^2)	Safety factor
Vpro	None	65.2	32	2	47.7	348	142	90	2.2	1.4
Vpro	V shield	165.1	83	2	60.3	387	182	87	1.1	1.8
Vpro	SHR	165.4	83	2	65.4	406	196	81	1.1	1.8
Vpro	H31	227	114	2	86.8	406	209	81	1.1	2.5
SV4	None	450	225	2	52.1	33.7	30	94	1	1.7
DV20	None	868	434	2	59.3	24.3	23	96	1	1.6

4.1.9 Scale-up of the recombinant protein production process

As described in paragraph 3.2.8 and 3.2.9, a scale-up to 25 L and 100 L of the downstream process was performed in order to assess the reproducibility of yields and contaminants reduction. The entire production process together with the viral inactivation and reduction steps is schematically represented in Figure 26.

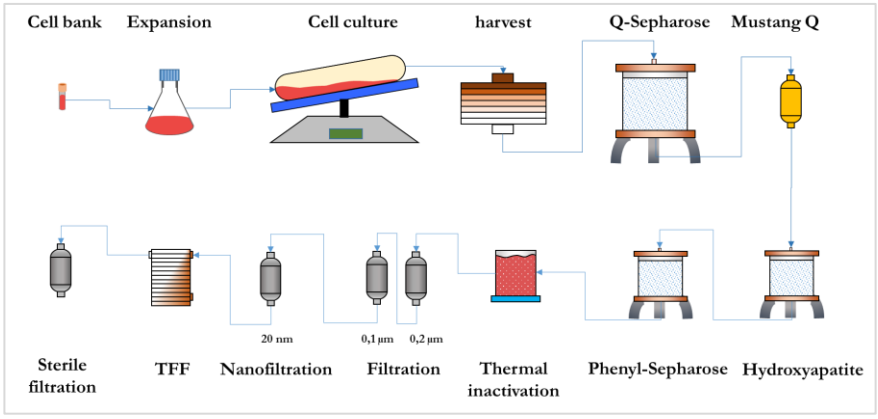


Figure 26: Schematic representation of the 340 kDa-protein production process

The cell culture was harvested by depth filtration followed by sterile filtration and was subsequently loaded on the Q Sepharose Fast Flow anion-exchanger equilibrated with 50 mM Tris-HCl, 400 mM NaCl, pH 7.0 taking advantage of the low isoelectric point of the protein of interest (pI 5.1). The elution was achieved by increasing the ionic strength with 50 mM Tris-HCl, 650 mM NaCl, pH 7.0 after a washing step with 50 mM Tris-HCl, 50 mM arginine pH 7.0 in order to reduce the HCPs and residual DNA content. The HCP content in the non-purified bulk was 100 mg/L and the average residual DNA content of 13 mg/L. With Q sepharose FF capture step the HCPs and DNA Log reduction were 2.8 and 1.7 respectively (Table XVI).

The fraction eluted from the Q Sepharose step was processed on a Mustang Q XT membrane chromatography filter in order to further increase the DNA reduction (Table XVI). The FT from the Mustang Q step was adjusted to a concentration of 50 mM sodium phosphate and processed by chromatography on a Macroprep Hydroxyapatite column based on complex interactions of proteins with the positively charged C-sites (calcium ions), and negatively charged P-sites. The sample was loaded assuming a dynamic binding capacity of 2 mg/mL at a linear flow of 76 cm/h. The decrease in conductivity during the washing step induced a pH increase leading to the concomitant elution of protein of interest. This chromatographic step reached a POI yield of 73% with a HCPs Log reduction of 0.6 and a rDNA Log reduction of 3.5.

The concentration of NaCl and arginine in the fraction of interest were adjusted to 2 M and 50 mM, respectively. The sample was then loaded on a Phenyl-Sepharose (high substitution) column. The addition of 2 M NaCl allows a complete binding of the protein of interest to the resin whereas the addition of 50 mM arginine reduce the interactions between the HCPs with the hydrophobic functional groups of the resin. The protein of interest was eluted with WFI in approximatively 1.7 CV at a protein concentration of 0.7 mg/mL. The eluted fraction represents 70% of the loaded protein of interest and with a

HCPs reduction of 0.5 Log. The final polishing of rDNA was achieved in this chromatographic step, obtaining a Log reduction of 0.2. The eluted fraction obtained from the HIC step was processed for viral thermal inactivation at 60±2 °C for 4.0±0.1 hours under continuous stirring followed by 0.1 µm filtration and viral removal through filtration at 20 nm. The final product was concentrated to 2.6 mg protein/mL and formulated by tangential flow filtration on a 0.1 m^2 PES cassette with a 100 kDa cut-off. The buffer exchange was performed against 7 buffer volumes of sterile filtered 10 mM sodium phosphate, 150 mM NaCl pH 7.0 at constant volume. The viral inactivation and removal steps together with the diafiltration, did not have significant effect in HCP reduction, in particular the Log reduction of these steps was 0.4. Regarding the clearance of rDNA Log reduction, the steps effective in rDNA removal were 0.1 µm filtration step and TFF with a Log reduction of 0.3 and 0.7, respectively. A total recovery of the protein of interest was obtained in the viral inactivation step whereas the viral removal and diafiltration steps resulted in a yield of 94 and 95%, respectively.

The yield of purified protein of interest at the end of the process was 41%, corresponding to approximately 43 mg of purified protein/L of cell culture. Regarding contaminant reduction, the concentration of HCPs/mg 340 kDa-protein in the final drug product was 147 ppm, with a cumulative Log reduction of 4.3, while residual DNA concentration at the end of the process was 0.06 ng/mg protein of interest, with a Log reduction of 7.

Table XVI: Summary of the downstream process in terms of total and step yields, protein of interest production and contaminants reduction

Purification step	Vol *(L)*	POI *(g/L)*	POI content *(g)*	Step yield *(%)*	Total yield *(%)*	HCD (ng/mg)	HCD *(Log red)*	HCPs *(ppm)*	HCPs *(Log red)*
Non purified bulk	100.00	0.104	10.4	-	-	253846	-	930728	-
IEX *(Q-Sepharose)*	9.60	1.08	10.4	100	100	5065	1.7	1626	2.8
IEX *(Mustang Q)*	10.18	0.99	10.1	97	97	1310	0.6	1626	0.0
HA *(CHT-I)*	2.14	3.46	7.4	73	71	0.57	3.5	688	0.6
HIC *(Phenyl Sepharose)*	6.48	0.77	5.0	68	48	0.53	0.2	335	0.5
Viral clearance *(heat inactivation)*	6.48	0.77	5.0	100	48	0.53	0.0	211	0.2
Viral clearance *(0.1 µm filtration)*	6.20	0.77	4.8	96	46	0.28	0.3	211	0.0
Viral clearance *(20 nm nanofiltration)*	7.00	0.64	4.5	94	43	0.28	0.0	163	0.1
TFF	1.65	2.60	4.3	95	41	0.06	0.7	147	0.1

4.2 Development of a systematic approach for the purification of a 13 kDa human protein produced in *E. coli*

4.2.1 Introduction

Purification of recombinant proteins for therapeutics or analytical applications requires the use of several chromatographic steps in order to achieve a high degree of purity. A range of techniques is available such as anion and cation exchange chromatography, which can be carried out at different pHs and used at different steps, hydrophobic interaction chromatography, size exclusion chromatography and affinity chromatography. Until now it is virtually impossible to select separation and purification operations for proteins in a fully rational manner due to a lack of fundamental knowledge on the molecular properties of the materials to be separated and the lack of an efficient system to organize such information (50).

For this reason, in the second part of my PhD project, the DoE and HTPD technology were used as a systematic approach for the optimization of the purification process of additional recombinant proteins. These two innovative techniques were used as a standardized “tool box” in order to introduce the

purification steps of a 13 kDa protein expressed in *E. coli* as inclusion bodies. The intention was to propose a comprehensive approach based on a step by step "decision tree" in order to point out a well-defined path in the purification and formulation of the protein of interest. As shown in the diagram reported in Figure 27 the approach starts when the information about the protein of interest and the contaminants in the feedstock are acquired. The information about the protein of interest are obtained from its sequence. In this case the theoretical biochemical characteristics of the protein can be gathered using for example the software CLC Main workbench or the Protparam tool. Regarding contaminants information, in particular the HCPs, a literature investigation is necessary in order to find the main biochemical properties of host proteins to address the subsequent purification steps.

A first optional starting step of the approach consist in an initial assessment of the protein's stability in the purification environment. A stability "tool box" based on a specific full factorial DoE is necessary to investigate the role of pH, ionic strength, excipients, incubation time, temperature and specific buffers on HCPs and POI stability. This assessment is more than ever essential in the last part of the process development where the drug product need to be formulated.

After this analysis, the systematic approach goes on with the purification steps investigation. Initially, the choice of the purification path depends on the biochemical characteristics of the protein of interest. For instance, if the protein is engineered with a specific tag, the affinity purification could be the first choice for its purification. Otherwise, if a protein mixture is clearly defined in terms of molecular weight composition a SEC could be a good choice if the process volumes are modest and the purification step does not need to be scaled up. In all other cases IEX, mixed mode chromatography or HIC are considered the first option for the capture chromatography. Normally the capture step of the protein of interest relies on IEX which exploits the divergences between solution pH and pI of the proteins. In an industrial process development, the choice of the IEX as a first step of purification is

strongly suggested in order to implement the concept of the continuous downstream processing of biopharmaceuticals. A shift to continuous operation can improve productivity of a process and substantially reduce the footprint. Moreover continuous operations also allow robust purification of labile biomolecules and this approach can easily applied to chromatography (51). Hence, it is easy to figure an eluate from IEX step containing an adequate amount of a salt used to increase the ionic strength (i.e. NaCl), which is thus available to be directly loaded on a HIC resins needing of a high ionic strength in order to ensure the salting out effect. Therefore, the second DoE platform proposed for the capture set-up combines a matrices of different variables such as buffers pH, resin matrix and exchangers, ionic strength, in order to identify the best condition for the binding of the protein of interest by FT analysis. In this case, the third DoE "tool box" is presented by the set-up of a Response Surface Methodology (RSM) platform to establish the best elution condition for the protein of interest.

Typically a chromatographic step like the capture requires a "bind and elute" mode, but, if the FT analysis reveals that a specific condition tested allows the binding of the contaminants but not of the protein of interest, the approach can be converted in a "flow through" mode for the purification step.

This rational approach, proposed for the chromatographic capture step, with its DoE, can be conceptually applied in every chromatographic step as the intermediate and polishing steps.

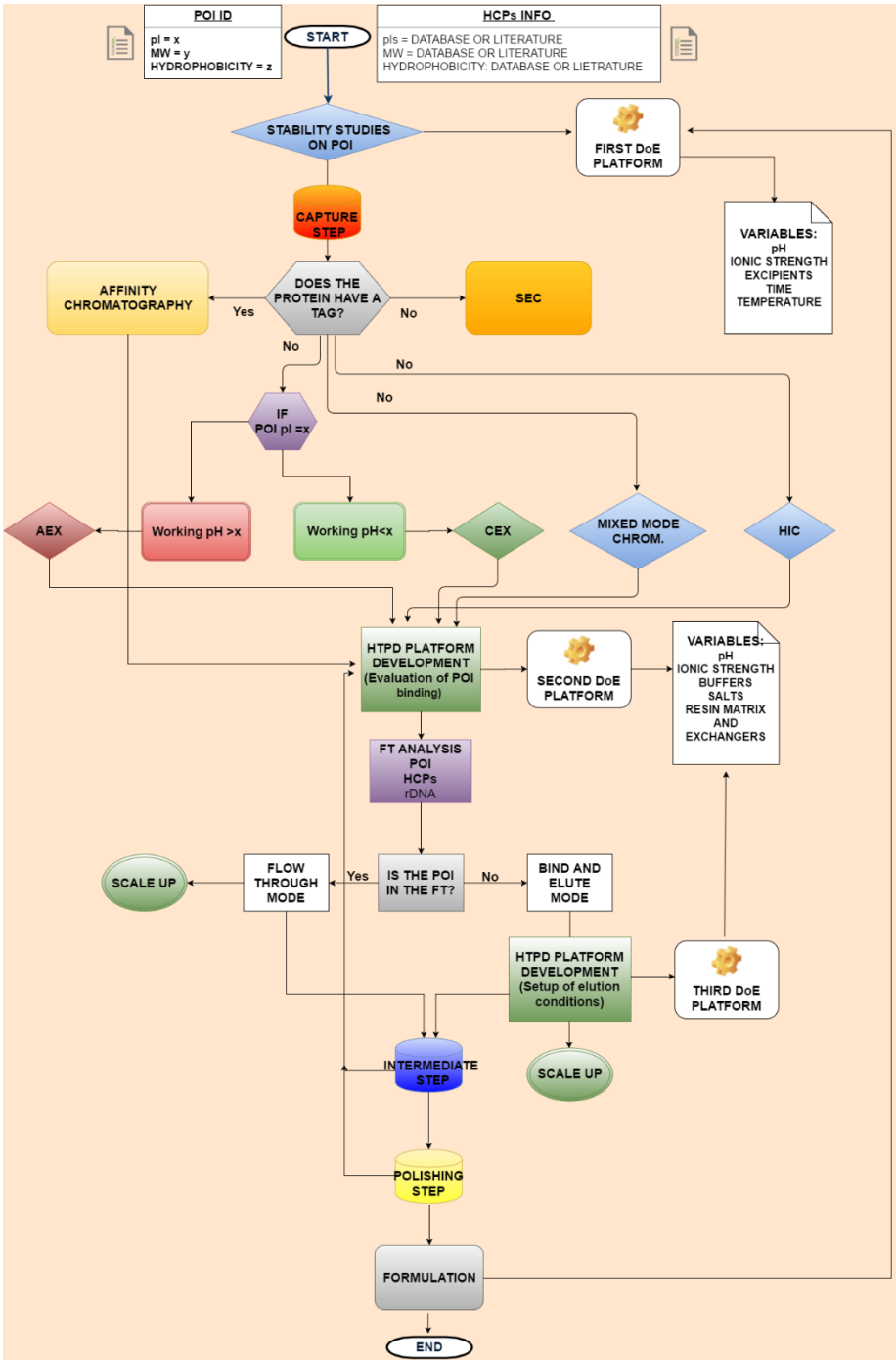


Figure 27: Schematic representation of the “decision tree” for the development of a systematic purification approach

The protein chosen to test the mentioned approach is a 13.6 kDa protein expressed in *E.coli* as inclusion bodies and used in rescue of the retinal function in glaucoma. The 13.6 kDa mass is referred to its monomeric form but the biologically active structure is a homodimer of about 27 kDa. As mentioned for the 340 kDa protein, the software CLC Main workbench and the bioinformatics tool Protparam allowed to obtain the main biochemical properties from the amino acid sequence, see Table XVII. The protein of interest is a basic and highly hydrophobic protein. The purification development is complicated by these characteristics and the instability of the molecule in neutral, low ionic aqueous buffers.

Table XVII: Biochemical characteristics of the 13 kDa protein monomer

Length	120 aa		
Weight	13.6 kDa		
Isoelectric point	9.8		
Aliphatic index	62		
Half-life: aa N-terminal glutamic acid	30 hours mammals	>20 hours yeast	>10 hours *E. coli*
Extinction coefficient at 280 nm	Non reduced cysteines 22550 $mM^{-1}cm^{-1}$		Reduced cysteines 22190 $mM^{-1}cm^{-1}$
Extinction coefficient absorption at 280 nm 0.1% (=1g/L)	Non reduced cysteines 1.653		Reduced cysteines 1.627

As described in the introduction of this chapter, also the information of the main *E. coli* HCPs are essential for the set-up of the approach. Some information about these contaminants were found in the literature (50-52).

The protein, after the refolding, was solubilized in a buffer containing 0.1 M Tris-HCl, 1 M arginine pH 9.5. In order to proceed with the protein purification, arginine must be removed due to its ability to modulate binding and elution in IEX and HIC chromatography (49). A dialysis/diafiltration step

was thus required. In order to maintain the same pH of the refolding buffer, four basic buffers were screened in order to identify the best in terms of total protein content and reduced amount of protein precipitate. The analyses performed, as described in the paragraph 3.3.2, were: total protein content, POI content and turbidity. The results are shown in Figure 28. The graph shows how the best conditions were obtained by decreasing arginine content with Tris-HCl pH 9.5: in this condition, the total protein reduction was about 30% and 90% of the remaining total protein content was composed by the POI. A drastic effect was achieved using the other buffers: the substitution of Tris with carbonate, glycine or phosphate caused a dramatic precipitation of the HCPs and the POI. The effect was significative using phosphate buffer where an additional negative effect on protein stability was caused by pH decrease up to pH 8. These results allowed to choose Tris-HCl buffer in order to reduce the amount of arginine in refolding buffer and to proceed in subsequent purification set-up without its interference.

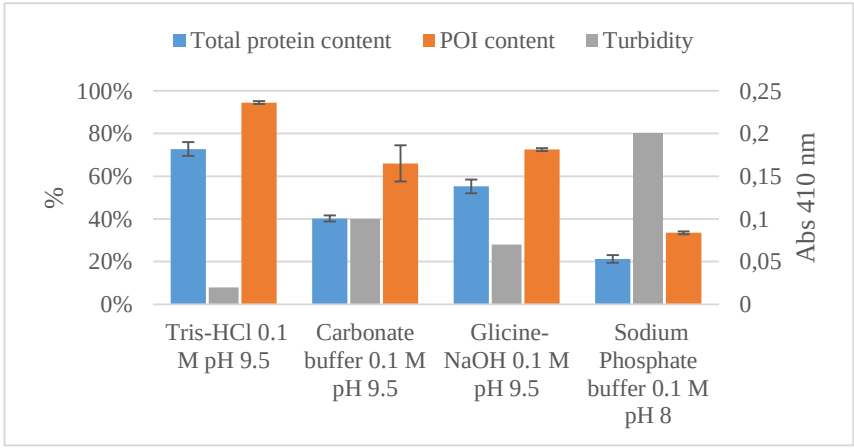


Figure 28: Screening of four different basic buffers in order to reduce arginine concentration from refolding buffer

4.2.2 Platform set-up for protein stability testing (platform n°1)

The first platform mentioned in the decision tree approach was the "protein stability test". As described above, a step committed to investigating

the protein stability in the purification environment and eventually in the last step of protein formulation is essential in order to predict the protein behavior in terms of aggregation, precipitation, denaturation and loss of activity. Considering the first part of the downstream process, corresponding to the initial purification steps, some variables such as pH, ionic strength, temperature, time and the starting total protein concentration could affect protein stability. Table XVIII resumes the main factors of the platform. This table was compiled using the parameters requested in the upper part and subsequently all the data were inserted in MODDE software in order to propose the list of the full factorial experiment.

Table XVIII: Summary of the main factors needed for the set-up of the stability testing

Ionic strength	pH	Time	Temperature	Protein Concentration
Choose the type of salt (i.e. NaCl or $(NH_4)_2SO_4$)	Choose the pH range (2 to 12)	Choose the incubation time (the variable must be quantitative multilevel)	Temperature range: from 2-8 °C to R.T. (the variable must be quantitative multilevel)	Choose the concentration range)
Define the concentration range	Choose the acid or base to add (HCl, NaOH, CH_3COOH etc.)			
NaCl from 0 to 1 M	**4 to 10.5 (HCl and NaOH)**	**0, 1 and 2 hours**	**2-8 °C and R.T.**	**From 0.75 to 0.1 mg/mL**

In order to test how these factors can alter protein composition in 0.1 M Tris-HCl buffer used during the chromatographic steps, a DoE based on a fractional factorial V+ resolution was performed.

The results obtained from the screening are shown in Figure 29. First, considering the fit plot histogram, the model of the ANOVA analysis showed very high values in terms of R^2, Q^2, model validity and reproducibility and this implies a good model. As a matter of fact, a high degree of response variation (R^2 of 0.913) was explained by the model, it was highly predictive (Q^2 of 0.813) and was also considered valid and highly reproducible. The coefficient plot shown in Figure 29b describes the main terms of the model and the interactions

able to improve it. A stability effect was observed increasing the pH but otherwise the opposite effect was obtained increasing the ionic strength with NaCl. Also the protein concentration had a negative effect on the total protein stability although this effect was lower compared to the ionic strength. The incubation time and the temperature, considered as singular factors, did not play a significant effect on the model. Conversely, a significant effect was observed when these two factors interacted each other. When there is an interaction between two factors, the effect of one depends on the levels of the other factor.

a) b)

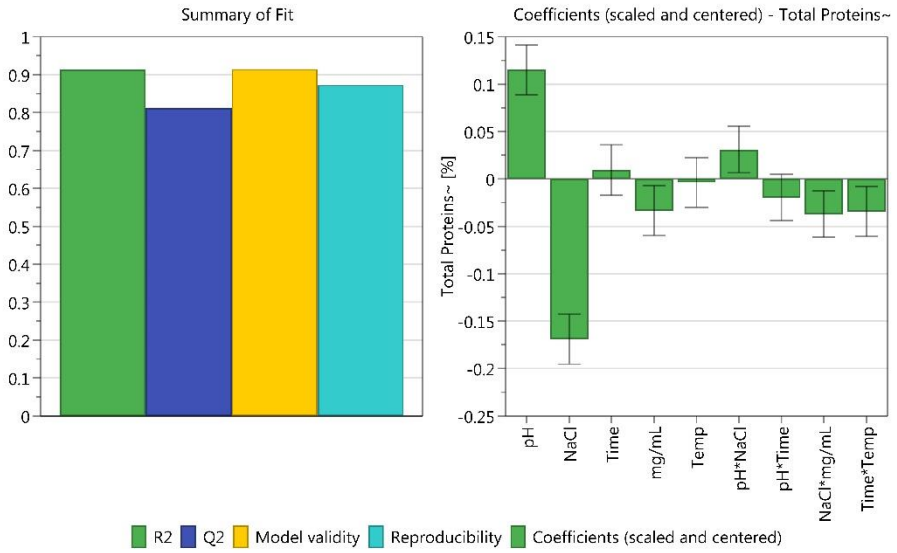


Figure 29: Summary of fit (a) and coefficient plot (b) of the model proposed for the stability testing

Figure 30 shows all the interactions of the variables identified in the model. In particular, the incubation time and the temperature showed a strong interaction, i.e. the two lines intersect each other. Considering the results obtained from R.T. samples, increasing the incubation time, the stability of the total proteins was reduced but at lower temperature this effect was the opposite.

A weak interaction was identified for the ionic strength and pH: as a matter of fact, the two lines are not parallel although, in the range of ionic strength used, do not intersect. In particular, starting from high or low pH values when NaCl concentration was increased the total protein content decreased. The same trend was observed for the more evident interaction between protein concentration and ionic strength (Figure 30b).

The contour plot in Figure 30c resumes the results obtained. In all the tests performed, the increase of the ionic strength and the decrease of the pH of the samples caused the precipitation of the total proteins and this effect was more evident when the total protein concentration was higher.

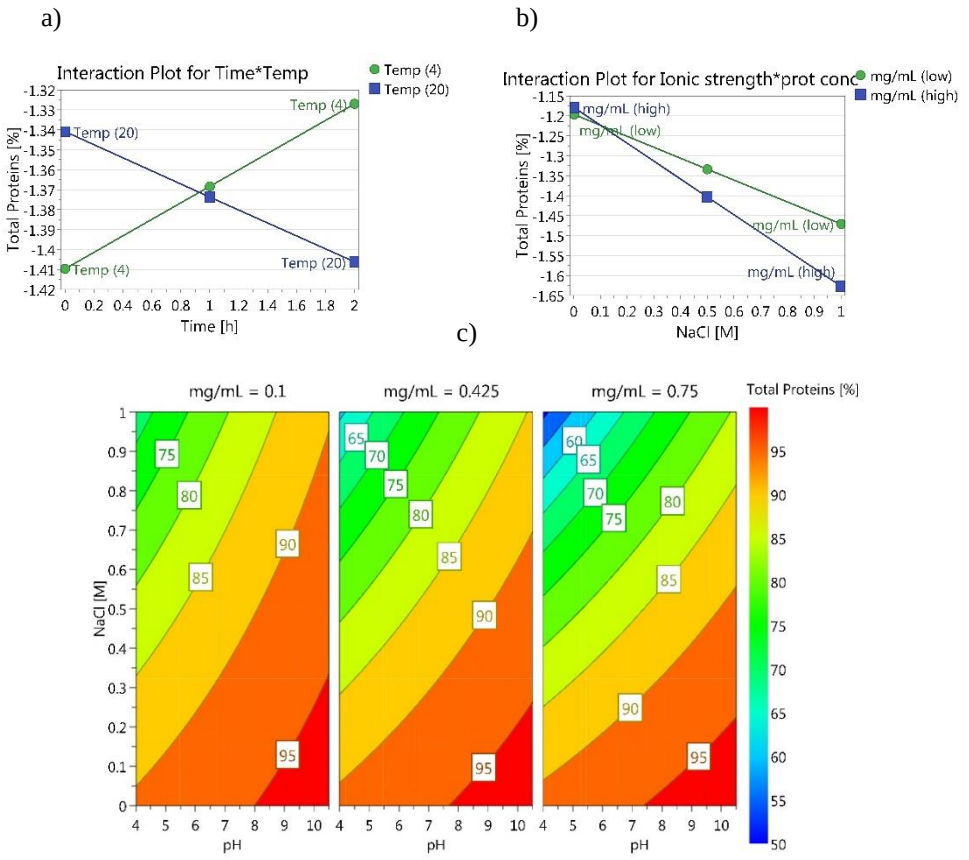


Figure 30: Interaction plot for time vs temperature a) and concentration vs ionic strength b). c) Contour plot considering the three significant variables (pH, protein concentration and NaCl concentration)

4.2.3 Chromatographic capture step: HTPD platform development (platform n°2)

As shown in the decision tree reported in Figure 27, the systematic approach continues with the introduction of the capture step for the purification of the protein of interest. In this step the aim was to identify, through HTPD approaches, a set of chromatographic media able to recover the protein of interest and to remove part of the contaminants. Due to its high isoelectric point the protein could be captured with a CEX at pH below 9.6 or an AEX at pH above the pI. Another option was to test resins, such as hydroxyapatite or Eshmuno HCX based on a mixed mode interaction.

These considerations allowed to set-up an HTPD approach matrix as described in Materials and Methods in paragraph 3.3.4. The only three variables considered in order to evaluate the binding of the protein of interest were pH, ionic strength and total protein loading. The results obtained from the screening are reported in Table XIX in form of contour plots generated by MODDE. All the graph are represented with the pH values on the x axis and ionic strength on the y axis and all the contour plots of total protein content and POI content refers to a 1:1 ratio of total protein loaded against the volume of resins.

The effect of buffer pH on the cationic resin screening was relevant because the protein of interest increased the binding at decreasing pH up to 7, due to its high positive charge. SP Sepharose, Nuvia S and Fractogel SO_3 showed the same trend with about a 50% of POI binding at pH 7 without addition of NaCl. Increasing the salt concentration and the pH, the yield of the POI decreased. Unfortunately, at low pH the Tris buffer could not be used with the cationic resins since its charge is mainly positive and caused a "shield effect" on the cationic exchangers. This effect could be observed in the total protein content contour plots where the maximum total binding obtained was about 30% and this value decreased up to 10% reducing pH and ionic strength. The low binding of HCPs on these cationic resins could be also related to the

negative charge of most of these proteins at neutral and basic pH as described in the literature (50-52).

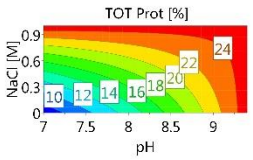
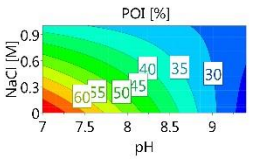
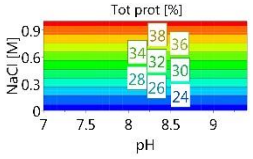
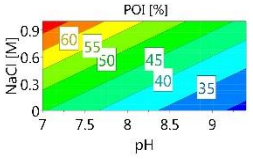
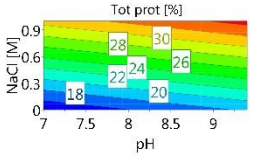
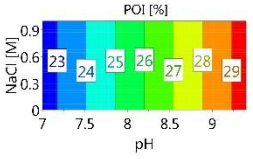
Using Eshmuno S and the weak cation exchanger CM Sepharose FF, the POI binding increased at low pH, as for the resins mentioned before, and at high ionic strength. This latter condition for binding was not easy to explain but could be probably attributed to a combination of uncontrolled factors such as the nature of matrix, the "shield effect" of Tris buffer, the pI of HCPs and POI, and the ionic strength. A same puzzling binding behavior of the POI was observed on Poros XS where the binding of the POI was achieved increasing the pH. In this case, the main confounding effect could be attributed to the polymeric matrix. As a matter of fact, the polystyrene divinylbenzene matrix of Poros XS is a common matrix used in Reverse Phase Chromatography (RPC) in order to bind and separate HCPs from the POI. Hence, in this case the "matrix effect" could explain the unconventional binding of the HCPs and of the POI.

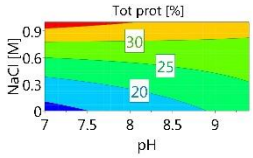
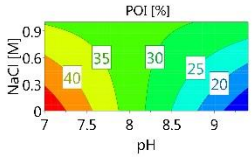
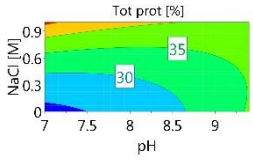
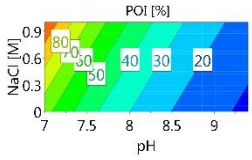
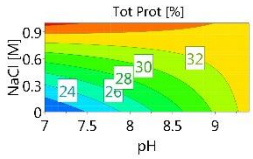
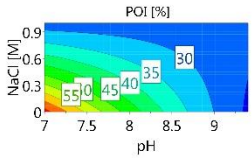
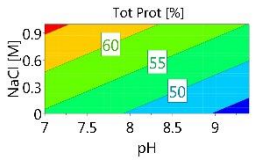
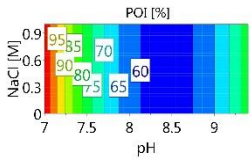
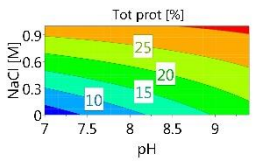
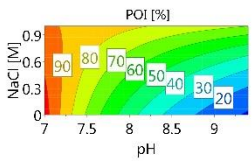
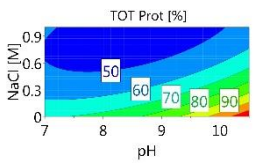
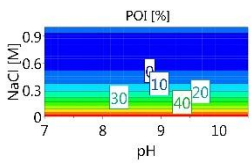
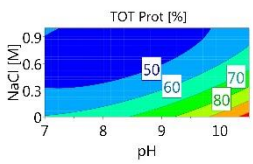
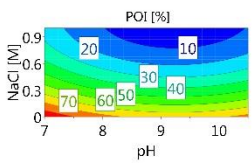
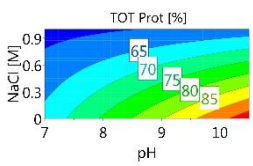
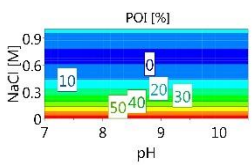
A very high degree of binding of the POI was observed in the two mixed mode chromatographic media. In HA a quadratic effect was observed in the POI binding reaching a maximum and a high purity at low pH and low ionic strength. In Eshmuno HCX a high binding of the POI was observed at low pH and at all NaCl concentrations of while the HCPs binding increased only with the addition of NaCl in the samples. These behaviors could be explained only by the presence of a cationic exchanger and a strong hydrophobic group such as the phenyl group. In fact, both the exchangers affect the binding of the POI because it is very basic and hydrophobic, therefore, it binds these ligands at low pH and at high ionic strength. Differently, the HCPs binding was affected mainly by the phenyl group of the matrix at high concentration of NaCl.

Regarding the effects on anionic chromatographic resins, the outcome of pH and ionic strength was more or less identical for the three resins (Nuvia Q, Poros HQ and Q Sepharose). The binding of both the POI and HCPs increased by increasing the pH and reducing the ionic strength.

In order to test a scale-up of a capture step, an anionic exchanger was chosen because the quaternary ammonium group was the only exchanger able to bind the POI at high yields and at the best stability condition identified in paragraph 4.2.2 (pH 9.5 at low ionic strength). Unfortunately, this condition has some disadvantages in terms of binding of contaminants. In fact, HCPs and rDNA bind very strong to the resin and they must be removed in the elution step. On the other hand, this choice was ambitious because allowed to test the capability of the step by step systematic approach to propose a process able to obtain a highly pure POI. Perhaps, a chromatographic step, such as a cationic interaction on SP Sepharose at pH 7, could allow an almost complete binding of the protein of interest with a reduced amount of HCPs and rDNA. However, this approach was affected by a high decrease of the POI (due to precipitation in the first step of buffer exchange up to pH 7) and by a reduced yield in the chromatographic step.

Table XIX: Contour plots of the eleven resins tested for the introduction of the capture step

SP		
ES		
PS		

NS		
CM		
FS		
HA		
EX		
NQ		
Q		
PQ		

4.2.4 Optimization of the elution conditions on the anionic exchangers: DoE platform n°3

The three resins identified as the best option for the capture step were tested with a third DoE platform approach in order to identify the main variables that could affect the elution of the POI and HCPs. The variables considered in this platform were ionic strength, pH and buffer composition (100 mM of phosphate or Tris buffer). The approach was based on a full factorial interaction model and the shared POI response surface of the three resins is represented in Figure 31.

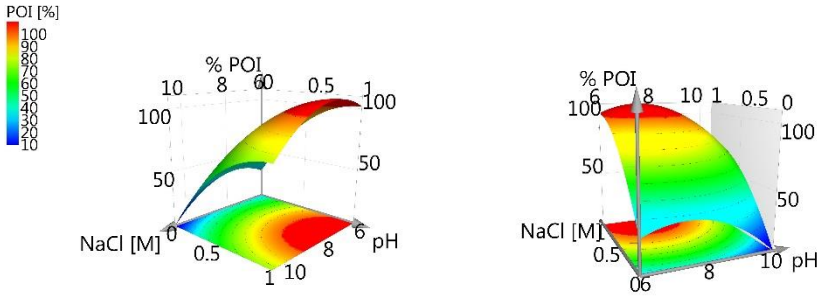


Figure 31: Response surface plot of the full factorial model proposed for the set-up of the elution conditions in the anionic exchangers

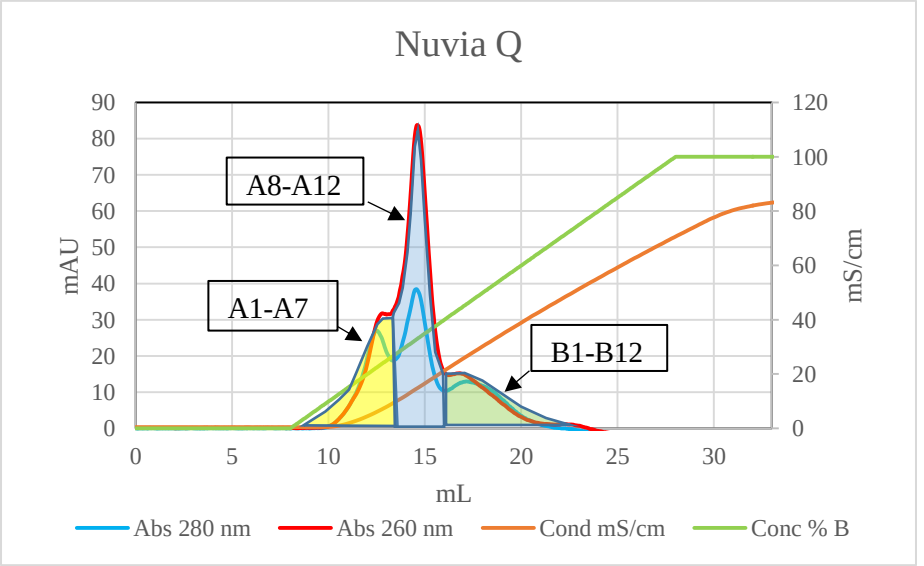
The protein of interest eluted with the highest yield at 1 M NaCl and at pH 6 in 100 mM phosphate buffer. Hence, the increase of ionic strength had a positive effect in POI elution as well as the decrease of pH. In particular, this effect was coherent with the increase of the positive repulsive charges on the protein of interest. This effect is evident at pH 6 and 0 M NaCl where about 20% of the POI was eluted. The main effect on protein elution was due to these two factors only, whereas the phosphate buffer was useful to partially change the Tris buffer content and to shift the buffer capacity at acid pH.

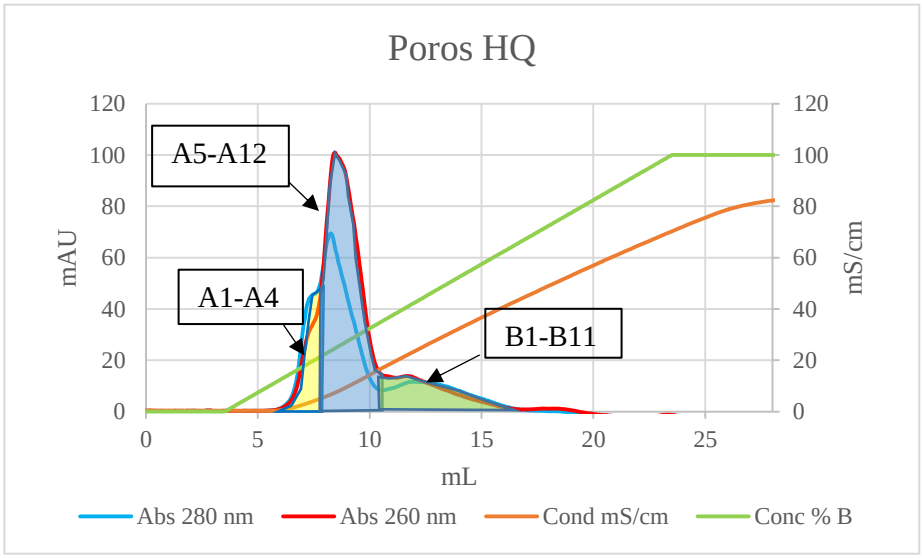
The data obtained from HCPs Log reduction showed how all the conditions tested had a very low Log reductions value, therefore, the model generated was not valid and predictive. As a matter of fact, all the elution

conditions of the platform were too drastic or too weak to separate the HCPs from the POI. Hence, it was necessary to perform a gradient elution step to identify the best conditions to reduce the contaminants level.

4.2.5 Scale-up of the anionic capture step

As mentioned in the previous paragraph, a gradient elution on the three columns was necessary in order to find the best elution condition. The binding capacities of the three resins were approximately similar, in particular the total protein binding capacity at the 5% breakthrough was about 0.75 mg/mL, while the POI was about 0.3 mg/mL. Figure 32 reports the three chromatographic profiles of Nuvia Q, Poros HQ and Q Sepharose FF performed on a 1 mL resin using a linear gradient from 0 to 100 mM phosphate buffer, 1 M NaCl, pH 6. The three profiles shared the same shape characterized by three peaks at different retention times. In the Nuvia Q purification the fraction A1 to A7 referred mainly to a mixture of protein and rDNA, while in Poros HQ and Q Sepharose they mainly contained proteins since the absorbance at 280 nm was about twice higher than at 260 nm. In all the three chromatograms the second peak was mainly rDNA because the A_{260nm}/A_{280nm} ratio was about 1.8, while the third peak consisted of proteins.





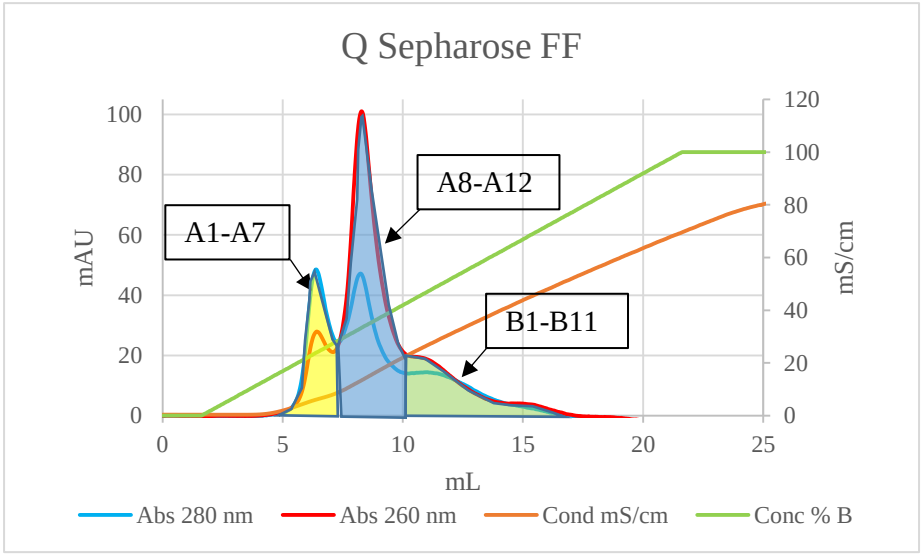


Figure 32: Chromatograms of the Nuvia Q, Poros HQ and Q Sepharose FF chromatographies performed with 10 CV gradient elution

The plot in Figure 33 reports the results obtained from ELISA test, HCPs and total protein content of the fractions collected from the three purifications. The

analysis show that the POI eluted in the last peak of the three chromatograms (it was approximately collected in fractions B1 to B12) while 80% of HCPs were collected up to fraction A12. Moreover, the ELISA test showed that the initial protein content of the POI corresponded to about 25% of the total protein content. Table XX resumes the Log reductions for rDNA and HCPs, and POI yield in the last chromatographic fraction: the POI was fully recovered in the last fraction for all the three resins (possessing a quaternary ammonium exchanger) allowing a five-fold reduction of HCPs content.

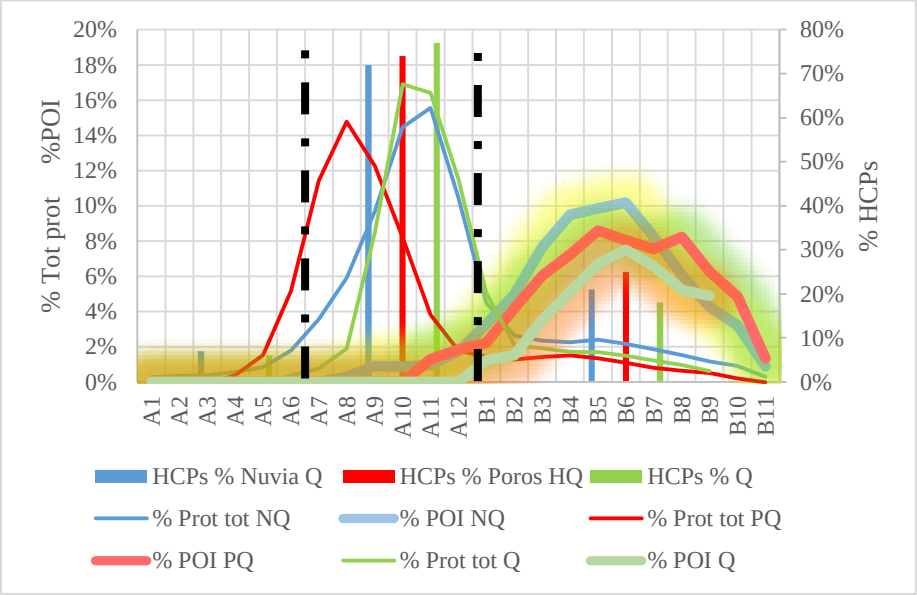


Figure 33: Results obtained from ELISA test, HCPs and total protein content of the fractions collected from the three purifications. The thin lines represent the total protein content, the thick lines the POI content, while the bars represent the HCPs percentage content

Table XX: Summary table of the three anionic exchangers purifications in terms of contaminants reduction and overall recovery

	NQ	PQ	Q
	B1-B12	B1-B12	B1-B12
HCPs Log red	0.69	0.60	0.75
rDNA Log red	3.50	3.00	2.90
POI yield (%)	98%	95%	96%

The best Log reduction in terms of rDNA was apparent for Nuvia Q resin, with a value of 3.5. This value, together with the best yield obtained, allowed the introduction of this resin in the purification process. The purification on Nuvia Q was optimized introducing a stepwise elution; in particular, the results obtained in the 1 mL-scale allowed to identify the NaCl concentrations required for contaminants removal and POI elution. A first elution step was assessed at 150 mM (15 mS/cm corresponding to 15% of buffer B) and the elution of the POI was performed at 370 mM NaCl (37 mS/cm corresponding to 37% of buffer B). At the end of the chromatographic run the resin was regenerated with 100 mM phosphate buffer, 1 M NaCl, pH 6. Figure 34 shows the scale-up performed on a 5 mL resin. All the results obtained in the small scale were fully reproducible on the 5 mL-column stepwise elution.

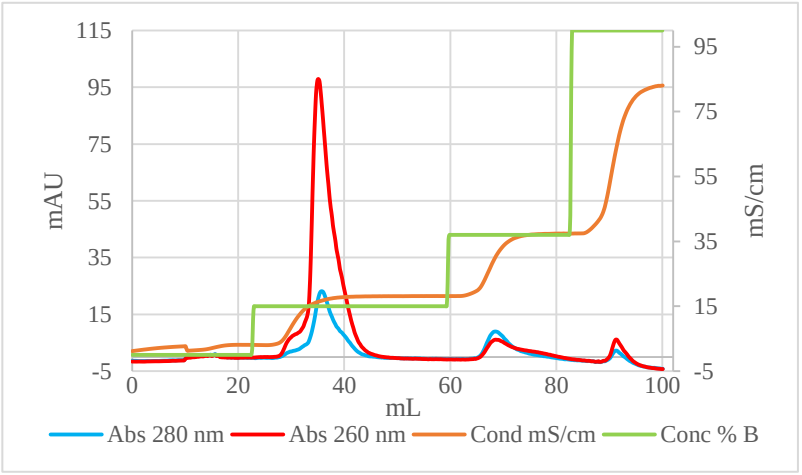


Figure 34: Stepwise elution on Nuvia Q resin during the purification of the 13.6 kDa protein

4.2.6 Introduction of a polishing/intermediate step for the purification of the 13 kDa protein

The same approach proposed for the determination of elution conditions on the anionic resins in capture step was performed in order to optimize the elution on HA. As described in paragraph 4.2.3, this resin showed a high binding capacity of the POI in the 7 to 8 pH range, hence, a DoE was performed to set up the elution step. The platform was set up as described in paragraph 3.3.7 using as starting sample the fraction eluted from Nuvia Q containing the POI. Unfortunately, all the conditions tested did not allow the elution of the POI; as a matter of fact, the POI was eluted only using a harsh regeneration step with NaOH 1 M. Due to the too strong binding of the POI, the HA was eliminated from the process development.

The CIM multus DEAE monolithic column, a weak anion exchanger with similar binding characteristics of strong cation exchanger, was thus investigated since many information on interaction between the POI and anion exchangers were available from platform n°2.

The peak eluted from Nuvia Q, containing the 13 kDa protein, was diluted twenty-fold with 0.1 M Tris-HCl pH 10.5 and loaded on the column. The same gradient elution described in paragraph 3.3.6 was performed. Three main groups of peaks were identified during the elution by the gradient step: one at low conductivity and the others at the end of the gradient when the conductivity was very high and the pH was completely shifted from 10.5 to 6 (Figure 35a). The SDS-PAGE analysis showed that there were not significant differences in the profile of the three fractions (Figure 35b). Also the quantification of the HCPs demonstrated that no significant reduction of the protein contaminants content was obtained.

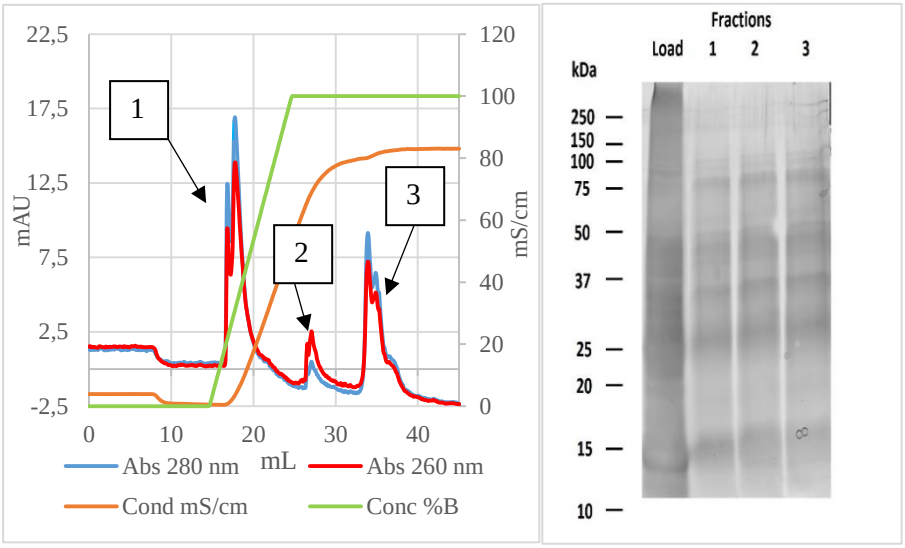


Figure 35: a) Chromatographic profile of the CIM Multus DEAE column during the purification of the 13.6 kDa protein. b) SDS-PAGE of the fractions collected

An additional resin tested for the polishing step was the Phenyl Sepharose HP. In order to evaluate the stability of the POI after adding the salts responsible of the salting out effect, a DoE approach based on two variables was performed. The factors analyzed were the type of salts used (NaCl or ammonium sulfate) and their concentrations (range from 0 to 2 M). Figure 36 show the coefficient plot of the model variables generated by MODDE.

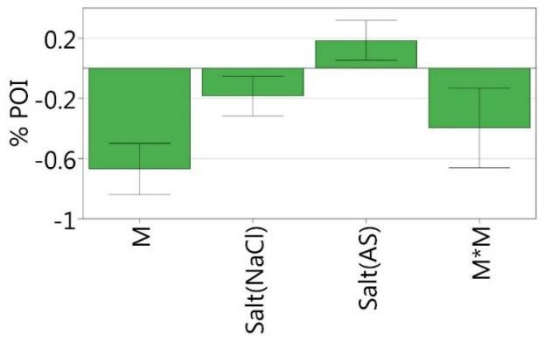
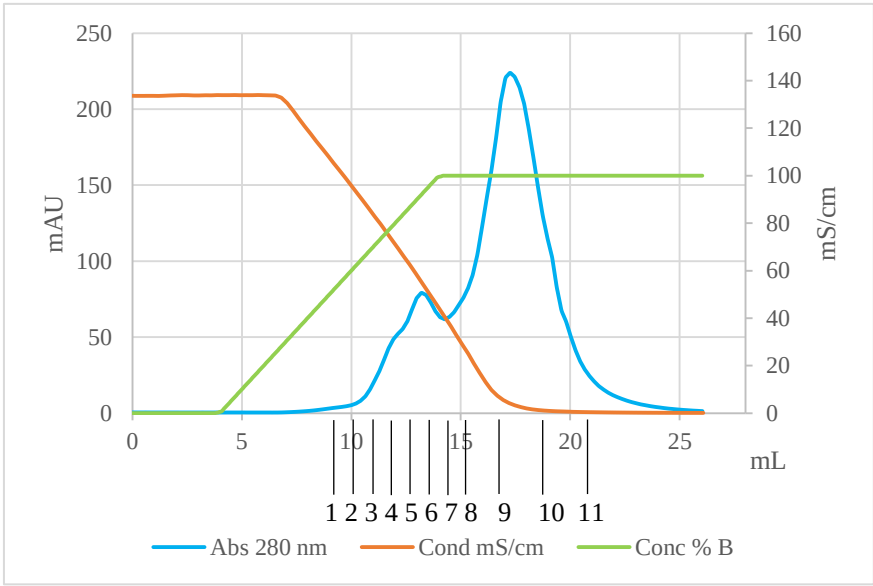
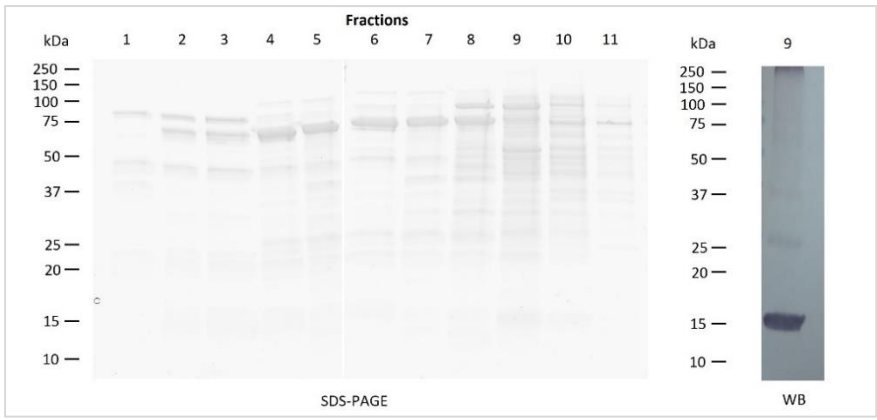


Figure 36: Coefficient plot of the stability test performed before the HIC step in order to evaluate the effects of NaCl and ammonium sulfate on protein precipitation. “M” refers to salts concentrations expressed as molarity

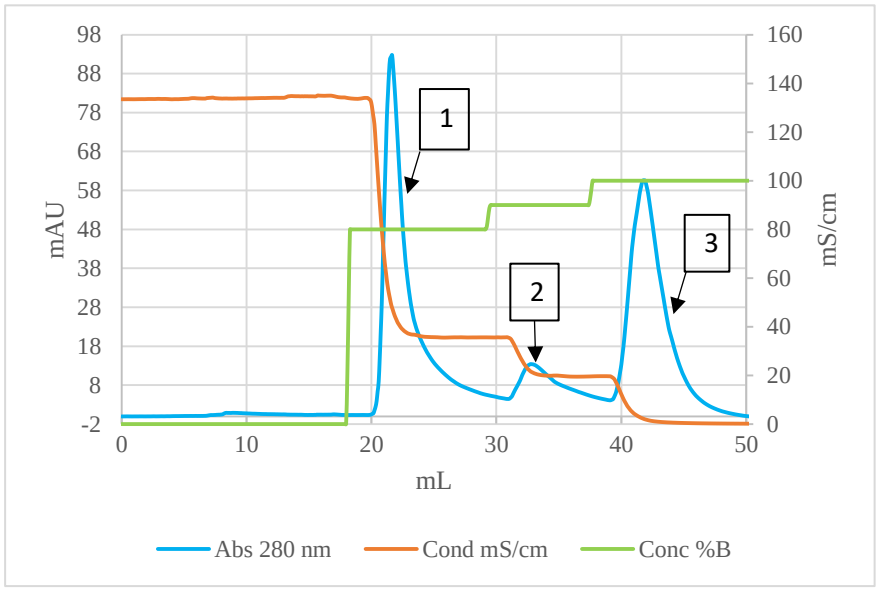
The graph shows that an increase in salts concentration affected the precipitation of the POI. The POI content was not significatively affected up to the concentration of 1 M of the two salts but at 2 M of NaCl the POI content dropped to 30%. At 2 M ammonium sulfate the POI content decreased up to 70%, hence, the NaCl concentration had a stronger negative effect on POI stability than ammonium sulfate. A binding test on Phenyl Sepharose HP demonstrated that 1 M NaCl did not allow the full binding of the POI, while at 1 M ammonium sulfate the binding was complete. Subsequently, three mg of total protein were loaded on the HIC column and the proteins were eluted with a gradient of 10 CV of WFI (Figure 37a). The fractions collected from the elution step were analyzed by SDS-PAGE and WB analysis (Figure 37b) and these allowed to set-up the stepwise elution. In particular, the HCPs were removed in the first two elution steps at the conductivity of 36 and 20 mS/cm without any loss of the POI (Figure 37c fractions 1 and 2). The latter was eluted further reducing the conductivity up to 0.2 mS/cm (Figure 37c fraction 3): this fraction contained the 70% of the POI and the HCPs Log reduction was about 1, while the rDNA Log reduction was about 1.5.

a)





b)



c)

Figure 37: a) Chromatographic profile of the elution gradient performed on Phenyl Sepharose FF. b) SDS-PAGE and WB of the fractions collected during the gradient. c) Stepwise elution of the HIC column

In order to further reduce the contaminant content, all the samples obtained from the cationic resin screened for the set-up of the capture step were analyzed in term of HCPs reduction. Actually SP Sepharose FF resin was chosen for a flow through purification step. As a matter of fact, at pH 8 with 150 mM NaCl, corresponding to HIC step elution condition, the binding of the POI was about 50% while the total protein binding was about 75%. Anyway, this step allowed

only a very low reduction of HCPs (Log reduction 0.3) and a rDNA reduction of 0.1. The SDS-PAGE and WB analysis in Figure 38 show the profile of SP FT and eluate.

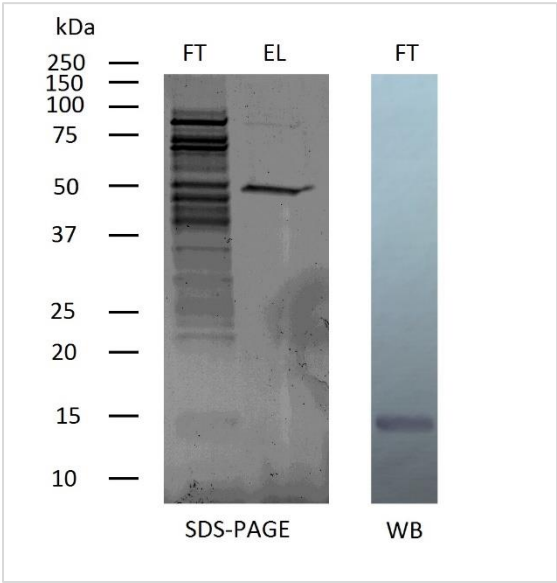


Figure 38: SDS-PAGE and WB of the SP Sepharose FT and eluate

At the end of these three purification steps the sample contained some HCPs bands and also additional contaminants at the molecular weight of 35, 45, 62, 75 kDa and others high MW aggregates. Only with a chromatographic step on a RPC source 15 (GE) the protein was completely purified: RPLC displacement chromatography for the purification of the POI from its variants and *E. coli* impurities is well documented. The SP FT was loaded on the RPC resin (3 mL) equilibrated with 0.1 M CH_3COOH and subsequently the elution was performed with 30 CV of 0.1 M CH_3COOH, 80% acetonitrile. The protein was eluted at high degree of homogeneity as confirmed by SDS-PAGE in Figure 39 and then was concentrated up to 1 mg/mL in 10 mM acetate buffer, 75 mM NaCl, pH 5. The yield of the protein of interest, at the end of the process, was ~13%, corresponding to approximately 6 mg of purified protein/L culture. The HCPs content at the end of the process was about 200

ppm and the rDNA content 0.01 ng/mg of POI. Table XXI resumes all the purification steps in terms of yield, HCPs and rDNA reduction.

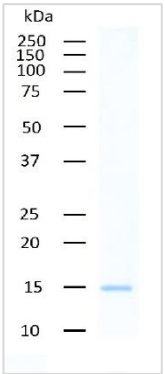


Figure 39: SDS-PAGE of the POI at the final stage of purification

Table XXI: Summary of the downstream process development for the 13 kDa protein

STEP	POI Yield (%)	HCPs Log red	rDNA Log red
Dialysis/TFF	**90%**	**0**	**0**
Capture - Nuvia Q	**98%**	**0.7**	**3.5**
Intermediate Phenyl Sepharose HP	**70%**	**1.0**	**1.5**
Polishing 1 SP Sepharose FF	**65%**	**0.3**	**0.1**
Polishing 2- RPC	**40%**	**0.8**	**0.1**
Dialysis/TFF	**80%**	**0**	**0**
OVERALL	**13%**	**2.8**	**5.2**

The DP obtained was tested for bioactivity on the RAT.C6 cell line and compared to a commercial preparation of the 13 kDa protein. As shown in Figure 40 the purified POI had the same bioactivity profile of the standard one. In particular, the two proteins showed two peaks of activity at about 5 and 15 µg/mL; the protein purified with the proposed process induced 86% of cell proliferation compared to the commercial standard, get as 100%.

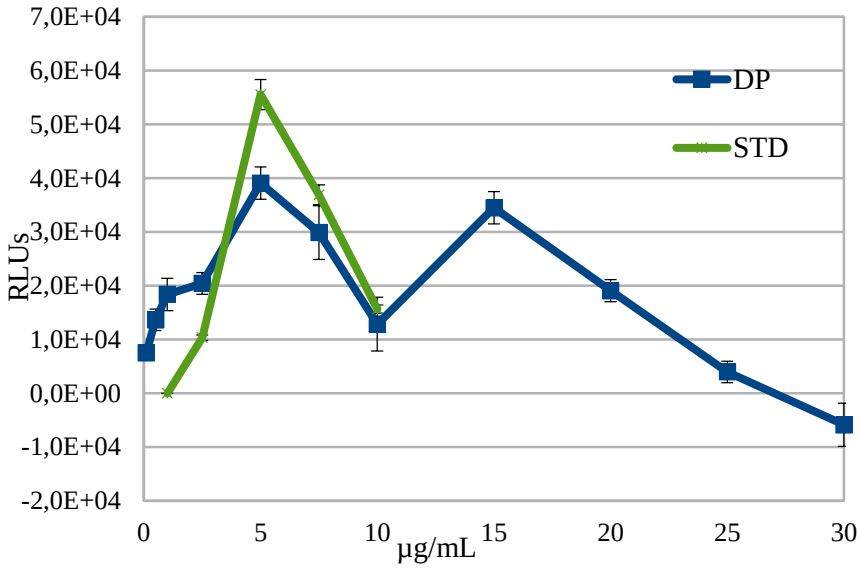


Figure 40: Bioactivity tests of the DP compared to the 13 kDa commercial protein used as reference. Results represent the average of three independent experiments

5. Discussion

The biomanufacturing industry is always looking for production processes that can be rapidly developed and can produce consistently and reproducibly large quantities of pharmaceutical-grade biomolecules at moderate costs. In order to fulfill this demand, standards process platforms in both upstream cell culture and downstream purification are going to become widely established in industry large-scale production of these biomolecules.

Downstream process of biopharmaceuticals depends on chromatographic techniques. In particular, the capture, the intermediate and the polishing steps have to ensure high purity degrees, to reduce product- (e.g. protein variants) and non-product-related (e.g. host cell proteins, rDNA or endotoxins) impurities in order to administrate the purified macromolecules in humans. These steps are usually based on ion exchange or hydrophobic interaction chromatographic principles. As a general rule, the development of these steps requires a "trial and error" sequential design decisions often obtaining results in suboptimal performance. Furthermore, large and time-consuming experimental sets are often needed to define the process parameters in order to obtain satisfactory results.

For these reasons a detailed process knowledge is becoming an essential and integral part of any production step design strategy. The aim is no longer to merely find the optimal parameter set, but to define the operating space using specific DoE strategies. These tools can generate the required process knowledge faster and with less resources, thus increasing the process understanding. Two potential solutions have emerged: HTPD and model-based design. The introduction of high-throughput methods into process development workflows improves efficiency, reduces the development time and the sample amount required to set-up the upstream phases and optimizes the chromatographic steps or stability studies.

For instance, the use of high-throughput screening based on 96-wells plates and statistical software for the DoE development allowed quick selection of most suitable chromatography medium and identification of

promising binding and elution conditions for the chromatographic steps. This gave a fast and confident start to the purification process development.

In this PhD project, both HTPD and model-based design approaches were combined in order to optimize the purification steps of a downstream process or to constitute a key "tool box" of a systematic approach for the downstream process development.

The HTPD and the model design approach were tested in the first part of the work where a complete process development for the production of a 340 kDa human recombinant protein was proposed. The non-optimized process, performed on a 25 L scale, allowed to obtain a product not compliant with EU GMP specifications. As a matter of fact, this process was only tested for its feasibility in terms of cells cultivation, identification and choice of the purification steps, evaluation of single step purification performance and investigation of the product formulation. In this work the non-optimized process was used as a preliminary scaffold in order to improve the downstream process so that it could be adopted in a fully GMP application. At first, the centrifugation harvest step was substituted with a scalable depth filtration step in order to size a procedure able to process industrial scale volumes of cells culture. The second part of the optimization focused on contaminants removal, such as HCPs and rDNA, during the purification steps. rDNA is comprised of DNA fragments and longer length molecules originating from the host organism that may be present in samples from recombinant biological processes. This contaminant could be easily reduced by benzonase treatment. The Benzonase enzyme is a dimer of identical subunits with molecular weight ~30 kDa each (with a weight totaling ~60 kDa) that can be used for digest all forms of nucleic acid by hydrolyzing them into smaller oligonucleotides of <10 base pairs in length. Benzonase treatment (9–90 U/mL) is typically carried out in batch mode and this enzyme is normally added to a process feed in presence of Mg^{2+} (1-2 mM) in a pH range of 6–10 and at temperatures of 0–42 °C. Regulatory authorities do not regulate how much residual endonuclease can be in a product. However, biotechnological products manufacturers using it in

their processes need data to demonstrate safety/toxicity status and measure residual endonuclease that might be present in final preparations for human use. For example Merck & Company's EU patent of VAQTA hepatitis A vaccine indicates that residual Benzonase enzyme is lower than 0.0001 ng/dose (53). It is important to note that the endonuclease is a process additive and not a drug, excipient, or active pharmaceutical ingredient. Benzonase removal from a process stream can be accomplished by several downstream unit operations, such as an irreversible thermal inactivation (~15 min at a temperature >70 °C and 0.02 N NaOH) or the addition of additional chromatographic and TFF steps. Such conditions could negatively affect the integrity of the drug product or reduce the POI yield. Moreover, removal can be demonstrated by showing a lack of residual nuclease activity (which does not detect residual nonactive enzyme) and using an ELISA assay for detection of total residual Benzonase molecules (both active and nonactive) (54). For these reasons, in this PhD work a DNA hydrolysis step was not introduced.

It is widely reported that arginine facilitates refolding, suppresses aggregation, increases reversibility of thermal unfolding, solubilizes insoluble pellets and reduces non-specific binding of proteins, in particular aggregates (55). In this work a washing step with 50 mM arginine during the capture on Q Sepharose FF proved the effectiveness on HCPs reduction of 1.6-fold compared to the non-optimized process and the rDNA reduction of 1.7 Log. This approach confirmed that arginine weakens HCPs interaction with anionic and hydrophobic matrices.

This process development also introduced a disposable membrane chromatographic step stated as a powerful alternative to polishing columns, particularly for process-scale recombinant protein purification, because of advantages such as high throughput, faster processing time, reduced buffer consumption, and elimination of column packing and packing testing activities. Membrane adsorbers are not new in the purification background and although available in several chemistries, their application has been limited to AEX in flow-through or isocratic mode with mostly quaternary amine "Q"

chemistry. The results obtained in this work, with the introduction of an AEX "flow-through" mode membrane, allowing an additional twofold HCPs reduction, agree with the results obtained by Zhou and Tressel (56) and by Etzel and Riordan (57) in terms of impurities reduction. The production process was finalized with the introduction of a viral inactivation and removal step. In particular, using the model based on the assumption of gradual pore blockage by particulates in the feed stream as described by Badminton et al. (42) was identified the best filter and its size in order to perform the 20 nm filtration.

The HTPD approaches and the DoE model design experiments were tested in the optimization of HA and HIC steps of the 340 kDa protein production process. These two combined approaches allowed to determine the minimal phosphate buffer concentration able to maximize the elution of the POI and to reduce the amount of contaminants in HA (3000-fold rDNA content reduction and four-fold HCPs reduction). The approaches were equally useful in HIC to define arginine and NaCl concentration able to increase POI recovery without contaminant content. These two approaches allowed not only to identify the best protein elution condition but also to explore and predict the entire design space with a statistically significance.

On the basis of these results, the aim of the second part of this PhD project focused on the introduction of a standardized approach to purify recombinant proteins. The "tool box" described above, acquired a pivotal role in the development of the method: as a matter of fact, each step of the purification program was characterized by a predefined set of variables to test with HTPD approaches and analyzed by the statistical software. Each recombinant protein purification process is unique due to different variables such as the hosts, the refolding step, the post-translational modifications and the presence of tags: accordingly, it is not possible to define in advance a common design for each element of the "tool box" of the approach. However, it is possible to define the objective of each step and, on this basis, define if the test could be a screening, robustness or an optimization study.

The decision tree approach was tested setting up a complete purification process of a 13 kDa recombinant protein expressed in *E. coli* as inclusion bodies. After the refolding step, the properties of the POI and of the contaminants were collected in order to propose a rational pathway for the purification and to interpret the obtained data. A starting platform tested the stability of the protein before protein purification: it is crucial to investigate the variables that could affect the stability of the protein during the purification process in order to avoid conditions that could negatively affect it. In this case the objective of the platform was a screening based on a full factorial V+ resolution design. In only one day five variables (and their ranges) were screened and the obtained results allowed to conclude that the increase of NaCl and protein concentration reduced protein stability as well as the interactions between NaCl and protein concentration and the incubation time with temperature. These results showed that higher concentration of NaCl causes a salting out effect and the hydrophobic regions of the POI and HCPs interact each other's creating an insoluble complex. This platform was powerful in order to define the operability ranges during all the purification process, that were set at basic pH and at low protein concentration. Moreover, the approach critically revised previous conclusions about drastic increment of ionic strength and pH reduction.

A complex full factorial design was then proposed for the capture step investigation. Eleven resins with different chemical exchangers or matrices were screened. This approach had to be interpreted at many levels in term of contaminants reduction, of purification approach ("bind and elute" or flow-through mode) and of confounding effects. In order to explain the interaction of the POI with the resins eleven different models were proposed. The platform allowed to point out the inadequacy of the POI purification on cationic exchangers because of the need to decrease the pH in order to increase the positive charge on the protein surface is not compatible with amine based buffers. The latter acquire a very pronounced positive charge that causes a shield effect on the exchangers reducing the POI interaction. Moreover, this

approach allowed to identify how for some resins such as CM sepharose FF, Eshmuno S and Poros HS the binding was not easy to explain but could be probably attributed to a commingling of uncontrolled factors such as the nature of matrix, the "shield effect" of Tris buffer, the pI of HCPs and POI, and the ionic strength. The resin chosen was the Nuvia Q resin, based on a quaternary ammonium, because the binding was achieved at the best POI stability conditions and because allowed to better reduce contaminants during the scale-up steps. The optimization of the capture step elution was made by a third DoE platform where ionic strength, pH and buffer composition were tested. Also in this case the model has proved to predict the main effect on protein elution.

A same approach to test the elution condition was proposed for an intermediate step with HA. The results showed the impossibility to employ the HA as a mixed mode chromatographic media because the POI binding was too high. This effect is due to the strong interaction between phosphate and the positive charge of the POI at low pH and high ionic strength. An alternative to test, in order to reduce this interaction, might be the addition of calcium chloride before loading the POI (and not in elution buffer). In fact, in this latter case, the Ca^{2+} ions did not have any effect in competing with the POI for phosphate group binding, while the early addition of the salts it may convert the HA resin in a full anionic resin reducing the interaction at low pH. Future studies will investigate this effect as the possibility to deeply study different mixed mode chromatography such as Eshmuno HCX, due its suppleness and its capability in aggregate reduction.

The intermediate step was committed to Phenyl Sepharose HP where a DoE approach as in the first platform was tested to investigate the effect of ammonium sulfate and NaCl in the salting out step before the loading on the HIC column. Differently from NaCl, ammonium and sulfate both belong to the Hofmeister series and are among the most stabilizing ions. For the POI, it was also confirmed the effect of ammonium sulfate, which is commonly used to precipitate and store proteins for long standing or preserves the native state of proteins for purification steps.

Although a final polishing step was not able to obtain the desired purity, an iterative approach was proposed in order to reduce HCPs and rDNA. The information obtained from the capture step platform, where the study was performed in order to propose a "bind and elute" mode, were used to identify SP Sepharose FF as resin to be used in a flow-through mode. This approach showed that the HTPD-model based approach generate a massive amount of information that the experimenter can use not only in the steps where these data were obtained but also in others because this approach has a predictive behavior.

The process described in the second part of the work allowed to obtain a pure and active preparation of the 13 kDa protein containing an amount of 200 ppm of HCPs and 0.01 ng/mg of rDNA.

The decision tree presented in this PhD work was a combination of *in silico* and *in vitro* analyses proposed to develop a strategy for downstream bio-processing biomolecules. Significant DoE investments in experimental and computing facilities have resulted in scientific data being generated in unprecedented volumes and velocities. Machine learning techniques have proven to be invaluable in the commercial world therefore these approaches could be implemented to extract insights from current and future scientific datasets, thereby enhancing scientific productivity and providing maximal science impact from existing DOE investments.

The DoE is gaining wider acceptance as another valuable tool for process optimization in the pharmaceutical industry. Using this kind of approach the experimental work and the analyses are reproducible and the time required for process development may be cut in half or decreased even further (58). The starting point of the approach was to understand the physico-chemical properties of potential contaminants and by-products of the host expression system. The knowledge obtained can direct the engineering of an effective and efficient downstream bio-processing operation. Care must be taken in choosing the appropriate equipment and technologies to ensure product purity, integrity

and safety, while creating an economic and efficient process based on sound manufacturing science.

6. References

1) C. Ghia, S. Shah, G. Rambhad, A. Mubashir, S. Upadhyaya (2015): ***Biologics, biosimilars, intended copies and the era of competitive medicine.*** Apollo medicine. 12, 103-111

2) ***http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CBER/ucm133077.html***

3) A. D. Bandaranayake, S. C. Almo (2014): ***Recent advances in mammalian protein production.*** Febs Lett. 588, 253-260

4) S.K. Niazi (2006)***: Handbook of biogeneric therapeutic proteins. Regulatory, Manufacturing, Testing and Patent Issues.*** Taylor & Francis. Pp 45-102

5) S. Behme (2009): ***Manufacturing of pharmaceuticals proteins. From technology to economy.*** Wiley-VCH Verlag GmbH & Co. KGaA. Pp 35-99

6) A. A. Shukla, Uwe Gottschalk (2013). **Single-Use disposable technologies for biopharmaceutical manufacturing.** Trends in Biotech 31 (3): 147-154

7) ***Guidelines of 5 November 2013 on Good Distribution Practice of medicinal products for human use:***
http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2013:343:0001:0014:EN:PDF

8) B. G. Gouveia, P. Rijo, T. S. Gonçalo, C. P. Reis (2015): ***Good manufacturing practices for medicinal products for human use.*** J Pharm Bioallied Sci. 7(2), 87–96.

9) ***Directive 2001/83/ec of the european parliament and of the council of 6 november 2001 on the community code relating to medicinal products for human use:***
http://ec.europa.eu/health/files/eudralex/vol1/dir_2001_83_cons/dir2001_83_cons_20081230_en.pdf

10) ***Regulation (ec) no 726/2004 of the european parliament and of the council of 31 march 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency:*** http://ec.europa.eu/health/files/eudralex/vol1/reg_2004_726/reg_2004_726_en.pdf

11) ***EudraLex – Volume 4 Good manufacturing practice (GMP) Guidelines:*** http://ec.europa.eu/health/documents/eudralex/vol-4/index_en.htm

12) ***Electronic code of federal regulation (FDA):*** http://www.ecfr.gov/cgi-bin/text-idx?SID=3ee286332416f26a91d9e6d786a604ab&mc=true&tpl=/ecfrbrowse/Title21/21tab_02.tpl

13) ***International council for harmonization web site:*** http://www.ich.org/home.html

14) ***ICH harmonised tripartite guideline derivation and characterization of cell substrates used for production of biotechnological/biological products Q5D:*** http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5D/Step4/Q5D_Guideline.pdf

15) ***Volume 4 EU guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use: Annex 2. Manufacture of Biological active substances and Medicinal Products for Human Use:*** http://ec.europa.eu/health/files/eudralex/vol-4/vol4-an2__2012-06_en.pdf

16) ***https://www.quora.com/What-is-a-brief-description-of-the-mass-production-process-for-pharmaceutical-drugs***

17) J. Conner, D. Wuchterl, M. Lopez, B. Minshall, Rabi Prusti, D. Boclair, J. Peterson, C. Allen (2014). ***Biotechnology Entrepreneurship.*** Chapter 26: The biomanufacturing of biotechnology products. Elsevier.

18) ***www.gelifesciences.com***

19) A. Noyes, J. Basha, J. Frostad, S. Cook, D. Millard, J. Mullin, D. LaCasse, R. S. Wright, B. Huffman, R. Fahrner, R. Godvarti, N. Titchener-Hooker, K. Sunsara, T. Mukhopadhyay (2015). ***A modular approach for the ultra-scale-down of depth filtration.*** J membrane sci. 496, 199-210.

20) ***www.pall.com***

21) ***Volume 4 EU guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use. Part II: Basic requirements for active substances used as starting materials:*** http://ec.europa.eu/health/files/eudralex/vol-4/2014-08_gmp_part1.pdf

22) ***ICH harmonised tripartite guideline: Good manufacturing practice guide for active pharmaceutical ingredients Q7:*** http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q7/Step4/Q7_Guideline.pdf

23) Z. F. Cui, H. S. Muralidhara (2010). ***Membrane technology. A practical guide to membrane technology and applications in food and bioprocessing.*** Chapter 6: Membrane applications in monoclonal antibody production. Elsevier

24) N. Fraud, R. Faber. C. Kiss, W. Demmer, H-H. Hoerl, S. Fisher-Fruehholz (2009). ***Hydrophobic-Interaction membrane chromatography for large scale purification of biopharmaceuticals.*** Bioprocess Internat June 2009 Pp-30-35

25) L. Schwartz, K. Seeley. ***Introduction to tangential flow filtration for laboratory and process development applications.*** Pall corporation literature library

26) ***ICH harmonised tripartite guideline: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A(R1):***
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5A_R1/Step4/Q5A_R1__Guideline.pdf

27) ***FDA guidance for industry: Q8 pharmaceutical development:*** http://www.fda.gov/OHRMS/DOCKETS/98fr/05d-0021-gdl0002.pdf

28) A. S. Rathore, R. Mhatre (2009*).* ***Quality by design for biopharmaceuticals. Principles and case studies.*** John Wiley & Sons, Inc.

29) ***ICH harmonised tripartite guideline: pharmacetical development* Q8(R2):**
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf

30) R. A. Lionberger, S. L. Lee, L. Lee, A. Raw, L. X. Yu (2008). ***Quality by design: concepts and ANDAs.*** The AAPs Journal. 10 (2): 268-275

31) L. Taevernier, E. Wynendaele, M. D'Hont, B. De Spiegeleer (2015). ***Analytical quality-by-design approach for sample treatment of BSA-containing solutions*.** Journal pharmaceut anal. 5 (1): 27-32

32) ***Design of experiments in protein production and purification.*** GE Healthcare handbook

33) D. C. Montgomery (2001): ***Design and analysis of experiments.*** John Wiley & Sons, Inc. Pp 170-500

34) S. Muthukumar, A. S. Rathore (2013): ***High throughput process development (HTPD) platform for membrane chromatography.*** J Membr sci 442: 245-253

35) S. Hansen, E. Skibsted, A. Staby, J. Hubbuch (2010). ***Selective protein quantification using multivariate calibration. An analytical tool for high-throughput experimenting and inline monitoring of chromatographic separations.*** Extended Reports from the 1st International Conference on High-Throughput Process Development

36) P. Sandblad, A. Florén, A. K. Hellman, T. Bergander (2010). ***An analytical approach to batch uptake characterization of chromatography media.*** Extended Reports from the 1st International Conference on High-Throughput Process Development

37) T. Bergander, M. Ahnfelt, G. Malmquist, E. Brekkan, C. Hemström Nilsson, and K. Łącki (2010). ***Process characterization to establish a control strategy for management of chromatography resin lot-to-lot variability***. Extended Reports from the 1st International Conference on High-Throughput Process Development

38) V. Kumar, A. Bhalla, A. S. Rathore (2014). ***Design of experiments applications in bioprocessing: Concepts and approach.*** Biotech progress. 30 (1), 86-99

39) M.J. Havenga, L. Holterman, I. Melis, S. Smits, J. Kaspers, E. Heemskerk, R. van der Vlugt, M. Koldijk, G.J. Schouten, G. Hateboer, K. Brouwer, R. Vogels, J. Goudsmit (2008). ***Serum-free transient protein production system based on adenoviral vector and PER.C6 technology: high yield and preserved Bioactivity.*** Biotech bioeng. *100*, 273– 283

40) L. Eriksson, E. Johansson, N. Kettaneh-Wold, C. Wiksröm, S. Wold (2008). ***Design of experiments. Principles and applications.*** Umetrics academy

41) ***High-throughput process development with predictor plates. Principles and methods***. GE healthcare handbook

42) F. Badminton, E. Honig, M. Payne, R. Wilkins (1995). ***V_{max} testing for practical microfiltration train scale-up in biopharmaceutical processing.*** Pharm Technol. 19: 64-76

43) ***http://web.expasy.org/protparam/***

44) J. E. Hansen, O. Lund, N. Tolstrup, A. A. Gooley, K. L. Williams, S. Brunak (1998). ***NetOglyc: prediction of mucine type O-glycolsilation sites based on sequence context and surface accesibility.*** Glycoconj j. *15*, 115-130

45) M.G. Pau, C. Ophorst, M.H. Koldijk, G. Schouten, M. Mehtali, F. Uytdehaag (2001). ***The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines.*** Vacc 19 (17-19), 2716-2721

46) J. Kyte, R. F. Doolittle (1982). ***A simple method for displaying the hydropathic character of a protein.*** J. Mol biol 157 (1):105-132

47) ***http://www.who.int/biologicals/biotherapeutics/rDNA_DB_final_19_Nov_2013.pdf***

48) P. Galliher, E. Fowler (2001). ***Validation of Impurity Removal by the CAMPATH 1-H Biomanufacturing Process.*** IBC's Biopharmaceutical Production Week, 2001 Nov 12–15, San Diego

49) T. Arakawa, K. Tsumoto, K. Nagase, D. Ejima (2007): ***The effects of arginine on protein binding and elution in hydrophobic interaction and ion-exchange chromatography.*** Prot. Expr and pur. 54, 110-116

50) J. A. Asenjo, B. A. Andrews (2004): ***Is there a rational method to purify proteins? From expert systems to proteomics.*** J Molec Recogn. 17, 236-247

51) A. Jungbauer (2013): ***Continuous downstream processing of biopharmaceuticals.*** Trends in Biotech 31 (8), 479-492

52) P. W. Woolston (1994): ***A physicochemical database for an expert system for the selection of recombinant protein purification processes.*** PhD Thesis, University of Reading.

53) R. A. Abound (1994): ***Patent WO/1994/003589A2***

54) E. Gousseinov, W. Kools, P. Pattnaik (2014): ***Nucleic acid impurity reduction in viral vaccine manufacturing.*** Bioproc Int. 12 (2), 59-68.

55) K. Tsumoto, M. Umetsu, I. Kumagai, D. Ejima, J.S. Philo, T. Arakawa (2004). ***Role of arginine in protein refolding, solubilization and purification.*** Biotechnol Prog. 20 (5): 1301-1308.

56) J. X. Zhou, T. Tressel (2006): ***Basic concepts in Q membrane chromatography for large scale antibody production.*** Biotechnol Prog. 22 (2): 341-349

57) M. R. Etzel, W. T. Riordan (2007). ***Membrane chromatography: analysis of breakthrough curves and viral clearance.*** Inside Bioprocess scale bioseparation for the biopharmaceutical industry. Shukla AA, Etzel MR, Gadam, editors. SCRC Press, Taylor and Francis Group

58) S. A. Weissman, N. G. Anderson (2015). ***Design of Experiments (DoE) and process optimization. A review of recent publications.*** Org. Process Rev. Dev. 19 (11): 1605-1633