Report:

"Development and optimization of stable CHO cell lines for expression of Recombinant antibodies for use in the diagnosis of cancer"

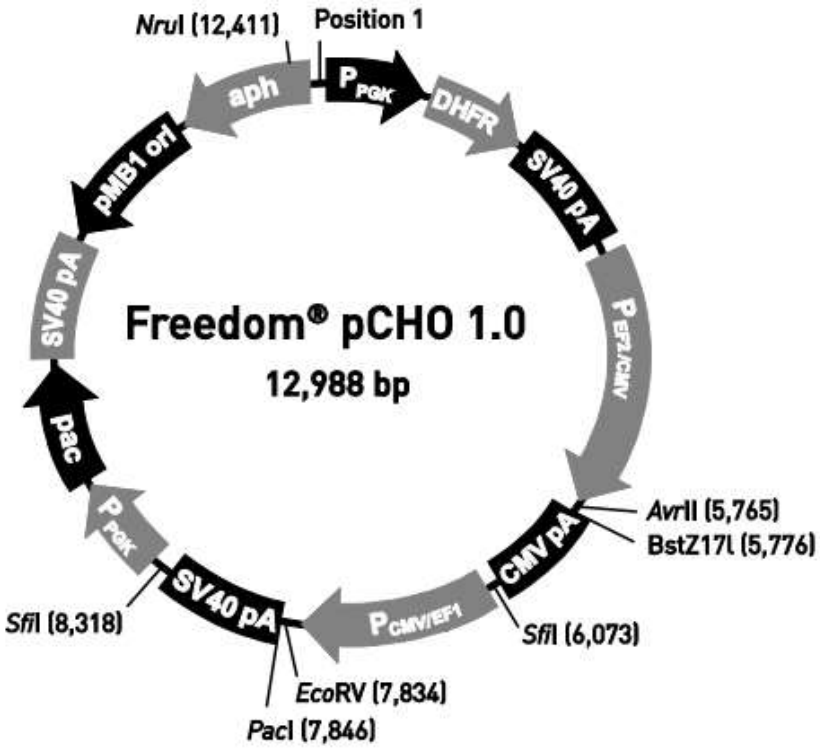

Figure 1. A map showing the elements of the vector Freedom™ pCHO 1.0 (Thermo Fisher Scientific US, 2022).

Author: Monica Pernsved
pernsved@chalmers.se
Examiner: Mikael Molin
mikael.molin@chalmers.se
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CHALMERS

Master´s Thesis at Department of Biology and Biological Engineering Chalmers University of Technology, Gothenburg, Sweden 2023

Establishment and evaluation of stable CHO cell lines for expression of recombinant antibodies for diagnosis of cancer

MONICA PERNSVED

Supervisors: Maria Lidqvist, Fujirebio Diagnostics AB, and Catharina Wising, Fujirebio Diagnostics AB

Examiner: Mikael Molin, Senior Researcher at Systems Biology/Biology and Biological Engineering, Director of studies at Biology and Biological Engineering at Chalmers

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Department of Biology and Biological Engineering Chalmers University of Technology

Gothenburg, Sweden

The research was performed at Fujirebio Diagnostics AB, Department of Bioreagent development group, Gothenburg, Sweden

Development and optimization of stable CHO cell lines for expression of Recombinant antibodies for use in the diagnosis of cancer

MONICA PERNSVED

Abstract

This master's thesis project aimed to investigate and evaluate the development of stable CHO cell lines for the expression of recombinant antibodies (rAbs), for use in the diagnosis of cancer and thereby increase the know-how of this technique at Fujirebio Diagnostics AB (FDAB). Three antibodies targeting cancer biomarkers; C192, Ov185, and SCC140 were selected for recombinant expression in an attempt to increase yield and to get more stable productivity, to meet the increased demand at the market. Biomarkers produced from cancer cells can be detected by antibodies in *in vitro* diagnostics.

In this project, the suspension cell line CHO-S adapted to high density serum-free culture was used in combination with the pCHO1.0 vector, constructed for the expression of two-subunit proteins, such as IgG. An alternative signal sequence (B), originating from human albumin was evaluated in comparison with the original signal sequence to increase antibody secretion from the cells further. Transient expressions with concentrations of 0.8-1.5 µg/ml were achieved 48 h post-transfection for pCHO 1.0 construct containing C192 with alternative signal sequence (C192B), Ov185 and Ov185 with alternative signal sequence (Ov185B). After selection with Methotrexate (MTX) and Puromycin stable expression with concentrations of 45 and 39 µg/ml were achieved for C192B and Ov185, respectively. The data indicate that a transient expression with concentrations of at least 0.8 µg/ml is a prerequisite for success in the selection phase, and if this is not achieved, that the transfection should be repeated.

To select high producing clones from the cell pool achieved, in the selection phase cloning by limiting dilution was performed on stably transfected pools. High producing C192B clones were obtained that produced up to 490 µg/ml in productivity assessment, corresponding to at least 10 times increase in expression as compared to the original hybridoma. Recombinant C192B was purified and characterised regarding immunoactivity, with high purity and with immunoactivity in comparison to hybridoma produced C192.

In this project two antibodies were successfully expressed in recombinant form and the technique can now be considered established at FDAB. Next step will be to scale up for manufacturing of rAbs at FDAB.

Acknowledgments

I would like to thank Fujirebio Diagnostics AB for the opportunity of writing my master´s thesis at the company for one year, at the Bioreagent development group. Furthermore, I would like to express my gratitude to the supervisors Catharina Wising and Maria Lidqvist for guiding and supervising me although my project. Always answering all my questions, endless help and support during the project have been valuable. I also want to thank everyone at Fujirebio, for your kindness and your willingness to always being helpful and make me feel included. I would like to thank my examiner Mikael Molin for taking the time to review my thesis and giving me feedback.

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List of Acronyms

List of acronyms that have been used throughout this thesis listed in alphabetic order:

AB – Assay Buffer
Abs - Antibodies
BSA – Bovine Serum Albumin
CHO – Chinese Hamster Ovary
CoV – Coefficient of Variation
CMV – Cytomegalovirus
DHFR – Dihydrofolate reductase
DNA – Deoxyribonucleic acid
DO – Dissolved Oxygen
DTT - Dithiothreitol
ER – Endoplasmic reticulum
ELISA – Enzyme-Linked ImmunoSorbent Assay
FBS – Fetal Bovine Serum
Fc - Fragment, crystallizable
FDAB- Fujirebio Diagnostics AB
HC – Heavy Chain
HRP – Horseradish peroxidase
ICC - Immunocytochemistry
IgG – Immunoglobulin G
IVD - *in vitro* Diagnostics
LAF – Laminar Air Flow
LB – Luria-Bertani
LC – Light Chain
kDa – kilo Dalton
mAb – monoclonal Antibody
MQ – Milli-Q (water)
MTX - Methotrexate
OD – Optical density
o/n – overnight
pAb - polyclonal Antibody
PBS – Phosphate-Buffered Saline
rAbs - recombinant Antibodies
RCB – Research Cell Bank
RT – Room Temperature
SCC - squamous cell carcinoma antigen
SDS-PAGE - Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophorese
SEC – Size Exclusion Chromatography
SFM - Serum Free Medium
SOC - Super Optimal Broth
SRP – Signal Recognition Particle
TGX – Tris-Glycine-eXtended
TMB – 3,3',5,5'-Tetramethylbenzidine

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1. Introduction

Early diagnosis of cancer can be lifesaving. With today's various possibilities of new treatments and medicines, several cancer forms can be cured when diagnosed at an early stage. Fujirebio Diagnostics AB (FDAB) is a biotechnology company and a global leader in the field of high-quality *in vitro* diagnostic (IVD) testing. FDAB has over 50 years of accumulated experience in developing and manufacturing diagnostic tests and is a global leader in the field of high-quality IVD products. FDAB has specialized in the production of monoclonal antibodies (mAbs) and antigens, products that are sold both separately and in test kits.

The overall aim of this master thesis is to express some of FDABs mAbs as recombinant antibodies (rAbs) to increase antibody yield and make productivity more stable over time. Having stable cell lines is important for example when establishing cell banks, in production planning, and up-scaling. In this project, a suspension cell line, CHO-S will be used. This cell line can be grown in high-density cultures in for example bioreactors, which in the future can be used to further increase the yield.

Comparison will be done between the mAbs produced by hybridomas and the corresponding recombinant format, to compare both productivity and immunoreactivity. Different steps in the process of establishing the cell lines will be studied, such as transfection effectivity, transient expression, and the selection of stable clones. An alternative signal peptide will be evaluated to increase secretion levels.

1.1 Aim

This master's thesis project aims to investigate and evaluate, the development of stable CHO cell lines for the expression of rAbs for use in the diagnosis of cancer and thereby increase the know-how of this technique at Fujirebio Diagnostics AB.
Three antibodies produced at FDAB; C192, Ov185, and SCC140 will be expressed as recombinant antibodies to increase yield and get more stable productivity.

The specific aims of this project will be to

- Clone the sequences of the heavy and light chains of one to three different mAbs into an expression vector.
- Clone and evaluate an alternative signal peptide.
- Compare the transient expression from different antibody constructs as well as different signal peptides.
- Evaluate the characteristics of the expressed antibodies and compare them to the hybridoma-produced antibody using different immunological methods such as ELISA and SDS-PAGE.
- Compare expression levels of rAbs to mAbs produced by classical hybridoma cell lines.

- Start selection of stable transformants and, if enough time is within the project, establish a stable CHO-S cell line expressing recombinant antibody.

Stable CHO cell lines for the expression of rAbs in this project will be established in a stepwise manner, following the procedure in figure 2.

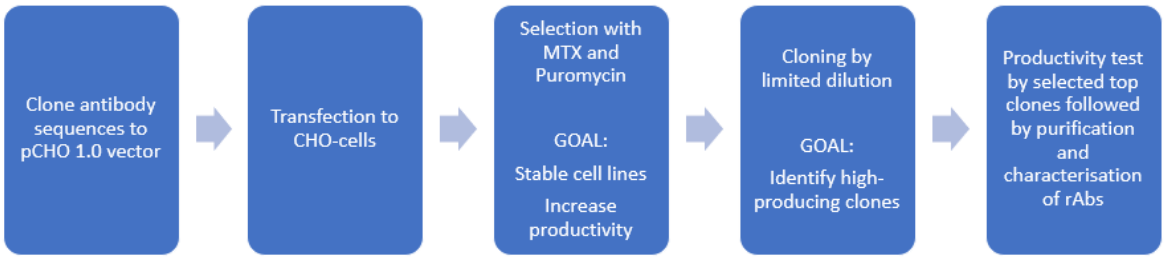

Figure 2. Illustrated stepwise manner in the project, to establish stable cell lines for expression of rAbs in CHO-cells.

1.2 Limitations

The project focuses on at least one of the three antibodies listed under the aims. If enough time within the project, all three will be investigated. An alternative signal peptide will be evaluated together with the original signal peptide.

2. Theory

This chapter is divided in subchapters describing antibodies and their characteristics and introducing the three mAbs studied in this project. This chapter is briefly describing biomarkers, and that the antibodies in the current project are targeting biomarkers for the diagnosis of cancer. The mammalian expression system is described containing the theory about the CHO cell lines, and the choice of the CHO-S cell line. Further the design and the elements in the vector Freedom™ pCHO 1.0 (Thermo Fisher Scientific US, 2022), used throughout this project is described. How to achieve an optimum in antibody expression, codon optimization and signal peptide optimization and the purpose of using it in producing rAbs is explained. The transfection in cells is explained with an introduction to liposome-mediated transfection, which was used in this project. Finally, cloning by limiting dilution is described followed by antibody purification and characterisation.

2.1 Antibodies

Antibodies (Abs) or Immunoglobulins (Igs) are secreted by a type of B cells, called plasma cells. The function of the Abs is to bind and neutralize foreign objects in the body, to protect against infection. Abs are composed of two heavy chains (HCs) and two light chains (LCs). The size of each HC is approximately 50 kDa and each LC 25 kDa. Abs have two structural regions: variable and constant regions. The HCs consist of three constant domains (CH1, CH2, and CH3) and a variable domain (VH), whereas the LC has one variable domain (VL) and only one constant domain (CL). Both the HCs and LCs are linked together by disulphide

bonds forming a Y-shaped structure. In mammalians, there are five major classes of Ig, that have various functions in the immune system but have the same structure. The five main isotypes in the classification are IgG, IgA, IgM, IgD, and IgE (D.R. Appling et.al, 2016). Each class is then divided into subclasses according to decreasing abundance, such as IgG1, IgG2, IgG3, and IgG4. Different subclasses have different Fc (Fragment, crystallizable) regions where the Fc is composed of two HC that contribute two or three constant domains depending on the subclass of the antibody. By binding to specific proteins, the Fc region ensures that each antibody generates an appropriate immune response for a given antigen. Subclasses IgG1, IgG2, IgG3, and IgG4, which are highly conserved, differ in their constant region, particularly in their hinges and upper CH2 domains. These regions are involved in binding to both IgG-Fc receptors (FcγR) of phagocytic cells and can activate the complement cascade via binding to CH1 complex (Vidarsson G et.al, 2014), see Figure 3.

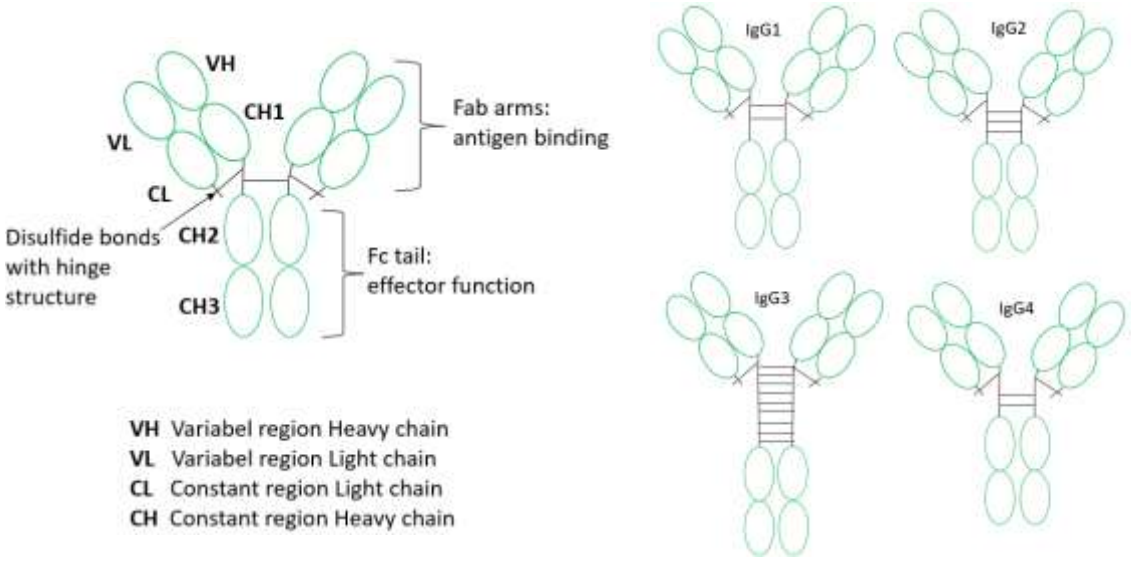

Figure 3. The typical antibody structure and the four subclasses of IgG isotypes IgG1, IgG2, IgG3, and IgG4. The overall structural organization of an antibody molecule is similar for all isotypes. It consists of two HC and two LC joined by disulphide bonds and different hinge structure. The antibody consists of two light polypeptide chains bound to the Fab arms of the HC, responsible for the specific binding to the antigen. The two heavy polypeptide chains bound to each other in the Fc tail are responsible for the activation of antibody effector functions.

2.1.1 Monoclonal and Polyclonal antibodies

MAbs are named monoclonal as they derive from a single clone and thus are directed against a single epitope. MAbs often have higher specificity and normally give a high lot to lot stability in comparison to polyclonal antibodies (pAbs). PAbs are produced from different clones of B lymphocytes and different antibodies with distinct specificity for a given antigen. PAbs have bigger variations from lot to lot and can generate cross-reactivity (André Frenzel et.al, 2013).

Although the hybridoma technique enables the production of large quantities of mAbs additional novel techniques are continuously being evaluated to meet the increased demands on productivity (Zhang. C, 2012). Here, we will evaluate the possibility to produce rAbs. The rAb technique has several advantages. It offers the possibility to modify existing clones

e.g., to facilitate fragmentation, change isotype or improve affinity and stability. The productivity of rAbs can be serum-free and reduces the use of animals in biomedical research and production.

2.1.2 Monoclonal antibodies in assays

C192 is a mAb that binds to Sialyl-Lewis that is a glycolipid present in elevated levels (CA19.9 Epitope) in Gastrointestinal cancers. CA19.9 is a marker for various gastrointestinal malignancies, such as gall bladder, pancreatic and colorectal cancers (CanAg CA19-9 EIA _C192, 2022).

Ov185 is a mAb that reacts specifically with CA125 that is a protein found on most ovarian cancer cells and that is secreted into the blood stream. Several studies have shown that CA125 is a useful tumour marker for ovarian epithelial malignancies. (CanAg® CA125 EIA_CA125, 2022).

SCC140 is a mAb that reacts with the squamous cell carcinoma antigen (SCC ag). That is a group of glycoproteins, belonging to the family of serine/cysteine protease inhibitors.
The SCC antigen is a serological marker of squamous cell carcinomas of the uterine cervix, lung, head and neck, vulva, and esophagus (CanAg® SCC EIA_SCC140, 2022).

The three antibodies, within the project, have the same isotype, IgG1, see Figure 3.

2.1.3 Recombinant antibodies

The current project aims to express rAbs in CHO-S cells. Here, rAbs will be generated by inserting HC and LC sequences of a selected mAbs into an expression vector for CHO-S cells. The advantage of rAbs has been proven and well documented (Ryu. J et al, 2022). Significant advantages are higher and more consistent yields with higher purity and can be manufactured in bioreactor in serum free medium.

2.2 Biomarkers

The antibodies in the current project are targeting biomarkers for the diagnosis of cancer. Biomarkers can be biological molecules such as proteins, antibodies, nucleic acids, or peptides and can be measured in tissues, blood, or other body fluids and are thus used in many different areas such as IVD testing. Biomarker research has been successfully developed for the past 50 years and the ability to use biomarkers, to detect cancer, has for long been the Holy Grail of cancer detection research (David F. Ransohoff, 2003).

2.3 Mammalian expression system

This section starts with an introductory subchapter containing general information about CHO cells and the CHO-S cell line used followed by description of the elements in the vector Freedom^{TM} pCHO 1.0 including the restriction sites and their various function. The next subchapter describes the theory about codon optimization. In the last subchapter the transfection in cells is described and the technique of liposome-mediate transfection, that is used in this project.

2.3.1 CHO cell line

In this project a Chinese hamster ovary (CHO) cell line, called CHO-S, will be used. CHO cells are the most common cells used in commercial production of recombinant proteins. CHO cells have a common ancestor with several lineages such as CHO-S, CHO-DXB11, CHO-K1 and CHO-DG44. CHO-S cells can grow in serum-free suspension cultures at high cell concentrations and are therefore suitable for bioreactor production (Kuo CC et.al, 2018). The cell line CHO-S is also known to reach high productivity of recombinant proteins and was therefore selected to be used in this project.

2.3.2 The elements of the vector Freedom™ pCHO 1.0.

The vector Freedom™ pCHO 1.0, is 12, 988 bp big, see Figure 4. It is included in the Freedom™ CHO-S™ Kit (Gibco™) used in this project. The vector is designed specially to express two genes of interest downstream of the vector´s two different hybrid cytomegalovirus (CMV), strong promotors and is thus especially well-suited for the expression of proteins with two subunits such as the antibody heavy and light chains in the production of recombinant antibodies.

The vector further includes two selection markers. Dihydrofolate reductase (DHFR) that leads cells to tolerate methotrexate (MTX) and the bacterial gene Pac that increases puromycin resistance. DHFR catalyses the reduction of 5,6-dihydrofolate to 5,6,7,8-tetrahydrofolate, which is essential for deoxyribonucleic acid (DNA) synthesis (Thermo Fisher Scientific US, 2022). The drug MTX is a folic acid antagonist that is actively transported into cells by the folate transporter. In the cell, it is converted to a high molecular weight polyglutamate metabolite by folylpolyglutamate synthase, which binds to DHFR and inhibits its activity and cells carrying the DHFR gene becomes more tolerate to MTX.

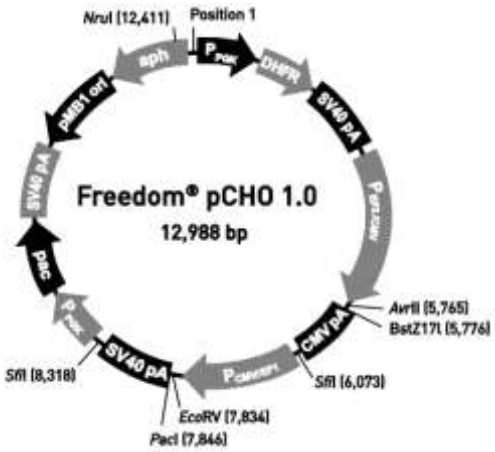

Figure 4. A map showing the elements of the vector Freedom™ pCHO 1.0 (Thermo Fisher Scientific US, 2022).

Puromycin is an amino nucleoside that blocks protein synthesis in mammalian cells by interfering with ribosomal function. Expression of the bacterial gene Pac, derived from *Streptomyces albonier*, results in the detoxification of Puromycin (Lacalle et.al 1989). Cells carrying the Pac gene from the pCHO vector are thus resistant to Puromycin. By increasing

the selection pressure of Puromycin and MTX in the media, high producing clones can be achieved. The cells that take up the vector become resistant against the drugs and can survive. The rest die and therefore the viability drops early in the selection phase. Those cells that are stably transfected survive and divide, thus the viability increases, and the cells divide despite selection pressure. By increasing MTX concentration further in the culture, the cells need several copies of the DHFR gene, to meet the increasing selection pressure. As a consequence, other genes in the vector amplifies along with the DHFR gene resulting increased expression of our gene of interest.

2.3.3 Signal peptide and optimization

The signal peptide also called the signal sequence, has a role in the first steps of the translation of the mRNA into a protein. A signal sequence is a short segment of amino acids that are located on the N-terminal of some proteins. The purpose of the signal sequence is to help the protein, to find their correct location outside the cell membrane. The signal sequence attracts signal recognition particle (SRP), that bind to the signal sequence and help it through a tunnel through the endoplasmic reticulum (ER) lumen and the protein to be translated right into the ER lumen. The signal sequence with the attached SRP helps the protein through the cellular secreting system through the cell membrane and out of the cell. Once the protein has been translated, and becomes mature, through the ER Lumen the signal sequence will get cleaved off the protein and never be a part of the final protein (You, M et.al, 2018).

The translocation of secretory proteins into the ER is a limiting step for protein secretion, which makes the secretory pathway to one of the critical steps in the production of stable and robust production of rAbs. Signal sequence can lead to increased protein secretion, as well as an improper signal sequence cleavage from the antibody chain can lead to light chain aggregation. Le Fourn et al. 2014, demonstrated in their study, how to reduce the improper signal cleavage by overexpressing SRP14, a protein of the signal recognition particle (SRP). Through this information the specific productivity of difficult-to-produce antibodies improved several-fold. Signal sequences from different species can be utilized to mediate an increased antibody secretion in CHO cells. Signal sequences are highly heterogeneous, and many are functionally interchangeable between different species (R. Haryadi et.al.2013). Therefore, regulation, reduction and optimization of the signal sequence can be one factor to succeed in the translocation step when producing rAbs.

For this master thesis, the original signal peptide and the signal peptide B, Serum albumin preproprotein from Homo sapiens, NP_000468 (You, M et.al, 2018), will be compared.

2.3.4 Codon optimization

Codon optimization is a technique used in the production of recombinant proteins, including antibodies, to improve the efficiency of protein expression in host cells. It involves altering the DNA sequence of the gene encoding the protein so that it contains codons that are more frequently used by the host cell's translation machinery. This can increase the rate of protein synthesis and potentially improve the yield of the protein. Codon optimization is especially important when producing proteins in heterologous systems, where the host cell may not be native to the protein being expressed. For example, if a protein is normally expressed in a human cell line but is being produced in a bacterial cell line for cost or convenience reasons,

the codon usage of the bacterial cells may differ from that of the human cells. This can lead to inefficient or low levels of protein expression. By optimizing the codon usage of the gene encoding the protein to match that of the host cells, protein expression can be improved. In this project, codons were optimized for CHO cells.

Codon usage biases are found in all eukaryotic and prokaryotic genomes, and it has multiple roles. For an example in the regulating gene expression and protein structure, through translation-dependent and translation-independent mechanisms. Synonymous codons are recognized with different efficiencies by cognate tRNAs. Codon usage bias correlates with levels of cognate tRNAs or with tRNA gene copy numbers. Codons with strong bias are found to be strongly enriched in highly expressed protein encoding genes, and codon optimization increases endogenous and heterologous gene expression in diverse eukaryotes and prokaryotes (Liu.Y, 2020).

Codon optimization can be performed using various software tools that analyse the codon usage of the host cells and suggest changes to the DNA sequence to improve expression. It is important to note that codon optimization is just one factor that can influence protein expression and yield, and other factors such as the choice of expression vector and host cells, as well as the optimization of culture conditions, may also need to be considered.

2.3.5 Transfection

Transfection is the introduction of foreign genetical material, DNA or RNA, *in vitro* or *in vivo* into mammalian cells. It is a powerful analytical tool that gives possibilities to study different genes functions, regulation, and protein function. DNA is a hydrophilic molecule and cannot pass the hydrophobic cell membrane which makes it quite challenging. When the foreign genetic material is integrated in the host genome, the transfection can be considered as stable. Transfection can be either transient or stable. This depends on the nature of the generic materials and what is going to be analysed, see Figure 5.

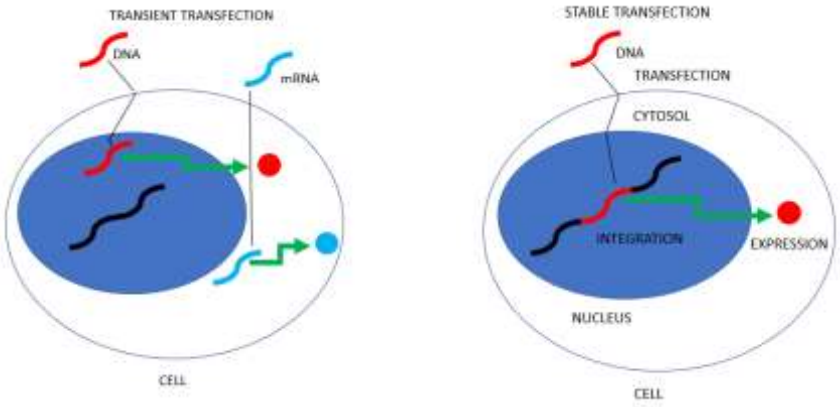

Figure 5. Transient and stable transfection in a cell is described. In the transient transfection there is no integration of the foreign gene into the host genome, and no replicate is expressed. The cells will express the gene for a finite period until the gene is lost. In the stable transfection the foreign gene is integrated into the host DNA, leading to a permanent expression of the foreign gene.

In the transfection process a subset of the transfected cells will become stably transfected. Stable transfection is mostly used for study different genes functions for therapy, regulation, and protein function. Transient transfection is suitable for studying pathways, functional and

promotor studies. There are several commercially available transfection kits. There are three main types of transfection techniques; Lipid-based reagents, transfection using Calcium phosphate ($CaPO_4$) or Electroporation that uses the electricity to transfect the DNA into the cell. Different transfection techniques can be chosen depending on the analysis aim to consider and what cell is studied (Kim TK, Eberwine JH, 2010).

2.3.6 Lipoplex-mediated transfection

For this study the lipid-based reagent Freestyle Max (Thermo Fisher Scientific US,2022) was used. This transfection is a liposome-mediate transfection, also called lipofection. It uses cationic lipids or non-lipid polymers to form micellar liposomes. Liposomes in the reagent Freestyle Max (Thermo Fisher Scientific US,2022), bind negatively charged DNA, as the liposomes are positively charged and can surround the DNA and form lipoplexes. The lipoplexes positively charged surfaces typically enables uptake into the cells via endocytosis. The endocytosis resulting in a double-layered micellar vesicle with a lipoplex within an endosome. The DNA needs to be released out of the endosome before the endosomal degradation performs by lysosomes. Before transfection to get this complex formation, the DNA incubates with the reagent (Parker, A et.al.2003). A Lipoplex is formed which in turn binds to the cell membrane which is negatively charged. The image in Figure 6 shows the principle of Lipoplex-mediated transfection.

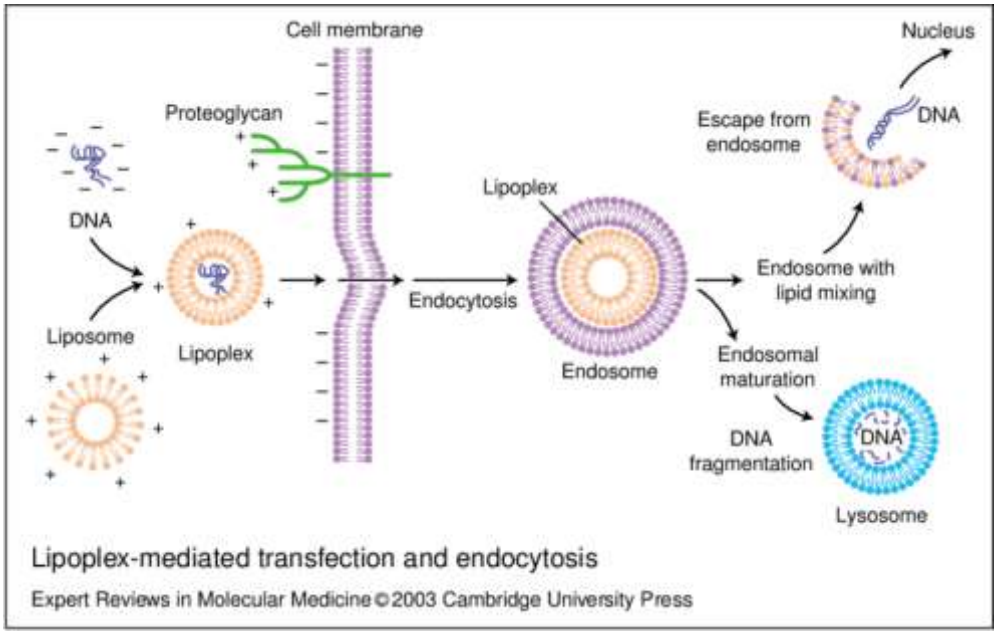

Figure 6. Illustrated phases of liposome-mediated transfection when a lipoplex enters a cell through endocytosis (Parker, A et.al.20).

2.4 Cloning by limiting dilution

To identify high producing clones and thereby achieve high productivity in the final cell line, cloning by limiting dilution was performed. In general, cloning by limiting dilution method is used to isolate high-producing cell clones. There are several methods for cloning cells, and limiting dilution is a simple, commonly used method. However, the limiting dilution method is time-consuming and when the cloning process is started the process needs to be continued until the top clones are selected and evaluated, in a process that is ongoing for about 5-6 weeks (Tsuyoshi Nakamura, Takeshi Omasa, 2015).

2.5 Antibody purification and characterisation

After the antibody purification, there is many various methods for quality control and characterisation of the purified antibody, to confirm that the antibodies of interest have been successfully purified. In this section some of the methods will be explained that is used in this project.

2.5.1 Antibody purification

Antibody purification's main objective is to purify from contaminating protein, nucleic acid, lipid, carbohydrate, and cell growth media components. Therefore, the purification techniques need to assure the elimination of these contaminants and maintaining them with maximal accuracy and reproducibility. Purification of recombinant protein has several advantages, as the protein is isolated into a cloned single cell and is scarce in natural biological sources. And not been defined in terms of kinetics physical-chemical properties or three-dimensional structure. Only the recombinant counterpart will be available for characterisation.

Antibody purification for IgG used by Protein A affinity chromatography is a method developed in the 1970s (SEETHARAM, Ramnath, et al., 1991). The protein is extracted from the pathogenic bacteria, *Staphylococcus aureus* (*S. aureus*), that is bonded to the cell wall of bacteria, and in another form is secreted to the culture supernatant. Protein A strongly interacts with the contact regions of the antibodies Fc region of IgG, and weaker interacts with the Fab region, see figure 3. These properties have a commercially importance, because of their more advantageous binding properties like weaker IgG binding and milder elution conditions. Furthermore, Protein A treatment is a powerful condition method to remove IgG from serum and plasma matrices facilitating the separation of other immunoglobulin classes such as IgA, IgM, IgD, and IgE. When the other compounds have been washed out of the column, the mAb-Protein A bonds are removed by washing the column at a lower pH (Mahshid Zarrineh et al. 2020).

2.5.2 Size exclusion chromatograph

Size exclusion chromatography (SEC) is a method that is used both as quality control and characterization of antibodies. The mAbs in SEC and other particles are separated based on size. Where the large particles elute faster than small particles that have a longer travel distance through the column. The column in SEC is detected by measuring absorbance at 280

nm, where the relative amount is shown in the chromatogram and the percentage of normal size mAbs in comparison to other particles is calculated (Leopold K Kostanski et al.2004).

2.5.3 Immunoactivity of antibody

In this project, an ELISA was used to measure the immunoactivity of the recombinant antibodies. The assay is composed and developed at FDAB, an ELISA-based method for estimation of relative antibody immunoactivity. Where the composition of immobilised antigens, biotin labelled mAbs, reference mAbs, rAbs of interest, horseradish peroxidase (HRP) -labelled mAbs, and tetramethylbenzidine (TMB) are evaluated. A signal is showing how much from the HRP-labelled mAbs is inhibited when reference mAbs respectively mAbs of interest compete with the labelled mAbs about antigen binding. The signal inhibition is measured in (%) and is related to the signal obtained when only labelled mAbs bind to the antigen. The lower the signal the more of the mAbs of interest bound. It is calculated with the following formula:

$$Signal\ inhibition = \frac{\left(\left(Abs\ 0\ \frac{\mu g}{ml} - background\right) - \left(Abs\ 100\ \frac{\mu g}{ml} - background\right)\right)}{\left(\text{Abs } 0\frac{\mu g}{ml} - background\right)} * 100 \qquad (1)$$

where “Abs 0 µg/ml” is absorbance from only labelled mAbs, and “Abs 100 µg/ml” is absorbance from reference mAb or mAb of interest in combination with labelled mAbs. The “background” corresponds to absorbance from capture antibodies and antigens.

The relative immunoactivity (%) is then compared and evaluated according to the following formula:

$$Relative\ immunoactivity = \frac{Signal\ inhibition\ (mAb\ of\ interest)}{Signal\ inhibition\ (mAb\ reference)} * 100 \qquad (2)$$

where signal inhibition (mAb of interest) and signal inhibition (mAb reference) are determined from Eq. (1).

2.5.4 SDS-PAGE

In this project, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is used to study the antibodies before and after purification. SDS-PAGE is a gel electrophoresis method where proteins are separated based on size. The proteins are separated in a polyacrylamide gel which is a highly cross-linked gel with pore size small enough for retarding migration of proteins. A sample of proteins are heated and loaded on the gel. SDS is a negatively charged detergent which together with the heating helps breaking secondary and tertiary structures of the proteins. The SDS molecules also cover all proteins in the samples, making them negatively charged. When the proteins are loaded on the gel and an electric field is applied, the negatively charged proteins start migrating towards the anode side of the gel. Large proteins are exposed to stronger electrical forces but will migrate slower than smaller proteins due to gel pore size. Each protein’s individual size is determined by including a reference sample containing proteins with known size and can be verified by for an example by a Precision Plus Protein Standard Unstained as a reference to evaluate the proteins size. An mAb IgG showing one band in approximately at size 150 kDa and reduced known size showing two band one for HC approximately at 50 kDa and a band for the LC approximately at 25 kDa (Liu, H et al. 2007).

The proteins can be separated to a reduced or non-reduced sample, to evaluate the protein size. In a non-reduced run, the sulphur bridges remain between the immunoglobulin's light and heavy chains, and in a reduced run, a reducing agent dithiothreitol (DTT) is added, whereby the sulphur bridges of the immunoglobulin are broken. In SDS-PAGE a commonly buffer system, is Tris-Glycine eXtended (TGX) stain-free gels do not need to be stained but can with the ChemiDouc Touch system, which detects UV fluorescence. For stain-free to visualize to work, the investigated protein must contain tryptophan as tryptophan is intrinsically fluoresce and enhance the trihalo compound. Tryptophan reacts with trihalo compounds in the gel in a UV-induced reaction whereupon a fluorescent signal is detected by the system.

3. Methods and materials

This chapter will describe the methods and the materials that was used. The materials from the Kit were purchased from Life technologies 2020 Freedom™ CHO-S™ (Thermo Fisher Scientific US, 2022). The kit included a special designed vector, Freedom™ pCHO.10, that was used to express one or two genes of interest. The vector is described in detail in subchapter 2.3.2.

The plasmids used in this project was already done, where the HC and LC were inserted into the Freedom™ pCHO 1.0 vector. The vector Freedom™ pCHO 1.0 and the antibodies HC gene blocks were digested with restriction enzymes AvrII (Thermo Fisher), BstZ17I/Bst1107I (Thermo Fisher), and the LC were digested with restriction enzymes Eco32I/EcoRV (Thermo Fisher), PacI (Thermo Fisher). All the elements of the vector features are presented in Appendix A1 and A2 and the antibodies with signal sequences cloned in the project are presented in Appendix A4.

The projects experimental flowchart, in the expression of rAbs schedule, can be followed throughout the project in the Figure 7.

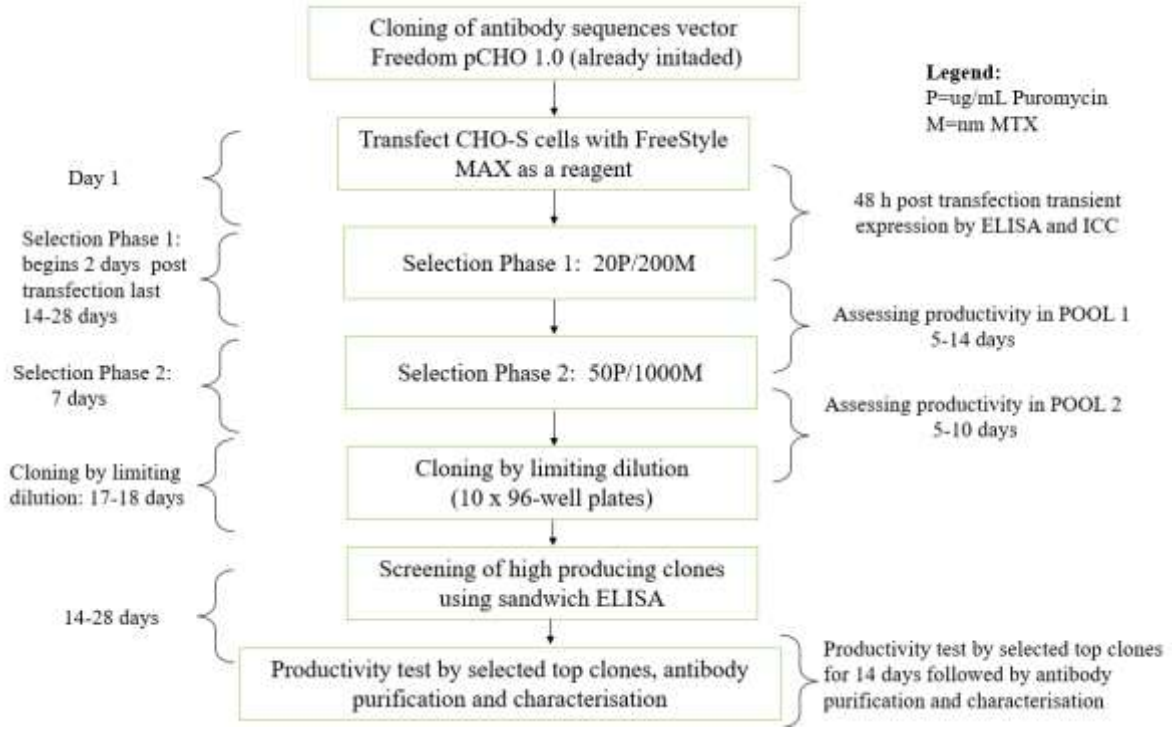

Figure 7. The experimental flowchart in the expression of rAbs schedule that was followed throughout the project.

3.1 Cultivation of CHO-S™ cells

The kit provided CHO- S™ cells, are CHO-derived cells adapted to high density, serum-free suspension culture in chemically defined medium that can produce high levels of secreted recombinant protein and achieve IgG titers over 3g/L. Serum free medium (CD FortiCHO medium), transfection reagent (FreeStyle™ MAX) and OptiPRO™ SFM that are used for transfection.

A CHO-S research cell bank (RCB) of 22 vials was established early in the project to ensure a safe storage of the cells. $1x10^7$ cells were frozen in each vial and stored in liquid nitrogen. The CHO-S cells were cultured at a maximum of 25 passages, and after that a new ampoule was thawed. Throughout the project, cells were cultured at 37°C, according to 3.1.1. All cell handling was performed under laminar Air Flow (LAF), to avoid contamination.

3.1.1 Thawing procedure and cell culturing

The thawing procedure was performed each time cells were thawed. The cryovial cells were taken from the liquid nitrogen -180°C and thawed quickly in a water bath 37°C. An additional step was done by washing the cells in 10 ml CD FortiCHO medium by centrifugation at 200xg for 5 min. Prewarmed complete medium was prepared with 99 ml CD FortiCHO medium and 1 ml GLUTAMAX (Gibco). The CHO-S cells were seeded into a new sterile 125ml shaker flask and pre-warmed complete CD FortiCHO medium was added. Cells were seeded out at $1\text{-}2x10^5$ cells/ml incubated at 37°C, 8% CO_2 with shake 150 rpm. After each incubation time, cell density and viability, were measured by either counting manually in Bürker chamber or using cell counter Nexcelom K2, according to 3.1.2. The CHO-S cells were incubated until the cells reached the cell density and viability that was needed, depending on which experiment was to be performed, but not $>2x10^6$ cells/ml.

3.1.2 Cell counting

Cell counting was performed using the Cellometer K2 automatic cell counter from Nexcelom or manually in a Bürker chamber with depth 0.1. In Cellometer K2, a sample with unknown cell density and viability was analysed by adding ViaStain AOPI stain to the sample. The stain-coloured living cells green and dead cells red. Then, the sample-stain solution was loaded into a counting chamber which in turn was inserted to the cell counter Nexcelom K2 device. The device was thereafter able to detect the number of living respectively dead cells, resulting in a read-out of viability (%) along with cell density (cells/ml) of living cells. In manual counting both total number of cells and number of living cells was counted. Viability was then calculated as number of living cells divided by total number of cells multiplied by 100.

3.1.3 Freezing cells

CHO-S cells were frozen, at different stages, throughout the project. When the cells reached cell growth of $<2x10^6$ cells/ml with a viability of 95%, the cells were ready to be prepared to freeze with approximately $1x10^7$ cells per cryovial. The CHO-S cells were transferred to sterile, conical centrifuge tubes and centrifuged at 200xg 5min. Freezing medium containing 10% Dimethyl Sulfide, CD Forti CHO and GLUTAMAX was prepared. The centrifuged

CHO-S cells were resuspended in the pre-determined volume of the chilled freezing medium. The cryovials were stored in the refrigerator for 30 min and stored overnight in -80°C, followed by a long-term storage in liquid nitrogen at -196°C.

3.2 Molecular cloning

This subchapter describes the preparation of the plasmid DNA before the transfection. Starting by DNA purification, continuing with molecular cloning of antibody sequences into pCHO 1.0. The molecular cloning was performed in two steps, starting with cloning of HC into the AvrII/Bst cloning site, followed by cloning of LC using EcoRV/PacI. When the master thesis project started, LC cloning was already performed, and the project started off with HC cloning. The chapter further describes methods of ligation, transformation, PCR screen amplification with agarose gel. Followed by subchapters including the transfection and analyzing methods; Immunocytochemistry (ICC), sandwich ELISA.

3.2.1 DNA purification

The existing Freedom™ pCHO 1.0 vector with the antibodies, see Table:3:1.

Table 3:1. The following combinations of antibody sequence vs signal sequences were evaluated in the project.

Name of construct	**Antibody sequence origin**	**Signal sequence**
Ov185	Ov185	Original signal sequence
Ov185+B	Ov185	Signal sequence B
C192	C192	Original signal sequence
C92+B	C192	Signal sequence B

For each DNA purification of the plasmids, the same procedure was performed. A start culture was made with 5 ml LB-medium, see table 3:2, containing kanamycin (50 µg/ml,) and one colony with the *E.coli* strains from LB with kanamycin plates were incubated, shaking at 37°C, 250 rpm for 7 hours. Then 500 µl start- culture was then added to 5 ml LB medium containing kanamycin (50 µg/ml) and incubate, shaking at 37°C, 150 rpm overnight (o/n). Bacterial cells from 25 ml culture were harvested by centrifuged 6000xg for 15 min at 4°C. The plasmids were purified with QIAGEN Plasmid Midi Kit (QIAGEN) and the DNA pellet was dissolved in 100 µl Milli-Q (MQ water).

Table 3:2. LB medium

Component	**Per 1 L**
Tryptone	10 g
Yeast Extract	10 g
NaCl	5 g
RO water	Up to 1 L

3.2.2 Molecular cloning of the antibody sequences into pCHO 1.0

The antibody HC gene blocks were digested with restriction enzymes AvrII (Thermo Fisher) and BstZ17I (Thermo Fisher). The gene blocks for antibody HC were digested with restriction enzymes Eco32I/EcoRV (Thermo Fisher) and PacI (Thermo Fisher). The vector pCHO 1.0 was cut with the same restriction enzymes. The mixtures were incubated at 37˚C for 10 min and run on 1% agarose gel at 90V for 30 min. Finally, the fragments were extracted from the gel using a QIAquick gel extraction kit (QIAGEN) and eluted with 100 µl MQ. The antibodies with signal sequences cloned in the project in table 4.1 and 4.2 in Appendix 4. Used amount components for the insert and vector can be seen in Table 3:3 and Table 3:4, respectively.

Table 3:3. The corresponding amount components of the insert depending on the ratio from the Equation 1

***Insert* Ov185 with B signal and HC**	**Volume (µl)**	**Total volume (µl)**
200 ng HC	20	30
AvrII/XmaJI	1	
BstZ17I/1107	1	
10x Fast digest green buffer	3	
Autoclaved MQ	5	

Table 3:4. The corresponding amount components of the vector pCHO 1.0

***Vector* pCHO 1.0**	**Volume (µl)**	**Total volume (µl)**
~2000 ng pCHO 1.0	6	20
AvrII/XmaJI	1	
BstZ17I/1107	1	
10x Fast digest green buffer	2	
Autoclaved MQ	10	

3.2.3 Ligation

The digested gene fragments were ligated into the linearized vector Freedom™ pCHO 1.0 with T4 ligase (Thermo Fisher). The ligation was made for the insert-vector ratios 3:1, insert, and vector in two 1,5 ml Eppendorf tubes. The amount of insert used in ligations was 200 ng/µl for Ov185 with the B signal with HC. The vector pCHO 1.0 amount was 200 ng/µl. The corresponding amount insert depending on the ratio was calculated with Equation 1. Ligation was performed according to T4 ligase (Thermo Fisher) protocol for sticky ends and blunt ends. The ligation was incubated at room temperature (RT) o/n.

$$Amount\ of\ insert\ (ng) = \frac{size\ of\ insert}{size\ of\ vector} * (insert{:}\ vector\ ratio) * \ (amount\ of\ vector\ (ng)) \qquad (3)$$

3.2.4 Transformation JM109 Competent cells with Chemical transformation

In the transformation process JM109 Competent cells were thawed on ice and 5 µl ligation mixture was added to the cells and mixed gently by tapping the tube. Cells were incubated on ice for 30 min, heat-shocked at 42°C for 45 sec (critical step) and placed on ice. Then 250 µl Super Optimal Broth (SOC) medium were added and the transformed cells were incubated at 37°C, 225 rpm for 1 hour. Two volumes of the culture, 200 µl and 50 µl, were plated out onto LB-kanamycin (50 µg/ml) agar plates and incubated upside down in 37°C o/n.

3.2.5 Transformation screen with PCR

Eight colonies from transformation of the antibody with the HC, from the incubated plates were screened for insertion with PCR. See Table 3:5 for the sequencing primers in vector pCHO 1.0. PCR mixture was made with Taq DNA polymerase (VWR), see Table 3:6, and divided into 15 µl samples.

Table 3:5. Sequencing primers in vector pCHO 1.0, used for the antibody HC.

Primer	Primer sequence	Location
Forward primer SU1 in pCHO 1.0	5'-GTCTGAGCCTCCTTGTCTTG-3'	begins ~270 bp upstream of AvrII/BstZ17I insertion site
Reverse primerSU1 in pCHO 1.0	5'-AGAAGACACGGGAGACTTAG-3'	begins ~90 bp downstream AvrII/BstZ17I insertion site

Table 3:6. Components of PCR mixture

Component	Per tube (µl)	Reactions	Total volume (µl)
10xKey Buffer	1,5	*12	18
MgCl2 25 mM	1,2	*12	14,4
dNTP mix	0,3	*12	3,6
Primer Forward	0,3	*12	3,6
Primer Reverse	0,3	*12	3,6
Tag DNA polymerase	0,075	*12	0,9
MQ	11,3	*12	135,6

DNA was added to the samples, 0,5 µl of empty vector and vector with Ov185 genes and picked colonies. The PCR samples run in the PCR program according to the Table 3:7. After the amplification samples were applied to agarose gel to evaluate the theoretical size, counting based on the primer location, from Table 3:5: 1386bp+270+90bp=1746bp. With 5 µl low mass ladder and 5 µl of the PCR samples run on 1,5% agarose gel run for 40 min at 90V.

Table 3:7. PCR program

Cycles	Duration of cycle	Temperature
1	5 min	94 ^{0}C
30	30 sec	94 ^{0}C
30	30 sec	56 ^{0}C
30	1 min	72 ^{0}C
1	5 min	72 ^{0}C

Two colonies were chosen for purification with QIAGEN Plasmid Mini-Prep (QIAGEN) and the pellet was dissolved in 200 µl TE buffer pH 8,0. and sent for sequencing by Eurofins.

3.2.6 Transfection

The transfection procedure was the same throughout the project, was proceeded by the liposome-mediated transfection, with different plasmids. Transfection with FreeStyle MAX as a reagent was mixed with CHO-S cells from the research cell bank (RCB) with >95% viability. The CHO-S cells were seeded with a cell density of 1x10^6 cells/ml in 30 ml pre-warmed CD FortiCHO medium. The CHO-S cells were prepared 24 h before transfection to reach optimum cell density (1x10^6 cells/ml) for transfection. The transfection mix was prepared by first adding 50 µg plasmid DNA to 1,5 ml OptiPRO Serum Free Medium (SFM) and adding 50 µl FreeStyle MAX reagent to1,5 ml OptiPRO SFM into separate 50ml tubes. The diluted FreeStyle MAX reagent was added to the diluted DNA and mixture were incubated for 10 min at room temperature. The FreeStyle MAX solution with the DNA mix, was then added dropwise into a 125 ml flask containing CHO-S cells prepared 24 h before transfection. Cells were incubated at 37˚C, 8% CO_2 with shake 150 rpm for 2-3 days until evaluated regarding transfection efficiency and expression.

3.2.7 Transient expression

1ml of the 48h post-transfection culture was evaluated for density and the viability of the cells by cell counting with the cellometer K2, according to 3.1.2. The 1ml 48h post-transfection cells was centrifuged at 300g 5min, the supernatant was saved to perform ELISA, and the pellets were resuspended by 500 µl sterile 1%PBS and 5 glass probes (Deckgläser/coverslips) was prepared for immunocytochemistry (ICC) and incubated in RT for 2-3 days.

3.2.8 Immunocytochemistry

Cell was mounted on poly-lysine slides and fixed with cold methanol in a glass cuvette for 10 min, followed by blocking in 1% fetal bovine serum (FBS) in Phosphate-Buffered Saline (PBS) for 30 min 37^0 C. The slides were washed 3x5 min with PBS, the cells were stained in the dark for 1 hour 37^0 C using antibody Alexa Fluor 488 goat anti-mouse IgG (H+L), with two different concentrations:10 µg/ml and 1µg/ml in blocking solution followed by a second wash step. The cells were further stained with DAPI diluted 1:1000 in PBS for 5 min and mounted with cover glass.

Table 3:8 Antibody solution with Alexa Fluor 488 goat anti-mouse IgG (H+L).

Component	**10 µg/ml**	**1 µg/ml**
1% Blocking buffert	1400 µl	1260 µl
Alex Fluor 488 Goat anti mouse	7 µl	140 µl (from the 10 µg/ml antibody solution)

3.2.9 Transfection efficiency using fluorescence microscopy

The transfection efficiency was studied using ICC according to Table 3:8. Fluorescence microscopy was used to count the number of transfected cells (Alexa Fluor 488 positive) and the total number of cells (DAPI positive). The experiments were performed from cells from 48h post-transfection cells and using in comparison cells from Mock (non-transfected cells) and hybridoma cells expressing C192 was used as positive control.

3.2.10 Evaluation of antibody concentration by ELISA

Throughout the project, sandwich ELISA was used to measure rAbs secreted into the medium. Each sample with unknown concentration was diluted in Assay Buffer (AB) to fit within the mAb concentration range of the standard curve. Two dilutions were done per sample, see Appendix A.3 for the plate design. 96-well plates were coated with Goat-anti-mouse-IgG+IgM. The plate was washed x1 with Wash Solution whereafter duplicates of 20 µl diluted rAb sample was added. Along with the samples, duplicates of blanks containing only AB and a standard curve of IgG1 ranging from 0.1 to 0.5 µg/ml was added. 200 µl AB was added to each well before the plate was incubated in a plate shaker at RT for 1 h. After incubation, the plate was washed x3 with Wash Solution whereafter 100 µl Rabbit-anti-mouse diluted in 1 % BSA-PBS + 0.1 % Tween20 was added per well. The Rabbit-anti-mouse antibodies were diluted 1:12. Once again, the plate was incubated on a plate shaker at RT for 1 hour. Next, the plate was washed x6 with Wash Solution and then 100 µl TMB substrate was added per well. After 3-10 min incubation in a plate shaker at RT, the HRP-TMB-induced colour shift was read in a Bio Tek ELx808 spectrophotometer at 620 nm. The computer software automatically calculated the average rAb concentration and coefficient of variation (CoV) for each pair of duplicates, dilution excluded. If the CoV for a duplicate exceeded 6 % or if a dilution yielded a concentration outside standard curve range, the sample was kept and analysed again to achieve a more reliable result.

3.3 Selection of stable transfectants

48 h after transfection, the selection of stable transfectants were performed in two phases, with a combination of Puromycin and MTX, see Table 3:9 and 3:10. The procedure is illustrated in Figure 6 and 7. Selection phase 1 was performed in 1-3 weeks followed by selection phase 2, until the cells reached viability >90%. After each phase, 3 vials of cell POOL 1 and 2 respectively were frozen to secure the cells. Supernatant was saved from the pools to determine antibody concentrations. Selection phase 1 continued until viability

reached >85% (as suggested in the manual) and selection phase 2, until the cells reached viability >90%.

Table 3:9 Growth medium during selection.

Component	**100 ml**
CD FortiCHO medium	98 ml
GLUTAMAX	1 ml
Anti-clump Agent	1 ml

Table. 3:10 Selection pressure for the Selection in Phase 1 and Phase 2 (added to growth medium).

Drµgs Selection Phase 1	**20P/200M**
Puromycin	2 µl/ml
MTX (0.1 mm stock)	2 µl/ml

Drµgs Selection Phase 2	**50P/1,000M**
Puromycin	5 µl/ml
MTX (1 mM stock)	1 µl/ml

48 h post-transfection cells were counted according to 3.1.2. Supernatant from 1 ml of 48 h post-transfection cells was saved after centrifuged 300xg for 5 min, and antibody content measured by sandwich ELISA was done. The transfected cells were seeded into new sterile T-flasks at a cell density of $5x10^5$ cells/ml with a selection pressure of 20P/200M, according to the selection phase 1 as illustrated in Figure 8.

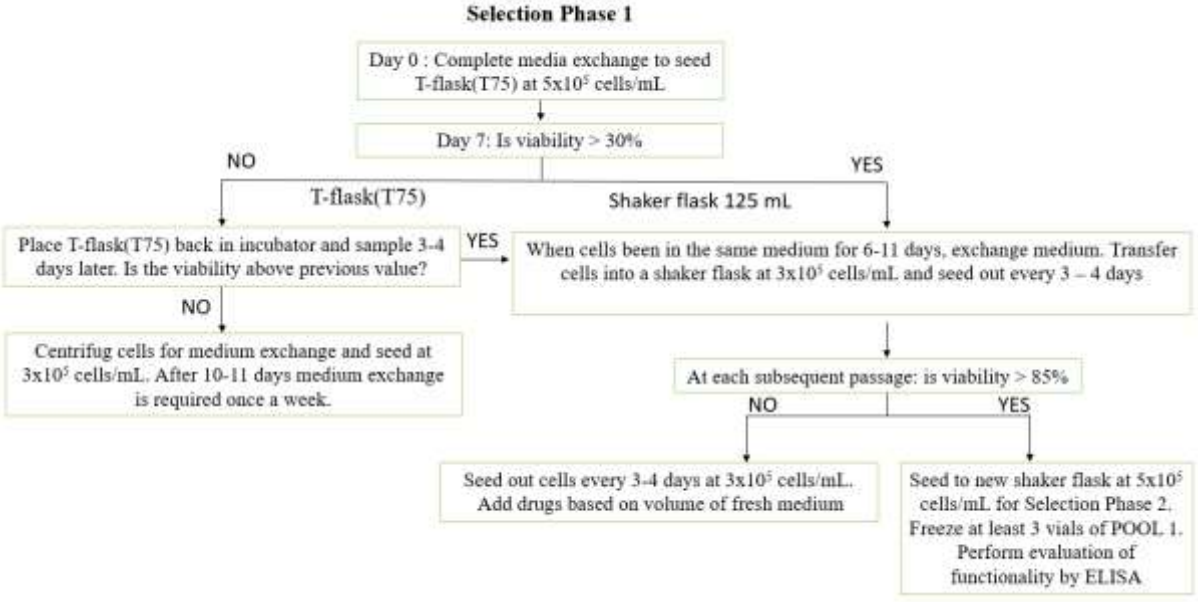

Figure 8. Schematic protocol that was followed in selection phase 1 for each transfection that was performed.

In the beginning of selection phase 1, the T-flasks were incubated static at 37 ^{0}C with and 8% CO_2 but as the phase continued, cells were moved to shaker flasks and cultured shaking at 150rpm. Selection phase 1 was performed for 21 days when the cells viability exceeds 85% and a POOL 1 was freezed and stored.

Selection phase 2 was performed directly after selection phase 1, following the procedure illustrated in figure 9.

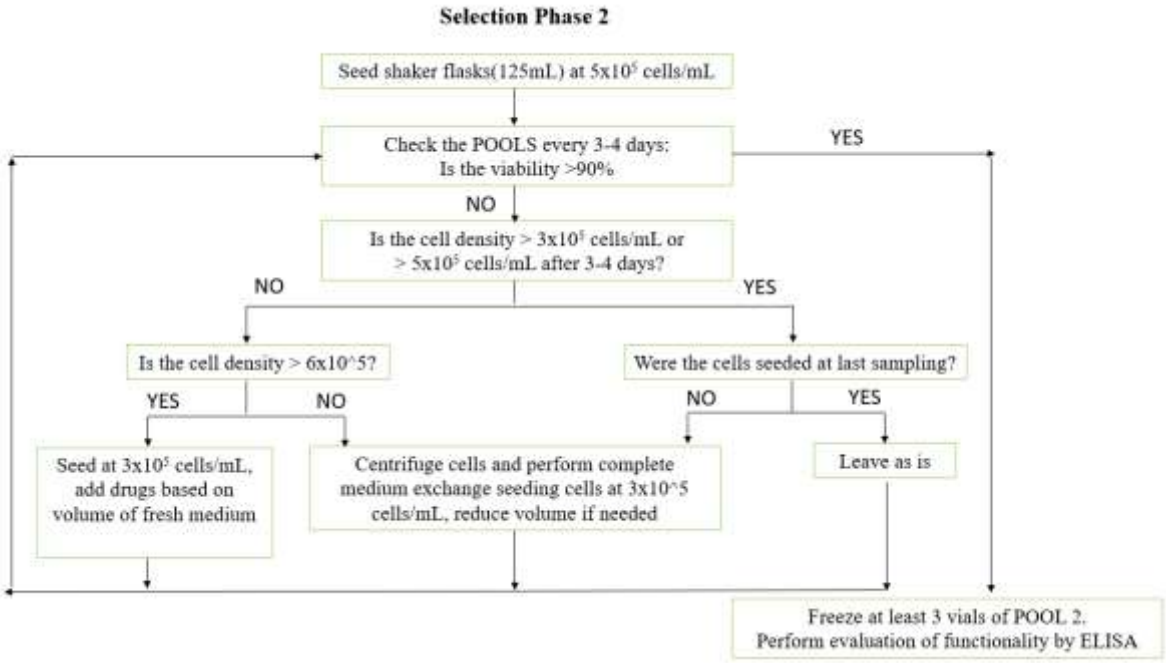

Figure 9. Schematic protocol that was followed in selection phase 2 for each transfection that was performed.

3.4 Cloning by limiting dilution

In the project, cloning by limiting dilution was performed with C192 with B signal (C192B) and Ov185 (Ov185) with original signal sequence. In a stepwise manner, illustrating the procedure in figure 10 for 2-3 weeks, followed by a productivity test by selected top clones for 14 days.

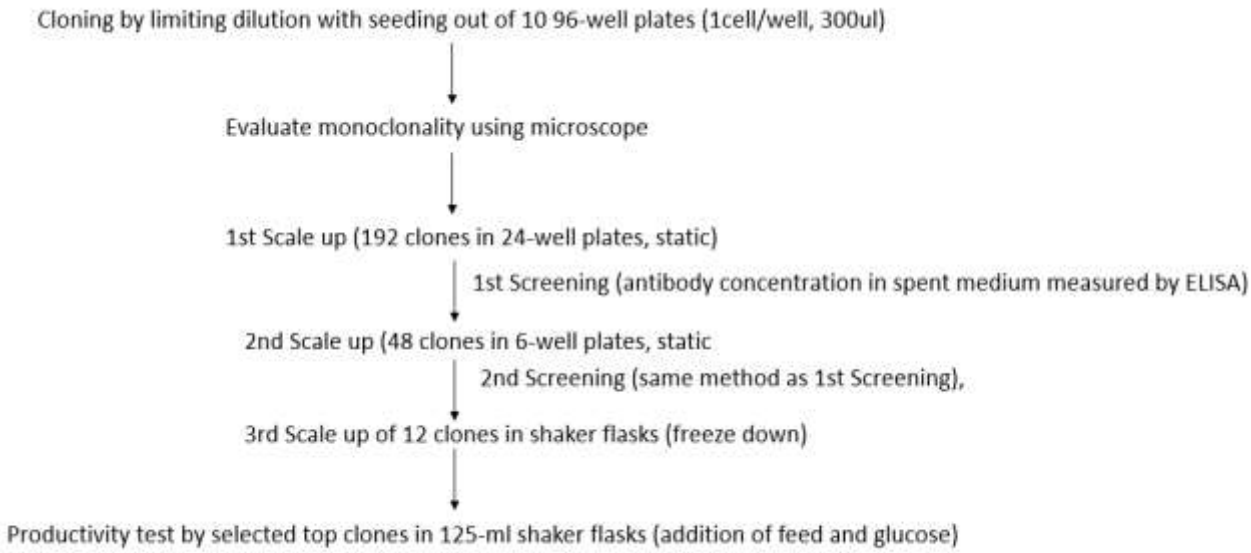

Figure 10. Illustrated stepwise manner of cloning by limiting dilution.

Cloning was performed after selection phase 2, using cells from POOL 2 with >90% viability. Cell cloning was performed by limiting dilution at a concentration of 1 cell/well (200 /well) in improved cloning medium into 10 plates of 96-wells (Falcon 353072). The plates were incubated at 37^0C with a humidified atmosphere of 8% CO2. After 5-7 days wells with single colonies were identified under microscope. When the clones reached at least 50% confluence, 192 clones were transferred to 24-well plates (Falcon 353047) with 400 µl prewarmed medium. The plates were then incubated again at 37^0C, 8% CO2 for 4-5 days.

When the cells reached at least 50% confluence a 1st screen by sandwich ELISA was performed to measure the antibody concentration in the cell supernatants. Medium from the 192 clones were diluted 1:100, in AB to fit within the mAb concentration range of the standard curve of IgG1, 0.1 µg/ml. The 48 clones with the highest concentrations (µg/ml) were chosen to continue to 2and Scale up in 24-well plates (Falcon 353046). The 48 clones were seeded out in prewarmed medium with GLUTAMAX. Total amount of cells in 24-well plates of each 48 clones were pipetted into 8 plates of 24-wells with 2 ml/well and incubated for 4 days. 2nd screening was performed by a sandwich ELISA, to measure the antibody concentration. 48 clones were screened by the final dilution 1:1000, where each sample with unknown concentration was diluted in AB to fit within the mAb concentration range of the standard curve of IgG1, 0.1 µg/ml.

Approximately 12 clones with the highest concentrations (µg/ml) were selected to continue to 3rd scale up into 125 ml Erlenmeyer Flasks with 30 ml/flask with cloning medium with GLUTAMAX. Total amount of cells in 6-well plates was added to each Flask incubated, shaking for 5 days. Cell counting by cell counter Nexcelom K2 according to 3.1.2. One passage was performed after 5 days with the 12 clones to $3x10^5$ cells/ml into new 30 ml/flask with cloning medium with GLUTAMAX. Incubated, shaking until the viable cell density reaches $1.5x10^6$ cells/ml.

3.4.1 Productivity test

Productivity test was performed on selected top clones, by a feeding process for 14 days. Cells were counted according to 3.1.2 and seeded out at a concentration of $3x10^5$ cells/ml in 125 ml Erlenmeyer Flasks with 30 ml/flask of cloning medium including GLUTAMAX. Feeding for 14 days of the selected clones was performed each second or third day, with a feeding composition. Feeding was prepared sterile under LAF according to Table 3:11 and Table 3:12.

Table 3:11 Feeding medium

Improved feeding medium	
Improved feeding medium	67.31g
Sodium Bicarbonate	2.2g
up to 1L with MQ	
0.22 µm filtration (Corning 430517)	

Table 3:12 Glucose

Glucose 35w/v %	
Glucose	35.07g
Up to 100 ml with MQ	
0.22 µm filtration	

Each clone was feeded with 1.5 ml feeding medium and 270 µl Glucose 35 w/v%, sterile under LAF. At day 14, the clones were centrifuged at 4500xg 10min and the cell supernatant was saved in 50 ml tubes in the refrigerator. Antibody concentration in supernatants were measured in a sandwich ELISA, on dilutions 1:1000, 1:2000, 1:4000 and1:8000. Were each sample with unknown concentration was diluted in AB to fit within the mAb concentration range of the standard curve of IgG1, 0.1-0.5 µg/ml.

3.5 Antibody purification and characterisation

In the project due to limitation of time, only scaled up selected clones of C192 with B signal (C192B) were continued with antibody purification and characterisation. The antibody purification and characterisation were performed in a stepwise manner, following the procedure in figure 11.

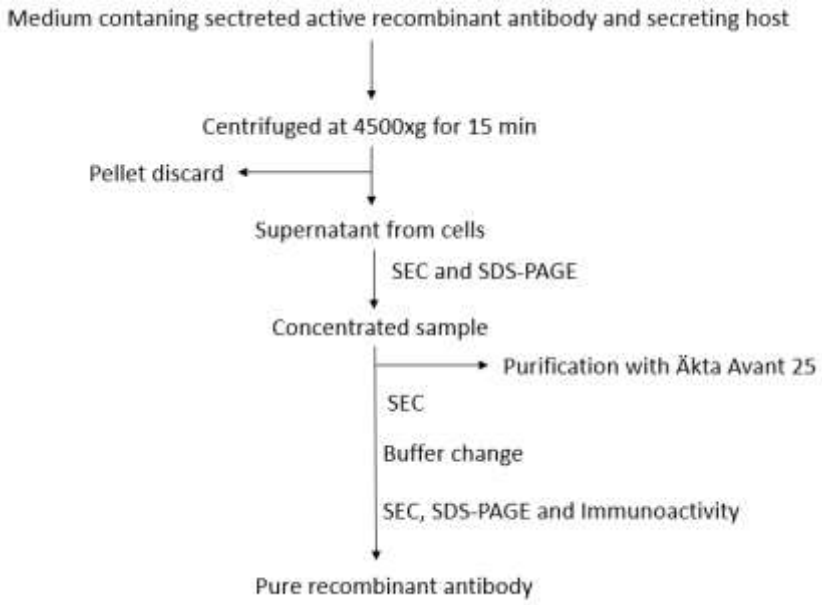

Figure 11. Illustrated stepwise manner the antibody purification and characterisation of rAbs.

3.5.1 Antibody purification

Antibody purification was performed on an ÄKTA Avant 25 chromatography system from Cytiva (GE Healthcare). First the cell supernatant from the top 11 clones of C192B were centrifuged at 4500xg for 15min and filtrated with 0.22 µm Millex-GV syringe filter. Thereafter, about 30 ml/clone of the top 11 clones of C192B were purified through Protein A affinity chromatography. As a reference to the purification results a mAb C192 from the bioreactor at the production department of FDAD were purified as well. The purified antibodies were pooled by pipetting from the 96-Deep well plates with printed GRD (VMR) into a new 50 ml tube. 1 ml of the pooled antibodies was filtered with a 0.22 µm Millex-GV syringe filter and centrifuged, this sample was used for SEC. The pooled antibodies concentrations were estimated after purification, by measuring absorbance at 260/280 nm, using the Nanodrop 2000C. Furthermore, the antibodies were eluted via a filter tube with 3x buffer changed, centrifuged at 4000xg for 15 min after each buffer change. Into a storing

buffer containing (10 mM potassium phosphate,150 mM KCI and 5% Sucrose). The buffer exchanged antibodies were filtered with a 0.22 µm Millex-GV syringe filter and concentration determined again at 260/280 nm by Nanodrop 2000C. Purified and buffer changed selected clones of C192B were stored in -76 ºC.

3.5.2 SEC

For C192B both spent medium and purified were analysed using size exclusion chromatography (SEC). SEC evaluated the purity of the mAb elute in terms of percentage intact C192B and C192 versus percentage aggregated and/or decomposed C192B and C192. The analysis was performed on an Ultimate 3000BioRS, with ≥ 30 µl filtered with a 0.22 µm Millex-GV syringe filter sample of antibody. And ≥ 20 µl of each filtrated sample were pipetted to each 1.1 ml glass vial and seal with a PTFE Sept and set into the chromatograph. In the system there was a spectrophotometer measuring absorbance at 280 nm for detection of antibodies with buffer 50 mM Sodium phosphate, 150 mM NaCl, pH 7.1, with a running time approximately 10min/sample. The results from the absorbance measurements were analysed by integration of peaks according to the Thermo Scientific Chromeleon 7.3.2 Software.

3.5.3 Immunoactivity of antibody

Immunoactivity of the purified selected clones of C192B and the reference mAb C192 from bioreactor was evaluated with an ELISA-based immunoactivity assay. The Ref and Test MAb samples were diluted to 100 µg/ml and the ability to inhibit the CA19.9 ELISA.
10 µl were pipetted of each Ref and Test Mab with adding each dilution volume from the example calculation table to Tracer Diluent to obtain Ref and Test Mab 100 µg/ml.

The assay used streptavidin coated wells in a 96-well plate format where ProGRP antigens were immobilized by biotin-labelled ProGRP PAb. Competing antibody was an HRP labelled C192 Mab (Ref and Test). Binding of HRP-C192 to ProGRP was then detectable at 450nm by adding TMB HRP-substrate followed by Stop Solution. Reference was C192 Mab. Absorbance measurement results were utilised to calculate percent signal inhibition (Eq. (1)) for reference respectively test antibodies, followed by relative immunoactivity (Eq. (2)) for C192B.

3.5.4 SDS-PAGE

Before and after the purification the selected clones of C192B and reference C192 from the bioreactor were analysed with SDS-PAGE to evaluate the antibodies sizes. 0.5 µg/band per clone were prepared with a volume of 10 µl/lane. In the reduced run, a reducing agent DTT was 10 µl/band added, and 10 µl/lane MQ. For the non-reduced band 10µl/lane per clone with 40 µl/band MQ. The samples were denatured in 70^0 C for 10 min. The gel cassette was prepared with TGX stain-free gels and 1L running buffer of 100 ml Tris/glycine/SDS Buffer(10x) with 900 ml RO_H2O. In each gel cassette first a sample of 5µl/band MQ of Precision PLus Standard Unstained pipetted, followed by sample of clones were pipetted 10 µl/band. The antibodies were analysed stain-free as both reduced and non-reduced. Results were scanned with the ChemiDoc apparatus.

4. Results

In this chapter the project results are described in a stepwise manner, about how to establish stable CHO cell lines for the expression of rAbs, (same as from the section 1.1 Aim), see Figure 12, starting with the establishment of RCB, and followed, by the molecular cloning of the antibodies sequences into pCHO 1.0 vector, with PCR screen amplification results visualised on agarose gel, and DNA preparation for transfection. Various analysis methods were used, we used ICC to visualise the transfection rate from 48 h post transfection. Selection phases with Methotrexat and Puromycin were used to achieve stable cell lines and to increase the productivity with recombinant expression. Sandwich ELISA was performed to determine the antibody concentrations after each analytical method. Selection was followed by cloning by limiting dilution to identify high-producing clones. Lastly a productivity test by selected clones was performed followed by antibody purification and characterisation of rAbs.

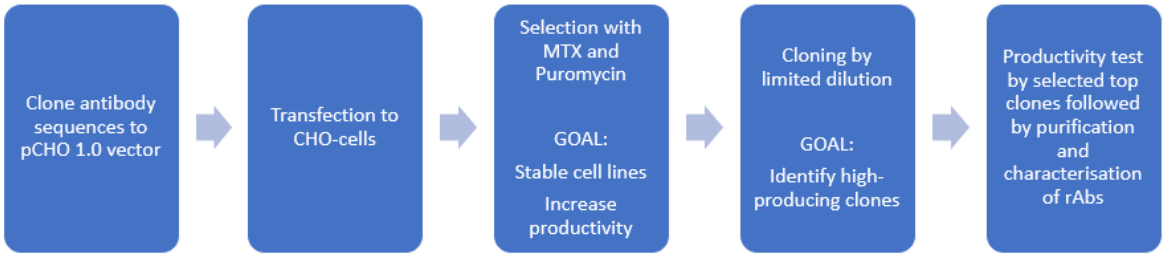

Figure 12. Illustrated stepwise manner, although the project, to establish stable cell lines in CHO-cells for expression of rAbs.

4.1 Establishment of Research Cell Bank

A RCB was performed with a result of 22 cryovials (1,5 ml) that were successfully freezed, according to 3.1.2, and has been used throughout the project. The cells have performed well up to 25 passages. Based on recommendation in the manual of the kit, CHO-S cells were split before reaching density> $2x10^6$ cells/ml. To keep cell viability high cells were counted and split every 2-3 days into new passages, following the procedure described in chapter 3.1.

4.2 Molecular cloning

Most of the DNA constructs were already at place prior to the start this master thesis work. The antibodies with signal sequences cloned in the project are shown in table 4:13 and 4:14 in Appendix 4. Except the cloning of Ov185 with B signal and HC that was finalized, with the sequencing primers in vector pCHO 1.0 according to Table 3:5 (from section 3.2.5)

Table 4:13. Sequencing primers in vector pCHO 1.0, used for the antibody HC.

Primer	**Primer sequence**	**Location**
Forward primer SU1 in pCHO 1.0	5'-GTCTGAGCCTCCTTGTCTTG-3'	begins ~270 bp upstream of AvrII/BstZ17I insertion site
Reverse primerSU1 in pCHO 1.0	5'-AGAAGACACGGGAGACTTAG-3'	begins ~90 bp downstream AvrII/BstZ17I insertion site

Re-cloning of insert Ov185 with B signal and HC into the vector pCHO 1.0 with Ov185 with B signal and LC was performed, since a STOP codon was confirmed in the sequence of the previous cloning. Transformants were screened for insert using PCR and agarose gel. Transformations with 5 µl of blunt-end ligation with new fresh ligase and buffers worked and formed colonies. S.O.C medium was used during the transformation. 8 colonies were generated when cloning Ov185 with B signal and HC. After the amplification samples were loaded onto an agarose gel to evaluate the theoretical size of approximately 1750 bp (see calculations in section 3.2.5). 5 µl low mass ladder and 5 µl of the PCR samples were run on 1,5% agarose gel. In Figure 13, where 6 colonies from Ov185 transformation evaluated with agarose gel to find positive clones, where the heavy chain gene was inserted into the vector. The selected colonies 1 (Lane 4) and 2 (Lane 5) have an insert of the correct size.

Sequencing confirmed that the insert was correct and colonies 1 and 2 was further used in the project.

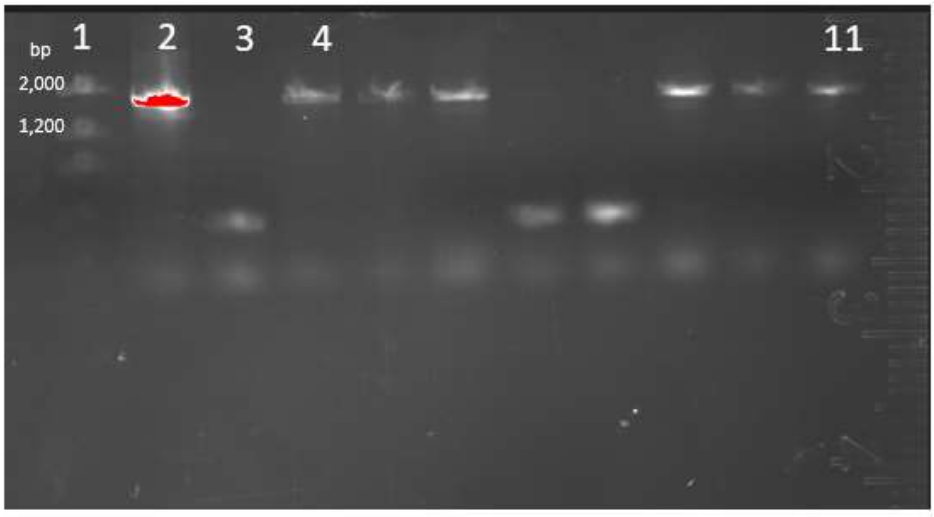

Figure 13. PCR screen of the transformant amplifications on agarose gel, from colonies (Lanes) transformed with Ov185 with B signal and the HC. Lane 1. Low DNA Mass Ladder, Lane 2. Positive control, Lane 3. Negative control. Lane 4-11 are the transformed colonies.

4.3 DNA preparation for transfection

The required amount of DNA for each plasmid to perform a successful transfection is 50 µg, regardless of whether the plasmid has been engineered to express 1 or 2 subunits. This process was performed several times with different outcome of concentrations, for the plasmids that was used in this project. When using a new QIAGEN Plasmid Midi Kit (QIAGEN), the concentration outcome was much better, and the optimum concentrations of 50 µg, were achieved to continue the project and perform transfections under optimum conditions.

4.4 Establishment of stable cell line for expression of recombinant antibodies.

This last subsection describes the main work of this project. The experimental flowchart according to Figure 11, showing the expression of rAbs with a schedule, that was evaluated and improved through the project. The schedule describes the transfection procedure, the evaluation of the transfection expression levels by ICC with fluorescence microscopy and evaluation of functionality by sandwich ELISA. The workflow of selection in two-phases, followed by performing POOLs productivities. Lastly, cloning by limiting dilution into 96 wells with final dilution to 1cell/well and screening clones, until a selection of high-producing clones were achieved. Productivity test was performed at the high-producing clones, by feeding schedule for 14 days followed by antibody purification and characterisation.

4.4.1 Evaluation of transfection grade

The transfection grade was evaluate from the 48 h post-transfection. Immunostaining (ICC), using goat-anti mouse Alexa flour was performed to study the intracellular expression of rAb. Non-transfected cells CHO-S cells and hybridoma cells expressing C192 from the FDAB manufacturing department, were used as negative and positive controls. The transfection grade was calculated as the proportion of Alexa flour stained cells compared to non-stained cells in each sample. Result of the 48 h post-transfection, using the rAb C192 with B signal is illustrated in the Figure 14, A and B. The transfection grade of 25% was obtained, calculated as the quota between 35 green cells stained with Alexa and 140 blue cells stained with DAPI. Different concentrations of the plasmid of C192 with B signal were prepared by ICC on glass probes, according to 3.3.4. None of the non-transfected cells stained positive in this experiment whereas all the hybridoma cells were stained (C, D).

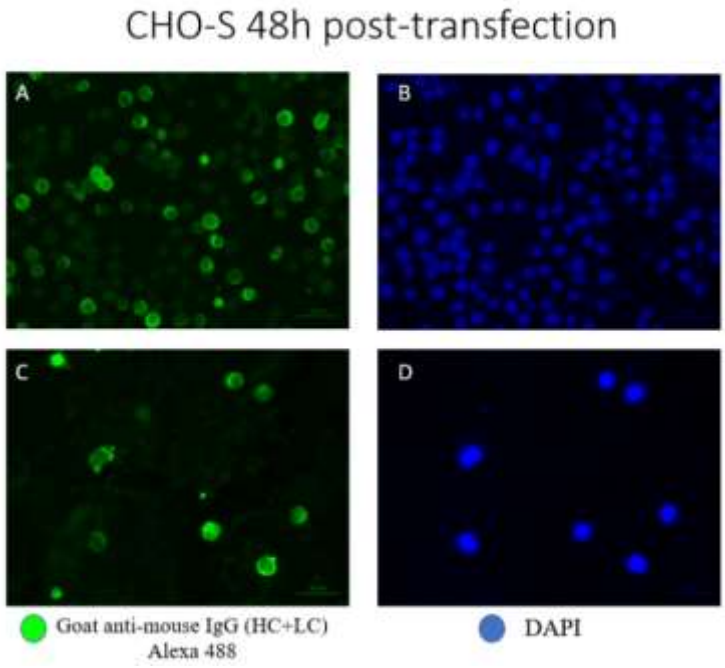

Figure 14. A) B) One representative CHO-S cells from 48 h post-transfection using pCHO rAb C192 B HC and LC, with transfection efficiency with 25%. C) D) C192 hybridoma as positive control. All cells stained positive with Alexa flour as expected.

4.4.2 Selection in two-phases

To obtain stable cell lines with high production levels of rAbs, selection in two-phases was performed. The selection was performed in CD FortiCHO medium supplemented with GLUTAMAX and Anti-clump Agent and the two drugs Puromycin and MTX. The process can be followed by the schematic protocols in Figure 8 and 9.
In Figure 15, viability during selection phase 1 of C192 with B signal and with original signal sequence is presented and Ov185 with b signal and with original signal sequence. As expected, the viability of the cells dropped when drugs were added, where both C192 and Ov185 had the same expression. Where transfection was successful, cells survive, divide and the viability thus increases, as seen for C192B, starting from day 10 and continually increasing viability and are following the schematic protocol in Figure 6. Cells from POOL 1 were frozen and selection phase 2 was performed. C192 with original signal dropped in viability and never recovered which might indicate an unsuccessful transformation. Additional transfections will be needed.

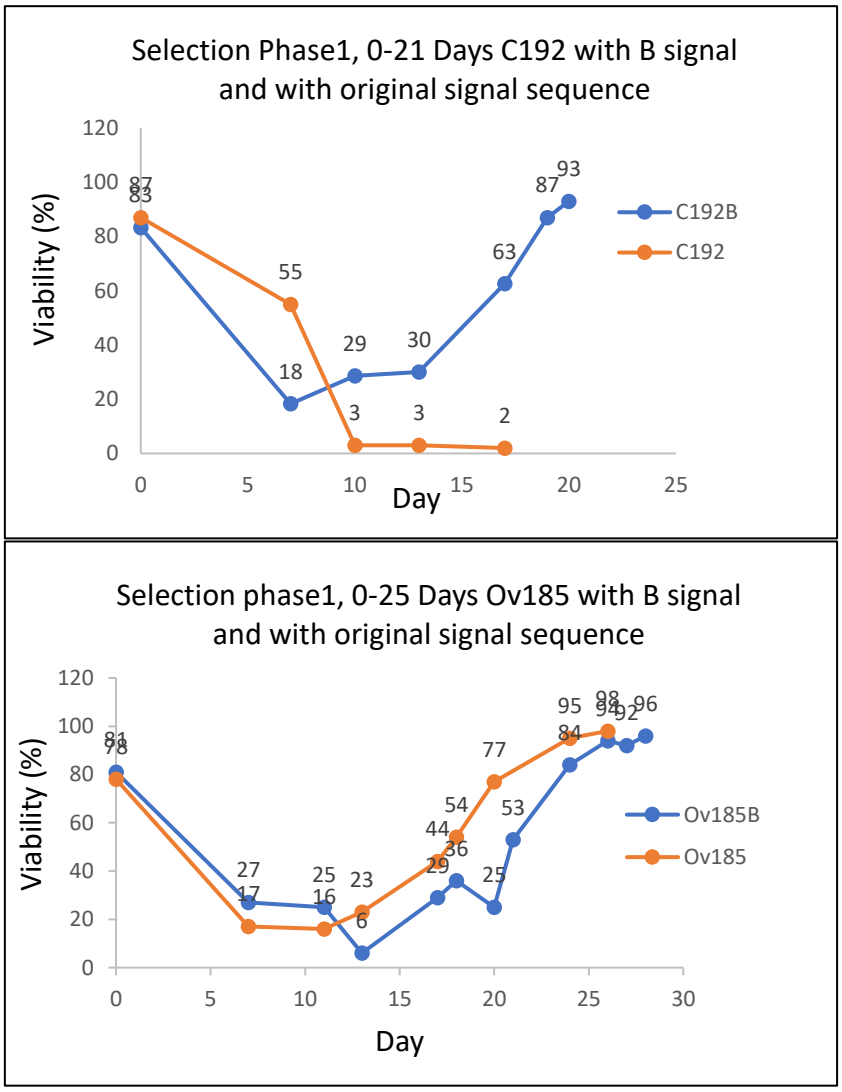

Figure 15. Selection Phase 1 of C192 with B signal and original signal sequence and Ov185 with B signal and original signal sequence, showing viability in (%) over 21-28 da

In Figure 15 viability of Ov185 with the B signal and with the original signal sequence are presented for the Selection Phase 1, from day 0 to day 28, where the viability dropped at day 7 and recovered in the same manner as C192 with the B signal sequence

Selection phase 2 was performed only for 5-7 days with a stable viability of 95-98%, for C192 with B signal and original signal sequence and Ov185 with B signal and original signal sequence. Cells from POOL 2 were frozen according to the schematic protocol in

4.4.3 Evaluation of antibody concentration by ELISA

ELISA was used to measure the amount of rAbs secreted into the medium. The experiment was performed with supernatant cells from both 48 h post transfections and from the selection phases. Each sample with unknown concentration was diluted in AB to fit within the mAb concentration range of the standard curve of IgG1, 0.1- 0.5 µg/ml. Two dilutions were done per sample, see Appendix A.3 for the plate design.

Different dilutions are illustrated by an ELISA plate for determination of concentration in supernatant in Appendix A.3. Concentrations that are < 0,1 µg/ml, indicates that the transfection wasn't successful, and a new transfection was suggested to be performed. Another transfection was performed with C192 with original signal with no success. Concentrations closer to 1 µg/ml or above probably indicates a successful transfection and might lead to success in the selection of stable clones. Furthermore, concentrations from the selection phases were evaluated and pools were established, and frozen. In Figure 12 both Ov185 and Ov185B had similar outcome, but the concentrations in POOL 2 for Ov185B gave no data (Table:4.14). Due to limitation of time in the project a decision was made to move on to cloning procedure for C192B and Ov185 POOL 2.

Table 4:14 Results from ELISA with evaluated concentrations after 48h-post transfection and POOL 1 and POOL 2 from the selection phases. Where C192 with original signal sequence, no transfection succeeded and therefore (-) no data available for POOL 1 and POOL2. Ov185 with B signal POOL 2 where (-) no data available as well.

Concentration µg/ml	**48 post - transfection**	**POOL 1**	**POOL 2**
C192 with B Signal sequence	1.5	38.1	44.7
C192 with original signal sequence	< 0.1	-	-
Ov185 with B signal sequence	0.8	34.0	-
Ov185 with original signal sequence	0.8	22.7	38.7

4.5 Cloning by limited dilution

To identify high producing clones and thereby achieve high productivity in the final cell line, cloning by limiting dilution was performed to isolate high-producing cell clones. Cloning was performed after selection phase 2, using cells from POOL 2 with >90% viability. According to Figure 10, illustrating the stepwise manner of cloning by limiting dilution. High producing clones were screened two times by sandwich ELISA. The screening was performed after 1st and 2nd Scale up. The 1st screen of 192 clones, gave concentrations of 2-15 µg/ml. 48 clones with the highest concentrations (µg/ml) were chosen to continue to 2nd Scale up in 6-well plates (Falcon 353046). 2nd screen of 48 clones, gave concentrations of 5-20 µg/ml.

Approximately 12 clones with the highest concentrations (µg/ml) were selected to continue to 3rd Scale up into 125ml Erlenmeyer Flasks with 30 ml/flask with cloning medium with GLUTAMAX, incubated, shaking for 5 days.

4.5.1 Productivity test

After cloning by limiting dilution to achieve the highest producing rAbs selected, top clones were moved on a productivity test. Experiments were performed on both C192 with B signal (C192B) and Ov185 with original signal sequence (Ov185) from POOL 2 from the selection phase 2. Results in Figure 16 illustrates the concentrations from the productivity assessment of the selected clones. The clones were fed with a feeding composition of medium and glucose according to Table 3:11 and Table 3:12, schematically each second or third day, for 14 days. Supernatants were concentration determined at day 7 by sandwich ELISA, with the final dilutions; 1:300, 1:600, 1:1200.

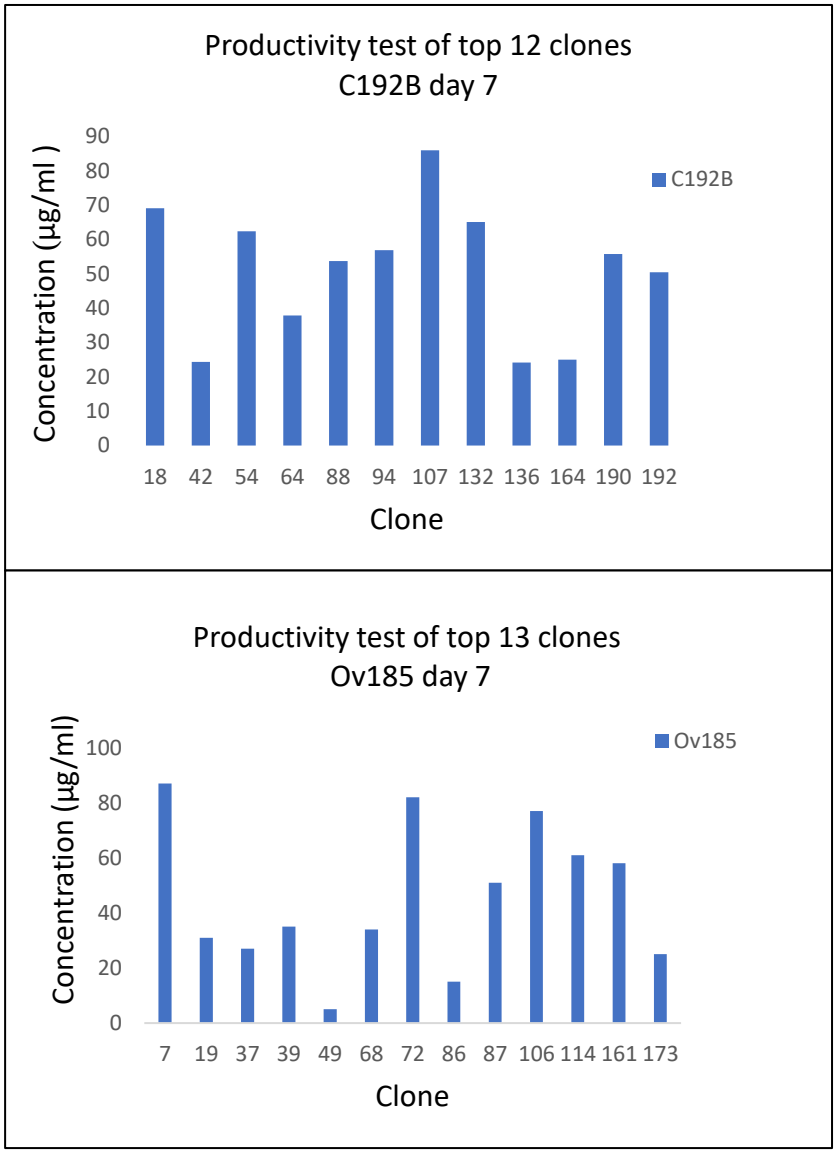

Figure 16. Concentrations from the productivity test of selected top clones, for C192B and Ov185 antibodies, after 7 days of feeding clones with feeding medium and glucose.

As seen in the Figure 16 the concentrations after 7 days from the productivity test of selected top clones, were similar for C192B and Ov185. C192B concentrations gave 8 selected top

clones > 50 µg/ml, with one top clone of 86 µg/ml. Ov185 concentrations gave 6 selected top clones > 50 µg/ml, with two top clones > 80 µg/ml.

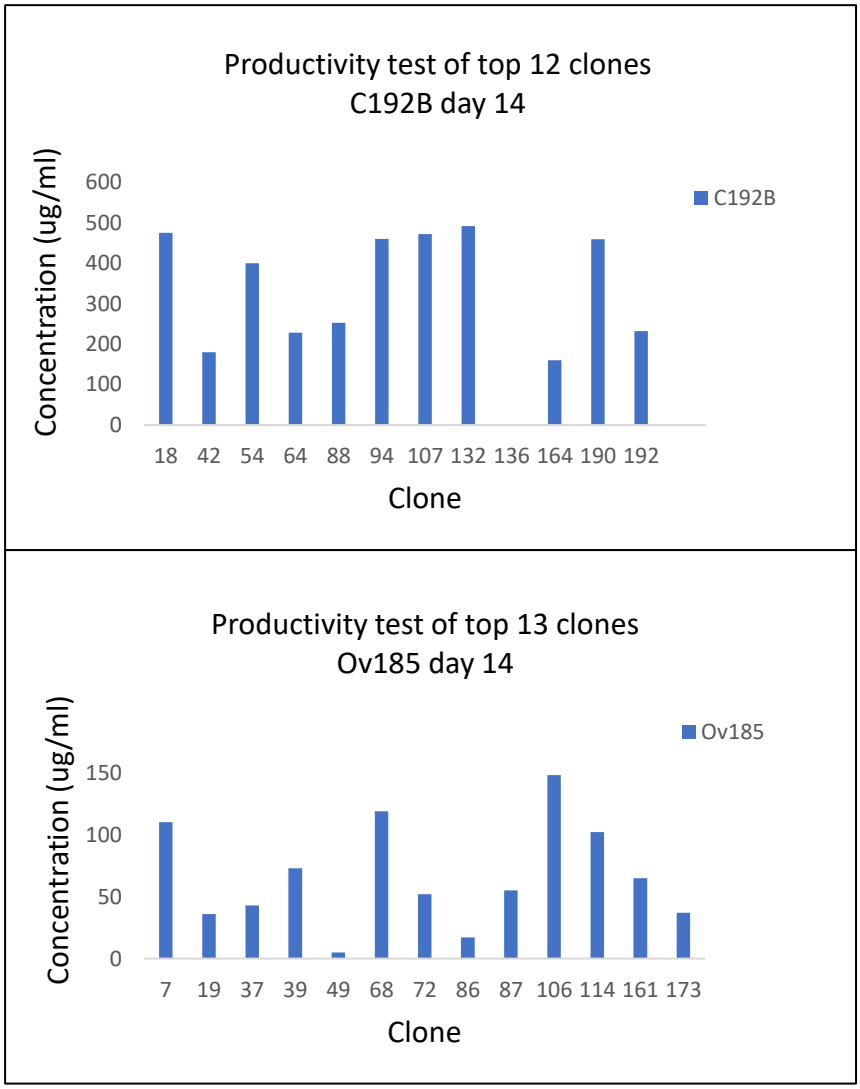

Figure 17. Concentrations from the productivity test of selected top clones, for C192B and Ov185 antibodies, after 14 days of feeding clones with feeding medium and glucose.

The concentrations after 14 days from the productivity test of selected top clones, gave more variable concentrations. C192B concentrations gave 6 selected top clones up to 490 µg/ml. Ov185 concentrations gave 4 selected top clones up to 100 µg/ml. This can be explained from next illustrated results in Figures 18-21. The cell growth and the viability in % were measured at day 7 and day 14 with the cell counter Nexcelom K2. In Figure 21 illustrates that Ov185 viability dropped to 50% after day 7. Accidentally after that the 13 clones were left in the incubator non-shaking. The productivity assessment was continued for 14 days, and as a result after the incident, the concentrations were not as high as for Ov185 after 14 days.

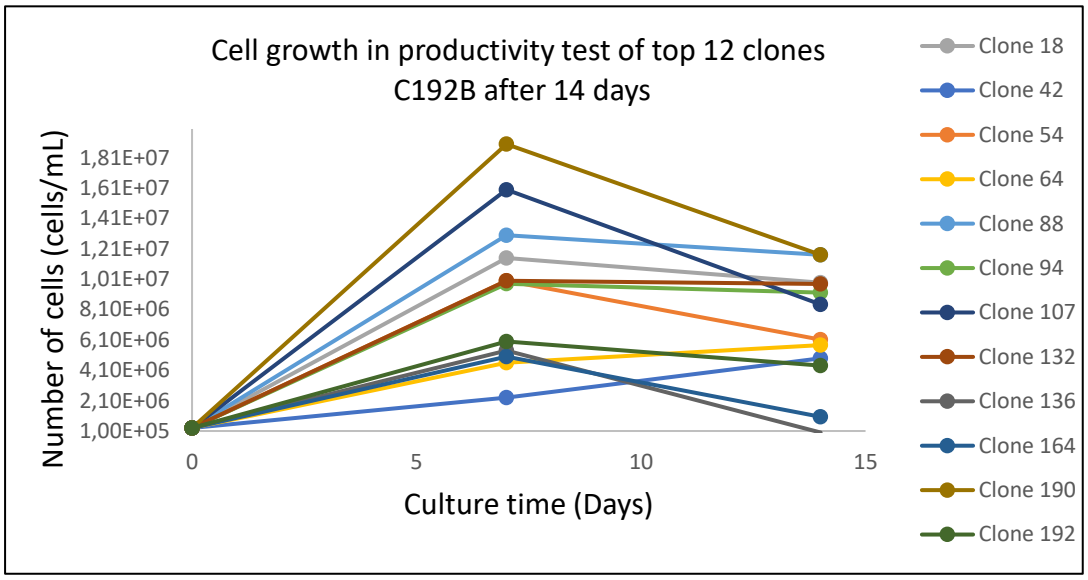

Figure 18. Productivity test of top clones for C192B antibodies illustrating the cell growth after 14 days of feeding, with feeding medium and glucose. The number of cells were counted by the cell counter Nexcelom K2 at day 0, 7 and 14, and the starting seed was 3x10^5 cells/ml.

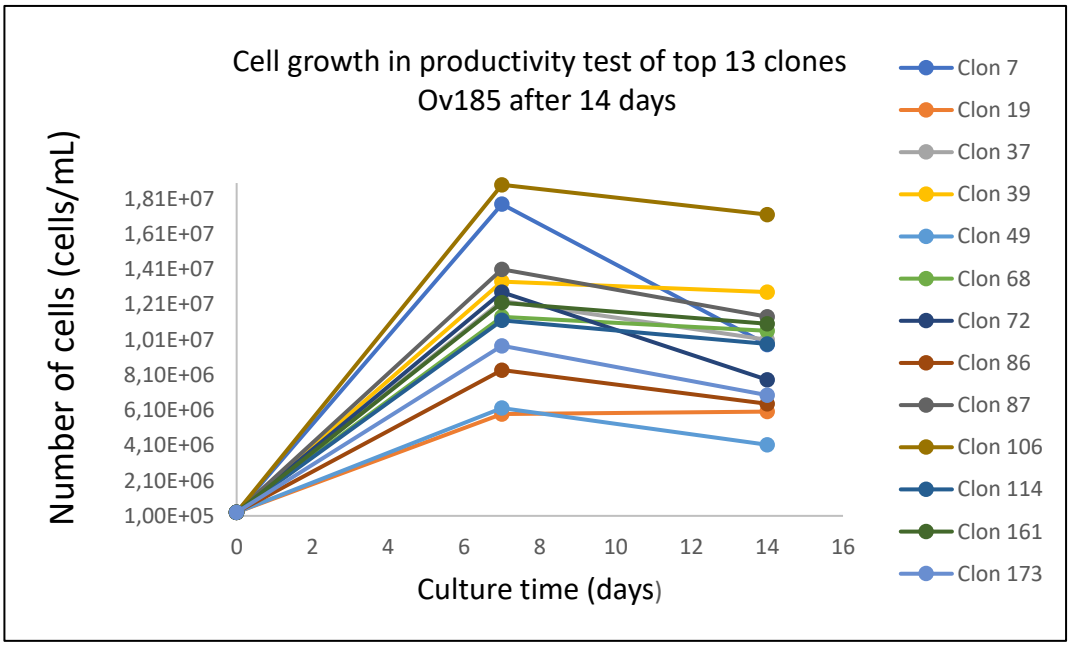

Figure 19. Productivity test of top clones for Ov185 antibodies illustrating the cell growth after 14 days of feeding, with feeding medium and glucose. The number of cells were counted by the cell counter Nexcelom K2 at day 0, 7 and 14, and the starting seed was 3x10^5 cells/ml.

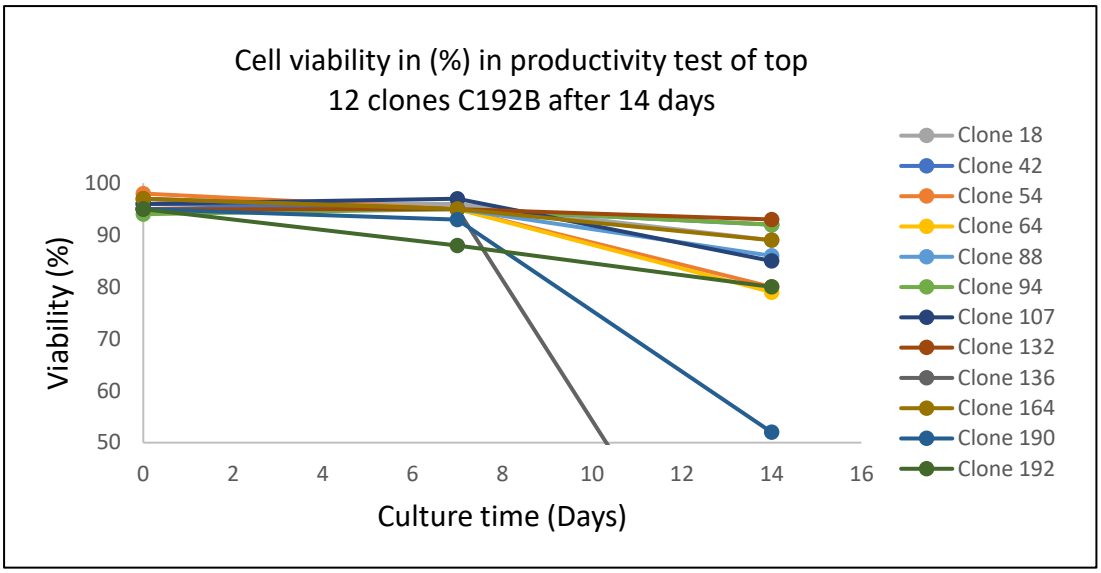

Figure 20. Productivity test of top clones for C192B antibodies illustrating the viability in (%) after 14 days of feeding with feed medium and glucose. The viability of cells was measured by the cell counter Nexcelom K2 at day 0, 7 and 14.

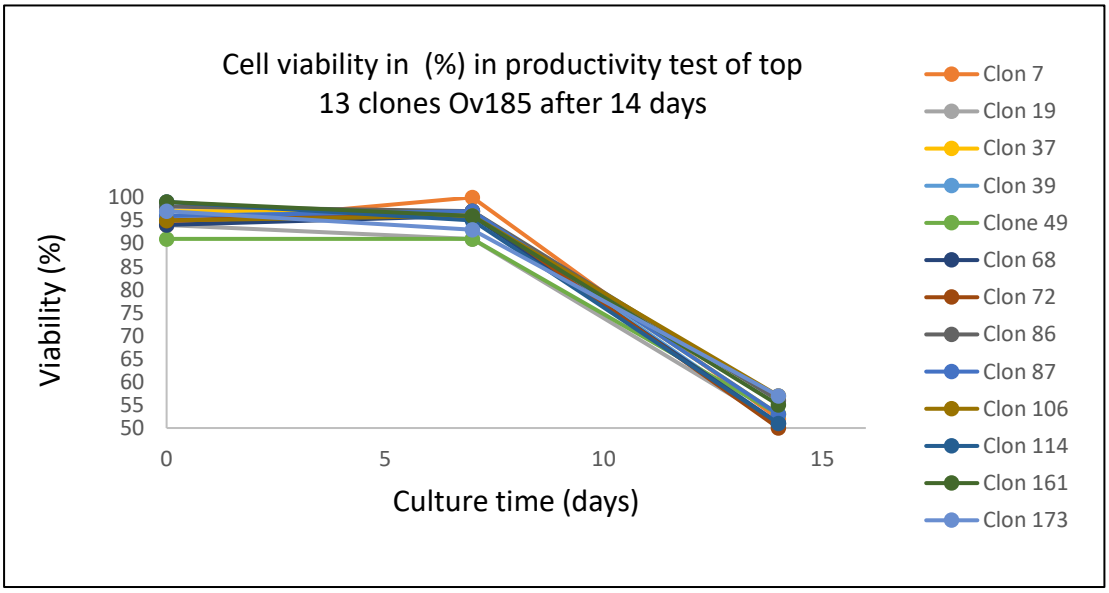

Figure 21. Productivity test of top clones for Ov185 antibodies illustrating the viability in (%) after 14 days of feeding with feed composition of medium and glucose. The viability of cells was measured by the cell counter Nexcelom K2 at day 0, 7 and 14.

4.6 Antibody purification and characterisation

In the project due to limitation of time, only the top 12 selected clones of C192B were antibody purified and characterized. The purification was performed by Protein A affinity chromatography. 30 ml/clone from the top 11 clones of C192B were purified. (At start there was 12 clones, but one clone had infection at day 8 therefore only 11 clones). As a reference for the purification was a mAb C192 from the bioreactor at the manufacturing department at the company was used as well. Characterisation was evaluated by SEC with both before and

after purified with buffer change, immunoactivity of antibodies were evaluated and SDS-PAGE.

4.6.1 SEC

Antibody purification for IgG used by Protein A affinity chromatography. SEC was performed of the top 11 clones of C192B mAbs and the reference mAb C192 from the bioreactor was performed both before and after the purification with the buffer change. SEC makes it possible to evaluate the purity of the mAbs eluted in terms of percentage intact C192B and C192 versus percentage aggregated and/or decomposed C192B and C192. The analysis was performed on an Ultimate 3000BioRS, with ≥ 30 µl filtered with a 0.22 µm Millex-GV syringe filter samples of antibodies. ≥ 20 µl of each filtrated sample was pipetted to each 1.1 ml glass vial and sealed with a PTFE Sept and set into the chromatograph. The result is illustrated in Figure 22, where one of the clones are shown as representative, as all 11 top clones gave the same purification results. In the upper right corner, the thumbnail, shows the entire chromatogram including retention times corresponding to low molecular weight substances (not protein), which absorb at A280 nm. Purity percentage of the selected clones after purification was >99 %.

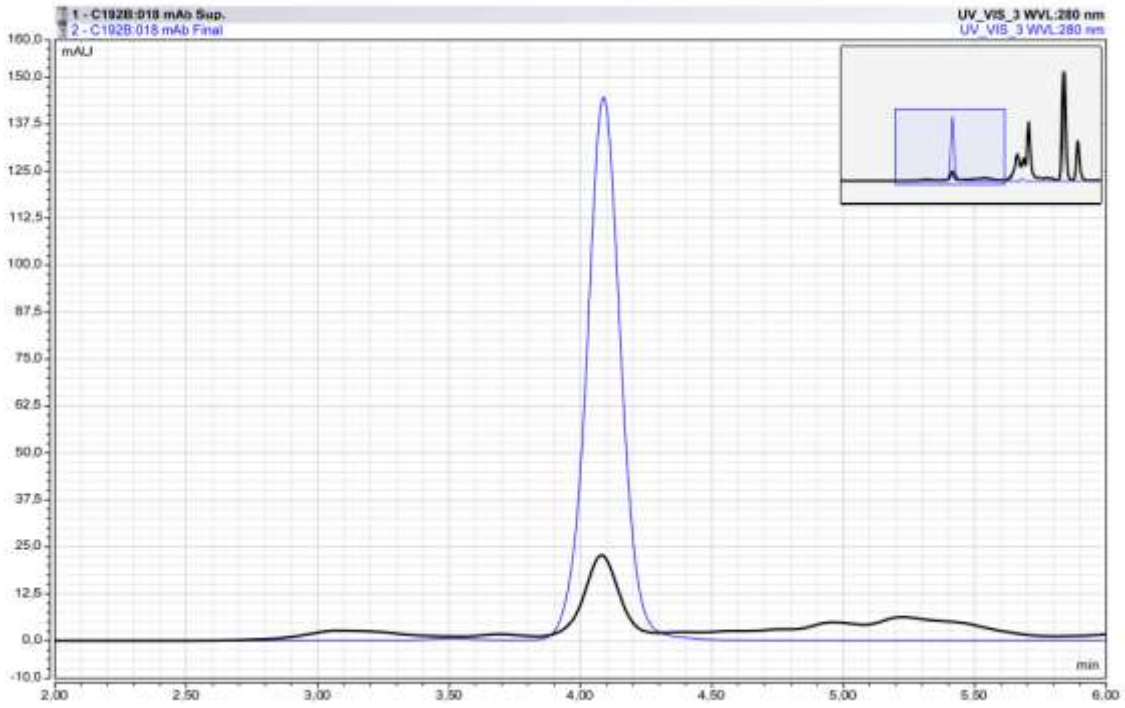

Figure 22. Zoomed-in chromatogram from SEC analysis of clone 18 C192B, i.e., before purification in black line, after purification in blue line. At the y-axis: A280nm in milli-absorbance unit (mAU) and the x-axis: retention time in min. In right upper corner, black curve shows the entire chromatogram. The peak around 4.1 min, consist of recombinant full length antibody. Additional peaks seen before purification are impurities such as medium proteins of varying size. Blue curve shows pure antibody with a single peak around 4.1 min indicating a successful purification.

4.6.2 Immunoactivity of antibody

To measure the immunoactivity of the selected top 11 clones of C192B, an ELISA-based assay method was used for estimation of relative antibody immunoactivity. The composition of immobilised antigens, biotin labelled mAbs, reference mAbs, rAbs of interest, HRP-labelled mAbs, and TMB were evaluated. Thus, the signal is showing how much the signal from the HRP-labelled mAbs is inhibited when reference mAbs or rAbs of interest compete with the labelled mAbs about antigen binding. The signal inhibition is measured in (%) and is related to the signal obtained when only labelled mAbs bind to the antigen and is calculated by equations (1) and (2). The results are presented in the Table 4:15.

Table 4:15 Relative immunoactivity measured in (%) of the purified scale up selected top 11 clones of C192B and the ref C192 from the bioreactor.

CLON C192B	Relative immunoactivity (%)
18	98
42	81
54	81
64	96
88	100
94	107
107	103
132	121
164	82
190	96
192	70
Ref 192	90

4.6.3 SDS-PAGE

SDS-PAGE was performed, before and after purification of the top 11 clones of C192B. FDAB produced C192 mAb was used as reference. The antibodies were analysed under both reduced and non-reduced conditions. Before the purification the concentrations of the samples were various. After the purification the samples had a buffer exchange and in that experiment the samples had the same concentrations of 0.5 µg/lane. A standard containing proteins with known size were used. As expected, the samples have a clear band approximately 150 kDa under non-reduced conditions, while the reduced samples show two bands, one for heavy chain approximately at 50 kDa and a band for the light chain approximately at 25 kDa. As expected, the samples are clear from medium components after the purification.

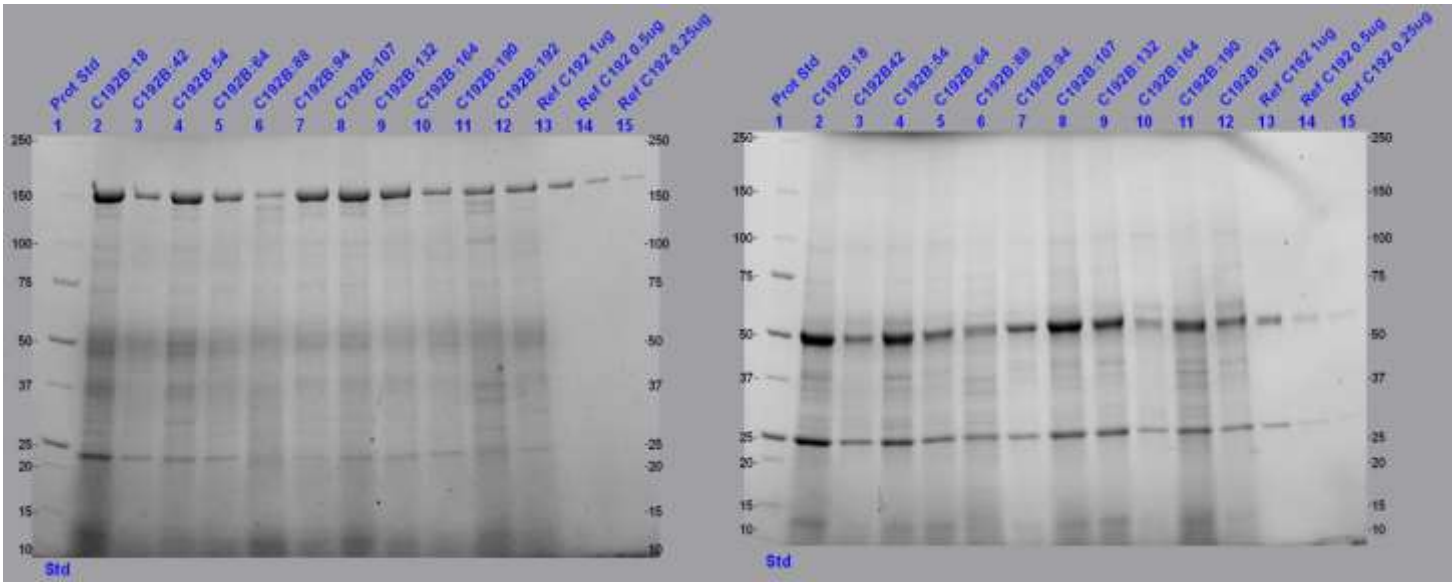

Figure 22. SDS-PAGE illustrating the scale up selected top 11 clones of C192B before the purification by Protein A affinity chromatography. To the left is the non-reduced bands at 150 kDa and to the right there is the reduced bands at 50 kDa and 25 kDa. The first lane is a Precision Plus Protein Standard Unstained to verify the antibodies size, and lanes 2-12 are the C192B clones. The last lanes 13-15 are ref mAbs C192 with variable concentrations.

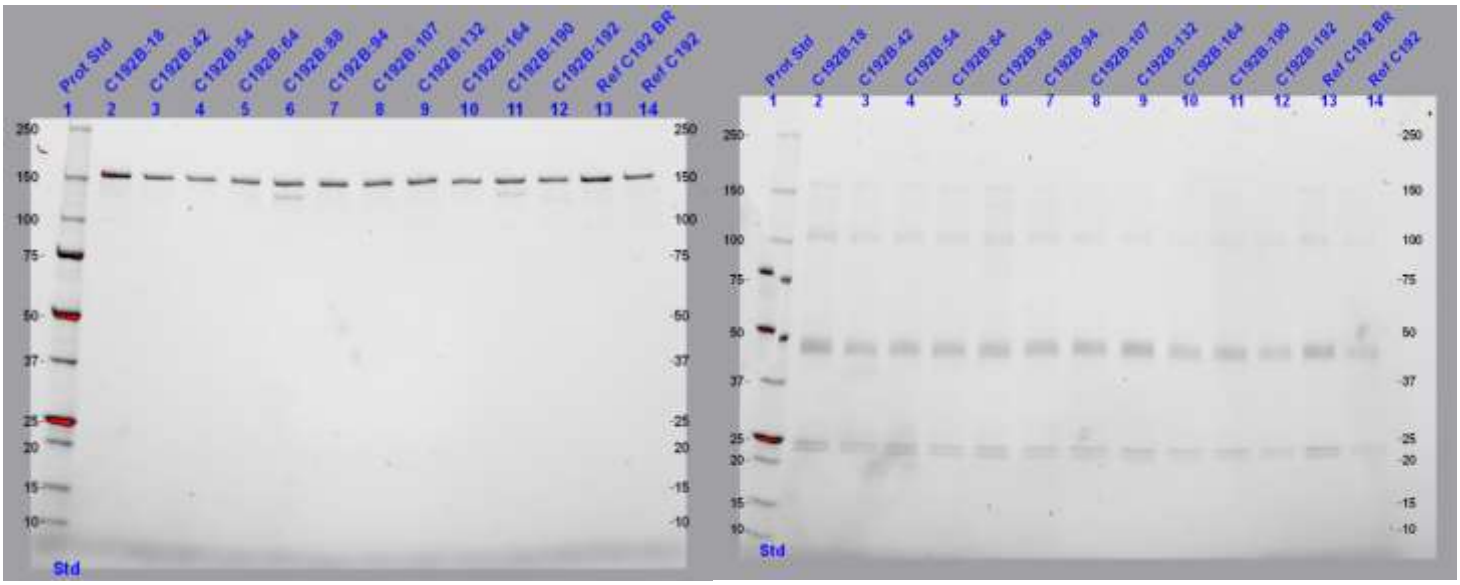

Figure 23. SDS-PAGE illustrating the scale up selected top 11 clones of C192B after the purification Protein A affinity chromatography. To the left is the non-reduced bands at 150 kDa and to the right there is the reduced bands at 50 kDa and 25 kDa. The first lane is a Precision Plus Protein Standard Unstained to verify the antibodies sizes, and lanes 2-12 are the C192B clones. The last lanes 13 and 14 are ref mAbs C192, all samples had the same concentrations 0.5 µg/lane.

5. Discussion

The purpose of this master thesis was to express some of FDAB mAbs as recombinant rAbs, to increase productivity and make stable cell lines. The host cell line selected in this project was a suspensions CHO cell line (CHO-S). Using a suspension cell line makes future upscaling in bioreactors possible, and the cell line is also already adapted to serum free media eliminating the need for serum in production. Additionally, an alternative signal sequence from human albumin previously shown to be beneficial for protein expression in the CHO cell (You et.al, 2018) was evaluated. However, we were not able to draw the same conclusion from this study due to time limitations in the project, and our focus to move on with the results that were accomplished.

The cell lines expressing rAbs were created by introducing genes for a specific antibody into a host cell genome, using an expression vector. The vector used in this project, pCHO 1.0 (Thermo Fisher Scientific US, 2022), is designed specially to express two genes of interest downstream of the vector´s two different hybrid CMV promotor and is thus perfect for the expression two-subunit proteins such as antibody light and heavy chain.

After transfection, the transient expression was measured using sandwich ELISA. Though only a small number of transfections were performed, we could draw the conclusion that too low transient expression indicates that the transfection wasn't successful and should be repeated to be successful in the selection phase. The transfection is probably the most critical step in the process. The purity of the DNA preparation is of big importance and the cells need to be at an optimal density and in exponential growth phase before transfection. There are different methods for transfection, in this project liposome-mediated transfection with the reagent FreeStyle MAX was used. One suggestion would be to try another reagent to improve transfection efficiency. A GFP reporter gene cloned either into the restriction sites for HC or LC in pCHO1.0, might be a valuable tool for optimisation of one transfection procedure.

A key step in the selection of a stable cell line is to select cells where the plasmid has become integrated into the host genome. To achieve this the vector further includes two selection markers; Dihydrofolate reductase (DHFR) to tolerate methotrexate (MTX) and bacterial gene Pac to tolerate puromycin. By increasing the selection pressure in the media, high producing clones can be achieved. In this project this was done in two phases with increasing concentrations of MTX and Puromycin. Since only a small proportion of the cells have become stably transfected, the majority of the cells die when the drugs are added to the growth media and the cell viability dropped remarkably in the beginning of the selections phase, in all transfections. For those cells that are stably transfected survive and divide, thus the viability increases despite the selection pressure. In one of the transfections, the cells did not recover after this initial drop, indicating that the transfection was not successful, and no stable clones were achieved. As a consequence of the selection pressure with MTX the DHFR gene gets amplifies together with the antibody sequences, leading to increased antibody expression. This was clearly seen as an increased antibody expression throughout the selection phases.

Next step was to select high producing clones from the pool of stable transfectants using cloning by limiting dilution. The cells in the pools have differences in expression level, which was clearly seen in the ELISA screen. By starting with a large number of clones, the risk to miss out high producing clones get minimized. The cloning process is time- consuming and an automated single-cell-based system can be used to fasten the process.
The cloning process was first done as described in the manual, resulting in low number of clones. Method development was done with another improved cloning medium, and this media was then successfully used in the project.

Approximately 12-15 high-producing clones, were selected to be evaluated in a productivity test. High producing clones were obtained that produced up to 490 µg/ml. Antibodies were purified by Protein A affinity chromatography, by SEC, to exclude contaminating protein, nucleic acid, lipid, carbohydrate, and cell growth media components. Antibodies from all clones were successfully purified to at least 99% purity by SEC and verified by SDS-PAGE. Further on, immunoactivity of the rAbs were evaluated, where the relative immunoactivity was calculated and proven in comparison to hybridoma produced antibody

6. Conclusion

The purpose and aim of this project was to investigate and evaluate, the development of stable CHO cell lines for the expression of rAbs and to develop a new method to increase yield and get more stable productivity. Stable cell lines were established for two antibodies, C192B and Ov185. High producing C192B clones were obtained that produced up to 490 µg/ml in productivity assessment, corresponding to at least 10 times increase in expression as compared to the original hybridoma. There are both financial and manufacturing progress improvements by producing rAbs. By shortened time with less cell culture and less medium it is possible to achieve high-producing rAbs with the final rAb titer of >1 g/L (Xu WJ et. al, 2023).

Proof of concept has been ascertained regarding expression of antibodies in a rAbs in stable CHO-S cell lines at the company. The technique can now be considered established at FDAB and based on experiences and optimization done in this project, additional antibodies can be expressed in recombinant form at FDAB. Next, step will be a scale-up study for manufacturing of rAbs at FDAB.

7. References

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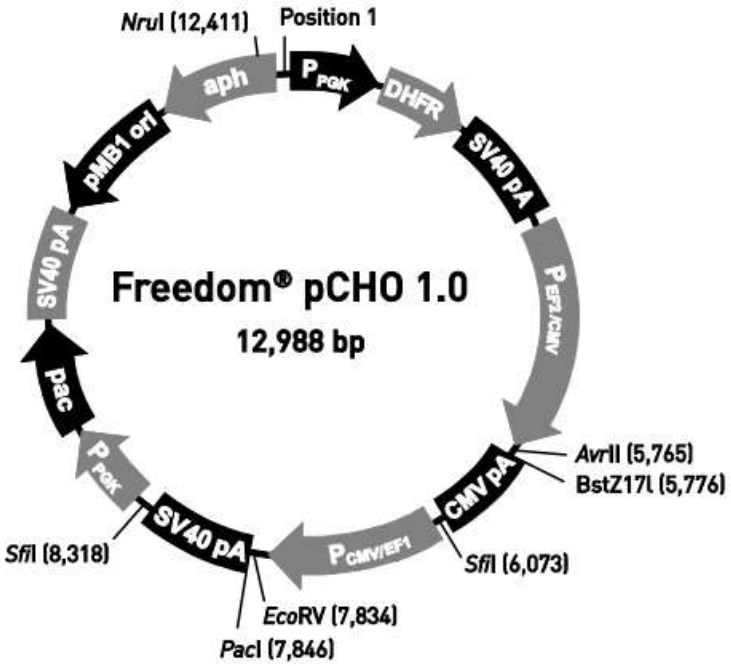
Appendix A.1



PGK (phosphoglycerate kinase) promoter:	117–623
DHFR (dihydrofolate reductase) gene:	730–1,293
SV40 polyadenylation signal:	1,404–1,631
EF2/CMV hybrid promoter:	1,728–5,517
CMV polyadenylation signal:	5,773–6,052
CMV/EF1 hybrid promoter:	6,093–7,565
SV40 polyadenylation signal:	7,848–8,094
PGK (phosphoglycerate kinase) promoter:	8,351–8,839
Puromycin resistance gene (pac):	8,860–9,459
SV40 polyadenylation signal:	9,583–9,823
pMB1 origin of replication:	10,495–11,168 (c)
Kanamycin resistance gene (aph):	11,684–12,499 (c)

(c) = complementary strand

Figure A.1. A map showing the elements of the vector Freedom™ pCHO 1.0 (Thermo Fisher Scientific US, 2022).

Appendix A.2

Features

The Freedom™ pCHO 1.0 vector contains the following elements. Features have been functionally tested, and the vector has been fully sequenced.

Feature	Benefit
EF2/cytomegalovirus (CMV) hybrid promoter	Allows efficient, high-level expression of your recombinant protein
*Avr*II and *Bsf*Z17I restriction enzyme sites	Entry points for gene insertion behind the EF2/CMV promoter (see page 17 for sequencing primers used for analyzing EF2/CMV promoter-gene of interest junction)
CMV polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
CMV/EF1 hybrid promoter	Allows efficient, high-level expression of your recombinant protein
*Eco*RV and *Pac*I restriction enzyme sites	Entry points for gene insertion behind the CMV/EF1 promoter (see page 17 for sequencing primers used for analyzing CMV/EF1 promoter-gene of interest junction)
Simian virus 40 (SV40) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
*Sfi*I restriction enzyme sites	Allows for removal of the CMV/EF1 expression cassette when using vector for single-subunit expression
Dihydrofolate reductase (DHFR) gene	Allows selection of transfected CHO-S™ cells using methotrexate (MTX) (Kaufman *et al.*, 1985)
Puromycin resistance gene (*pac*) (Puromycin N-acetyl-transferase)	Allows selection of transfected CHO-S™ cells using Puromycin (de la Luna & Ortin, 1992; Lacalle *et al.*, 1989; Vara *et al.*, 1985)
pMB1 origin of replication	Allows high-copy number replication and growth in *E. coli* (Lin-Chao *et al.*, 1992; Yanisch-Perron *et al.*, 1985)
Kanamycin resistance gene (*aph*) (aminoglycoside phosphotransferase, also known as kanamycin kinase type I)	Allows selection of transformants in *E. coli* (Oka *et al.*, 1981; Vakulenko *et al.*, 1987)

Figure A.2 The features of the vector Freedom™ pCHO 1.0 (Thermo Fisher Scientific US, 2022).

Appendix A.3

One example of Elisa plate illustrating the determination of concentrations in cell supernatants, with different dilutions

Strip	1	2	3	4	5
A	Assay buff	IgG1 0.4	C192 48h 1:1	Ov185 48h 1:1	C1921 pool1 1:1200
B	Assay buff	IgG1 0.4	C192 48h 1:1	Ov185 48h 1:1	C192B pool1 1:1200
C	IgG1 0.1	IgG1 0.5	C192 48h 1:10	Ov185 48h 1:10	C192B pool2 1:300
D	IgG1 0.1	IgG1 0.5	C192 48h 1:10	Ov185 48h 1:10	C1921 pool2 1:300
E	IgG1 0.2	C192B 48h 1:1	Ov185B 48h 1:1	C192B pool1 1:300	C1921 pool2 1:600
F	IgG1 0.2	C192B 48h 1:1	Ov185B 48h 1:1	C192B pool1 1:300	C192B pool2 1:600
G	IgG1 0.3	C192B 48h 1:10	Ov185B 48h 1:10	C192B pool1 1:600	C192B pool2 1:1200
H	IgG1 0.3	C192B 48h 1:10	Ov185B 48h 1:10	C192B pool1 1:600	C192B pool2 1:1200

Figure A.3.
Plate design for ELISA with dilution rates, to determinate concentration of supernatants from 48 h post transfections of C192 with B signal and C192 with original signal. 48 h post transfection of Ov185 with B signal and Ov185 with original signal. And after 21 days POOL 1 for C192 with B signal and 25 days from POOL 2 for C192 with B signal, counting from the selection phase 1 day 0.

Appendix A.4

The antibodies with signal sequences cloned in the project

Table 4.1: The antibodies with signal sequences cloned in the project

Name of construct	**Antibody sequence origin**	**Signal sequence**
Ov185	Ov185	Original signal sequence
Ov185+B	Ov185	Signal sequence B
C192	C192	Original signal sequence
C92+B	C192	Signal sequence B

Table 4.2: Design of the six different gene fragments for cloning of C192, Ov185 and SCC140 into Freedom™ pCHO 1.0 vector, that was done in an earlier experiment, but used in this project.

Restrictionenzyme sequence	UTR	Signal sequence	Gene	Restriction enzyme sequence
AvrII	Kozak	Original	Heavy chain	Bst11070I
AvrII	Kozak	B	Heavy chain	Bst11070I
EcoRV	Kozak	Original	Light chain	PacI
EcoRV	Kozak	B	Light chain	PacI