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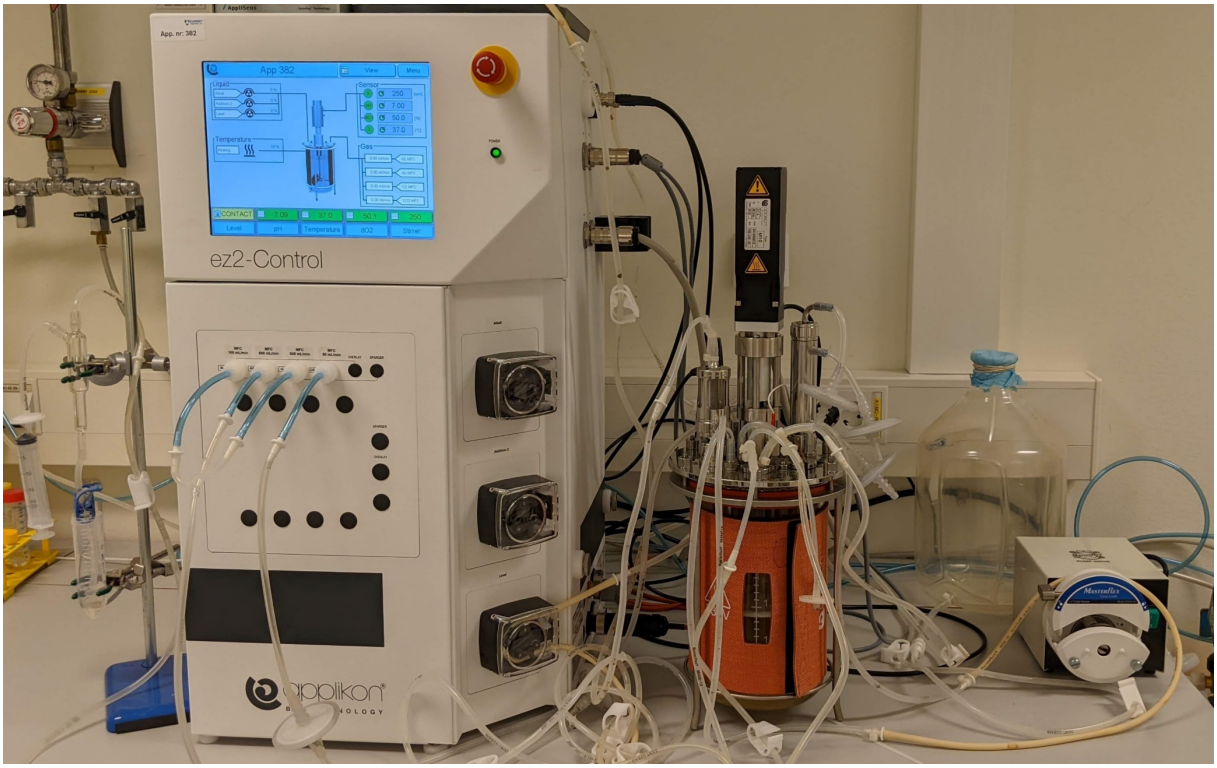

Serum-free production of mAbs: from flask cultivation to perfusion bioreactor cultivation

Adaptation of two hybridoma cell lines towards serum-free cultivation in perfusion bioreactor

Master's thesis in Biology and Biological Engineering

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BIOLOGICAL ENGINEERING

CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover:
[Photography of the 3 L stirred-tank bioreactor and the accompanying control unit used for cultivation of hybridoma cells. More information in 2.3.4]

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Abstract

Monoclonal antibodies (mAbs) are known for their binding affinity and specificity to specific epitopes of an antigen, allowing for various applications in therapeutics and diagnostics. Today, most mAbs are produced from hybridoma cell lines developed by the hybridoma technique introduced by Köhler and Milsten in 1975[1]. Fujirebio Diagnostics AB (FDAB) Gothenburg, Sweden, is a company in close contact with the *in vitro* diagnostics industry. FDAB specialises in production of monoclonal antibodies and antigens, sold both separately and in prepared test kits.

This project contributes to an ongoing project at FDAB with the objective to cultivate mAb-producing hybridoma cells serum-free in perfusion bioreactors. A serum-free production ensures a stable production of mAbs independent on serum availability and/or quality. Cultivation in perfusion reactors intends to scale up the production to a controlled environment with high antibody productivity. In this project, hybridoma cells producing mAbs named E146 are adapted to two commercially available serum-free media from Gibco, namely Chemically Defined (CD) Hybridoma and Hybridoma Serum-Free Medium (SFM). Furthermore, the project evaluates if hybridoma cells producing mAbs named C192 are cultivable in a stirred-tank perfusion bioreactor. Downstream processing for quality control and characterisation of E146/C192 mAbs includes procedures/methods such as purification, size exclusion chromatography (SEC), immunoactivity assay, and isoelectric focusing (IEF).

The results show that E146-producing cells cultivated in CD Hybridoma allow for higher specific antibody productivity (pg mAb $cell^{-1}$ day^{-1}) compared to standard medium and Hybridoma SFM. It is however to be elucidated whether a discovered change in charge distribution among the charge isoforms of E146 affect the quality of the product. E146-producing cells are cultivable in Hybridoma SFM as well but was not a favourable medium from an economical or specific antibody productivity point of view.

The bioreactor cultivation of C192-producing cells encountered various difficulties, such as aggregation and cellular degradation. Aggregation occurred after rapid transitions from log phase to stationary phase and decline phase. Therefore, closer monitoring and regulation of cell specific perfusion rate (CSPR) is suggested to further investigate what causes cell aggregation. Furthermore, a change in charge distribution is seen for charge isoforms of C192 as well, but it does not affect the antigen-antibody binding ability of the mAbs.

Keywords: Monoclonal antibodies, hybridoma cells, serum-free culture medium, stirred-tank perfusion bioreactor.

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List of abbreviations

AB – Assay Buffer
AEC – Anion Exchange Chromatography
BSA – Bovine Serum Albumin
CD Hybridoma – Chemically Defined Hybridoma
CEC – Cation Exchange Chromatography
CoV – Coefficient of Variation
CSPR – Cell Specific Perfusion Rate
CV – Column Volume
DO – Dissolved Oxygen
DW – Dry Weight
ELISA – Enzyme-Linked ImmunoSorbent Assay
FBS – Fetal Bovine Serum
FT – Flow Through
GRP – Gastrin Releasing Peptide
HRP – Horse Radish Peroxidase
Hybridoma SFM – Hybridoma Serum Free Medium
IEF – IsoElectric Focusing
Ig - Immunoglobulin
kDa – kilo Dalton
mAb – monoclonal Antibody
MWCO – Molecular Weight Cut-off
OD – Optical density
PBS – Phosphate-Buffered Saline
pI – isoelectric Point
QC – Quality Control
RT – Room Temperature
SCLC – Small Cell Lung Cancer
SDS-PAGE - Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SEC – Size Exclusion Chromatography
STR – Stirred-Tank Bioreactor
TMB – 3,3',5,5'-Tetramethylbenzidine

1 Introduction

A monoclonal antibody (mAb) is an immunoglobulin (Ig) protein with binding affinity to one specific epitope of an antigen. Ever since Köhler and Milstein introduced the hybridoma technique in 1975[1], mAbs have been established as a powerful tool in therapeutics and diagnostics[2]. The hybridoma technique allows for an amplified and simplified production of mAbs with high affinity and specificity towards the desired antigen[1]. Fujirebio Diagnostics AB (FDAB) Gothenburg, Sweden, is a company in close contact with the *in vitro* diagnostics industry to which they provide a wide range of antibodies and antigens, sold both separately and in prepared test kits. Today, the production of monoclonal antibodies at FDAB is performed by letting hybridoma cells grow in plastic bottles whereafter the secreted monoclonal antibodies are purified and concentrated to generate a small volume with highly concentrated mAbs. The medium in which the hybridoma cells grow contains serum extracted from bovine fetuses to enhance the growth. There is an ongoing development project at FDAB in Gothenburg with the aim to scale up the production of monoclonal antibodies and simultaneously adapt the hybridoma cells to cultivation in serum-free medium. The upscaling is to take place in perfusion bioreactors with the ambition to increase the production rates and hence enable FDAB to expand their businesses. A serum-free cultivation is intended to ensure a stable production of high quality mAbs independent on serum availability and/or quality.

Upscaling and serum-free cultivation however entail several issues to be addressed. Hybridoma cells have varying sensitivity towards changes in culture medium and cultivation environment. Therefore, each hybridoma cell line needs to be adapted individually, with careful monitoring of cellular growth and antibody production. Furthermore, quality of produced mAbs is of uttermost importance to in the end ensure stable and reliable diagnostics results. The antibodies produced must therefore be characterised and undergo elaborate quality controls.

1.1 Aim

The aim of this master's thesis is to contribute to the above-mentioned project at Fujirebio Diagnostics AB in two separate projects. The first project is called "Serum-free production E146" and will evaluate if hybridoma cells producing the antibody E146 are cultivable in a serum-free medium. The second project is called "Bioreactor production C192" and will develop a process for production of an antibody named C192 in a perfusion bioreactor. Hybridoma cells producing C192 are already adapted to cultivation in serum-free medium.

1.2 Limitations

This project will not include the upscaling of all available cell lines at Fujirebio, nor investigate serum-free cultivation of all cell lines. Two specific hybridoma cell lines are instead evaluated individually. The project will neither consider how the upscaling of the specific cell line influences the long-term business value of the antibodies produced. Furthermore, there are many serum-free media commercially available but only two are evaluated in this project. Lastly, the only type of bioreactor evaluated in this project will be a 3 L stirred-tank perfusion bioreactor.

1.3 Specification of issues under investigation

For cells producing E146, the following issues will be addressed:

- How is cell density (cells mL^{-1}), viability (%), specific growth rate (day^{-1}), and specific antibody productivity (pg mAb $cell^{-1}$ day^{-1}) affected by cultivation in serum-free medium?
- Can a cell bank be established with cells cultivated in serum-free medium?

- How is antigen-antibody binding ability, size, and net charge of E146 affected by serum-free cultivation?

The following issues will be addressed when cultivating C192-producing cells in a perfusion bioreactor.

- How can the cultivation be scaled up from cell culture flask to bioreactor?
- How is cell density (cells mL^{-1}), viability (%), specific growth rate (day^{-1}), and antibody production (mg mAb mL^{-1}) affected by cultivation in bioreactor?
- How is antigen-antibody binding ability, size, and net charge of C192 affected by bioreactor cultivation?

2 Theory

This chapter is divided into four subchapters. The first subchapter covers how mAbs are developed and explains their characteristic structure. Furthermore, the subchapter introduces the two mAbs produced in this project. The second subchapter introduces cultivation of hybridoma cells from a theoretical point of view. Important theory when monitoring cell growth, such as cell density, viability, specific growth rate, and specific antibody productivity is presented. The practical part of hybridoma cell cultivation is then explained in subchapter three. This subchapter covers general cell cultivation practice, introduction to cell culture media, hitherto established cultivation strategies, and lastly an introduction to bioreactor cultivation at FDAB. The fourth and last subchapter presents downstream processing methods used when purifying, quality controlling, and characterising the produced mAbs.

2.1 Monoclonal antibodies

In this subchapter, it is described how development of monoclonal antibodies is performed. Furthermore, mAb's characteristic structure is described and the two mAbs E146 and C192 are introduced.

2.1.1 Development of monoclonal antibodies

Development of monoclonal antibodies is nowadays mostly done using the hybridoma technique introduced by Köhler and Milstein in 1975[1]. The technique fuses antibody-producing cells, such as spleen cells, with mouse myeloma cells to create a cell line of so called hybridoma cells. The hybridoma cells can, because of the fusion, produce the monoclonal antibody of interest and simultaneously divide itself infinitely, allowing for eternal production of mAbs. The methodology of the technique starts with injection of antibody targets, so-called antigens, to a mouse. Antigen injection triggers an immune response where the plasma cells of the spleen produce antibodies towards the antigen. The antibody-producing plasma cells are then isolated and fused with cancerous plasma cells, so called myeloma cells, to create hybrid cells. At this stage, there is a mix of different hybridoma cells, each producing a wide range of antibodies with different capability for antigen binding. Therefore, a screening assay looking for a hybridoma cell producing the most suitable mAb is performed[2]. The suitability of the mAbs can for instance be evaluated in terms of specificity, affinity, stability, and solubility[3]. Hybridoma cells are then cultivated in larger scale to produce the monoclonal antibody of interest.

2.1.2 Structure of monoclonal antibodies

Antibodies, mAbs included, constitute of two heavy chains and two light chains, as visualised in Figure 1. The chains are linked with disulphide bonds such that the characteristic Y-shape is formed. Antigen binding site is situated at the variable region of the two Fab regions.

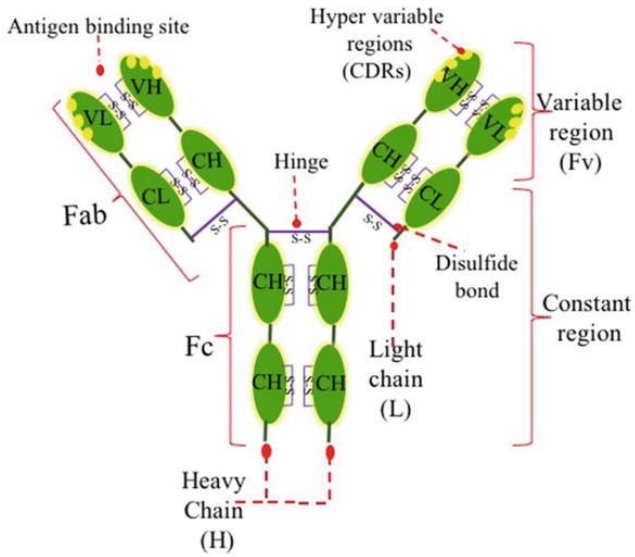

Figure 1: Structure of general (monoclonal) antibody. The antibody consists of two heavy (H) polypeptide chains bound to each other in the Fc region, and two light (L) polypeptide chains bound to the Fab regions of the heavy chains. Antigen binds to the variable (V) region of the heavy respectively light chains. Both type of chains also contain a constant I region. S refers to disulphide bonds between the chains, CDRs means complementarity determining regions[4].

Antibodies are divided into classes and subclasses. Classes are for instance IgG, IgM, and IgD where IgG is the most common class. Each class is then divided into subclasses according to decreasing abundance, such as IgG1, IgG2, IgG3, and IgG4. Different classes have different Fc regions and different functional properties. Subclasses instead differ in hinge structure and in their heavy constant region[4]. Due to the varying nature among different classes and subclasses of mAbs, information on specific antibodies used in this project is provided below.

2.1.3 Monoclonal antibodies E146 & C192

E146 is a monoclonal antibody developed for binding to a ProGRP 31-98 epitope. ProGRP 31-98 is a precursor to Gastrin Releasing Peptide (GRP) which in turn is a hormone secreted from Small Cell Lung Cancer (SCLC). E146 is therefore used to detect and monitor therapy response to lung cancer. 31-98 refers to the specific carboxy-terminal region of the hormone where E146 binds.[5] Finally, E146 is documented at FDAB as an IgG1 antibody that is secreted extracellularly during cell growth. The cells grow solubilised in culture medium. Cells for production of E146 are available in a serum-based cell bank, stored at -170 ºC.

C192 is also an extracellularly secreted IgG1 mAb. C192-producing cells grow suspended in culture medium and are available in a serum-free cell bank at FDAB, stored at -170 ºC. The mAbs are used to detect various gastrointestinal malignancies, such as gall bladder, pancreatic, colorectal, and gastric cancers. C192 binds to sialyl Lewis epitopes on the CA19-9 antigen[6].

2.2 Hybridoma cell cultivation theory

This subchapter covers theory on how hybridoma cells grow and how their growth is monitored.

2.2.1 Hybridoma cell growth

Hybridoma cells have four different growth phases when cultivated under optimal culture conditions. First the lag phase where the cells adapt to the surroundings without dividing. Thereafter the log phase where the number of cells increases exponentially. The cells then enter the stationary phase where they once again divide very slowly, or not at all. In a batch cultivation, the transition to stationary phase would for instance origin from nutritional deficit, production of toxic compounds, or space shortage. Lastly, if cells are kept in the stationary

phase for a while the cells start dying, the cellular growth has thus reached the decline phase[7]. Noteworthy, it has been reported that different types of hybridoma cells differ considering in which phase mAbs are produced. The antibodies are for instance potentially produced mostly in the lag phase and beginning of log phase. They can additionally also have a peak in mAb production towards the end of the exponential phase. There is also a third alternative where the cells continuously produce antibodies until transition to the stationary phase[8]. When monitoring growth of mAb-producing hybridoma cells there are three well established factors to observe: cell density, cell viability, and specific antibody productivity[8-12].

2.2.2 Cultivation monitoring

Cell density expresses how many cells there are in a sample per volume unit of medium. There is a wide range of methods for measuring cell density[11, 13, 14]. These methods are divided into two groups. One group where the exact number of cells in a small sample volume is counted, allowing for distinction between living and dead cells. The other group of methods do not count individual cells and can therefore not distinguish between living and dead cells. Manual counting in a hemacytometer is a long-standing but still widespread method for counting of individual cells. The method is affordable and gives reliable results, but with certain reproducibility difficulties. Manual counting is also relatively time consuming and hence not suitable for high throughput. Other methods for counting of individual cells are automatic cell counting and flow cytometry. Automatic cell counting is based on image analysis algorithms and is more expensive than manual counting. Furthermore, the method is reported to not be as accurate and precise as manual counting but allows for higher throughput. Flow cytometry is a highly reproducible method that is based on fluorescent labelling of cells that are counted when flowing through a cytometer. The method allows for higher throughput compared to manual counting, but it suffers from being even more expensive than automatic cell counting in addition to still lacking accuracy and precision. All three methods mentioned above allows for counting of both living and dead cells in a sample[13].

A method which does not count individual cells is optical density (OD). In OD, light absorption of a sample, for instance at 780 nm, is measured and related to a standard curve with known cell densities for cell density determination. With this method, it is possible that other molecules and particles that are not accounted for absorb light, hence introducing uncertainties to the cell density value. On the contrary, the method allows for high throughput while possibly only requiring ordinary laboratory materials and instruments[11]. Another method for measuring cell density without counting individual cells is to analyse the dry weight (DW) of a sample. DW is measured by removing liquid from the sample followed by weighting of the remaining solids. A drawback with this method is that a relatively large sample volume is needed to obtain a measurable weight. Another drawback is that DW is evaluated collectively for all solids in a sample, introducing variability depending on sample constituents[14].
Cell viability denotes percentage living cells in a sample and is hence an indication of cell culture condition[7]. Viability is calculated by dividing number of living cells by total number of cells and multiplying by one hundred[8]. Viability is hence calculated from cell density measurements, subject to the condition that cell density is estimated from a method where individual cells (both living and dead) are counted.

Furthermore, specific growth rate of the cultivated cells is also estimated from cell density measurements. Specific growth rate declares how many times the cells in a culture divide per time unit and per cells available in the cultivation environment. Specific growth rate is only applicable during the log phase. There are many models available for estimation of specific

growth rate, based on various mass balances etc[15, 16]. To simplify, cell density N_2 (cells mL^{-1}) at a particular time t_2 (days) is expressed as

$$N_2 = N_1 e^{x(t_2 - t_1)} \tag{1}$$

where N_0 (cells mL^{-1}) is the cell density at a previous time t_1 (days), and x (day^{-1}) is the specific growth rate. Equation (1) then implies that

$$x = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \tag{2}$$

in day^{-1}.[8]

The last factor to monitor during cell growth is specific antibody productivity. Specific antibody productivity is expressed as amount mAbs produced per cell and per day (µg mAb $cell^{-1}$ day^{-1}). The productivity is estimated by dividing antibody concentration (µg mL^{-1}) with cell density (cells mL^{-1}) and cultivation days. Antibody concentrations are frequently determined with sandwich enzyme-linked immunosorbent assays (ELISA) in 96-well plate formats[17]. Sandwich ELISAs allow for concentration determination without modifying the analysed antibody. The method starts with immobilising capture antibodies on the surface of each well of the well plate. Capture antibodies then in turn immobilises the mAbs of interest by binding to their Fc region. Thereafter, a mixture of various enzyme-labelled polyclonal antibodies is added. Labelled antibodies with binding specificity to the mAbs of interest then bind to the variable regions of the mAbs. The enzyme label, situated on the Fc region of the secondary antibody, then cleaves an added substrate which in turn induces a colour shift. The intensity of the induced colour increases the more substrate is cleaved, meaning that higher concentration of mAb of interest results in higher colour intensity. Colour intensity is measured by a spectrophotometer. A standard curve containing antibodies with known concentration then relates the signal to antibody concentration[18].

Furthermore, there is also a possibility to measure antibody concentration in a sample by measuring the absorbance at 280 nm. At this wavelength, aromatic amino acids present in the antibodies absorb light and are via Lambert-Beers law related to antibody concentration. However, the method is mostly applicable on purified samples of antibodies where the background noise from potentially interfering particles is low[7, 18, 19]. The method will therefore be more thoroughly explained later.

2.3 Hybridoma cell cultivation practice

This section starts with an introductory subchapter about general cell cultivation practice. It is then followed by subchapters about cell culture medium, common cell cultivation environments, and lastly a subchapter introducing bioreactor cultivation at FDAB.

2.3.1 General cell cultivation practice

Practises for cultivation of hybridoma cells is a diverse topic that is heavily dependent on cultivation purpose, production scale, individual cell characteristics, etcetera. A general principle is however to cultivate hybridoma cells in a sterile workspace. An aseptic workspace ensures that laboratory technicians are protected against potentially harmful material in the cell culture while contaminants, such as bacteria, viruses, and fungi, are kept away from the cell

culture. Hybridoma cell lines are generally stored in a cell bank consisting of many separate vials stored in a liquid nitrogen freezer at -170 ºC. In the freezer, the cells are inactive and do not grow or produce antibodies. When a mAb is to be produced, a vial of cells is thawed and suspended in cell culture medium in a suitable cultivation environment. The cells then start following the four growth phases described earlier. While growing, the cells will thus secrete mAbs to the culture medium, but they will also consume nutrients from the medium while occupying more and more space in the cultivation environment. Additionally, cells generally secrete toxic metabolites[20] such as lactate and ammonia to culture medium while growing. The cells also release additional toxic metabolites upon death[21].

If the cells are cultivated in a batch cultivation environment, the cells will after a while suffer from nutrient deficit, space deficit, and/or intoxication. To avoid that, cells in a batch cultivation are split to a new passage meaning that either all or some cells are moved from the previous environment to a new environment, to which fresh culture medium is added. Cell growth and production of mAbs thereafter continues in the new passage. When splitting to a new passage, medium volume and cultivation environment size could be increased to allow for increased maximum cell number and thus increased production of mAbs. Noteworthy related to splitting of cells is also that some cells grow freely in medium suspension, while some are adherent to cultivation environment surface. Such characteristics, together with cell specific characteristics, determines how and when the splitting is performed[20].

2.3.2 Cell culture medium

Traditionally, culture medium for animal cells is a complex mixture of inorganic salts and nutrients that are meant to resemble a natural animal environment. To enhance the growth of the cells, serum from for instance bovine or fetal calf is often added to the cell culture as well. Serum is chemically undefined but is known to contain hormone-like growth factors and additional nutrients[7]. There are however numerous concerns regarding usage of serum in cell cultivation. Firstly, the usage of serum raises ethical concerns since it is extracted from pregnant cows during slaughter. Another concern is the uncertainties a chemically undefined component of a medium entails. This uncertainty needs to be addressed by variability studies coupled to every new batch of serum that is acquired. Furthermore, protein concentration is high in serum which potentially implies more complex downstream processing of produced mAbs. Lastly, serum is expensive relative other medium components, and it is a source of contaminants to both cell culture and laboratory technicians. Possible contaminants in cell culture are for instance prions, viruses, and mycoplasma[22]. For laboratory personnel, infection with bovine spongiform encephalitis (‘mad cow disease’) is one of the risks[7].

Considering the concerns with cultivation media containing serum, methods for serum-free production of monoclonal antibodies are investigated. One approach to solve the problem is to shift the manufacturing of mAbs from *in vitro* production towards *in vivo* production in humans[23] or model animals such as mice[22, 24]. Production directly in humans would be beneficial when manufacturing mAbs for therapeutic use[23] but is unnecessarily complex for diagnostics industry. Manufacturing of mAbs in mice was however once the preferred method. The method uses ascites induction, meaning that a tumour is induced in a mouse in which the antibodies then are produced. Due to ethical concerns in combination with the advent of satisfactory *in vitro* cultivation methods, production in mice has been discontinued[24]. Another method for producing mAbs without using serum is to adapt the hybridoma cells to cultivation in serum-free medium. The adaptivity of a cell line to a new medium is individual and is therefore evaluated separately for each cell line[12]. There are various media available on the

market to evaluate. Although the transition to serum-free medium has been investigated for more than four decades[25], there are still cell lines to be adapted[12, 26, 27].

2.3.3 Cultivation environments

Cultivation of hybridoma cells is performed in a wide range of cultivation environments. From small and simple 96-well plates[20] to large and more complex bioreactors with working volume of several thousand liters[28]. A 96-well plate format is beneficial when screening for suitable antibodies during hybridoma development[29] but is apparently less useful for production of larger quantities of mAbs because of low medium volume per well. The standard cultivation environment for hybridoma cells is however in T-flasks. T-flasks are plastic bottles with a favourably large surface area due to their cuboidal shape. Furthermore, T-flasks are available in different sizes allowing partly for starting of a cultivation by suspending cells from the freezer in a small flask, but also for scale-up projects where flask size is increased gradually over passages[30]. Flask cultures are commonly incubated at 5-7 % CO_2 at 37 °C[29, 31, 32]. Filtered caps on the T-flasks allow for gas exchange through the filter without risk for contamination. A drawback with T-flasks however is that they are normally not stirred or shaken during cultivation. Consequently, medium constituents and gases are not well distributed around all cells in the culture. Additionally, cultivation in T-flasks is not suitable when large volumes of mAb product is intended.

There are however larger-scale batch cultivation environments which allow for mixing of culture medium, namely roller bottles and spinner flasks. Roller bottles are cylindrical bottles with filtered caps where main benefits being their readiness to operate and their scalability. Therefore, they are commonly used as the final mAb production environment where numerous roller bottle cultivations take place simultaneously to produce large number of antibodies. They are incubated at the same temperature and percent CO_2 as T-flasks, but as the name indicates, they are also continuously rolled during incubation for uniform distribution of nutrients and gases[32-34]. Spinner flask cultures on the other hand are instead mixed by an impeller situated inside the flask, driven by a spinner apparatus. As for other batch environments, the flasks allow for gas exchange while incubated in 5-7 % CO_2 at 37 °C. Spinner flasks are just like roller bottles used as final mAb production cultivation environments[35]. A final joint notation on roller bottles and spinner flasks is however that release and accumulation of toxic compounds is still a concern due to both methods being batch cultivations[31, 35].

The demand on large production volumes of mAbs has pushed the development of cultivation environments beyond aforementioned batch modes, starting over three decades ago[31]. Although there have been attempts to modify existing batch cultivation environments to allow for higher mAb productivity, the results have not been fruitful enough for implementation into larger-scale production[32]. The most established solution to satisfy high production demands is instead various types of stirred bioreactors[28, 31, 32, 34, 36-40]. Bioreactor cultivation environments allow for beneficial monitoring and regulation of growth-influencing factors such as temperature, pH, stirring, cell concentration, and dissolved O_2 (DO). Consequently, bioreactors are thus used to establish an optimal cultivation environment with high cell viability, cell density, and mAb productivity, where release and accumulation of toxic metabolites is minimized[28]. Bioreactors can be run in batch mode just like other cultivation environments, but a major advantage is the possibility to readily switch to fed-batch, continuous, or perfusion mode.

In fed-batch mode, fresh medium is added continuously during the cultivation. Fed-batch mode allows for increased cell density and mAb productivity when compared to batch mode. Furthermore, fed-batch cultures are just like batch cultures easy to scale up and easy to

operate[32]. There are nevertheless disadvantages as well. There is still release and accumulation of toxic metabolites in a fed-batch culture[34]. To minimize this, continuous cultivation is an option. In this mode, there is partly an inlet of fresh medium but also an outlet of culture medium. The outlet thus contains a mixture of spent medium, mAbs, cells, etc. Typically, inlet and outlet flow rates are kept at equal levels such that cell density in the reactor is kept constant at a satisfactory level. This is achieved by not letting the dilution rate, i.e., the outflow rate divided by reactor volume, exceed the cell maximum specific growth rate. In this way, a steady state is developed allowing for stable production of mAbs. In addition, a steady state cultivation is appropriate when studying cell physiology or production kinetics. The drawback with a continuous culture however is low product yield due to cells being washed out by the outlet flow.

To amplify product yield but maintaining a highly controlled cultivation process while minimizing release and accumulation of toxic metabolites, perfusion cultivation is appliable. A perfusion system is a continuous system with an attached cell retention system, meaning that cells are separated from the outflow and instead recirculated to the reactor[28]. Perfusion cultivations support high cell density cultures with high viability numbers since cells are not washed out of the reactor. The cells are maintained at a high cell density and viability for a long time, allowing for higher mAb productivity but in a smaller scale relative batch and fed-batch cultures[32]. A high viability culture with an outflow contributes to minimized release and accumulation of toxic compounds. The major drawbacks with perfusion cultures are that large volumes of possibly expensive medium is consumed, while diluting the product and thus complicating product recovery[34]. However, residence time for mAbs in the bioreactor is reduced relative batch and fed-batch cultures which potentially improves product quality[32].

The joint flow rate of inflow and outflow at which a perfusion reactor is operating is called perfusion rate. Considering what is mentioned in the previous paragraph, perfusion rate is thus a crucial factor for maintaining high cell density, viability, and antibody productivity, without spending unnecessary amounts of medium or complicate downstream processing [32, 34]. One routinely used method for applying a suitable perfusion rate to a cultivation is to apply a Cell Specific Perfusion Rate (CSPR). With CSPR (nL cell^{-1} day^{-1}), each individual cell in the culture is provided same amount of new medium every day, regardless of total number of cells[37, 38]. CSPR is calculated according to Eq. (3):

$$CSPR = \frac{D}{X} \tag{3}$$

where X is cell density (x10^6 cells mL^{-1}) and D is dilution rate (L L^{-1} day^{-1})[37]. D is in turn calculated according to Eq. (4).

$$D = \frac{F * 24}{V_{reactor}} \tag{4}$$

with F being flow rate in L/h and $V_{reactor}$ is reactor liquid volume (L).

CSPR is typically set to 0.05-0.5 nl/cell/day, depending on the cells being cultivated. Optimal CSPR is hence to be determined for each cell line. An optimal CSPR is where the cells are sufficiently provided with medium to survive and produce mAbs, but where the mAbs are not unnecessarily diluted in expensive medium. One method for determining optimal CSPR is to

utilize the "Push-to-Low" approach. This approach starts at relatively high CSPR and then pushes the CSPR down to as low as possible. By keeping the cell density constant in the reactor, it is possible to determine minimum CSPR by monitoring when cell density starts decreasing. Suitable initial CSPR is suggested to be 0.2 nLcell/day[37] to 0.3 nL/cell/day[38].

2.3.4 Perfusion bioreactor cultivation at Fujirebio Diagnostics AB

For the scope of this project, it is of interest to develop a robust cultivation procedure, with potential of large-scale production of mAbs. Therefore, perfusion cultivation is considered advantageous. Furthermore, FDAB possess a stirred-tank bioreactor (STR) equipped with a marine impeller for stirring. For recirculation of cells, the STR is accompanied with a cell retention using high frequency resonant ultrasonic waves to separate cells from medium. STRs are the most conventional types of bioreactors and are especially suitable for non-adherent cells, which will come to be suitable for this project. However, STRs induce relatively high shear stress on cells in the reactor[28, 34]. Stirring rate is thus an important factor when developing a robust cultivation process.

Due to shear stress concerns, the project will investigate the tolerability of shear stress or more precisely the optimal stirring rate specifically for each hybridoma cell line being adapted to STR cultivation. Different impellers induce varying degrees of shear stress[41], but to avoid unnecessary cultivation rounds in the bioreactor, the optimal stirring rate will be evaluated through cultivation in spinner flasks. Optimal stirring rate in spinner flask is then used to calculate optimal stirring rate in the reactor. It is assumed that equal impeller tip speed in spinner flask and bioreactor induces equal shear stress to surrounding cells[41]. First, spinner flask impeller tip speed $v_{spinner}$ (m rpm^{-1}) is calculated according to Eq. (5).

$$v_{spinner} = \pi * d_{spinner} * n_{spinner} \tag{5}$$

where $d_{spinner}$ is diameter of spinner impeller (m), rotating in $n_{spinner}$ rotations per minute (rpm). Same equation applies for reactor impeller tip speed $v_{reactor}$ (m rpm^{-1}) (Eq. (6)).

$$v_{reactor} = \pi * d_{reactor} * n_{reactor} \tag{6}$$

where $d_{reactor}$ (m) is diameter of reactor impeller and $n_{reactor}$ (rpm) is impeller rotations per minute. Thereafter, $v_{spinner}$ is set equal to $v_{reactor}$, allowing for solving of $n_{reactor}$ according to Eq. (7).

$$\pi * d_{spinner} * n_{spinner} = \pi * d_{reactor} * n_{reactor} \rightarrow n_{reactor} = \frac{d_{spinner} * n_{spinner}}{d_{reactor}} \tag{7}$$

where $n_{reactor}$ (rpm) hence is optimal stirring rate for the bioreactor marine impeller, valid for a specific hybridoma cell line. Equation (7) assumes that impellers with equal tip speed induce equal shear stress to the surroundings. In other words, it is assumed that varying cultivation environment geometrics and/or different type of impellers do not affect Equation (7) greatly, which have been shown to not always be correct[41].

2.4 Downstream processing

This subchapter covers downstream processing procedures commonly performed after antibody production.

2.4.1 Harvesting, concentration, and purification

Downstream processing of mAbs starts with harvesting and concentration of cell culture medium. Harvesting is performed to remove larger particles such as cell debris from the mAb-containing medium. Concentration in turn is performed to decrease culture volume, potentially simplifying subsequent steps in the process. Harvesting and concentration can be done simultaneously by for instance centrifugation of culture medium, or separately through filtration followed by tangential flow filtration. Thereafter, the mAbs are purified. Purification is in two out of three industrial processes performed with Protein A affinity chromatography. Protein A located inside the chromatography column binds to the Fc region of IgG antibodies, thus allowing for separation of IgG mAbs from other compounds. 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[42].

2.4.2 Antibody quality control and characterisation

After purification, there is a wide range of methods for quality control (QC) and characterisation of the purified mAbs of interest. QCs are performed to confirm that the antibodies of interest have been successfully purified. The antibodies are also characterised, for instance in terms of size and isoelectric point (pI). QC and characterisation are combined by comparing results of mAbs of interest with reference mAbs. Reference mAbs can for instance be mAbs cultivated in standard medium, or in roller bottles instead of bioreactor. In that way, it is detected if an altered cultivation procedure induces any changes to the mAbs.

Purified antibodies are as indicated in 2.2.2 detectable and readily concentration determined in a spectrophotometer at 280 nm, assuming there are aromatic amino acids present in the mAb[7, 18, 19]. The spectrophotometer measures absorbance A_{280} at 280 nm, given by the Beer-Lambert law as:

$$A_{280} = \varepsilon_{280} * c * l \tag{8}$$

where ε_{280} is the absorptivity (mL mg^{-1} cm^{-1}) at 280 nm for the antibodies being analysed, c is the antibody concentration (mg mL^{-1}), and l is cuvette path length (cm)[19].
E146 and C192 are both reported by FDAB to contain aromatic amino acids. The method is therefore appropriate for concentration determination while verifying that purification elute contains antibodies.

Another quality control is size exclusion chromatography (SEC). In SEC, the mAbs and other particles are separated based on size. Small particles elute later than large particles due to longer travel distance through the column. The method is thus also able to separate mAb aggregates, decomposed mAbs, and other molecules from normal size antibodies. All constituents that are washed out of the column are detected by measuring absorbance at 280 nm after which their relative amount is reported in a chromatogram. The chromatogram is then used to calculate the percentage of normal size mAbs versus other particles. SEC thus evaluates the purity of a purified mAb sample[43].

There are other chromatography methods applicable as well, such as anion exchange chromatography (AEC), cation exchange chromatography (CEC), and hydrophobic interaction chromatography (HIC). Such methods are combinations of characterisation and QC. In AEC, the chromatography column contains positively charged particles which bind to negatively charged sites of antibodies and other particles. An antibody or particle with high negative net charge binds stronger to the column than an antibody or particle with lower negative net charge. It is therefore possible to use an elution gradient with increasing salt concentration to separate antibodies/particles with different net charge. Particles with weak bonding to the column elute at low salt concentrations, while particles that are strongly bond to the column elute at high salt concentration. CEC functions in the same way as AEC, but with a negatively charged column. HIC instead uses a hydrophobic column and separates antibodies and other particles based on their hydrophobicity. Just like for SEC, all constituents washed out of the column are detected through measuring absorbance at 280 nm[2, 7]. AEC, CEC, and HIC are reported as "polishing steps" when purifying antibodies, for instance suitable when Protein A chromatography is not applicable, when there are host cell impurities, or for viral clearance[44]. However, if the antibodies have been satisfactory purified, any of the three chromatography methods are instead used to compare charge/hydrophobicity properties of mAbs of interest with reference mAbs[45, 46].

FDAB have developed an ELISA-based method for estimation of relative antibody immunoactivity. In the assay, it is evaluated whether the antigen-antibody binding ability differs between the mAbs of interest and reference mAbs. The assay is composed of immobilised antigens, biotin labelled mAbs, reference mAbs, mAbs of interest, HRP-labelled mAbs, and TMB. The antigens are immobilised by the biotin labelled mAbs in the wells of a 96-well plate. It is then evaluated how much the signal from the HRP-labelled mAbs is inhibited when reference mAbs respectively mAbs of interest compete with the labelled mAbs about antigen binding. The inhibition is related to the signal obtained when only labelled mAbs bind to the antigen. The signal inhibition (%) is hence expressed as

$$Inhibition = \frac{(Abs\ (labelled\ mAb) - blank) - (Abs\ (inhib.mAb) - blank)}{(Abs\ (labelled\ mAb) - blank)} * 100 \quad (9)$$

where Abs (labelled mAb) is absorbance from only labelled mAbs, blank is absorbance from capture antibodies and antigens, and Abs (inhib. mAb) is absorbance from reference mAb or mAb of interest in combination with labelled mAbs. Relative immunoactivity (%) is then evaluated according to Eq. (10).

$$Relative\ immunoactivity = \frac{Inhibition\ (mAb\ of\ interest)}{Inhibition\ (reference\ mAb)} * 100 \quad (10)$$

where Inhibition (mAb of interest) and Inhibition (reference mAb) are determined from Eq. (9).

Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is another method used for combined QC and characterisation of mAbs. SDS-PAGE is a gel electrophoresis method where proteins are separated based on size. The separation takes place in a polyacrylamide gel which is a highly cross-linked gel with pore size small enough for retarding migration of proteins. However, before proteins are loaded on the gel, they are heated in the presence of SDS and eventually mercaptoethanol[2]. If mercaptoethanol is present, the proteins are reduced meaning that disulphide bonds are destroyed. If mercaptoethanol is not present, the

disulphide bonds remain intact[47]. 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 nevertheless 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[2]. In the case of antibodies in SDS-PAGE, there is a major difference between reduced and non-reduced mAb samples. In reduced samples, all chains are separated due to breaking of disulphide bonds. It is reported that reduced mAbs analysed with SDS-PAGE usually show one band at approximately 50 kDa, being the heavy chains, and one band at approximately 25 kDa, being the light chains. Non-reduced mAbs are on the other hand reported to give rise to a single band at approximately 150 kDa[47].

Another gel electrophoresis method for QC and characterisation is isoelectric focusing (IEF). IEF separates proteins based on their isoelectric points (pI), which is where the protein's net charge is zero. IEF gels contain a pH gradient such that when a potential difference is applied, proteins migrate through the gel until their net charge is zero. The pI of the analysed sample is then determined through comparing with a reference containing proteins with known pI[7]. Noteworthy, IEF is highly sensitive and therefore allows for detection and analysis of different charge isoforms. Charge isoforms are mAbs with small differences in pI, originating from small variations in amino acid sequence and/or post-translational modifications. There is a wide range of post-translational modifications reported to change the charge of mAbs. Some examples are sialylation, deamination, asialylation, succinimide formation[48], and phosphorylation[7]. Detection and analysis of charge isoforms is nevertheless important since variations potentially affects crucial antibody characteristics. A change in the variable region may for instance impair antigen binding specificity and/or affinity, while a change in the constant region may not be as severe[48].

3 Methods and Materials

3.1 Serum-free production E146

3.1.1 Strains and media

E146-producing cells were received from a cell bank at FDAB, kept in a liquid nitrogen freezer at -170 ºC. The project with E146-producing cells evaluated two serum-free media from Gibco, namely Chemically Defined Hybridoma, referred to as CD Hybridoma, and Hybridoma Serum Free Medium, referred to as Hybridoma SFM. In CD Hybridoma medium, 40 ml GlutaMAX supplement from Gibco was added per litre medium. The two serum-free media was compared to standard medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with 5 % fetal bovine serum (FBS) and 1 % DMEM supplement added to it.

3.1.2 Cultivation procedures

Serum-free cultivation of E146-producing hybridoma cells took place in two separate rounds. In both rounds, the cells were cultivated using T75 flasks with filtered caps, incubated in 8 % CO_2 at 37 ºC. Cell density, cell viability, and antibody concentration was estimated at the end of each cultivation passage. The cells were stored in vials containing $2.2x10^6$ cells per vial, with medium consisting of 90 % FBS and 10 % dimethyl sulfoxide (DMSO). To start a cultivation round, one vial of cells was thawed in tepid water. Thereafter, the cells were suspended in 8 mL refrigerator cold DMEM before centrifuged at 200×g for 5 minutes. The supernatant was discarded whereafter the cells were resuspended in cultivation medium. From here, the

cultivation strategy differed on a few points when comparing the two rounds. Each round will hence hereafter be described separately.

In the first round, the cells were resuspended in a T25 flask with medium containing DMEM with 1 % DMEM supplement and 20 % FBS. When the cell density was high enough (see 3.1) the cells were split into passage 1 (p1), consisting of one T75 flask with standard medium. The initial cell density in passage 1 was $5x10^4$ cells mL^{-1}. In passage 2, the serum-free cultivation started through splitting the cells to T75 flasks containing CD Hybridoma or Hybridoma SFM. The initial cell density was $6x10^4$ cells mL^{-1} for all cells in serum-free medium. As reference, the cells were cultivated in standard medium as well: one flask with starting cell density $6x10^4$ cells mL^{-1} (denoted "+") and one flask with starting density $4x10^4$ cells mL^{-1} (denoted "-"). In passage 2, only one flask of cells was set in CD Hybridoma due to lack of cells in passage 1. This flask was split into two separate T75 flasks in passage 3. For full flask setup during all ten passages, see Figure 2. Samples for determining antibody concentration with ELISA were taken at the end of each passage, except for at p0, p1, and p7. Antibody determination samples were stored at -18 ºC until analysis.

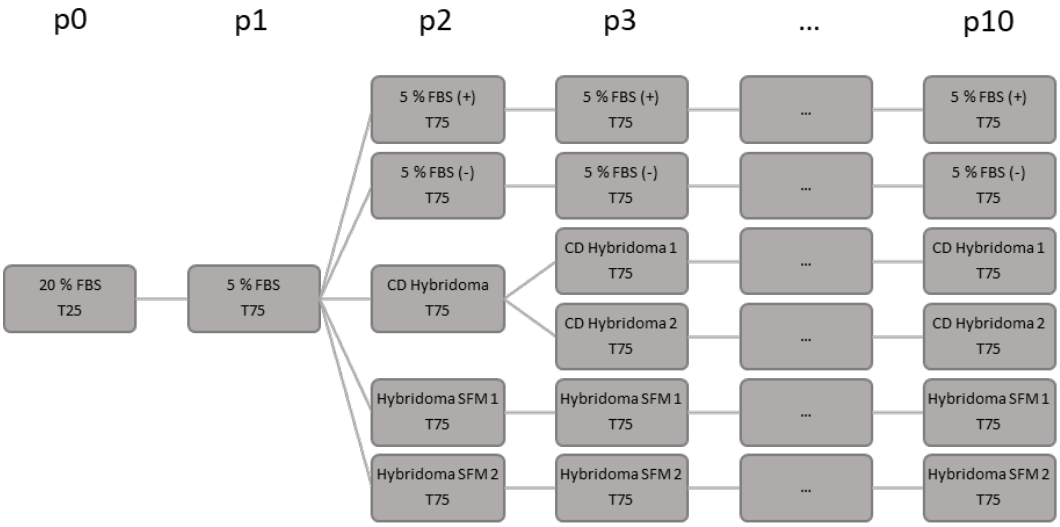

Figure 2: Experimental procedure for the first adaptation of E146-producing cells to serum-free medium. Stated in each box is cultivation medium and T-flask size. "+" denotes an initial cell density of $6x10^4$ cells mL^{-1}, "-" denotes an initial cell density of 4 $x10^4$ cells mL^{-1}. Initial cell density for cells in CD Hybridoma and Hybridoma SFM was 6 $x10^4$ cells mL^{-1}. The cells were cultivated for ten passages (p0 – p10).

In the second round of serum-free cultivation, the cells were resuspended in a T75 flask with standard medium after centrifugation. When possible, the cells were split to CD Hybridoma and Hybridoma SFM with the same initial cell density as in round one ($6x10^4$ cells mL^{-1}). Same setup of reference flasks (+ and -) was used again. The full flask setup for all passages in round two is seen in Figure 3. Samples for antibody concentration determination were taken from all passages, except for cells in p4 cultivated in standard medium (+ and -) and Hybridoma SFM. As in round one, the samples for antibody concentration determination with ELISA were stored at -18 ºC until analysis.

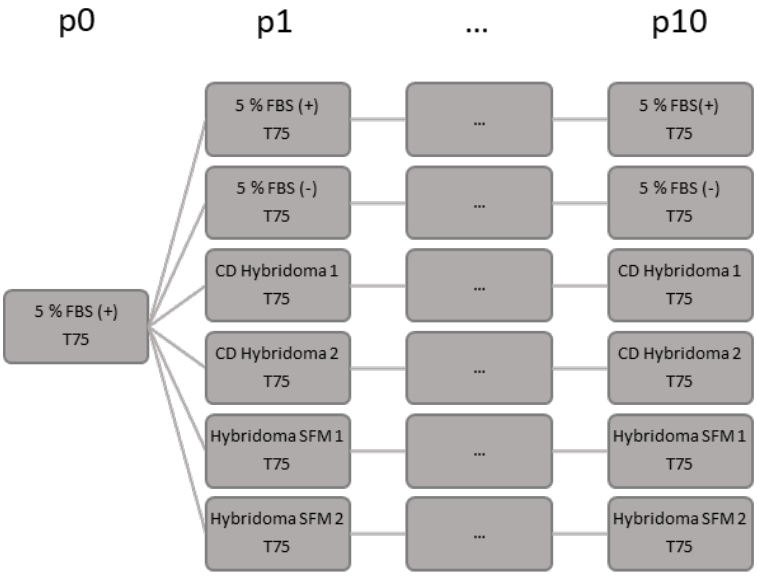

Figure 3: Experimental procedure for the second adaptation of E146-producing cells to serum-free medium. Stated in each box is cultivation medium and T-flask size. "+" denotes an initial cell density of 6x10^4 cells mL^{-1}, "-" denotes an initial cell density of 4x10^4 cells mL^{-1}. Initial cell density for cells in CD Hybridoma and Hybridoma SFM was 6 x10^4 cells mL^{-1}. The cells were cultivated for ten passages.

In parallel to the procedure seen in Figure 3, the cultivation was enlarged to allow for mAb QC and characterisation. From p5 of E146 in CD Hybridoma, and from p6 of E146 in Hybridoma SFM, another four respectively three T175 flasks of cells were cultivated. In p6, cells in CD Hybridoma and Hybridoma SFM were cultivated for 13 days to maximize the antibody production in that specific passage. Thereafter, all flasks containing the same medium were pooled before the cells were harvested by filtration through 2.0 µm glass fibre prefilters from Merck Millipore Ltd. The mAb-containing filtrates were kept at 4 ºC until further downstream processing.

Furthermore, it was investigated whether the cells survived a freeze-thaw cycle in CD Hybridoma respectively Hybridoma SFM. Cells from adaptation I were therefore frozen in p5 and p10. The cells were frozen in freezing solution consisting of 92.5 % cultivation medium (50 % fresh, 50 % spent) mixed with 7.5 % DMSO. However, to freeze cells they were first centrifuged at 200xg for 5 minutes. Thereafter, supernatant was taken for preparing freezing solution before the cells were resuspended in cultivation medium again. The cells were then counted whereafter being centrifuged for another 10 minutes at 200xg. Cell pellet was resuspended in 1.8 mL refrigerator cold freezing solution before refrigerated in +2-8 ºC for 30 minutes. Thereafter, the cells were frozen to -76 ºC for one day and then stored in a -170 ºC nitrogen freezer until use. All cells, both from p5 and p10, were frozen for more than ten days prior to thawing. Cells were thawed, like earlier, in tepid water and were then suspended in 37 ºC culture medium (CD Hybridoma or Hybridoma SFM). If number of thawed cells exceeded 450x10^4, the cells were suspended in a T75-flask, otherwise a T25-flask. The cells from p5 respectively p10 were then cultivated for five passages. Cell density, viability, and specific antibody productivity was estimated at the end of each passage.

During cultivation in T-flasks, the aim was to split the cells when there were 25-50x10^4 cells mL^{-1} present. However, deviations occurred, often due to no laboratory work being performed during weekends.

3.1.3 Antibody purification

For complete protocols for all downstream processing methods for E146, see Appendix B:. The antibodies were concentrated by filtration with Amicon Ultra 30K centrifugal filter devices. Thereafter, the E146 mAbs were purified through Protein A affinity chromatography. E146 mAbs produced in CD Hybridoma were purified on a manual chromatography system while Hybridoma SFM produced E146 were purified on an automated system. However, both systems used the same Protein A based column. As a reference to the purification results from the automated system, E146 mAbs from the production department of FDAD were purified as well. After purification, the mAb elutes from the purification were buffer exchanged to Storing Buffer. Antibodies cultivated in CD Hybridoma were buffer exchanged through dialysis while mAbs cultivated in Hybridoma SFM were put in Storing Buffer using Amicon Ultra 30 K centrifugal filter devices. Purified E146 was concentration determined by measuring absorbance at 280 nm. For E146 cultivated in CD Hybridoma, an Ultraspec 2100 pro spectrophotometer was used. The reference mAbs and the mAbs produced in Hybridoma SFM were instead concentration determined by a Nanodrop 2000c spectrophotometer. From this step, only purified and buffer exchanged E146 mAbs produced in CD Hybridoma were further quality controlled and characterised with the subsequent analyses. Until analysis, antibodies were stored at -76 ºC.

3.1.4 Analytical methods

Cell counting was performed either in a Cellometer K2 automatic cell counter from Nexcelom or manually in a Bürker chamber with depth 0.1 mm. In the automatic cell counter, 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 Cellometer 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^{-1}) 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.

Determination of mAb concentration (µg mAb mL^{-1}) was done with a sandwich ELISA in a 96-well plate format. E146 and C192 are both IgG1 antibodies which allowed for identical experimental procedures. The ELISA utilised the enzyme Horse radish peroxidase (HRP) in combination with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate for inducing a measurable colour shift. In short, each well was coated with goat-anti-mouse antibodies to which E146/C192 were bound. Thereafter, a mixture of HRP-labelled rabbit-anti-mouse antibodies were used together with TMB, allowing for HRP signal detection in a spectrophotometer at 620 nm. Full protocol for ELISA is available in Appendix B:.

The first quality control of the purified and buffer exchanged E146 mAbs (only E146 cultivated in CD Hybridoma) was size exclusion chromatography (SEC). SEC evaluated the purity of the mAb elute in terms of percentage intact E146 versus percentage aggregated and/or decomposed E146. The analysis was performed on an ÄKTA pure 25 L system from Cytiva. In the system there was a spectrophotometer measuring absorbance at 280 nm for detection of antibodies. The results from the absorbance measurements were analysed by integration of peaks between 6.5 mL and 18 mL. Peaks outside that range were neglected since 6.5 mL is less than the void volume of the column, and peaks after 18 mL originated from constituents of the Storing Buffer.

Anion exchange chromatography (AEC) was the second quality control of the antibodies, and it was also performed on the ÄKTA pure 25 L system. In AEC, the charge isoforms of E146 were separated based on their individual isoelectric point (pI). Results from the absorbance measurements at 280 nm was evaluated by integration of the two largest peaks between 5 and 17 mL elute volume. As a reference, AEC of E146 in standard medium (5 % FBS) was performed as well. Reference E146 mAbs originated from the production department of FDAB.

Immunoactivity of E146 produced in CD Hybridoma was evaluated with an ELISA-based immunoactivity assay. 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 E146 antibody. Binding of HRP-E146 to ProGRP was then detectable at 450 by adding TMB HRP-substrate followed by Stop Solution. Reference antibody was E146 from the same batch as reference in AEC. Absorbance measurement results were utilised to calculate percent inhibition (Eq. (9)) for reference respectively test mAb, followed by relative immunoactivity (Eq. (10)) for CD Hybridoma E146.

Isoelectric focusing (IEF) was performed on both purified and reference E146 to address the isoelectric point of the mAbs. Staining of the antibodies was done with a Colloidal Blue Staining Kit and the results were scanned with a ChemiDoc Touch Imaging system.

Purified and reference E146 mAbs were also analysed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate their sizes. The mAbs were analysed stain-free as both reduced and non-reduced. Results were scanned with the ChemiDoc apparatus.

3.2 Bioreactor production C192

3.2.1 Strains and media

C192-producing cells were received from a serum-free cell bank at FDAB, kept in a liquid nitrogen freezer at -170 ºC. The cells were cultivated in CD Hybridoma medium with 40 ml GlutaMAX supplement from Gibco added per litre medium.

3.2.2 Cultivation procedures

Bioreactor cultivation of C192-producing hybridoma cells was performed in a 3 L stirred-tank reactor (STR), named “Applikon Bundle”, from Applikon Inc. To regulate the cultivation, an ez2-Control control unit from the same company was used. The general procedure for reactor cultivation was to preculture cells in T175 flasks in CD Hybridoma medium and then inoculate the reactor. The reactor was at first run in batch mode to adapt the cells the new environment whereafter the perfusion rate was turned once the cells entered the log phase. Cell density (cells mL^{-1}), cell viability (%), and antibody concentration (µg mL^{-1}) was measured off-line during cultivation. Both fresh and used medium was stored at 4 ºC. Microbial contamination was avoided by autoclavation of the bioreactor system before inoculation into a closed environment.

Bioreactor cultivation of C192 was as mentioned performed in a 3 L STR from Applikon with an accompanying control unit. Setpoints for stirring rate, dissolved oxygen (DO), pH, temperature, and liquid level was set in the control unit interface. The control unit then automatically monitored and kept the parameters at their setpoints by varying each parameter’s actuator based on response from a PID controller. Stirring rate was actuated by a marine impeller with diameter 3 cm, situated close to the bottom of the reactor. Dissolved oxygen was measured by an AppliSens Clark-cell based DO sensor from Applikon Inc., submerged into the reactor. The DO was down regulated by gaseous nitrogen (N_2) and up regulated by primarily

air and secondarily gaseous oxygen (O_2). N_2, air, and O_2 was injected through a sparger situated underneath the stirrer. The pH was monitored by an AppliSens pH electrode from Applikon Inc. submerged into the reactor. pH was downregulated by letting gaseous carbon dioxide (CO_2) diffuse into the liquid from an inlet positioned in the reactor lid. CO_2 reacts with water to create carbonic acid, hence lowering pH. Reactor medium temperature was regulated with an external heating mantle responding to a thermometer which in turn was placed inside a metal pipe sited in the reactor. Liquid level was monitored, only during perfusion cultivation, by a level sensor. The level sensor was submerged into the reactor at a pre-selected height. Cell retention device used in the reactor was an Applikon BioSep using high frequency resonant ultrasonic waves to separate cells from medium.

Spinner flask cultivations were performed with cells thawed from a cell bank at FDAD. The cells were already adapted to serum-free medium, namely CD Hybridoma. Three separate spinner flasks with impeller diameter 7.5 cm were inoculated with $6x10^4$ cells mL^{-1} (75 % viability) and total volume of 300 mL each. The cells were pooled from five T175 flasks, all in p2. As a reference, cells were cultivated in a T175 flask with $6x10^4$ cells mL^{-1} (total volume 100 mL) in parallel. The spinner flasks were stirred at three rates: 140 rpm, 120 rpm, and 100 rpm. All spinner flasks along with the reference flask were incubated in 8 % CO_2 at 37 ºC. Samples for cell density, viability, and antibody concentration determination were taken every day except for on day 5 and 6. The optimal stirring rate in spinner flask cultivation was recalculated to bioreactor stirring rate using Eq. (7).

C192-producing cells for the first bioreactor inoculation, inoculation I, were thawed from a serum-free cell bank at FDAB, stored in a nitrogen freezer at -170 ºC. To thaw the cells, a vial containing $7.9x10^6$ cells were defrosted in tepid water. Thereafter, the cells were suspended in preheated CD Hybridoma medium in a T75 flask before incubated in 8 % CO_2 at 37 ºC. The cells were cultivated and amplified until p3 where four separate T175 flasks with cells were pooled and inoculated to a total volume of 1 L. Initial cell density in the reactor was $12x10^4$ cells mL^{-1} (80 % viability) and stirring rate was set to 250 rpm. Setpoints for dissolved oxygen (DO), pH, and temperature (T) was 50 %, 7, and 37 ºC, respectively. Cell density was measured daily by taking out a sample from the reactor. Samples for mAb concentration determinations were frozen three times a week. Because of sample taking, the medium volume in the reactor decreased every day. Therefore, the reactor was filled to 1.2 L three days after inoculation. The medium addition also intended to avoid nutrition shortage. After seven days, the CSPR was set to 6.5 nL $cell^{-1}$ day^{-1}, equal to a volumetric flow of 0.6 L h^{-1}. After the cultivation was terminated, the media was harvested with a glass fibre filter to remove cells and cell debris. Later the same day, the mAb-containing filtrate was concentrated.

In the second inoculation of the reactor (inoculation II), cells at that time growing at the production department of FDAB were used. The cells had been grown in 5 % FBS for four respectively seven passages upon obtention. C192 in p4 were split three times in CD Hybridoma and were hence in p7 upon inoculation. The cells in p7 were split once (to p8) in CD Hybridoma prior to inoculation. Totally, there were four T175 flasks in p8 and two in p7. To amplify cell concentration, cell suspension of the cells in p8 were centrifuged before resuspended in the same medium as cells in p7. Total volume in the reactor after inoculation was 1 L with cell density $9.2x10^4$ cells mL^{-1} and 72 % viability. Cell density and viability was measured daily, sample for mAb concentration determination was frozen three times a week. Set points for stirring rate, DO, pH, and temperature were 250 rpm, 50 %, 7, respectively 37 ºC. CSPR was set to zero. After four days, the reactor was filled with medium from 850 mL to 1 L, and after

eight days the reactor was filled from 800 mL to 900 mL. Additionally, the reactor was filled on day 12 (from 800 mL to 1 L) and day 15 (from 820 mL to 1.1 L).

Inoculation III consisted of cells from the serum-free cell bank at FDAB, thawed and precultured in the same way as prior to inoculation I. However, this time there were three T175 flasks with cells in p4 inoculated to a total volume of 1 L. Starting cell density in reactor was $12.8x10^4$ cells mL^{-1}. The cell density was determined once, five days after inoculation. Stirring rate, DO, pH, and temperature was set to 250 rpm, 50 %, 7, respectively 37 ºC. CSPR was once again set to zero.

Inoculation IV consisted of C192-producing cells in p7 from four T175 flasks. Cells were thawed from the serum-free cell bank. Starting cell density was $10x10^4$ cells mL^{-1} in a total volume of 1.5 L, viability was 71 %. Parameter setpoints were set according to earlier inoculations. At day three, the CSPR was set to 0.28 nL $cell^{-1}$ day^{-1}, equal to a pumping rate of 0.007 L h^{-1}.

In inoculation V, five T175 flasks with cells in p3 were inoculated to a reactor cell density equal to $20x10^4$ cells mL^{-1} in 1 L culture medium. Reactor was filled to 1 L on day three and then to 1.4 L on day four. Parameter setpoints were again set according to earlier inoculations.

3.2.3 Antibody purification

For full protocols related to antibody purification of C192, see Appendix C:. Harvested cell culture medium from inoculation I was concentrated using an Amicon ProFlux M12 tangential filtration system with three Pellicon 3 Ultracel filters, each with filtration area 0.1 m^2. Antibody harvest was concentrated to about 100 mL. C192 concentration was evaluated in an ELISA for both harvest and concentrate. C192 was then purified with affinity chromatography on 50 mL of the mAb concentrate. The purification was performed on an ÄKTA Avant 150 chromatography system from Cytiva. Antibodies were detected by measuring absorbance at 280 nm. After purification, C192 elute was buffer exchanged to Storing Buffer through dialysis following the same process as for E146. After dialysis, the mAb concentration was estimated by measuring absorbance at 280 nm, using the Ultraspec 2100 pro spectrophotometer. Thereafter, the elute was filtered with a 0.22 µm Millex-GV syringe filter and then concentration determined again at 280 nm. Purified and buffer exchanged C192 mAbs were stored in -76 ºC until QC and characterisation.

3.2.4 Analytical methods

For full protocols of analytical methods, see Appendix C:. SEC was once again used as a quality control of the purified and buffer exchanged antibodies. As for E146, SEC was performed on an ÄKTA pure 25 L system connected to a Superdex 200 Increase 10/300 GL column. The analysis was completed in the same way as for E146, with the same sample loading volume and same processing of absorbance measurement results.

Thereafter, an immunoactivity assay evaluated percent inhibition of bioreactor C192 respectively C192 produced in standard medium (5 % FBS) at the manufacturing department of FDAB. It also evaluated relative immunoactivity when comparing antibodies from the bioreactor with reference mAbs. The assay utilized HRP-labelled C192 mAbs as competing antibodies along with CA19-9 antigens for antibody binding. The antigens were immobilized in streptavidin coated wells of a 96-well plate by biotin-labelled C192. Detection of labelled C192 was done with addition of TMB followed by measurement of absorbance at 620 nm.

IEF and SDS-PAGE was performed on both purified and reference C192 to address the isoelectric point and size of the mAbs. In IEF, staining of the antibodies was done with a Colloidal Blue Staining Kit. For SDS-PAGE, the analysis was performed stain-free. As for E146, SDS-PAGE was done with both reduced and non-reduced antibodies. Results from IEF and SDS-PAGE were scanned with the ChemiDoc Touch Imaging system.

4 Results and discussion

This chapter is divided into two subchapters, namely "Serum-free production E146" and "Bioreactor production C192". In the first subchapter, results from serum-free cultivation and downstream processing of E146 is presented and discussed. Subchapter two discusses results from bioreactor cultivation and downstream processing of C192.

4.1 Serum-free production E146

Results from the serum-free cultivation of E146-producing cells are presented and discussed in two separate subchapters below. The first subchapter presents the results obtained during cultivation, that is viability, specific growth rate, and antibody productivity. Results from the downstream processing steps are covered in subchapter two.

4.1.1 Medium adaptation

Cell density (cells mL^{-1}) (bottom) and viability (%) (top) results, from p2-p10 of adaptation I and II, are presented in Figure 4. The figure shows that the cells were able to divide in both CD Hybridoma and Hybridoma SFM. It is hence shown that the cells adapted to the serum-free environments.

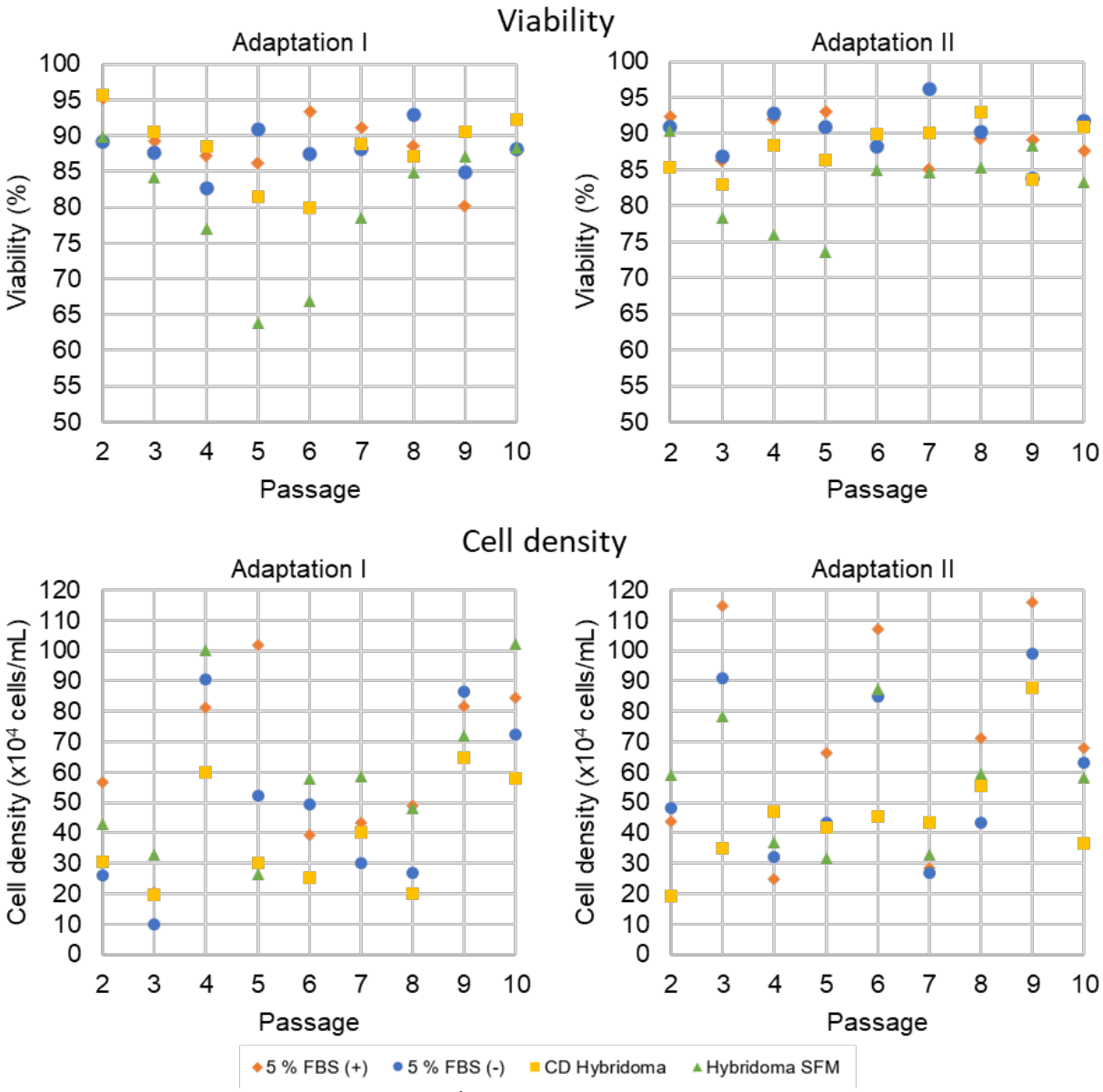

Figure 4: Viability (%) (top) and cell density (cells mL^{-1}) (bottom) in passage 2 (p2) to passage 10 (p10) for E146-producing cells cultivated in 5 % FBS, CD Hybridoma, or Hybridoma SFM during two rounds: adaptation I (left) and adaptation II (right). "+" denotes an initial cell density of 6x10^4 cells mL^{-1}, "-" denotes an initial cell density of 4 x10^4 cell mL^{-1}. Initial cell density for CD Hybridoma and Hybridoma SFM was 6 x10^4 cells mL^{-1}.

Cells cultivated in CD Hybridoma however had decreasing viability during p2-p6 of adaptation I. This decreasing trend was not seen for the cells cultivated in CD Hybridoma in adaptation II. Furthermore, there was a significant drop in viability for cells cultivated in Hybridoma SFM from p4 to p7 in adaptation I. Similarly, viability decreased in adaptation II during p3 to p5 for cells in Hybridoma SFM. An explanation to the viability decrease is adaptation to new medium, from medium containing 5 % FBS to either CD Hybridoma or Hybridoma SFM. In that case, the adaptation is slower from a viability point of view for cells cultivated in Hybridoma SFM compared to cells cultivated in CD Hybridoma. Another explanation may be inhomogeneous sampling during the first four passages of adaptation I.. However, all culture flasks of the first four passages (p0-p4) of adaptation I was poorly mixed before sampling, but no other viability decreased as significantly. There is neither a significant difference between the adaptation rounds considering viability when only observing p2 to p4.

Another notable difference considering cultivation methodology between the two rounds of adaptation is that the cells in adaptation I were cultivated in 20 % FBS in p0, 5 % FBS in p1, and thereafter either in 5 % FBS or serum-free medium. In adaptation II however, the cells were cultivated in 5 % FBS directly in p0 before adapted to serum-free medium or further cultivated in 5 % FBS in p1. There is a tendency, visualized in Figure 4, that adaptation II allows for higher viability during p2-p10. However, there is a risk that poor mixing interferes with the mentioned tendency. It is at least clear that a faster adaptation does not impair cell viability.

The average viability (%) considering p2-p10 for both rounds of adaptation is visualized in Figure 5. The figure suggests that the viability for cells cultivated in Hybridoma SFM is slightly lower than the viability for cells in CD Hybridoma or 5 % FBS. It is however shown through error bars based on standard deviation that the variability of the data is highest for cells cultivated in Hybridoma SFM. Furthermore, Figure 5 suggests small or no difference in average viability when comparing CD Hybridoma to 5 % FBS. The figure also points at a generally higher viability for cells in adaptation II which was denoted from Figure 4 as well.

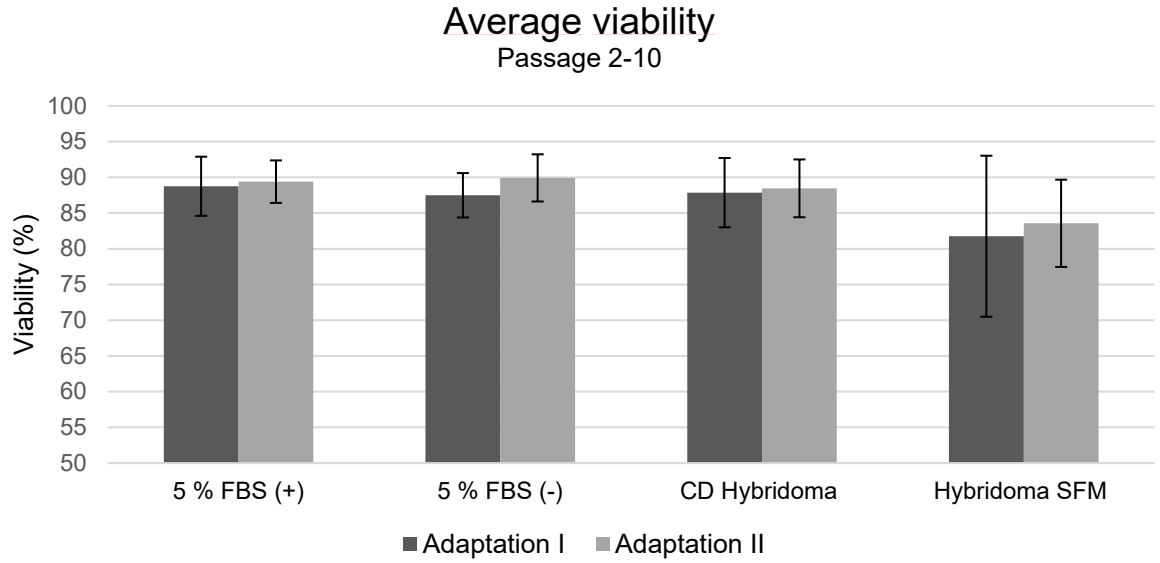

Figure 5: Average viability (%) of E146-producing cells over passage 2 to passage 10, cultivated in 5 % FBS, CD Hybridoma, or Hybridoma SFM. "+" denotes an initial cell density of $6x10^4$ cells mL^{-1}, "-" denotes an initial cell density of $4 x10^4$ cells mL^{-1}. Initial cell density for CD Hybridoma and Hybridoma SFM was $6 x10^4$ cells mL^{-1}. Results from Adaptation I in dark grey, results from Adaptation II in light grey. Error bars are based on standard deviation.

Specific growth rate (day^{-1}) over p2 to p10, estimated with Eq. (2), for adaptation I (left) and II (right) is shown in Figure 6. Variability is introduced to the data in adaptation I from previously mentioned mixing inadequacy. The figure shows that specific growth rate for cells cultivated in CD Hybridoma during adaptation II is lower relative the other cultivation conditions. This trend is also somewhat visualizable during adaptation I. Over time, there is no increase or decrease in specific growth rate, neither in adaptation I nor II.

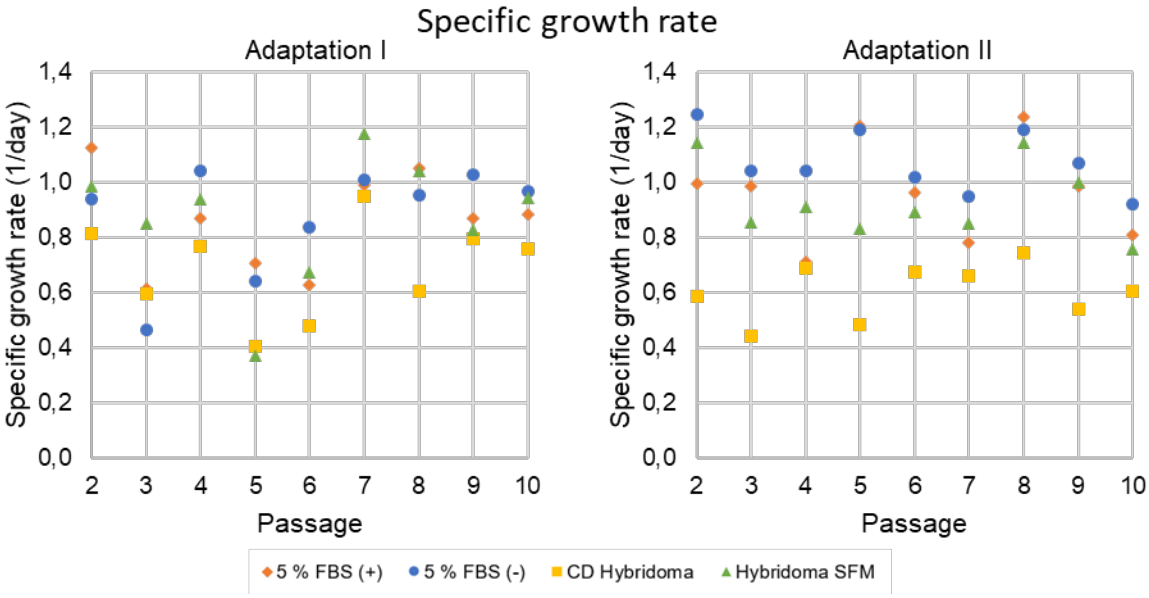

Figure 6: Specific growth rate (day^{-1}) for E146-producing cells during passage 2 to passage 10 when cultivated in 5 % FBS, CD Hybridoma, or Hybridoma SFM. "+" denotes an initial cell density of 6x10^4 cells mL^{-1}, "-" denotes an initial cell density of 4 x10^4 cells mL^{-1}. Initial cell density for CD Hybridoma and Hybridoma SFM was 6 x10^4 cells mL^{-1}. Results are presented separately for adaptation I (left) and adaptation II (right).

The specific growth rates (day^{-1}) are summarized in Figure 7. The figure enlights that cells cultivated in CD Hybridoma have lower average specific growth rate compared to other cultivation media. The three other cultivation media have similar average specific growth rates according to the figure. Joint for all four cultivation conditions is that the average specific growth rate is higher during the second round of adaptation. Noteworthy is once again poor mixing during adaptation I, possibly reflected in average specific growth rate.

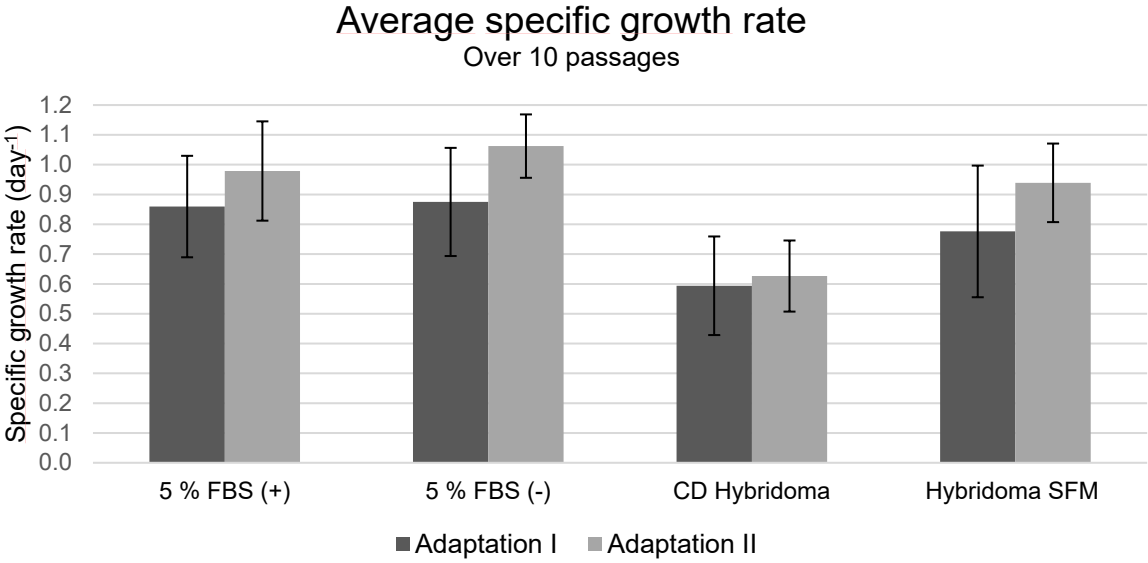

Figure 7: Average specific growth rate (day^{-1}) of E146-producing cells over ten passages, cultivated in 5 % FBS, CD Hybridoma, respectively Hybridoma SFM. "+" denotes an initial cell density of 6x10^4 cells mL^{-1}, "-" denotes an initial cell density of 4 x10^4 cells mL^{-1}. Initial cell density for CD Hybridoma and Hybridoma SFM was 6 x10^4 cells mL^{-1}. Results from adaptation I in dark grey, results from adaptation II in light grey. Error bars indicate the standard deviation (n=9).

Specific antibody productivity (pg mAb cell^{-1} day^{-1}) is introduced in Figure 8 in terms of average amount of mAbs produced per cell per day. Figure 7 showed, as mentioned, that cells cultivated in CD Hybridoma grow slowly in comparison. Remarkably, average specific

productivity is potentially highest for cells in CD Hybridoma. The variability is however high for all cases. Figure 7 and Figure 8 in combination suggest that a lower specific growth rate enhances antibody production.

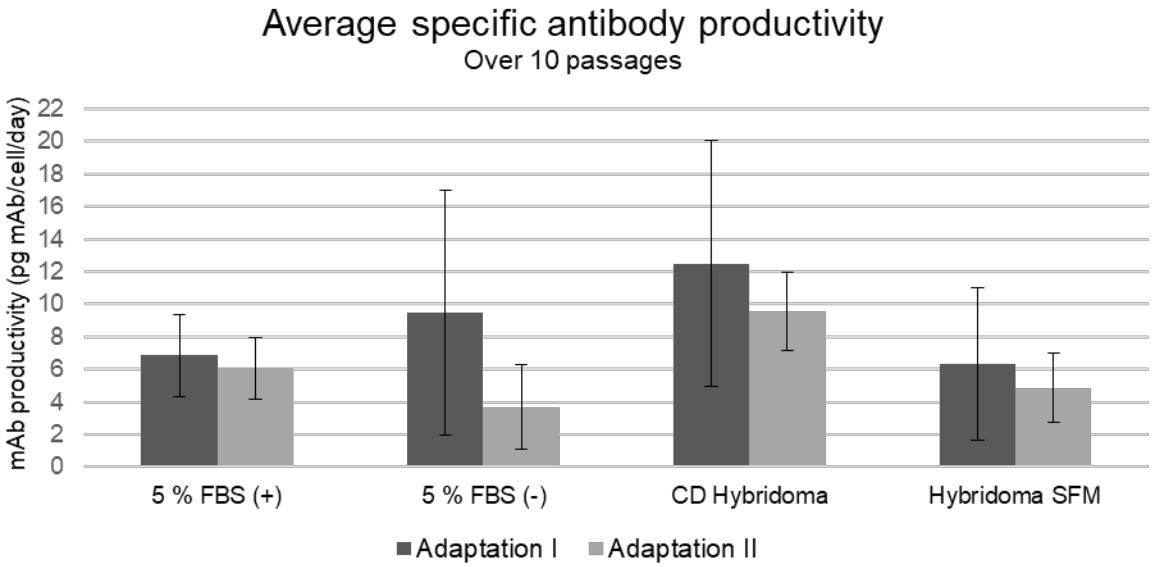

Figure 8: Average specific antibody productivity (pg mAb cell^{-1} day^{-1}) of E146 over p2-p10 for cells cultivated in 5 % FBS, CD Hybridoma, or Hybridoma SFM. "+" denotes an initial cell density of 6x10^4 cells mL^{-1}, "-" denotes an initial cell density of 4 x10^4 cells mL^{-1}. Initial cell density for CD Hybridoma and Hybridoma SFM was 6 x10^4 cells mL^{-1}. Results from adaptation I in dark grey, results from adaptation II in light grey. Error bars indicate the standard deviation (n=9).

Results from cell counting obtained during freezing of cells in p5 respectively p10 are presented in Table 1. The four different cultures were named 1-4. Note that there are two culture 1, two culture 2, etc., one cultivated in CD Hybridoma, and one cultivated in Hybridoma SFM.

Table 1: Number of cells (x10^4) frozen in p5 respectively p10. There are eight cultures frozen, four per culture medium. In each culture medium, two cultures were frozen in p5 and two in p10. The cultures consist of cells producing E146.

	Culture 1 (p5) (x10^4 cells)	**Culture 2 (p5) (x10^4 cells)**	**Culture 3 (p10) (x10^4 cells)**	**Culture 4 (p10) (x10^4 cells)**
CD Hybridoma	390	230	670	590
Hybridoma SFM	180	430	370	490

Culture 3 and 4 in CD Hybridoma and culture 4 in Hybridoma SFM were thawed in T75 flasks, the rest were thawed in T25 flasks. Cell viabilities (%) (top) and cell densities (cells mL^{-1}) (bottom) in p0-p5 from the freeze-thaw cycle investigation is presented in Figure 9. Viabilities and densities are presented separately for CD Hybridoma (left) and Hybridoma SFM (right). Culture 1 in Hybridoma SFM did not start growing and was hence discarded in p0. This culture had the lowest cell number when frozen, hence emphasizes the importance of high cell numbers when freezing cells. There is a drop in viability in p3 for all cultures in Hybridoma SFM and culture 1 in CD Hybridoma. The cells in p3 were grown for five days, which thus is indicated to be too long for the mentioned cultures. Cell density results show that cell density in p3 is around 90x10^4 cells mL^{-1} for cells in CD Hybridoma and around 80x10^4 cells mL^{-1} for cells in Hybridoma SFM. Consequently, the cells in p3 could have been split into a new passage earlier to possibly avoid the viability drop.

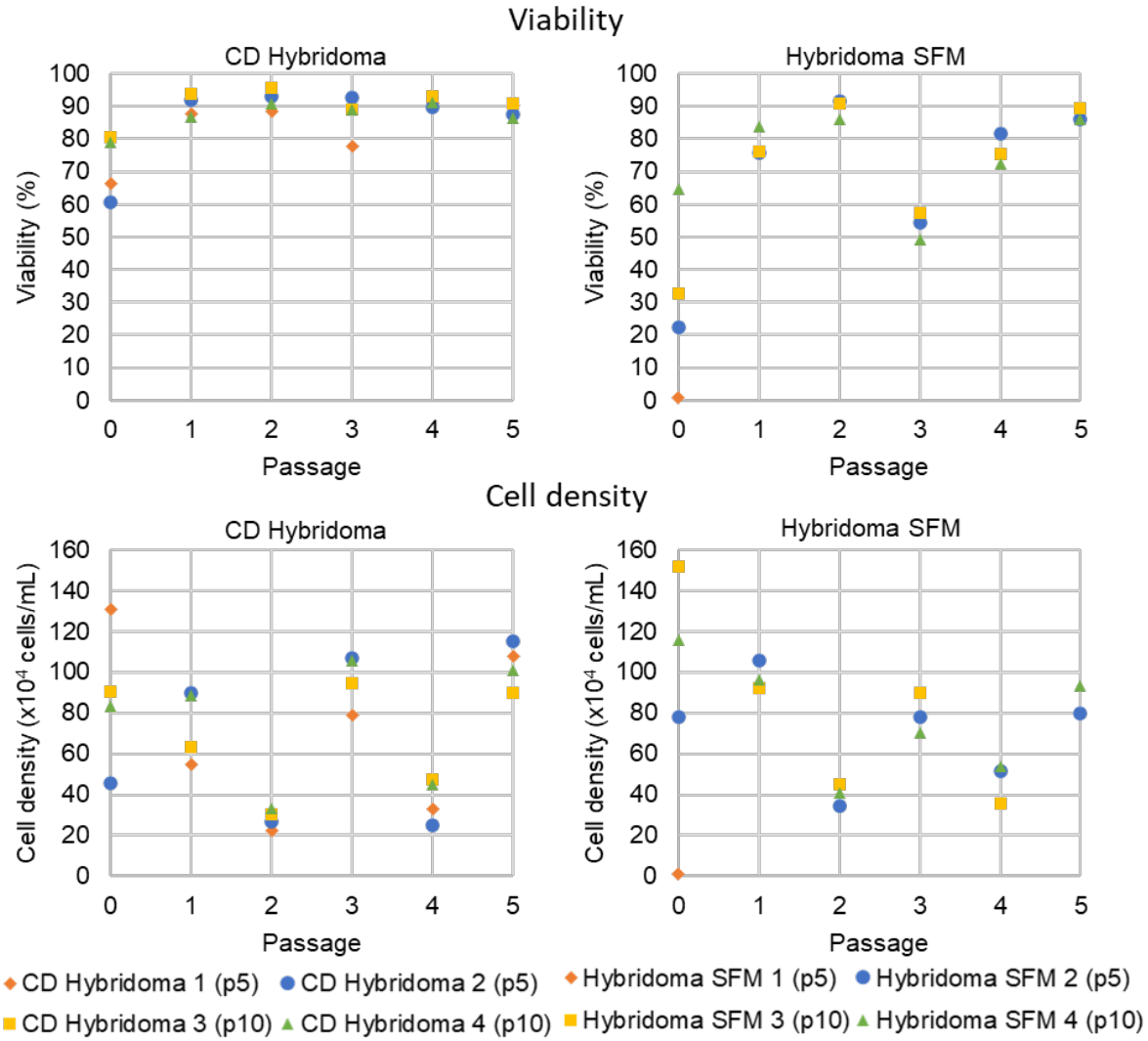

Figure 9: Cell viability (%) (top) and cell density (cells mL^{-1}) (bottom) for E146-producing cells in CD Hybridoma (left) respectively Hybridoma SFM (right) during five passages in a freeze-thaw cycle investigation. In total, eight cultures were monitored: four in CD Hybridoma and four in Hybridoma SFM. Two cultures in each medium were cultivated with cells thawed from earlier cultures in p5 and two from earlier cultures in p10. However, culture 1 in Hybridoma SFM was discarded after p0 due to no growth.

Average viability (%) over five passages was calculated and reported, partly separate for each culture (left), but also altogether on all four cultures in CD Hybridoma and culture 2-4 in Hybridoma SFM (right) (Figure 10). The viability for cells in CD Hybridoma is higher compared to cells in Hybridoma SFM, which was shown in adaptation I and II as well (Figure 5). Furthermore, average viability, all cultures included, for cells in CD Hybridoma is 85-90 % both during adaptation (Figure 5) and freeze-thaw cycle investigation. Cells in Hybridoma SFM however show a lower average viability (about 70 %) during cell-thaw cycle investigation compared to during adaptation (80-85 %). Noteworthy, the average viability was calculated for p2-p10 during adaptation while the average viability during freeze-thaw cycle investigation is calculated on p0-p5. Viability during p0 is low compared to viability during the following passages (Figure 9), thus decreasing the average viability in Figure 10. A final remark, there is no clear effect when comparing viability between cultures in the same medium in Figure 10.

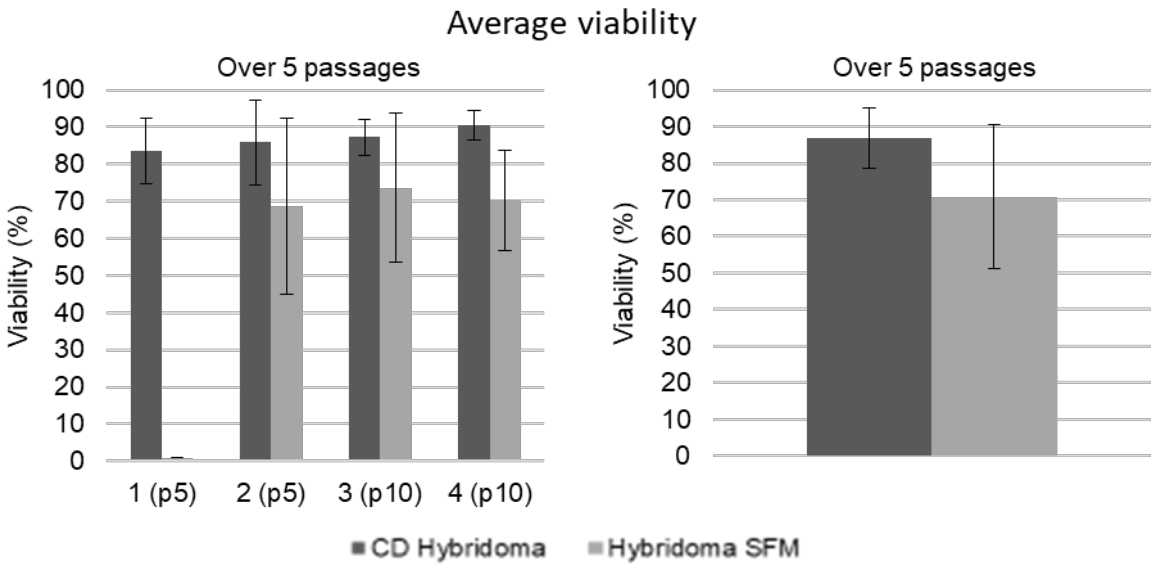

Figure 10: Average viability (%) over five passages in a freeze-thaw cycle investigation of E146-producing cells. To the left: each culture and medium separated. To the right: culture 1-4 averaged for CD Hybridoma and culture 2-4 averaged for Hybridoma SFM. For cells in CD Hybridoma (dark grey), the average in the right figure is calculated from four cultures where two originate from cells frozen in p5 and two from cells frozen in p10. For cells in Hybridoma SFM (light grey), the average in the right figure is calculated from three cultures where one originates from cells frozen in p5 and two from cells frozen in p10. Error bars indicate the standard deviation (n=5).

Specific growth rates (day^{-1}) for cells cultivated during freeze-thaw cycle investigation in CD Hybridoma (left) respectively Hybridoma SFM (right) are presented in Figure 11. The specific growth rates for cells in Hybridoma SFM vary more than cells cultivated in CD Hybridoma. There is a decrease in specific growth rate during p3 and p4 for cells in Hybridoma SFM. It should however be noted that the specific growth rates are based on cell densities estimated when splitting the cells. It is thus not accounted for cells that are formed but do not survive until the cells are split. Therefore, it is probable that the decrease in specific growth rate for cells in Hybridoma SFM is linked to the viability decrease seen in Figure 9. Additionally, cells are believed to have higher specific growth rate in Hybridoma SFM than in CD Hybridoma (Figure 6). Cells in Hybridoma SFM thus transition to the stationary phase earlier than cells in CD Hybridoma. That explains why the viability decrease is only present for the cells in Hybridoma SFM. In other words, the transition phase for cells in CD Hybridoma is not yet reached.

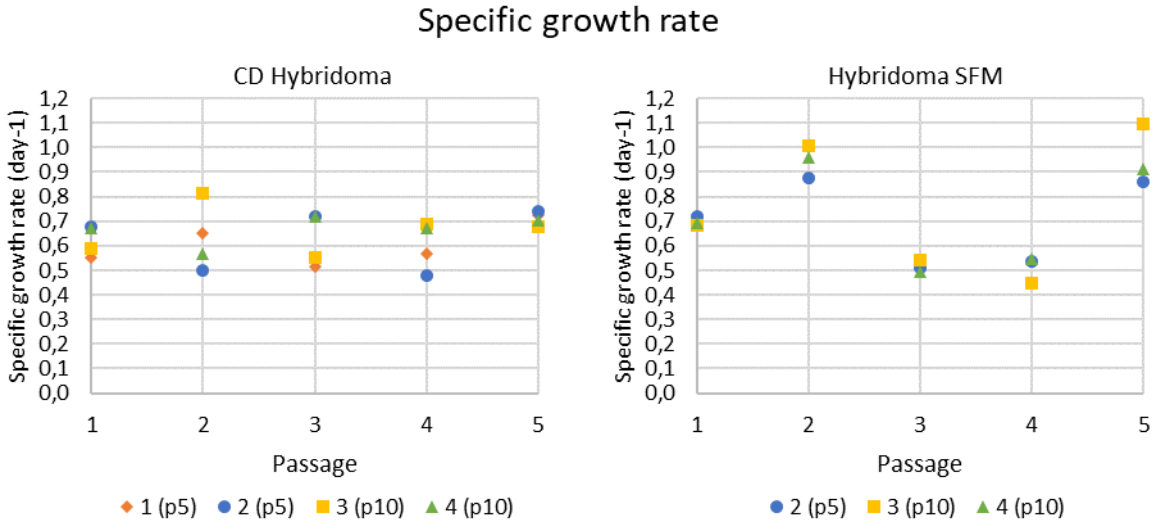

Figure 11: Specific growth rates (day^{-1}) for cells producing E146 cultivated during freeze-thaw cycle investigation in CD Hybridoma (left) respectively Hybridoma SFM (right). All cells were cultivated for five passages, data is shown for passage 1 to 5. Furthermore, data is presented for four cultures in CD Hybridoma: two frozen in passage 5 (p5) and two frozen in passage 10 (p10). For cells in Hybridoma SFM, specific growth rates from three cultures are reported: one frozen in p5 and two frozen in p10. Culture 1 in Hybridoma SFM was discorded in p0 due to no growth.

Average specific growth rate (day^{-1}) for cells in CD Hybridoma respectively Hybridoma SFM during freeze-thaw cycle investigation is shown in Figure 12. The figure partly presents average specific growth rate for each culture (left) but also average specific growth rate for all cultures together (right, culture 1 excluded for Hybridoma SFM). The average is based on specific growth rates for p1 to p5. Despite the drop in viability and specific growth rate in p3, cells cultivated in Hybridoma SFM are suggested to have slightly higher specific growth rate compared to CD Hybridoma. Consequently, the results align with what was seen during adaptation I and II (Figure 7). At last, there is no major difference in average specific growth rate between cells frozen in p5 respectively p10.

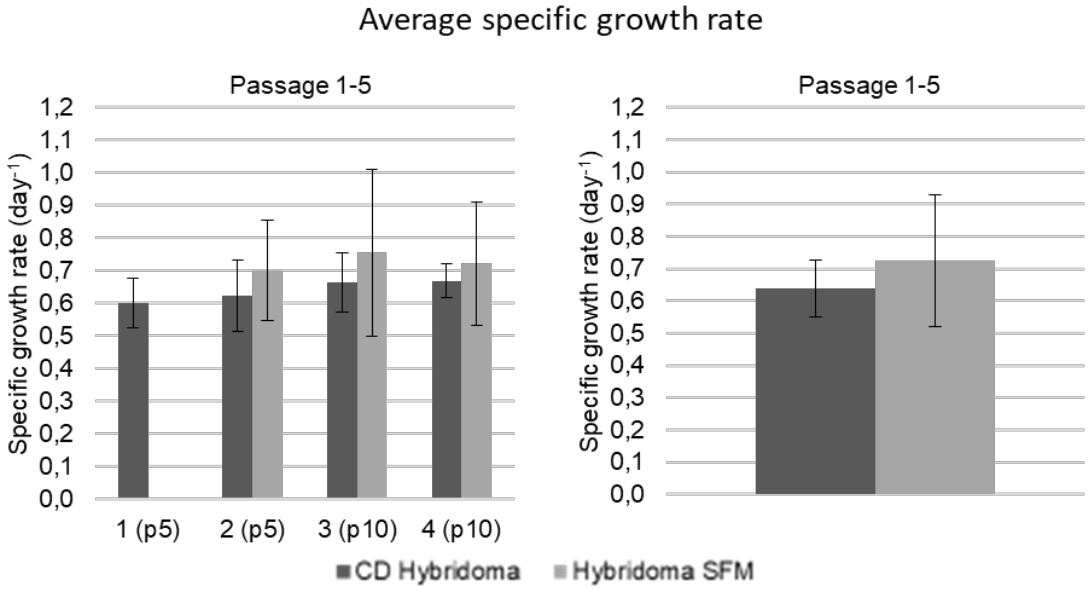

Figure 12: Average specific growth rate (day^{-1}) during freeze-thaw cycle investigation for cells producing E146 cultivated in CD Hybridoma (dark grey) respectively Hybridoma SFM (light grey). The average is based on cultivations from passage 1 (p1) to passage 5 (p5) for each individual culture (left) or for all cultures (right). Cells were thawed from four cultures: two frozen in p5 and two frozen in p10. Culture one was however excluded from Hybridoma SFM data in the right figure. Error bars indicate the standard deviation (n=5).

Each culture's average specific antibody productivity (pg mAb cell^{-1} day^{-1}) (p0-p5) is presented in Figure 13 (left) next to the average for all cultures (right). Culture 1 was once again not included in total average for Hybridoma SFM. The specific mAb productivity is significantly higher for cells cultivated in CD Hybridoma. It is also suggested that cells frozen in CD Hybridoma at p10 produce on average less mAbs compared to cells frozen in CD Hybridoma at p5. However, cells frozen at p10 in CD Hybridoma still produce more mAbs than cells frozen in Hybridoma SFM, despite a possibly decreased specific mAb productivity.

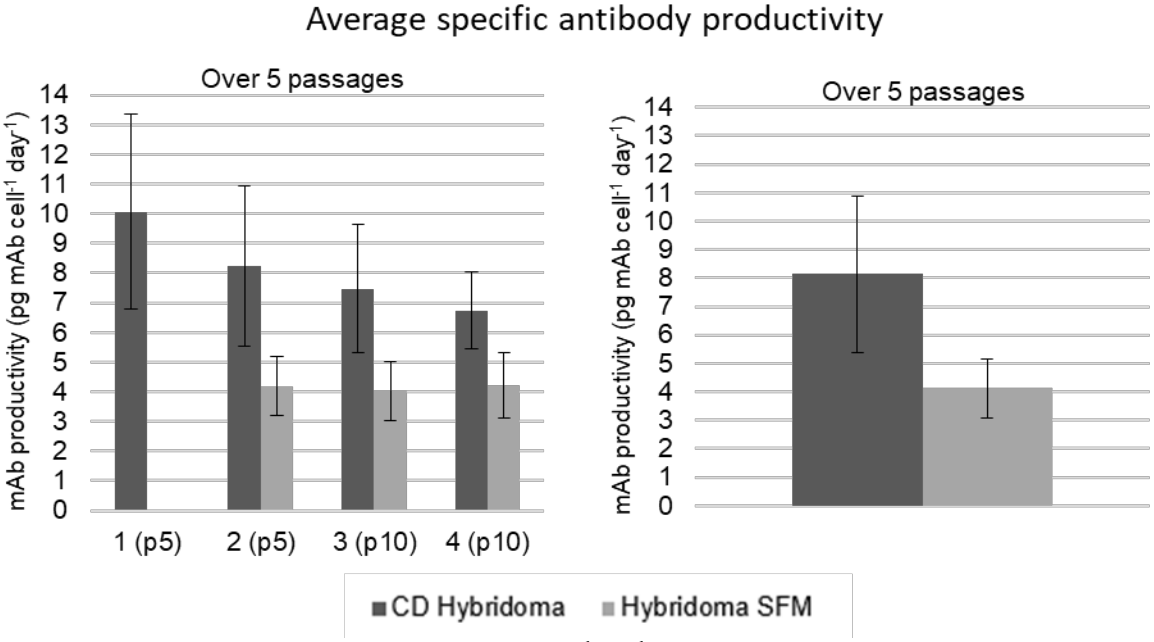

Figure 13: Average specific antibody productivity (pg mAb cell^{-1} day^{-1}) for each culture (left) and all cultures together (right). The cultures consisted of E146-producing cells that were part of the freeze-thaw cycle investigation where two cultures were frozen at passage 5 (p5) and two were frozen at passage 10 (p10). Culture 1 in Hybridoma SFM was however not included in total average specific antibody productivity (right). Error bars indicate the standard deviation (n=5).

4.1.2 Downstream processing E146

Cell culture harvest was concentrated from 300 mL to 7 mL for cells in CD Hybridoma, and from 200 to 4 mL for cells in Hybridoma SFM. Antibody concentration in concentrate was 3.1 mg mL^{-1} (CD Hybridoma) respectively 2.0 mg mL^{-1} (Hybridoma SFM). Despite CD Hybridoma harvest being concentrated 44 times versus Hybridoma SFM harvest being concentrated 51 times, antibody concentration is still higher in CD hybridoma harvest. However, it is expected that cells cultivated in CD Hybridoma produce larger amounts of mAbs, see Figure 8.

Chromatograms from Protein A affinity chromatography purifications are presented in Appendix D. The peak in the chromatogram in Figure D.1 occurred after elution and was hence thought to contain CD Hybridoma produced E146 mAbs. The elutes during this peak was therefore collected in six vials to a total pooled volume of 9 mL after addition of storing buffer. Chromatogram from the ÄKTA pure system used when purifying E146 produced in Hybridoma SFM is shown in Figure D.2. Here, elution started at approximately 35 mL. Total volume of fractionised elutes was 5 mL. It is however noted that the largest peak occurs at 27-30 mL during washing of the column. The large peak was not collected since it was not programmed in the method.

Buffer exchange of E146 produced in CD Hybridoma resulted in 7 mL elute with mAb concentration 2.9 mg mL^{-1}. Elutes from the ÄKTA pure system was buffer exchanged but there was, as mentioned, no additional Storing Buffer added to the filtrate after the second exchange. Instead, the filtrate and the FT was analysed on the Nanodrop with resulting mAb concentrations of 0.043 respectively 0.007 mg mL^{-1}.

It was believed that the mAbs were eluted in the large peak during the wash of the column. Troubleshooting was initiated by measuring pH of all buffers, and check that inlet tubes were submerged into the correct buffers. No deviations were noted. Noteworthy is that flow rate was set to 1 mL min^{-1} instead of recommended 0.3 mL min^{-1} when purifying E146 cultivated in Hybridoma SFM. The mistake was corrected for after 20 mL into the automated process. The exact same flow rates were used when the first sample of reference E146 were purified. This purification ought to elucidate whether early elution of mAbs was due to altered antibody properties or due to a malfunctioning chromatography methodology. The chromatogram in the chromatography report (not included in Appendix D) once again showed a peak during washing of the column. Antibody concentrations acquired from the Nanodrop concluded that there were 3.6 mg mL^{-1} E146 in the wash elute and 0.079 mg mL^{-1} E146 in the elution buffer elute. It is hence clear that the antibodies elute during the washing phase of the process, and that the early elution is not due to alterations in antibody properties. The early elution is instead believed to occur due to a malfunctioning chromatography method. It was investigated if the increased flow rate led to weaker binding of mAb to the column and thus early elution. This theory was however rejected through another purification run (not reported) with reference mAbs where flow rate was set to 0.3 mL min^{-1} throughout the whole process. Because of lack of purified antibodies, no further quality controls were conducted on E146 produced in Hybridoma SFM.

For E146 produced in CD Hybridoma however, QC and mAb characterisation were performed. SEC results show that the mAb elute is 99.38 % pure, see analysis report in Figure D.3. The results thus imply that over 99 % of all particles in the mAb elute have the same size and that there are less than 1 % degraded and/or aggregated antibodies in the elute.

Analysis reports from AEC performed for both CD Hybridoma mAbs and 5 % FBS mAbs are shown in Figure D.4 respectively Figure D.5. The reference results show that E146 mAbs yield two peaks in the chromatogram, with 76.93 % respectively 23.07 % of the total peak area. There are hence two charge isoforms of E146 present. Two peaks were seen for E146 cultivated with CD Hybridoma as well. However, the distribution of the total peak area differs in CD Hybridoma mAbs compared to reference mAbs. For mAbs produced in CD Hybridoma, the peaks were distributed as 81.82 % versus 18.18 %. A more negatively charged isomer binds more strongly to the column and higher salt concentration is required before it loosens. The charge isoforms of E146 produced in CD Hybridoma are hence shifted towards a larger ratio of isoforms with weaker negative net charge.

The IEF gel image for E146 produced in CD Hybridoma (lane 2) and reference E146 (lane 3) are presented in the figure to the left in Figure 9. Both mAbs have a similar series of bands around pI = 6.9. The result thus suggest that the isoelectric points of reference mAb charge isoforms are equal to the isoelectric points of the charge isoforms of CD Hybridoma produced mAbs. This result contradicts the result from AEC where a shift in charge distribution was seen.

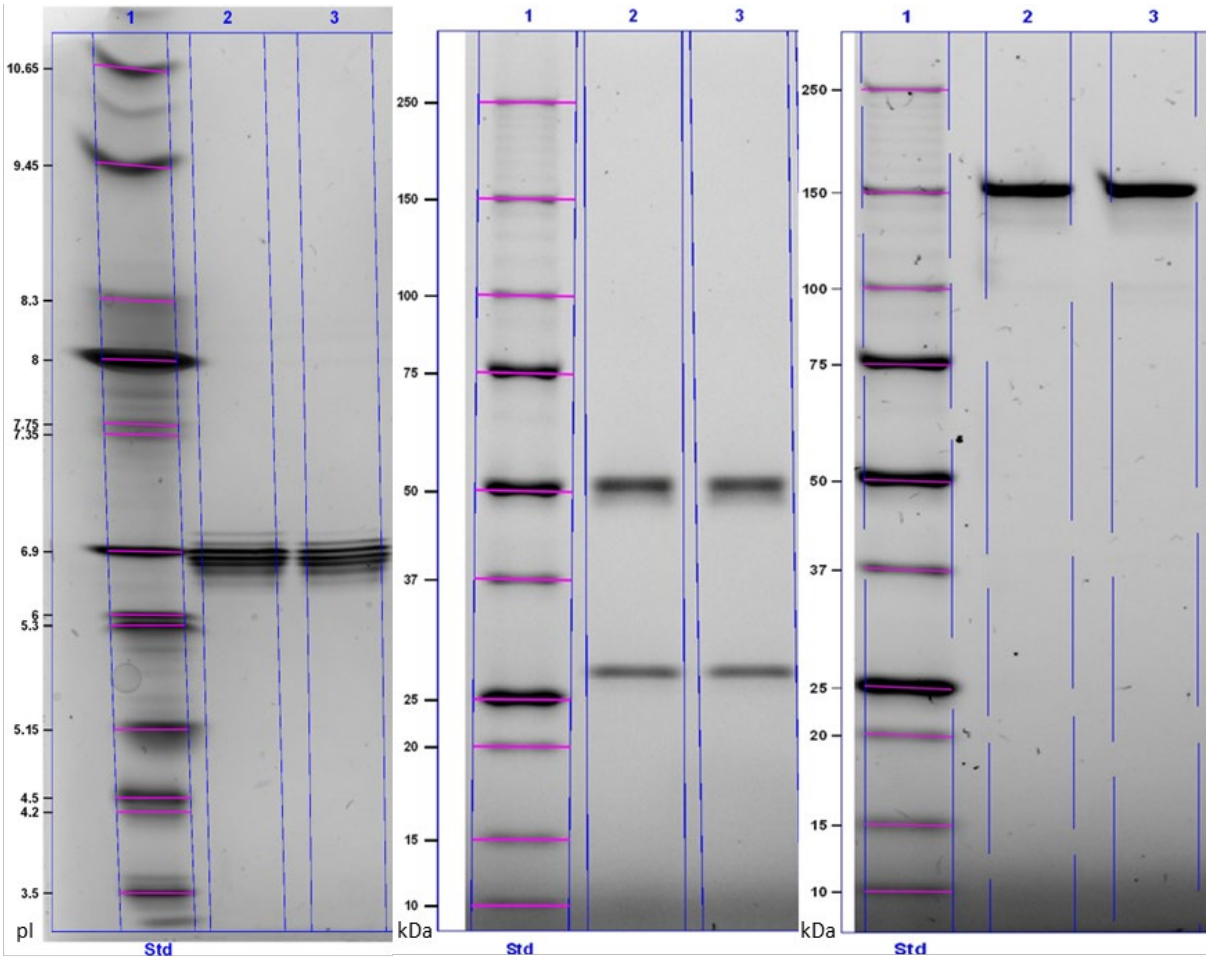

Figure 14: Images of IEF (left) and SDS-PAGE (middle and right) gels for E146-producing cells cultivated in CD Hybridoma (2nd lanes) respectively 5 % FBS (3rd lanes). The green and red arrows in the left image points out a difference in charge distribution among charge isoform of E146 when comparing CD Hybridoma mAbs and mAbs produced in 5 % FBS. The first lane in all three gels is loaded with reference proteins with known pI (left) or size (kDa) (middle and right).

Images of SDS-PAGE gels with CD hybridoma E146 (second lanes) and reference E146 (third lanes) are shown in Figure 14, reduced SDS-PAGE in the centre and non-reduced to the right. The gel with reduced mAbs reveals that the chains are separated with resulting bands at approximately 50 kDa for the heavy chains and approximately 28 kDa for the light chains. The result is equal for both types of mAbs. The gel with non-reduced mAbs also shows equal results for both mAbs. The bands of the non-reduced mAbs shows that both mAbs weigh approximately 150 kDa each.

Inhibition of E146 produced in CD Hybridoma respectively reference E146 was estimated two times. The first results estimated percent inhibition to 66.0 respectively 66.0 % for reference mAbs and CD Hybridoma mAbs (Eq. (9)). From that, relative immunoactivity was estimated to 99.9 % according to Eq. (10). However, CV for background was 11.2 % which is not within acceptance. The results from the second immunoactivity investigation estimated percent inhibition for reference mAbs and mAbs produced in CD Hybridoma to 72,5 respectively 66.4 %. Relative immunoactivity was calculated to 91.7 %. Once again, the CV was not within acceptance (9.4 %). The results are therefore interpreted with caution. From the first estimation, the results pointed at no change in antigen-binding ability when the mAbs are produced in CD Hybridoma. The second estimation do however point at a possible decrease in relative immunoactivity for E146 produced in CD Hybridoma.

Due to the high CVs in the immunoactivity assays, it is not elucidated whether the change in charge distribution seen in AEC affects the antigen-antibody binding ability of E146 produced in CD Hybridoma. It is possible that the shift seen in AEC originates from a post-translational modification induced by the change of cultivation medium. Earlier reports have shown that alterations in post-translational modification of mAbs potentially affect their quality. For further investigation of charge isoforms of E146, it is recommended to elute each peak from the AEC and analyse them individually[48].

4.2 Bioreactor production C192

Results from the bioreactor cultivation part of the project are presented and discussed in three separate subchapters. First, the results from spinner flask cultivation of C192. Secondly, results obtained during each bioreactor cultivation, and thirdly the results from downstream processing of harvest from inoculation I. The five bioreactor cultivation rounds are named inoculation I-V.

4.2.1 Spinner flask cultivations

Cell density (cells mL^{-1}) and viability (%) during spinner flask cultivations are presented in Figure 15. Cultivation in spinner flask 1 was terminated on day 1 due to infection. Likewise, spinner flask 2 was found with an infection on day 7 and was therefore discarded. Reference flask and spinner flask 3 were cultivated for eight days. Cells in reference had a shorter lag phase than cells in spinner flask 3 (two days instead of four). Also, viability was higher for cells in reference compared to spinner flask 2 and 3 during lag phase. A longer lag phase in combination with lower viability emphasises that the cells go through an adaptation phase when cultivated in spinner flask. When adapted, the cells grow to equal cell density and have equal viability as cells in reference. Cell density and viability decreased for cells in the reference flask on day 7. For spinner flask 3, cell density and viability increased continuously from day 4.

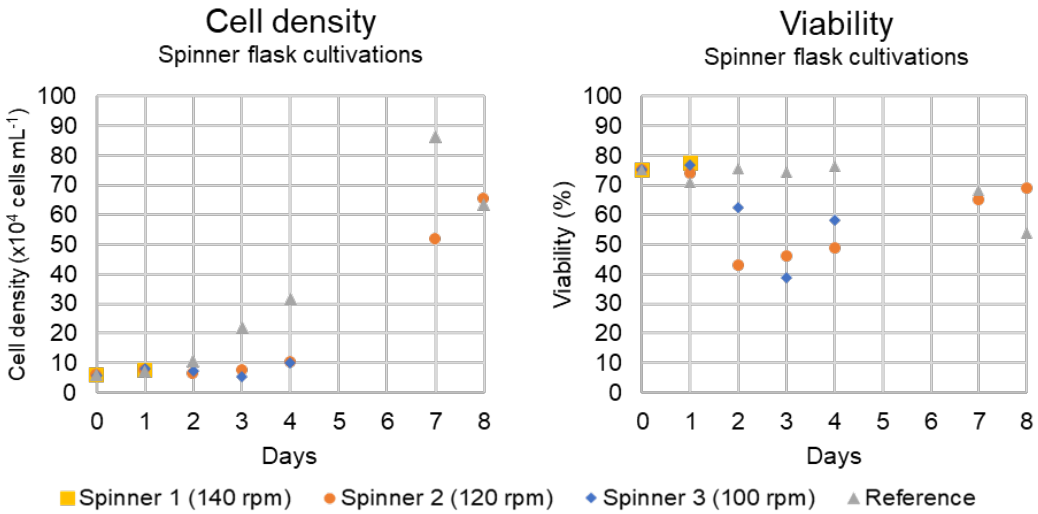

Figure 15: Cell density (cells mL^{-1}) and viability (%) for C192-producing cells in three spinner flask cultivations, with three different stirring rates, and a T175-flask reference cultivation. Spinner 1 and spinner 2 were discarded due to infection on day 1 respectively day 4.

The results from the spinner flask cultivations show that C192-producing cells are cultivable in spinner flasks at 120 RPM, which corresponds to 300 rpm in the bioreactor (Eq. (7)). The stirring rate in the reactor was however set to 250 rpm to ensure cells were not exposed to too high levels of shear stress. This number was concluded partly from the spinner flask cultivation results, but also from protocol at FDAB.

4.2.2 Bioreactor cultivations

The monitoring of dissolved oxygen (DO) from the first inoculation of the bioreactor is shown in Figure E.1, Appendix E. The figure shows DO (%) and amount of injected N_2 (mL), air (mL), respectively O_2 (mL). Temperature, stirring rate, and pH were successfully kept at 37 ºC, 250 rpm, respectively 7 during the whole process. They are therefore excluded from Figure E.1. Cell density in cells mL^{-1} (left) and viability (%) (right) for all days except for weekends (day 5 and 6) is presented in Figure 16. The cell viability was approximately 80 % from day 0 to day 8, whereafter it dropped to 0 % on day 9. Specific growth rate was estimated with Eq. (2) between day 4 and 7 to 0.42 day^{-1}, assuming log-phase in the region. The DO stabilised after approximately 2.5 days. At that point, the cells started to consume O_2 such that air was injected. The large DO peak at 3.75 days originates from change of the PID controller. After that, the PID controller settings were set back to the previous settings before DO level slowly decreased until stabilised on day five.

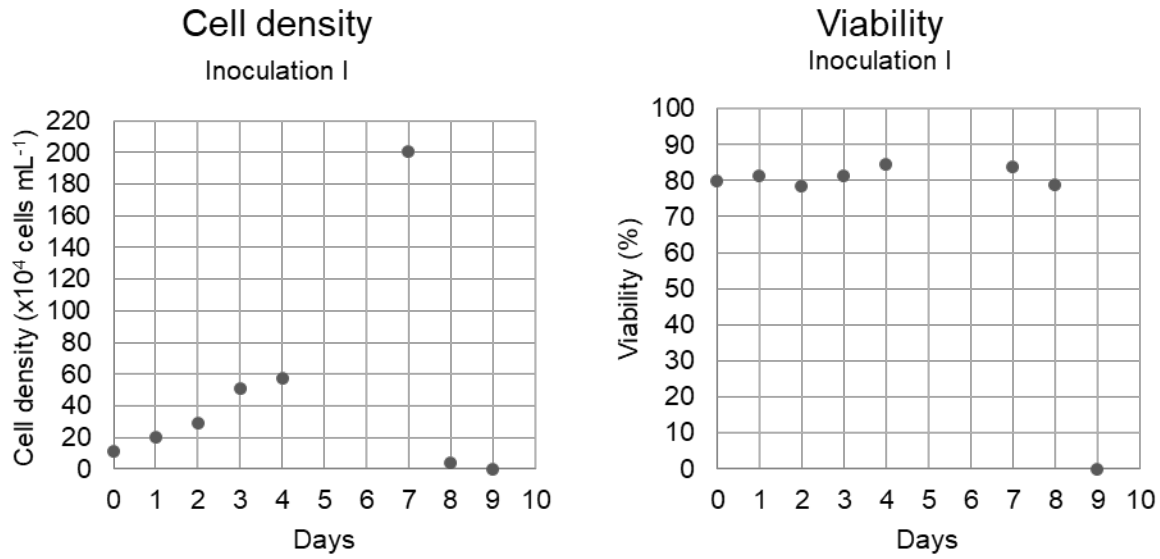

Figure 16: Cell density (cells mL^{-1}) (left) and cell viability (%) (right) for C192-producing in bioreactor during inoculation I, estimated by cell counting on sample from the reactor. The cells were not counted on day 5 or 6.

From day 5-7, the cells increased from $60x10^4$ cells mL^{-1} to $200x10^4$ cells mL^{-1} (Figure 16). It is estimated visually that the cells entered the log phase after four days in the reactor. There is a rapid oscillation in DO between day 5-7 (Figure E.1). It is speculated that the oscillation appears when the cells consume oxygen to proliferate. The oscillation phenomena will be further evaluated in terms of possible indicator for cellular growth during future cultivations. Contradictory, the DO oscillated between day 2-4 as well where cellular growth was not as rapid. On day 7, the perfusion rate was started with volume set at 1.1 L. The rate was however set too high, resulting in washout of cells from the reactor since the perfusion system was not able to handle such a high flow rate. On day 8, the cell culture medium was visually clear since very few cells were present (Figure 16). Since there were no cells present there was no oxygen consumed, and no more air or oxygen were thus injected. The cultivation was therefore terminated on day 9. On day 9, the outlet vessel contained 12 L medium with mAb concentration 11.1 µg mL^{-1}. Total mAb weight was thus 133 mg. Further downstream processing results of outlet is presented in 4.2.3.

As for inoculation I, monitoring of DO (%) is shown for inoculation II, now in Figure E.2 and Figure E.3. After three days of cultivation, the software was unexpectedly shut down. Therefore, a new monitoring process was started. The monitoring of the first three days is shown in Figure E.2 while the remaining days (starting approx. at day 4) are presented in Figure E.3. As for inoculation I, temperature, stirring rate, and pH were kept constant at setpoints and are therefore excluded from the mentioned figures. Cell density (cells mL^{-1}) and viability (%) is reported in Figure 17 for all days except weekends (day 2, 3, 9, 10, 16, and 17).

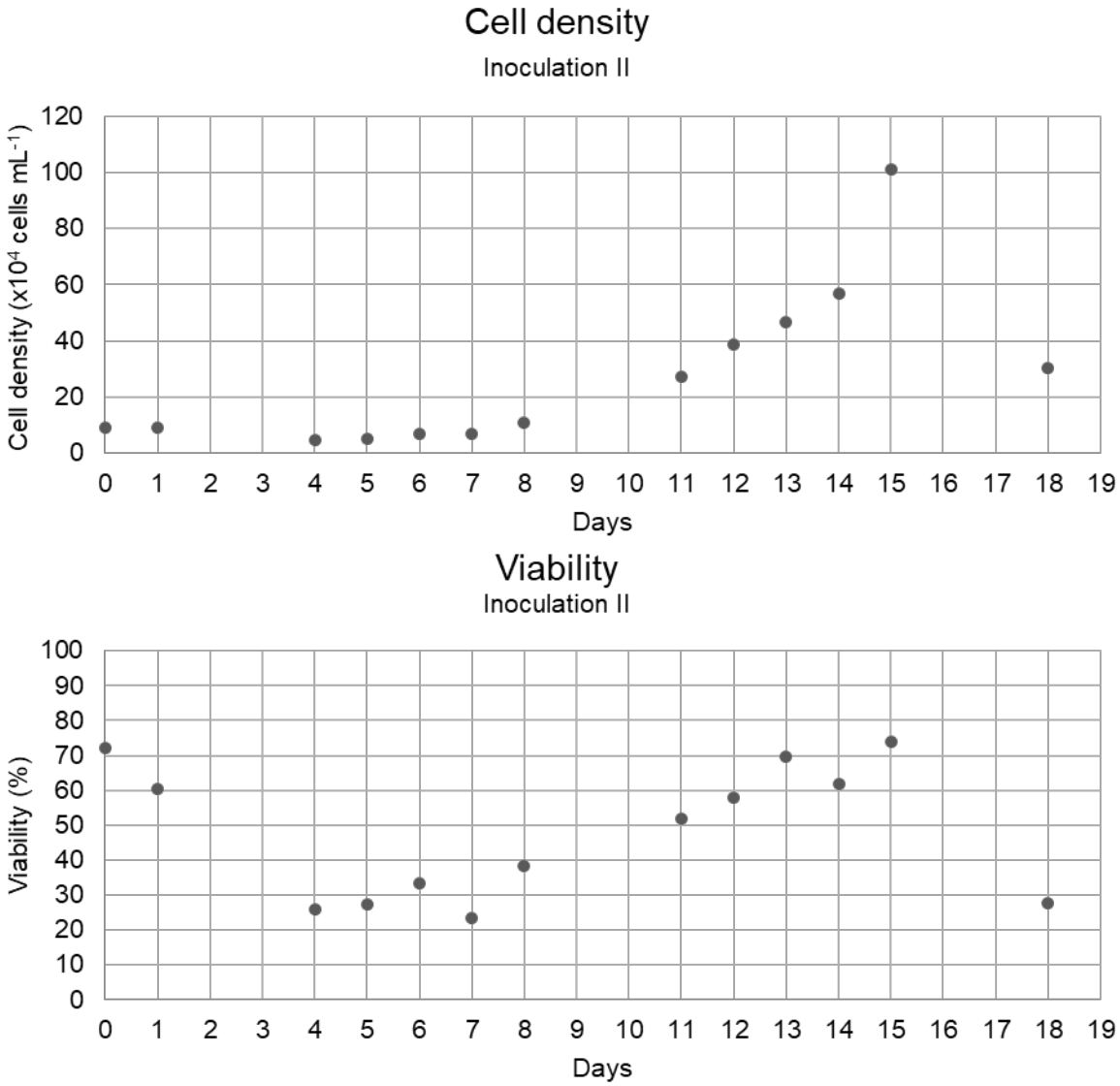

Figure 17: Cell density (cells mL^{-1}) (top) and cell viability (%) (bottom) for C192-producing cells in bioreactor during inoculation II, estimated by cell counting on sample from the reactor. The cells were not counted on weekends (day 2, 3, 9, 10, 16, and 17).

According to Figure E.2, DO reached 50 % after approximately a half day. There were however occasional peaks, not referring to oscillations described earlier, where DO reach up to 85 % and down to 0 % during the first three days. The peak formation in the DO curve then continues with increased intensity from day 4 in Figure E.3. According to user instructions for the DO sensor, fluctuating readings have many possible explanations, for instance depleted electrolyte solution and disturbances from air/O_2 bubbles. The origin of the DO fluctuations was not further elucidated in conjunction with inoculation II.

After about twelve days, the oscillation started and continued until day 17.5. During this time, cell density and viability increased (Figure 17). Cell density was not estimated on day 17 but it is speculated that when the cells stopped growing (somewhere between day 15 and 18), the oscillation stopped. In that case, the cells stopped growing on day 17.5 approximately. Due to low cell density and viability the cultivation was ended on day 19. Additionally, the DO signal was completely lost on day 19 but was later re-established without opening the reactor.

The cells in inoculation II were in lag phase for about eight to ten days. Thereafter, the growth rate increased slightly between day 9 or 10 to day 14. Between day 14 and 15, the cells grew from approx. $55x10^4$ cells mL^{-1} to about $100x10^4$ cells mL^{-1}, which is almost a duplication in one day. At this stage, the cells have entered the log phase and specific growth rate is estimated with Eq. (2) to 0.6 day^{-1} which is higher than during lag phase of inoculation I. Notable for inoculation II is the long lag phase and low viability. The viability dropped after day 1 to less than 30 % on day 4 (Figure 17). The inoculated cells were obtained from the production department of FDAB and were therefore not adapted to CD Hybridoma medium at obtention. Although the cells were cultivated in CD Hybridoma for one respectively three passages before inoculation, the cells required a longer lag phase in the reactor, possibly for adaptation to both medium and environment.

It is not fully understood why the cells entered the stationary phase and decline phase between day 16 and 18. However, due to continuously low viability numbers in combination with batch cultivation, it is believed that toxic metabolites accumulated in the reactor which inhibited cellular growth. It is also possible that the cells left the log phase due to nutrient deficit. Fresh medium was added on day 15, but perhaps not in sufficient volume. Also noted is the rapid decrease in cell density and viability after the log phase, which is shown for inoculation I as well. Perfusion mode was not initiated on day 15 since it was assumed to be too few cells in the reactor. However, it is thought that the cells would have benefited from continuous addition of fresh medium for prevention of toxic metabolites production. Furthermore, it is speculated that perfusion mode would have beneficially diluted and partially removed already accumulated toxic metabolites in the reactor.

Concentration of C192 increased during the whole cultivation and was estimated to 62.7 µg mL^{-1} on day 18 in approximately 1.05 L culture medium. Total amount of mAbs produced is thus 65,8 mg which is about half of what was produced in eight days in inoculation I. The antibody production is thus not enhanced during a long lag phase where the cells adapt.

Monitoring results for inoculation III are presented in the same way as for inoculation I and II, now in Figure E.4. Cell density (cells mL^{-1}) and viability (%) was, due to long weekend, estimated for the first time on day 5. At that time, there were nothing but visible debris in the reactor. It was concluded that the cells had been degraded during the weekend. Figure E.4 reveals that there were no air or oxygen injected to the reactor during the five days of monitoring, indicating that the cells never started growing. Noteworthy, there are no DO-peaks in the figure thus indicating a functioning DO sensor.

A possible explanation to the cellular degradation is accumulated toxic compounds in the reactor from inoculation II. The reactor was not cleaned between inoculation II and III. Most of the medium from inoculation II was instead pumped out and then diluted with fresh medium. The process was repeated a couple of times to further dilute the remaining medium from inoculation II. It is however hypothesized that dilution of spent medium was not enough

for removing toxic metabolites from the culture medium. Interestingly, total amount of C192 decreased in the reactor from day 0 to day 5, from 26,8 mg to 16.1 mg. It is therefore suggested that toxic metabolites affected the antibodies as well. Either by degradation or by changing their properties such that the antibodies were not detected in the ELISA.

DO monitoring during inoculation IV is shown in Figure E.5, with same parameters and variables included and excluded as for inoculation I. Cell density (cells mL^{-1}) and viability (%) was estimated on samples from the reactor for all days except during weekend (day 4 and 5), see Figure 18. The cells show an almost immediate transition from lag phase to log phase. The reactor was therefore put in perfusion mode on day 3. The cells nevertheless aggregated to visible aggregates on day 4 or 5, resulting in no viable cells on day 6. It is believed that the cells aggregated because of too high cell density. It is unclear if the aggregates would have formed if the CSPR had been kept constant during the cultivation. Since the cells are recirculated to the reactor in perfusion mode, it is possible that the cells would have aggregated at higher perfusion rates (constant CSPR) as well.

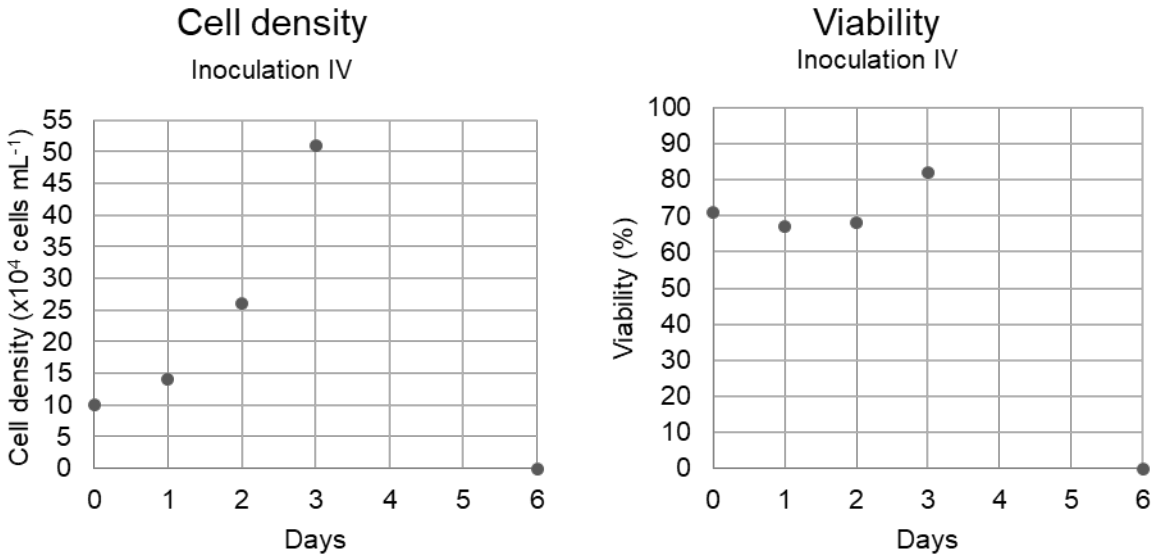

Figure 18: Cell density (cells mL^{-1}) (left) and viability (%) (right) for C192-producing cells estimated on samples from the bioreactor during inoculation IV. Samples were taken on day 0, 1, 2, 3, and 6.

Noteworthy for inoculation IV is the consumption of air and oxygen. The air injection started after 1.5 days and continued until day 6. Oxygen was injected slowly until approx. 5.2 days where the injection accelerated and continued until day 6. When the reactor was found with cell aggregates on day 6, the injection of air and oxygen was ongoing. It is therefore unclear why high volumes of air and oxygen had to be injected to the reactor to keep the DO at 50 %. There was no infection causing it. High injection volumes of air and later also oxygen caused formation of foam in the reactor. The foam caused the aggregates to stick to the pipes close to the reactor lid instead of being solubilised in the medium. The oscillation phenomena occurred from just before day 3 to day 5. It is plausible that the cells were in the log phase during that time and then aggregated just before day 6.

Antibody concentration in the reactor was determined on day 0, 1, 3, and 5. Interestingly, the total mAb weight was first decreasing from day 0 to 1, from 17.8 mg to 8.1 mg. Thereafter, the weight increased to 23.8 mg on day 3 and finally 25.0 mg on day 6. Unlike inoculation III, the

cells in inoculation IV grow while the amount of mAbs decreased. The mAbs are thus affected before the cells are.

Monitoring of DO during inoculation V is presented in Figure E.6 along with injection volumes of air, N_2, and O_2. Other parameters and variables monitored during cultivation were excluded from the figure. During the first 2.5 days, the DO was 70 %. The DO was not down regulated since there was no pressure over the nitrogen tube. Additionally, pH was approx. 6.7 during the first 2.5 days. The pH remained constant since the control unit had no way to upregulate pH. At approx. 2.5 days, the N_2 pressure was turned on and the gas injection volumes were set to zero, hence the abrupt changes after 2.5 h. DO and pH were then kept at 50 % respectively 7 for the rest of the time by continuous addition of nitrogen and carbon dioxide. Cell density was slowly decreasing during the first four days. At day five, there were no cells present in the reactor. The cells have earlier, during spinner flask cultivation and bioreactor inoculation 2, been noted to adapt slowly to a new environment. It is therefore believed that the cells died from a combination of environmental adaption and high respectively low DO and pH.

4.2.3 Downstream processing C192

Concentration of C192 produced in the bioreactor during inoculation I resulted in 102 mL elute with mAb concentration 0.46 mg mL^{-1}. Purification in turn resulted in two fractions of purified C192 with a total volume of 12 mL after pooling. Chromatogram from purification is included in Appendix D, Figure D.6. Elute volume was then decreased to 9.2 mL after buffer exchange and filtration, with antibody concentration 2.4 mg mL^{-1}.

Analysis report from SEC is shown in Figure D.7. The SEC results show that 99.3 % of the total peak area belongs to C192. As concluded for SEC of E146 elute, over 99 % of the molecules in the sample are the purified antibody.

The immunoactivity assay elucidates that the relative immunoactivity (Eq. (10)) is 100.4 % for C192 produced in the bioreactor. Percent inhibition for reference C192 and bioreactor C192 was 60.6 % respectively 60.8 %. The relative immunoactivity declares that the antigen (CA19-9) binding ability of C192 is not altered when the mAb-producing cells are cultivated serum-free in a bioreactor.
An image of the gel from IEF on C192 mAbs is shown in Figure 19 (left). C192 from the reactor (lane 2) is shown together with reference C912 (lane 3) and a reference containing proteins with known pI (lane 1). Clusters of bands are visible in the pI interval 8.3 to 9.45. There is a clear change in charge distribution among charge isoforms of C192 when comparing reference mAbs with mAbs from the reactor. Antibodies from the reactor have a higher pI than reference mAbs, meaning that mAbs from the reactor have higher positive net charge (smaller negative net charge). It is not elucidated from where the change in charge distribution originates, but it is shown that the antigen-antibody binding ability is not affected.

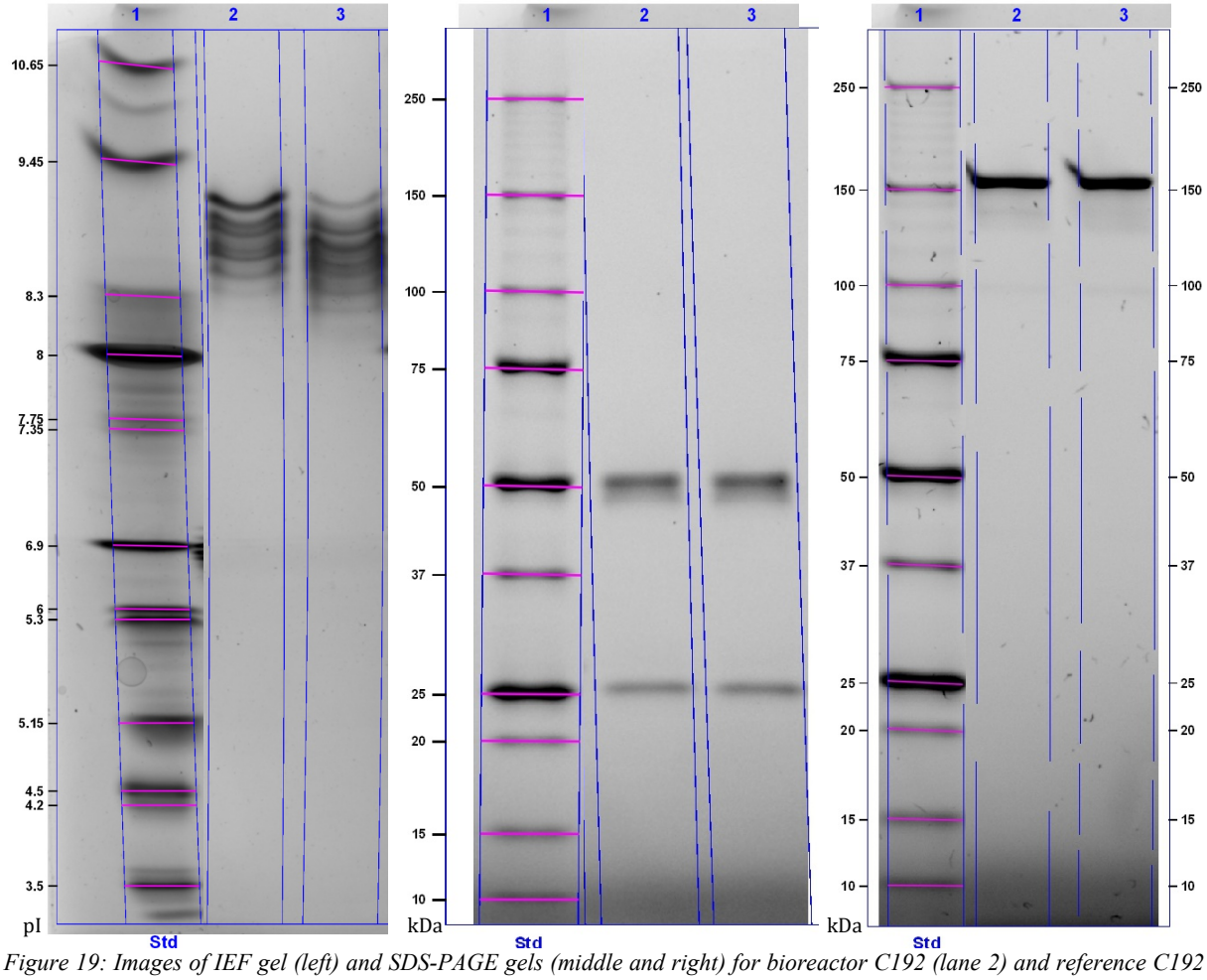

Figure 19: Images of IEF gel (left) and SDS-PAGE gels (middle and right) for bioreactor C192 (lane 2) and reference C192 (lane 3). Reference mAbs were cultivated in standard medium (5 % FBS) at the production department at FDAB. Lane 1 in each gel is loaded with a reference containing proteins with known pI (IEF) respectively size (SDS-PAGE). The image in the middle is from a reduced SDS-PAGE while the image to the right is from a non-reduced SDS-PAGE.

Images from SDS-PAGE, both reduced (middle) and non-reduced (right), are shown in Figure 19. The second lanes are loaded with reduced respectively non-reduced mAbs from the bioreactor. The third lanes are loaded with reference mAbs, reduced and non-reduced. In reduced SDS-PAGE, the light chains end up at 25 kDa while the heavy chains end up at 50 kDa. That is because the disulphide bonds have been broken by the mercaptoethanol. The results are consistent for both bioreactor mAbs and reference mAbs. SDS-PAGE with non-reduced mAbs shows one band at 150 kDa for both types of mAbs.

5 Conclusions

The conclusions are split into two subchapters, one related to serum-free production of E146 and one with conclusions from bioreactor production of C192.

5.1 Serum-free production E146

E146-producing cells grow and produce antibodies in both CD Hybridoma and Hybridoma SFM. Specific antibody productivity (pg mAb $cell^{-1}$ day^{-1}) is however higher for cells cultivated in CD Hybridoma when compared to Hybridoma SFM or standard medium (5 % FBS). The antibody productivity is believed to be enhanced by low specific growth rate. Noteworthy, a low specific growth rate in combination with high antibody productivity is potentially favourable from an economic point of view since less medium is consumed per antibody produced. The litre price for CD Hybridoma is reported by FDAB to be slightly lower when

compared to Hybridoma SFM. The choice of CD Hybridoma for production of E146 is thus beneficial from partly a specific antibody productivity point of view, but also from an economical point of view. There are however additional factors to consider when deciding on which culture medium to use, such as shipping availability.

There are above all two points to further elucidate considering cultivation of E146-producing cells in CD Hybridoma. Firstly, the charge distribution shift seen in AEC. Relative immunoactivity should be re-evaluated to ensure the shift do not change the relative immunoactivity of the antibodies. There are other more specific mAb characteristics, such as affinity, specificity, stability, and solubility to evaluate as well to ensure mAb quality[3, 48]. Secondly, the freeze-thaw cycle investigation should be remade to ensure cells cultivated in CD Hybridoma do not decrease in specific antibody productivity with increasing passages.

As a last note, the automated method for purification of E146 with the ÄKTA pure device should be further studied to untangle why the mAbs are eluted during column wash.

5.2 Bioreactor production C192

C192-producing cells show rapid transitions from log phase to stationary phase and then to decline phase for all inoculations (except inoculation III and V) when cultivated in batch mode in the bioreactor. Daily monitoring is therefore important such that CSPR is turned on and then kept constant when cell density increases. A suitable CSPR is believed to prevent the cells from aggregating. Another point of improvement for preventing the cells from aggregating is to operate the reactor at higher volume. When the cells aggregated in inoculation IV, the operating volume was approx. 1.2 L. If the cells aggregate during growth despite a constant CSPR and higher operating volume, a system for continuous reduction of cell density, also known as cell bleed[37], would be beneficial.

The cells were however also seen to be degraded, possibly due to accumulation of toxic metabolites. Therefore, it is of importance to inoculate the cells in a clean reactor with fresh culture medium. The production of toxic metabolites is possibly avoided by inoculating the reactor with a cell culture with high density that is properly adapted to the cell culture medium. In that way, an unnecessary long batch cultivation in the reactor is avoided. An alternative to preculturing in T-flasks is to preculture the cells in a spinner flask. In that way, the cells are adapted to stirring before inoculated into the reactor. This alternative has had success at FDAD for other cell lines, especially when cells are to be cultivated in a larger bioreactor. However, spinner flasks have been shown, not only in this project, to be easily infected. Furthermore, the lag phase is only believed to be too long in inoculation II when the cells were not fully adapted to culture medium.

One major problem throughout the project was regarding monitoring and operation during weekends. During the project it was not possible to count the cells on the weekends. The cultivation was instead only monitored through the software. However, the software did not allow the user to change the settings of the control unit and it was hence not possible to change the perfusion rate during the weekends. Optimally, cell density is estimated daily, also on the weekends, such that perfusion rate is appropriately varied. An alternative to daily manual counting is to consider an automatic cell counting device, for instance based on optical density[11].

From QC and characterisation (IEF) of C192 it is concluded that the charge distribution among the charge isoforms of C192 varies when comparing bioreactor mAbs and reference mAbs. It is speculated that the degradation/modification of mAbs seen in inoculation III and IV is an explanation. Even though the decrease in amount of mAbs was only seen in the beginning and not at all for the other cultivations, it is still possible that the degradation or modification is in progress during the whole cultivation. Alterations in terms of amino acid sequence and/or post-translational modifications when cultivated in bioreactor is a possible explanation to why the charge distribution changes. Consequentially, it is important to consider where the mAbs are modified to ensure that antibody quality is not decreased. The immunoactivity assay concluded that the difference in charge distribution does not affect the relative immunoactivity. The assay uses CA19-9, unlike other assays such as Protein A chromatography and sandwich ELISA where the specific antigen is not utilized. The modifications are thus not considered to impair the antibody's ability to bind to the antigen. There are however more mAb characteristics to investigate to ensure mAb quality[3].

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Appendix A: Buffers and solutions constituents

Table A.1 shows constituents and area of use for buffers and solutions, such as Assay buffer (AB) and Wash Solution, that are not explicitly described in Appendix B-D.

Table A.1: Constituents and area of use for 12 different buffers/solutions used throughout the project. Other buffers and solutions used in the project are stated explicitly in their respective context.

Buffer/solution	**Constituents**	**Area of use**
Assay Buffer (AB)	Tween 40 10 g/L – 10 mL/L DTPA titriplex V 2 mM – 10 mL/L Tris – 6.06 g/L NaCl – 9.0 g/L NaN_3 – 0.5 g/L Amaranth – 20 mg/L BSA – 15 g/L Bovine immunoglobulin – 0.5 g/L	ELISA
Wash Solution	NaCl – 225 g/L Germall II – 25 g/L Tween 20 – 1.25 g/L Tris – 15.14 g/L	ELISA, immunoactivity E146/C192
1 % Bovine serum albumin (BSA)-phosphate buffered saline (PBS) + 0.1 % Tween20	BSA – 10 g/L PBS x10 – 100 mL/L Tween20 – 1 mL/L	ELISA
Storing Buffer	KH_2PO_4 – 3.4 g/L K_2HPO_4 – 13.1 g/L KCl – 112 g/L Sucrose – 50 g/L	Storing buffer for mAbs
Cathode Buffer (10x)	Unknown, not prepared in-house	IEF
Anode Buffer (10x)	Unknown, not prepared in-house	IEF
IEF Sample Buffer	Unknown, not prepared in-house	IEF
SDS Sample Buffer (4x)	Unknown, not prepared in-house	SDS-PAGE
Running Buffer	Unknown, not prepared in-house	SDS-PAGE
Stop Solution	0.12 M 32 % HCl	IEF
HE4 Tracer Diluent	Confidential	Immunoactivity E146
CA19-9 Tracer Diluent	Confidential	Immunoactivity C192

Appendix B: Protocols for downstream processing E146

Appendix B constitutes of protocols used during downstream processing of E146. The protocols were used on reference mAbs cultivated in standard medium (5 % FBS), E146 produced in CD Hybridoma, and/or E146 produced in Hybridoma SFM.

B.1 E146 sandwich ELISA

Concentration determination of E146 was performed with sandwich ELISA according to the following procedure. Each sample with unknown E146 concentration was diluted in Assay Buffer (AB) to fit within the mAb concentration range of the standard curve. Two dilutions were done per sample. 96-well plates coated with Goat-anti-mouse-IgG+IgM were already prepared and thus available in-house. The plate was first washed x1 with Wash Solution whereafter duplicates of 20 µL diluted mAb sample was added. Along with the samples, duplicates of blanks containing only AB and a standard curve ranging from 0.1 to 0.5 µg mL^{-1} was added. 200 µL AB was added to each well before the plate was incubated in a plate shaker at room temperature 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 1:25000. Once again, the plate was incubated on a plate shaker at RT for 1 h. Next, the plate was washed x6 with Wash Solution and then 100 µL TMB was added per well. After 10 min incubation in a plate shaker at RT, the HRP-TMB-induced colour shift was read in a BioTek ELx808 spectrophotometer at 620 nm. The computer software automatically calculated the average mAb 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.

B.2 E146 Amicon Ultra 30 K concentration

Concentration of E146 was performed with Amicon Ultra 30K centrifugal filter devices. The filters had a molecular weight cut-off (MWCO) equal to 30 kDa and were enclosed in a centrifuge tube. Antibodies and other large molecules were hence kept in the filter upon centrifugation, while smaller molecules along with most of the medium constituted the flow through (FT). Each device was loaded with maximum 12 mL mAb harvest whereafter the devices were centrifuged in a Sigma 6-16k centrifuge (fixed-angle rotor) at 5000xgfor 30 minutes at RT. Between each centrifugation, FT and concentrate was collected into two separate flasks. After concentration, the mAb concentration in both FT and concentrate was evaluated in an ELISA. Concentrate from E146 in CD Hybridoma was kept at 4 ºC for two days prior to purification. Hybridoma SFM concentrate was stored at 4 ºC for one day and then frozen to -18 ºC for 13 days before purification.

B.3 E146 manual chromatography purification

Purification of E146 in CD Hybridoma was made on a manual chromatography system from Pharmacia Biotech consisting of a pumping unit, a UV-vis spectroscopy unit detecting at 280 nm, a fraction collector set at 1 mL per fraction, and a printer for continuous recording of absorbance at 280 nm. A 1 mL HiTrap MabSelect SuRe column from Cytiva was attached to the system. Prior to loading the mAb to the loop of the chromatography system, the mAb supernatant was diluted in 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer to a final mAb concentration of 0.5 mg mL^{-1}. It was then filtrated through a 0.2 µm AcroCap positive pressure device from Pall Corporation. Flow rate was set on the pumping system to approximately 0.5 mL/min. The column was prepared through first regenerating the gel with more than three column volumes (CV) of 0.1 M Citrate-NaOH, pH 3.0 buffer. Thereafter, the column was equilibrated with ≥ 5 CV of 1 M Glycine-NaOH, 0.15 NaCl, pH 8.6 buffer. The mAb

supernatant was then pumped through the column before unbound proteins were eluted from the column with ≥ 5 CV of 1 M Glycine-NaOH, 0.15 NaCl, pH 8.6 buffer. Then, the E146 antibodies were eluted through pumping 0.1 M citrate, pH 4.0 buffer through the system. The antibodies were collected by the fraction collector. Prior to pooling all mAb-containing vials, each fraction was diluted 2:1 in storage buffer (1 M Tris-HCl, pH 8). The column was neutralised and regenerated by washing with ≥ 1 CV of 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer, followed by ≥ 5 CV of 0.1 NaOH buffer, and lastly ≥ 1 CV of 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer. Last of all, the column was equilibrated with storing solution (100 mM Sodium acetate, 2 % benzyl alcohol, pH 5.2) for ≥ 5 CV.

B.4 E146 ÄKTA pure purification

Purification of E146 in Hybridoma SFM was made on an ÄKTA pure protein purification system from Cytiva, using the same 1 mL HiTrap MabSelect SuRe column as for E146 in CD Hybridoma. The system detected molecules by measuring absorbance at 280 nm, just like the manual system. Before the mAb was loaded on the chromatography system it was diluted to a final mAb concentration of 0.5 mg mL^{-1} in 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer. The mAb supernatant was also filtered through a 0.22 µm Millex-GV syringe filter unit. Furthermore, the ÄKTA system was prepared by pumping all connected buffers through the system, ending with 100 mM sodium acetate, 2 % benzyl alcohol, pH 5.2 buffer. The mAb solution was added to a flask which in turn was connected to an inlet tube. Thereafter, a method file was run allowing for automatic progression of the following process. First, the column was prepared through regenerating the gel with 3 CV of 0.1 M Citrate-NaOH, pH 3.0 buffer. The column was then equilibrated with 5 CV of 1 M Glycine-NaOH, 0.15 NaCl, pH 8.6 buffer. The mAb supernatant was then pumped to the system before unbound proteins were washed out of the column with 5 CV of 1 M Glycine-NaOH, 0.15 NaCl, pH 8.6 buffer. Thereafter, the E146 antibodies were eluted through pumping 5 CV of 0.1 M citrate, pH 4.0 buffer through the system. The antibodies eluted at this stage were collected by a fraction collector with fraction size 1 mL before each elute was diluted 2:1 in Storing Buffer. The column was neutralised and regenerated by washing it with 1 CV of 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer, followed by 5 CV of 0.1 NaOH buffer, and lastly 1 CV of 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer. Last of all, 5 CV of Storing Buffer (100 mM Sodium acetate, 2 % benzyl alcohol, pH 5.2) was pumped through the column. As a reference to the purification results from the ÄKTA pure system, E146 mAbs produced in standard medium at the production department of FDAB were purified as well. The reference was run with the same chromatography method apart from that elutes from the washing phase of the process were collected as well. For information about flowrates, see 4.1.2.

B.5 E146 Spectra/Por 4 buffer exchange

After purification, E146 mAbs were buffer exchanged to Storing Buffer. For E146 produced in CD Hybridoma, the elute was buffer exchanged through dialysis. The dialysis took place in a Spectra/Por 4 dialysis membrane with MWCO equal to 12-14 kDa, submerged into 5 L Storing Buffer. The buffer was replaced after one day. After dialysis, the elute was filtered with a 0.22 µm Millex-GV syringe filter and then fractioned prior to freezing at -76 ºC.

B.6 E146 Amicon Ultra 30 K buffer exchange

E146 in Hybridoma SFM was buffer exchanged through filtering, using an Amicon Ultra 30 K device with maximum loading volume of 4 mL. The mAb elute from the ÄKTA system was added to the filtering device, whereafter it was filled up to 4 mL with Storing Buffer. Thereafter, the device was centrifuged in a Sigma 6-16k centrifuge (fixed-angle rotor) at 7500xg for ten minutes. The FT was kept separately before another 4 mL Storing Buffer was added, and the

device was centrifuged for ten more minutes at 7500xg. FT was kept once again while the supernatant was kept undiluted until further analysis of concentration.

B.7 E146 Ultraspec 2100 pro/Nanodrop 2000c concentration determination

Purified E146 was concentration determined by measuring absorbance at 280 nm. For E146 in CD Hybridoma, an Ultraspec 2100 pro spectrophotometer from Amersham Biosciences was used accompanied by a 50 µL quartz cuvette with path length 1 mm. The device was blanket with Storing Buffer before mAb elute was measured three times. For E146 in Hybridoma SFM however, a Nanodrop 2000c spectrophotometer from Thermo scientific was used instead. The device required 2 µL sample and was blanked with Storing Buffer before FT respectively elute was measured. For reference sample, elutes from both washing phase end elution phase was concentration determined. Both methods automatically used the measured absorbances to calculate the antibody concentration by using the Beer-Lambert law, see Eq. (8). Extinction coefficient was set to 13,500 mL/mg/cm.

B.8 E146 ÄKTA pure 25 L SEC

SEC was performed on an ÄKTA pure 25 L system from Cytiva, connected to a Superdex 200 Increase 10/300 GL column, also from Cytiva. In the system there was a spectrophotometer measuring absorbance at 280 nm for detection of molecules. The system was prepared through an equilibration where 1 CV analysis buffer (50 mM Tris-HCl, pH 7.75 + 0.05 % NaN_3) was washed through the system with a flow of 1 mL/min. Thereafter, a connected 100 mL sample loop was washed with the recently mentioned buffer whereafter the loop was loaded with 500 mL mAb sample. An automatic process was started where the flow was kept at 1 mL/min. The process started with equilibration of 0.5 CV analysis buffer before 100 µL mAb sample from the loop was flown through to the column. To elute the antibodies, 1 CV of analysis buffer was washed through the system.

B.9 E146 ÄKTA pure 25 L AEC

AEC was also performed on the ÄKTA pure 25 L system but now with a ProPac SAX-10 4x250 mm column guarded by a ProPac SAX-10 Guard 4x50 mm column. Constituent buffers were 20 mM Tris, pH 7.6 buffer, from now on mentioned as Buffer A, and 20 mM Tris, 1 M NaCl, pH 7.6 buffer, from now on Buffer B. The flow rate was set to 0.8 mL/min. To prepare the column, 1 mL 20 mM Tris, pH 7.6 buffer was injected to the sample loop before the automated chromatography process was run. The process started with equilibration of the column for 2 CV with a buffer-mix of 95 % Buffer A and 5 % Buffer B. Then, the content of the sample loop was added to the system through washing with 100 µL of the same buffer-mix buffer before the whole system was washed with 95 % Buffer A and 5 % Buffer B for 1 CV. A linear gradient of 5 to 50 % of Buffer B, relative Buffer A, was then flown through the system for 3 CV, ending with a gradient delay of 50 % Buffer A and 50 % Buffer B for 1 CV. Finally, a reversed linear gradient, from 50 to 5 % Buffer B (relative Buffer A), was flown through the system for 0.2 CV before the column was re-equilibrated with 95 % Buffer A and 5 % Buffer B for 2 CV. While the preparation of the system took place, the elute of E146 cultivated in CD Hybridoma was diluted in dilution buffer, consisting of 95 % Buffer A and 5 % Buffer B, to a final concentration of 0.80 mg mL^{-1} and a final volume of 600 µL. Thereafter, the diluted sample was filtered through a 0.22 µm Millex-GV syringe filter unit whereafter it was loaded onto the attached 100 µL sample loop. Lastly, the automated chromatography process described above was started again. The reference E146 mAbs analysed with the same method.

B.10 E146 immunoactivity assay

Before immunoactivity analysis preparation started, all reagents were allowed to reach RT. Then, analysis preparation started with diluting both reference and test mAbs from adaptation to a final concentration of 1 μg mL^{-1}. The antibodies were diluted in HE4 Tracer Diluent to a total volume of 300 μL. Antigen stock solution, containing ProGRP antigen, was diluted in HE4 Tracer Diluent to a final concentration of 1500 ng L^{-1}. 220 μL of the diluted ProGRP was then added to 220 μL reference respectively test mAbs. The solutions were thereafter incubated in RT for 20 minutes. Meanwhile, 860 μL diluted ProGRP was added to 860 μL HE4 Tracer Diluent. Also, biotin-labelled ProGRP was combined with HRP-labelled E146 by adding 150 μL HRP-E146 to 3 mL ProGRP-biotin PAb. The streptavidin coated wells were washed once with Wash Solution before 100 μL sample per well was added as following: 4 x reference mAb+ProGRP, 4 x ProGRP diluted in HE4 Tracer Diluent, 4 x CD Hybridoma mAb+ProGRP, 4 x ProGRP diluted in HE4 Tracer Diluent, and lastly 4 x background consisting of 0 ng L^{-1} ProGRP reagent. Thereafter, 100 μL HRP-E146+ProGRP-biotin PAb solution was added to each well before the plate was incubated at a plate shaker at RT for two hours. After incubation, the plate was washed six times with Wash Solution whereafter 100 μL TMB was added to each well. The plate was incubated again, this time for 30 minutes in a plate shaker at RT. Thereafter 100 μL Stop Solution was added to each well to stop the reaction between HRP and TMB. Lastly, the plate was incubated for five more minutes at RT with constant shaking before the absorbance was measured in a BioTek ELx808 spectrophotometer at 450 nm.

B.11 E146 IEF

Isoelectric focusing (IEF) was performed on reference E146 and E146 produced in CD Hybridoma. Cathode Buffer (1x) was prepared by diluting 20 mL Cathode Buffer (10x) in 180 mL RO-H_2O. Furthermore, Anode Buffer (10x) was diluted to 1x by adding 12 mL Anode Buffer (10x) to 588 mL RO-H_2O. Anode Buffer (1x) and Cathode Buffer (1x) was refrigerated (4-10 ºC) before use. Reference E146 was diluted from 15.1 mg mL^{-1} to 2.5 mg mL^{-1} in Storing Buffer. E146 produced in CD Hybridoma was kept undiluted (2.9 mg mL^{-1}). 10 μL of each E146 sample was then added to 10 μL IEF Sample Buffer (2x). The analysis used an IEF gel with pH range 3-10. Each well in the gel was washed three times with Cathode Buffer before the wells were filled with Cathode Buffer. Thereafter, the gel equipment was assembled, and the inner chamber was filled with 200 mL Cathode Buffer. 5 μL sample was added per well along with 5 μL SERVA Liquid Mix IEF Marker 3-10. The outer chamber was filled with 600 mL Anode Buffer and the power cables were attached to the equipment. The analysis was run for 1 hour at 100 V, followed by 1 hour at 200 V and finally 30 minutes at 490 V. While the IEF was running, Fixing Solution was prepared by solving 6 g 12 % trichloroacetic acid (TCA) and 1.8 g 3.5 % sulfosalicylic acid in 50 mL RO-H_2O. When the IEF run was complete, the gel was fixated in Fixing Solution for one hour under constant cradling. During that time, Dye Solution was prepared by adding 10 mL methanol, 10 mL Stainer A (from Colloidal Blue Staining Kit), and 1 mL Stainer B (also from Colloidal Blue Staining Kit) to 29 mL RO-H_2O. The gel was then incubated in 50 mL Dye Solution for 30 minutes with constant cradling. After 30 minutes, the gel was decolorized with RO-H_2O until the next day. On the next day, the gel was scanned in a ChemiDoc Touch Imaging system.

B.12 E146 SDS-PAGE

SDS-PAGE was performed on reference E146 and E146 produced in CD Hybridoma. Both mAbs were run both reduced and non-reduced on two separate gels. The reduced samples were prepared by adding 10 μL 0.2 mg mL^{-1} mAb and 10 μL SDS Sample Buffer (4x) to 20 μL diluted dithiothreitol (DTT). The DTT was prepared by diluting 1 M DTT (20x) 1:10 in RO-H_2O. Non-reduced samples on the other hand were prepared by adding 10 μL 0.2 mg mL^{-1} mAb

and 10 µl SDS Sample Buffer (4x) to 20 µL RO-H_2O. All samples, both reduced and non-reduced, were then denatured at 70 ºC for 10 minutes. Meanwhile, 1 L Running Buffer was prepared from 100 mL Tris/glycine/SDS Buffer (10x) added to 900 mL RO-H_2O. Thereafter, the wells of the gels were washed with Running Buffer before the whole module was assembled. Before the samples were added to the wells, 5 µL Precision Plus Protein Standard Unstained ladder was added to one well per gel. The samples were then added, 10 µL per well, before the power was turned on at 86 V for 30 min followed by another 15 min at 200 V. Lastly, the gels were visualised in the ChemiDoc Touch Imaging System.

Appendix C: Protocols for downstream processing C192

Appendix C constitutes of protocols used during downstream processing of C192. The protocols were used on reference mAbs cultivated in standard medium (5 % FBS) and/or C192 cultivated in serum-free medium in bioreactor.

C.1 C192 sandwich ELISA

Concentration determination of C192 was performed according to sandwich ELISA protocol described for E146 in Appendix B.

C.2 C192 Amicon ProFlux M12 concentration

Concentration with the Amicon ProFlux M12 tangential filtration system was prepared by washing 10 L RO water through the system with inlet pressure 1.5 bar, outlet pressure 0.5 bar, and a resulting pressure difference (ΔP) equal to 1.0 bar. During the last 2-3 L litres of the washing, the clean water flux (CWF) was measured as a quality control of the filters. CWF was evaluated by first measuring volume flow through the system for one minute. Volume flow was then multiplied with a temperature correction factor and divided by ΔP, filter size in m^2, and number of filters. The temperature correction factor was 1.047. After the RO water wash, C192 harvest was loaded on the device. Total harvest volume was 12 L. The pump was started and both inlet pressure and ΔP was set to the same value in the interval 1-2 bar. Antibody harvest was then concentrated to about 100 mL. Lastly, the system was cleaned by washing with another 10 L of RO water and then circulation with 3 L 0.1 M NaOH for one hour. Inlet pressure, outlet pressure, and pressure difference was 1.5, 0.5, respectively 1.0 bar during cleaning. Antibody concentrate was frozen at -76 ºC until purification.

C.3 C192 ÄKTA Avant 150 purification

C192 was purified on an ÄKTA Avant 150 chromatography system from Cytiva, with an attached 5 mL HiTrap MabSelect SuRe column from Cytiva. Molecules were detected by measuring absorbance at 280 nm. Before purification, the mAb concentrate was diluted 1:1 in 1 M Glycine-NaOH, 0.15 M NaCl, pH 8.6 buffer, and filtrated through a 0.2 µm AcroCap filter. The automated purification process was performed according to protocol in Appendix A, chapter B.4, differing only at two points: the mAbs were eluted with 0.1 M Citrate-NaOH, pH 3.5 buffer, and the maximum fraction size was 5 mL. As before, each fraction was diluted 2:1 in Storing Buffer whereafter the fractions were pooled.

C.4 C192 Spectra/Por 4 buffer exchange

Buffer exchange of purified C192 was made according to protocol for E146 in Appendix B, chapter B.5.

C.5 C192 Ultraspec 2100 pro concentration determination

After dialysis, the mAb concentration was estimated by measuring absorbance at 280 nm, using the Ultraspec 2100 pro spectrophotometer. Thereafter, the elute was filtered with a 0.22 µm Millex-GV syringe filter before fractioned and frozen at -76 ºC. The concentration was then once again measured in the spectrophotometer at 280 nm.

C.6 C192 immunoactivity assay

Immunoactivity assay for C192 was performed with reagents at RT. The assay started with washing the wells once with Wash Solution. In total, 20 wells were needed for the analysis. After the first wash, 25 µL 120 U mL^{-1} CA19-9 antigens were added to 16 of the 20 wells. To the remaining four wells, 25 µL background reagent (0 U mL^{-1} CA19-9) was added. Thereafter, 100 µL biotin-labelled C192 was added to each well before the plate was incubated on a plate

shaker at RT for two hours. During incubation, unlabelled C192 mAbs (reference resp. test) were diluted to 100 µg mL^{-1} in CA19-9 Tracer Diluent. Final volumes were 250 mL. Also, HRP-labelled C192 was prepared by diluting 150 µL mAb reagent in 3 mL CA19-9 Tracer Diluent. After incubation, each well was washed three times with Wash Solution. Thereafter, 25 µL reference, test, or HRP mAbs were added to the 16 CA19-9 containing wells, as following: 4 x reference mAbs, 4 x HRP mAbs, 4 x test mAbs, and lastly 4 x HRP mAbs. To the background, 25 µL HRP mAbs were added per well. Before the plate was incubated on a plate shaker at RT for one more hour, 100 µL HRP mAbs were added to every well. After incubation, each well was washed six times with Wash Solution whereafter 100 µL TMB was added per well. The plate was then incubated for 30 minutes on a plate shaker at RT before the absorbance at 620 nm was measured in a BioTek ELx808 spectrophotometer. Absorbance measurement results were then used to calculate percent inhibition (Eq. (9)) for reference respectively test mAb, followed by relative immunoactivity (Eq. (10)) for C192 produced in the bioreactor.

C.7 C192 IEF

IEF of C192 mAbs was performed on undiluted C192 samples on the same gel as E146, hence following the same protocol (Appendix B, chapter B.11). Both reference mAbs and bioreactor mAbs were evaluated.

C.8 C192 SDS-PAGE

SDS-PAGE on C192 was performed stain-free on the same gel as E146. C192 was thus analysed both reduced and non-reduced for both reference and bioreactor mAbs. Protocol is available in Appendix B, chapter B.12.

Appendix D: Chromatography results

Appendix D contains all chromatograms obtained during the project, both for E146 and C192.

D.1 E146 CD Hybridoma manual chromatography purification

Chromatogram from purification of E146 produced in CD hybridoma on the manual affinity chromatography system is presented in Figure D.1. Column used was a 1 mL HiTrap MabSelect SuRe.

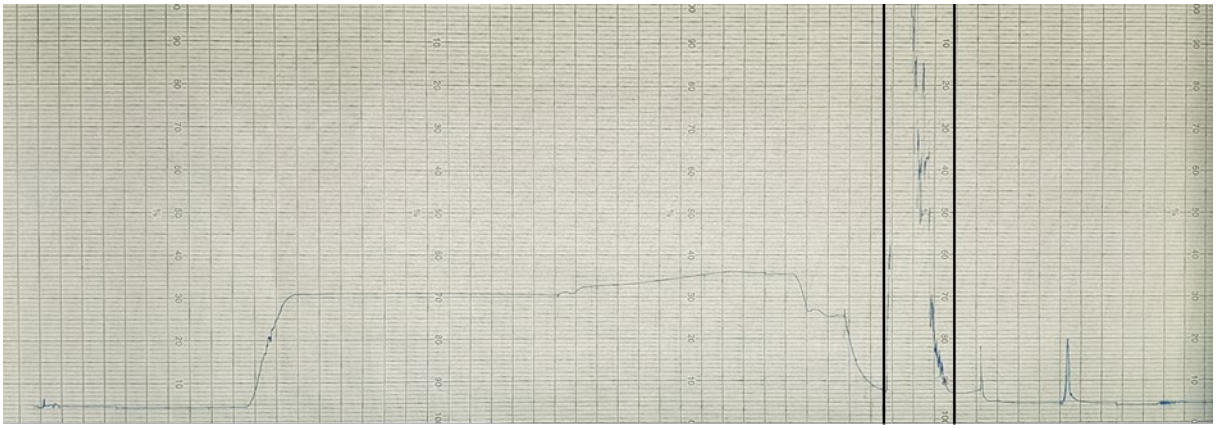


Figure D.1 Chromatogram from the manual affinity chromatography system used when purifying E146 mAbs produced by hybridoma cells cultivated in CD Hybridoma. The two black vertical lines enclose the peak where E146 was eluted. Elute was collected in six vials by the fraction collector to a total volume of 6 mL. Elute was thereafter diluted 2:1 in Storing Buffer.

D.2 E146 Hybridoma SFM ÄKTA pure purification

Figure D.2 shows the chromatogram from Protein A affinity chromatography performed on harvest from E146-producing cells cultivated in Hybridoma SFM.

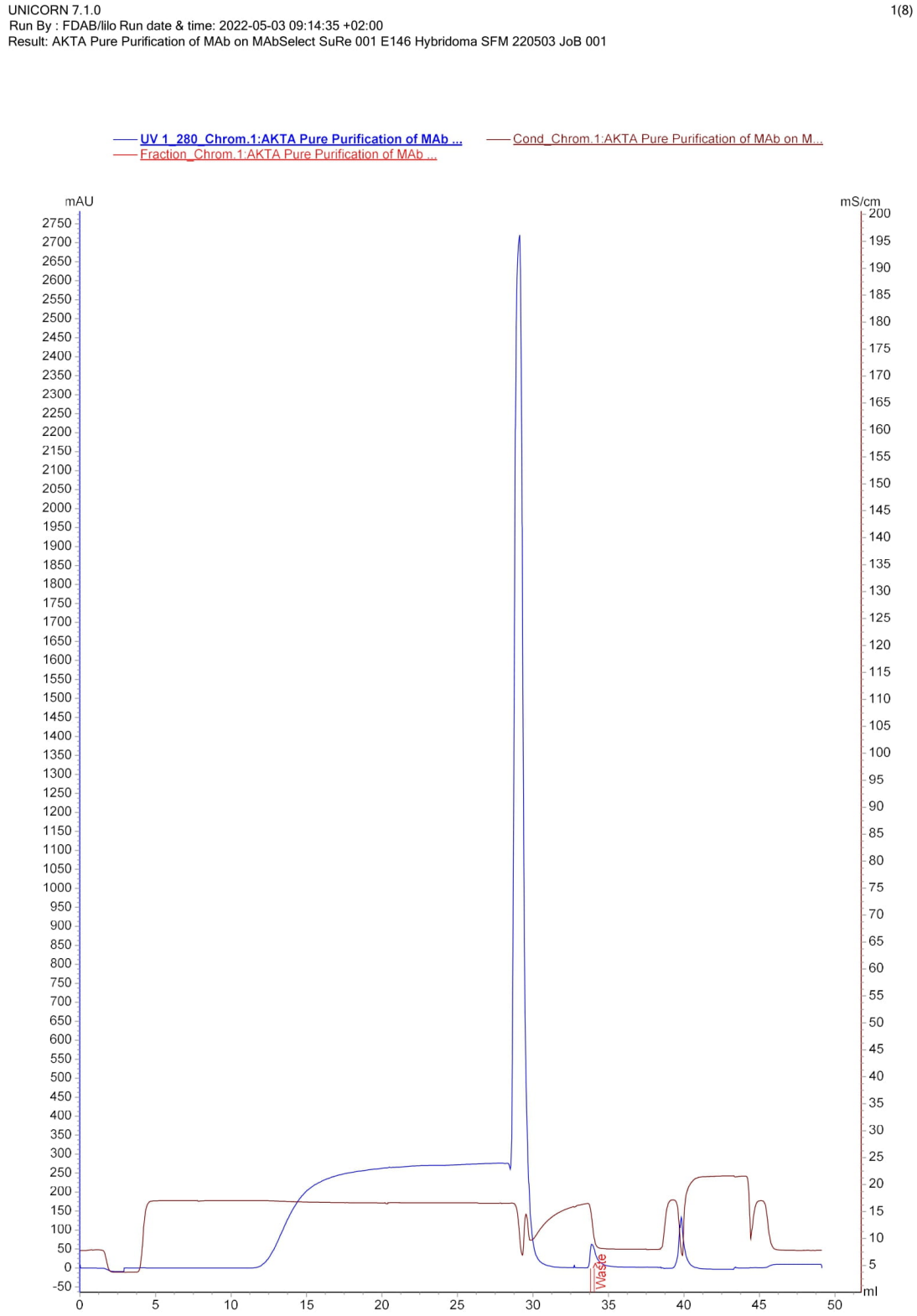
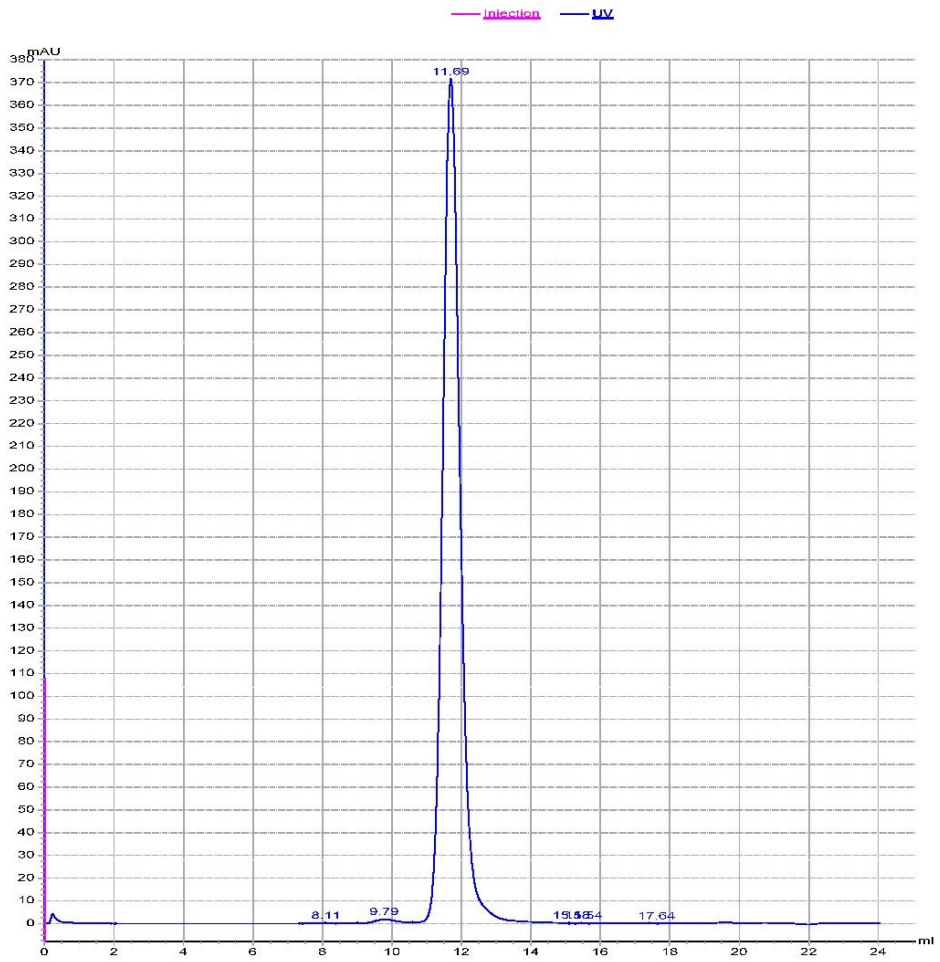


Figure D.2: Chromatogram from Protein A affinity chromatography for purification of E146 produced in Hybridoma SFM. The antibodies were unexpectedly eluted at 30 mL during the washing phase. Expected elution volume was 40 mL.

D.3 E146 ÄKTA pure 25 L SEC

Chromatography report from SEC on purified E146 produced by cells cultivated in CD Hybridoma is shown in Figure D.3.



UNICORN 7.1.0 2(2)
Run By : FDAB/lilo Run date & time: 4/27/2022 9:22:29 AM +02:00
Result: Superdex 200 10 300 GL, Analysis E146 CD hybridoma 220427 JoB 001

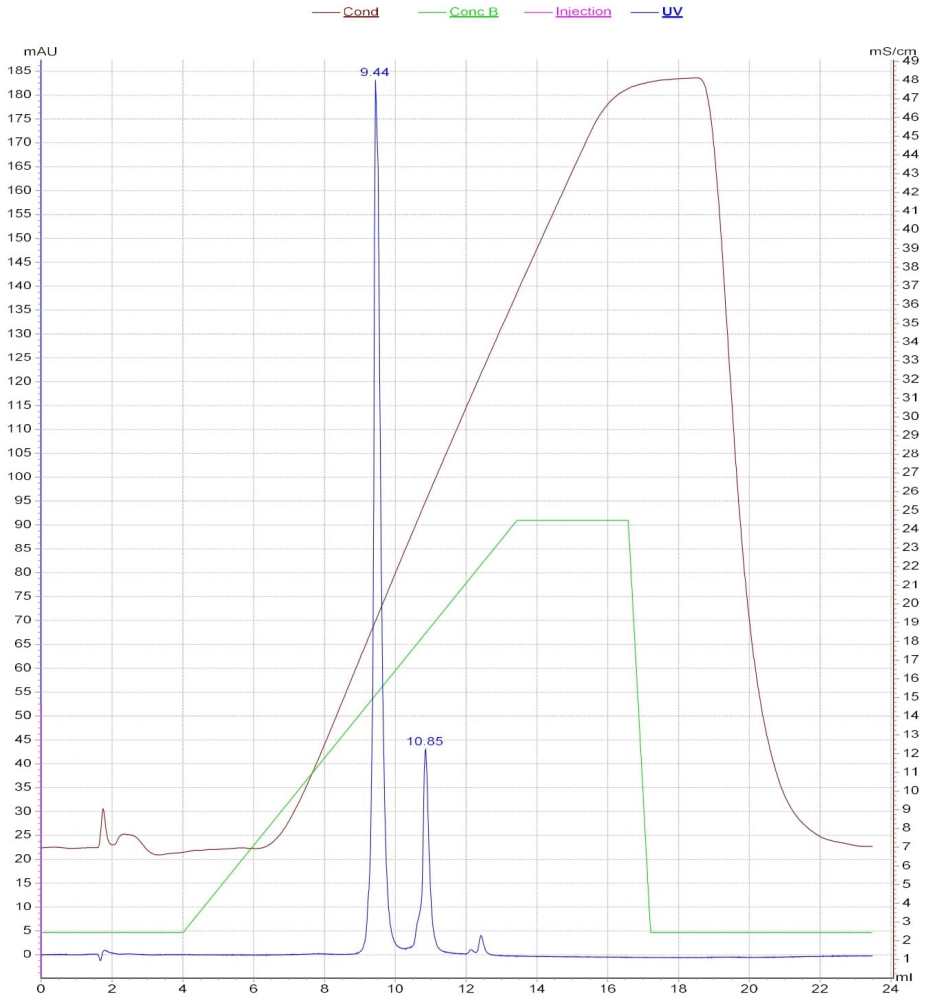
No	Retention	Area	% of total peak area	Height
	ml	ml*mAU		mAU
1	8.11	0.0188	0.01	0.092
2	9.79	1.2639	0.58	1.673
3	11.69	217.2921	99.38	371.430
4	15.18	0.0219	0.01	0.133
5	15.54	0.0188	0.01	0.096
6	17.64	0.0218	0.01	0.102

Calculation made in = Volume
Total number of detected peaks = 305
Total Area = 758.8873 ml*mAU
Total peak width = 7.03 ml
Area in evaluated peaks = 218.6372 ml*mAU
Ratio peak area / total area = 0.288102
Asymmetry level = 10.00
Calculated from = UV
Baseline name = UV@18,BASEM-Edited
Separation technique = GelFiltration
Column height = 30.00 cm
Column volume = 23.56 ml
Column void volume = 8.00 ml
Created = 4/27/2022 10:13 AM
Peaks rejected if:
Peak is less than the N largest = 20
Resolution algorithm = 3: ((Ret2 - Ret1) / ((2 * (WidthHalfHeight2 + WidthHalfHeight1))) / 2.354

Figure D.3: Report from size exclusion chromatography on purified E146 produced in CD Hybridoma. The peak at 12 mL corresponds to the purified mAbs.

D.4 E146 ÄKTA pure 25 L AEC

Chromatography report for AEC performed on E146 produced in CD Hybridoma is shown in Figure D.4.



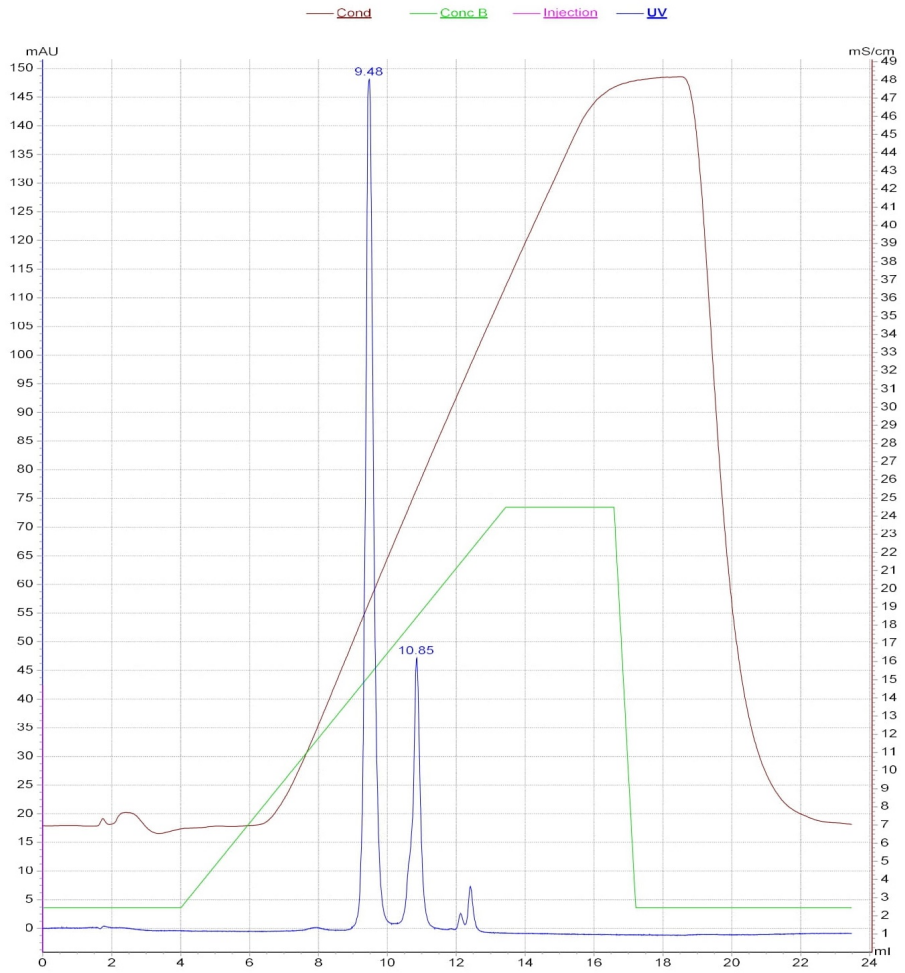
UNICORN 7.1.0 2(2)
Run By : FDAB/sogun Run date & time: 2022-04-28 09:56:31 +02:00
Result: ProPac SAX-10 E146 CD Hybridoma 220428 JoB 001

No	Retention	Area	% of total peak area	Height	Resolution
	ml	ml*mAU		mAU	
1	9.44	46.1080	81.82	183.180	-
2	10.85	10.2434	18.18	42.996	4.41

Calculation made in = Volume
Total number of detected peaks = 881
Total Area = 80.1163 ml*mAU
Total peak width = 2.35 ml
Area in evaluated peaks = 56.3513 ml*mAU
Ratio peak area / total area = 0.703369
Asymmetry level = 10.00
Calculated from = UV
Baseline name = Zero Baseline
Separation technique = AnionExchange
Column height = 25.00 cm
Column volume = 3.14 ml
Created = 2022-05-25 11:52
Peaks rejected if:
Peak is less than the N largest = 2
Resolution algorithm = 3: ((Ret2 - Ret1) / ((2 * (WidthHalfHeight2 + WidthHalfHeight1))) / 2.354

Figure D.4: Report from anion exchange chromatography performed on purified CD Hybridoma-produced E146. The distribution between the two largest charge isoforms is presented in the table below the chromatogram.

Report obtained from AEC performed on purified E146 produced in standard medium with 5 % FBS at the production department of FDAB is presented in Figure D.5.



UNICORN 7.1.0 2(2)
Run By : FDAB/sogun Run date & time: 2022-04-29 10:40:13 +02:00
Result: ProPac SAX-10, Analysis, E146, 48127, JoB 001

No	Retention	Area	% of total peak area	Height	Resolution
	ml	ml*mAU		mAU	
1	9.48	41.8118	76.93	148.158	-
2	10.85	12.5370	23.07	47.185	3.68

Calculation made in = Volume
Total number of detected peaks = 837
Total Area = 77.3450 ml*mAU
Total peak width = 2.25 ml
Area in evaluated peaks = 54.3488 ml*mAU
Ratio peak area / total area = 0.702680
Asymmetry level = 10.00
Calculated from = UV
Baseline name = Zero Baseline
Separation technique = AnionExchange
Column height = 25.00 cm
Column volume = 3.14 ml
Created = 2022-05-25 11:51
Peaks rejected if:
Peak is less than the N largest = 2
Resolution algorithm = 3: ((Ret2 - Ret1) / ((2 * (WidthHalfHeight2 + WidthHalfHeight1))) / 2.354

Figure D.5: Chromatography report from anion exchange chromatography performed on reference E146. The reference mAbs were produced in standard medium with 5 % FBS at the production department at FDAB. Charge distribution of charge isoforms is presented in the table below the chromatogram.

D.5 C192 ÄKTA Avant 150 purification

Figure D.6 presents the chromatogram from purification of C192 cultivated serum-free in bioreactor. The mAbs were purified with Protein A affinity chromatography.

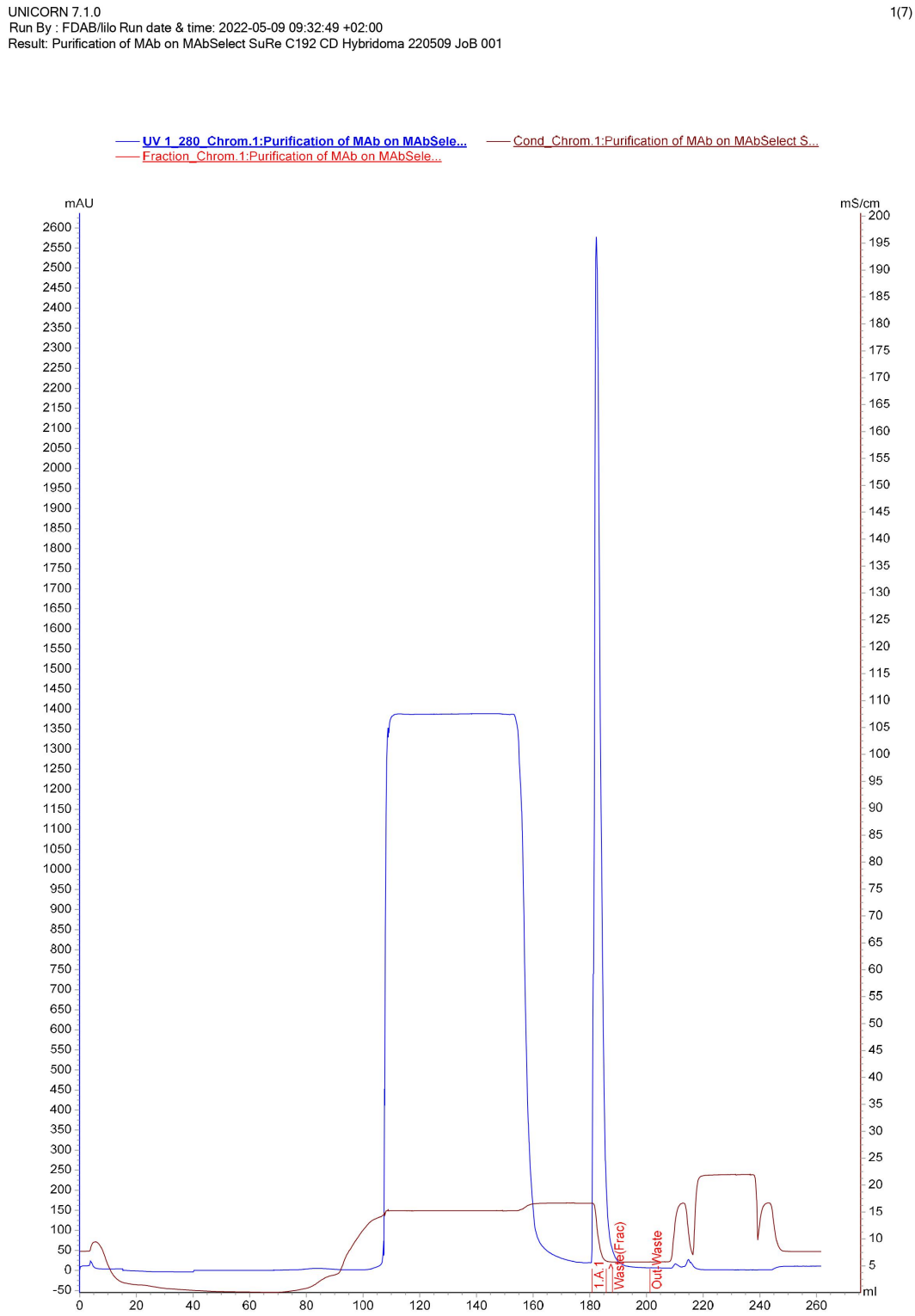
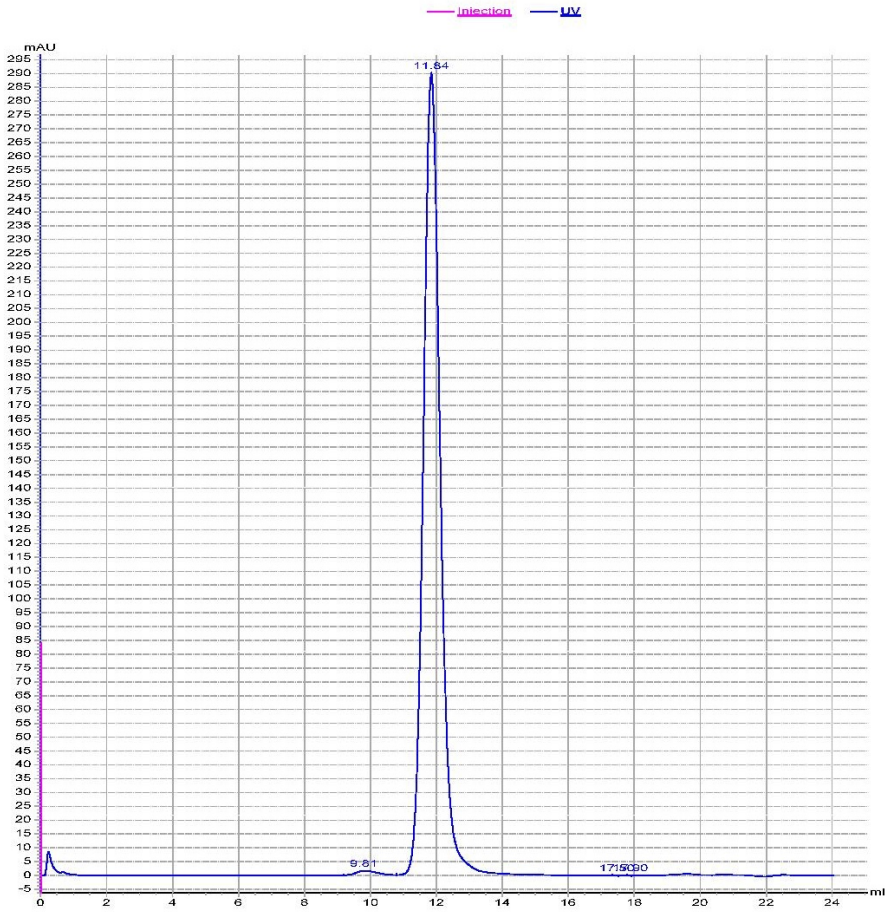


Figure D.6:Chromatogram from Protein A affinity chromatography for purification of C192 produced serum-free in bioreactor. The peak at 180 mL corresponds to the C192 mAbs.

D.6 C192 ÄKTA pure 25 L SEC

Chromatography report from SEC on C192 produced by cells cultivated in CD Hybridoma culture medium in a perfusion bioreactor is shown in Figure D.7.



UNICORN 7.1.0 2(2)
Run By : FDAB/kavaRun date & time: 5/12/2022 12:05:46 PM +02:00
Result: Superdex 200 10 300 GL, Analysis C192 CD Hybridoma 220512 JoB 001

No	Retention	Area	% of total peak area	Height
	ml	ml*mAU		mAU
1	9.81	1.1767	0.68	1.630
2	11.84	172.7882	99.30	289.959
3	17.50	0.0179	0.01	0.104
4	17.90	0.0167	0.01	0.153

Calculation made in = Volume
Total number of detected peaks = 256
Total Area = 810.9094 ml*mAU
Total peak width = 6.72 ml
Area in evaluated peaks = 173.9996 ml*mAU
Ratio peak area / total area = 0.214574
Asymmetry level = 10.00
Calculated from = UV
Baseline name = UV@18,BASEM-Edited
Separation technique = GelFiltration
Column height = 30.00 cm
Column volume = 23.56 ml
Column void volume = 8.00 ml
Created = 5/12/2022 1:18 PM
Peaks rejected if:
Peak is less than the N largest = 20
Resolution algorithm = 3: ((Ret2 - Ret1) / ((2 * (WidthHalfHeight2 + WidthHalfHeight1))) / 2.354

Figure D.7: Chromatography report from size exclusion chromatography on C192 produced serum-free in a bioreactor. The peak at 12 mL corresponds to the purified mAbs.

Appendix E: Bioreactor monitoring results

Appendix E contains DO-monitoring reports of inoculation I-V from the bioreactor software.

E.1 Inoculation I

Figure E.1 displays how DO (%) was regulated with air, O_2, and N_2 during inoculation I of the bioreactor. The gases are monitored in terms of amounts injected (mL).

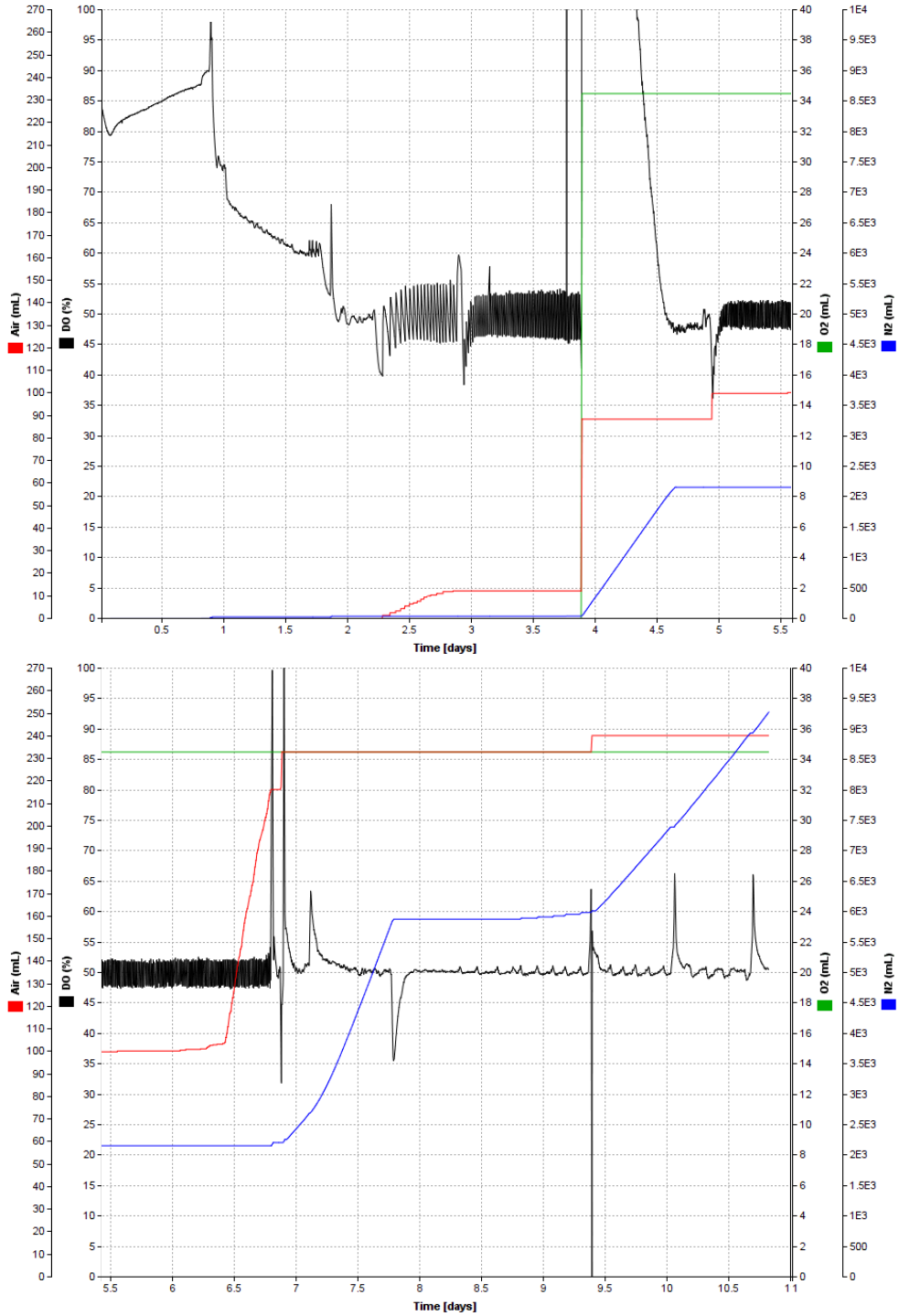


Figure E.1: Monitoring of DO (%) over 11 days during inoculation I of C192 in bioreactor. DO was upregulated with air (mL) and O_2 (mL). DO was downregulated with N_2 (mL).

E.2 Inoculation II

Figure E.2 and Figure E.3 displays how DO (%) was regulated with air (mL), O_2 (mL), and N_2 (mL) during inoculation II of the bioreactor. The first figure shows monitoring for the first three days while the latter shows from day 4-21. The gases are monitored in terms of amounts injected (mL).

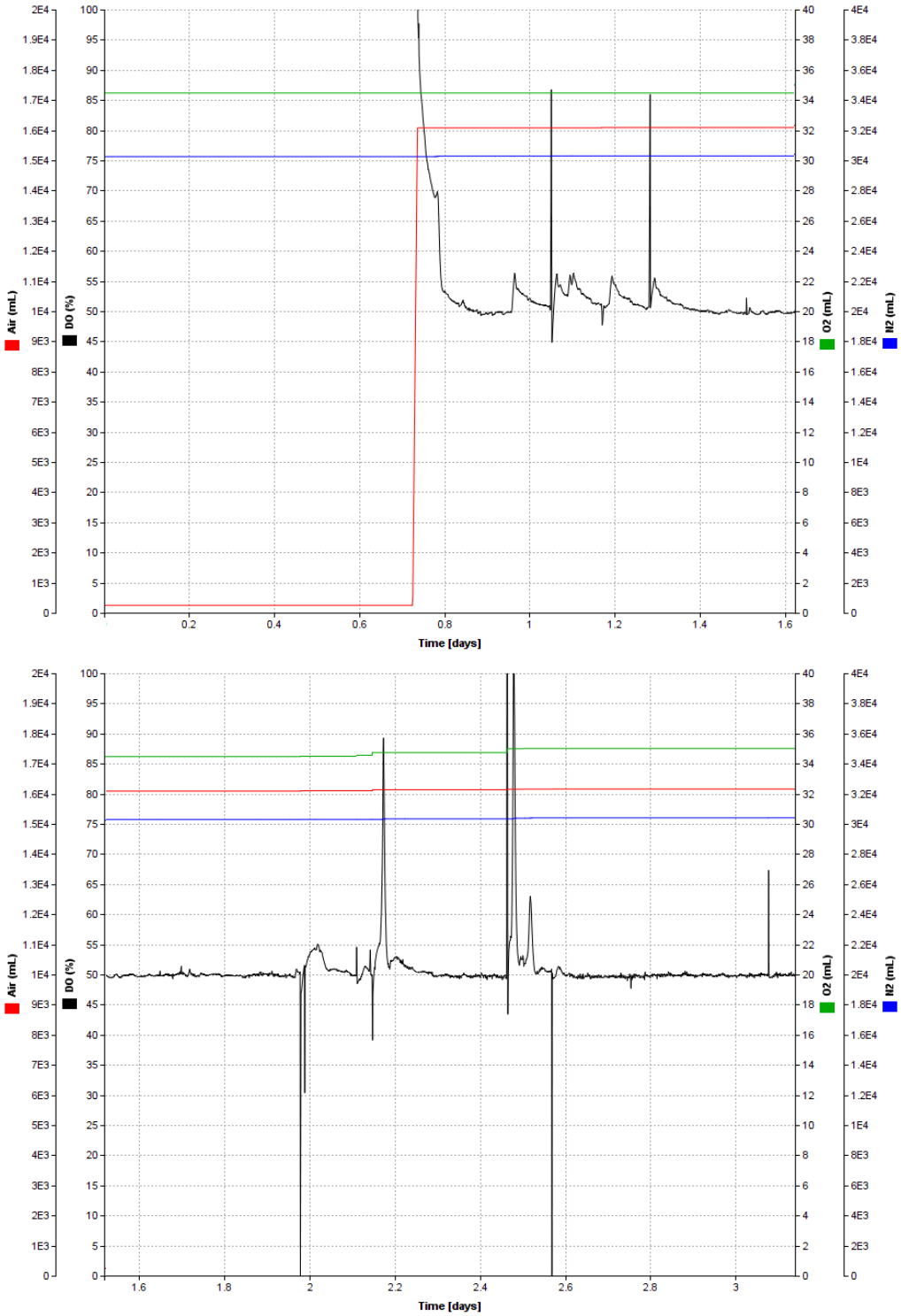


Figure E.2: Monitoring of DO (%), air (mL), O_2 (mL), and N_2 (mL) for the first three days of inoculation II with C192 in bioreactor. DO was upregulated with air (mL) and O_2 (mL). DO was downregulated with N_2 (mL).

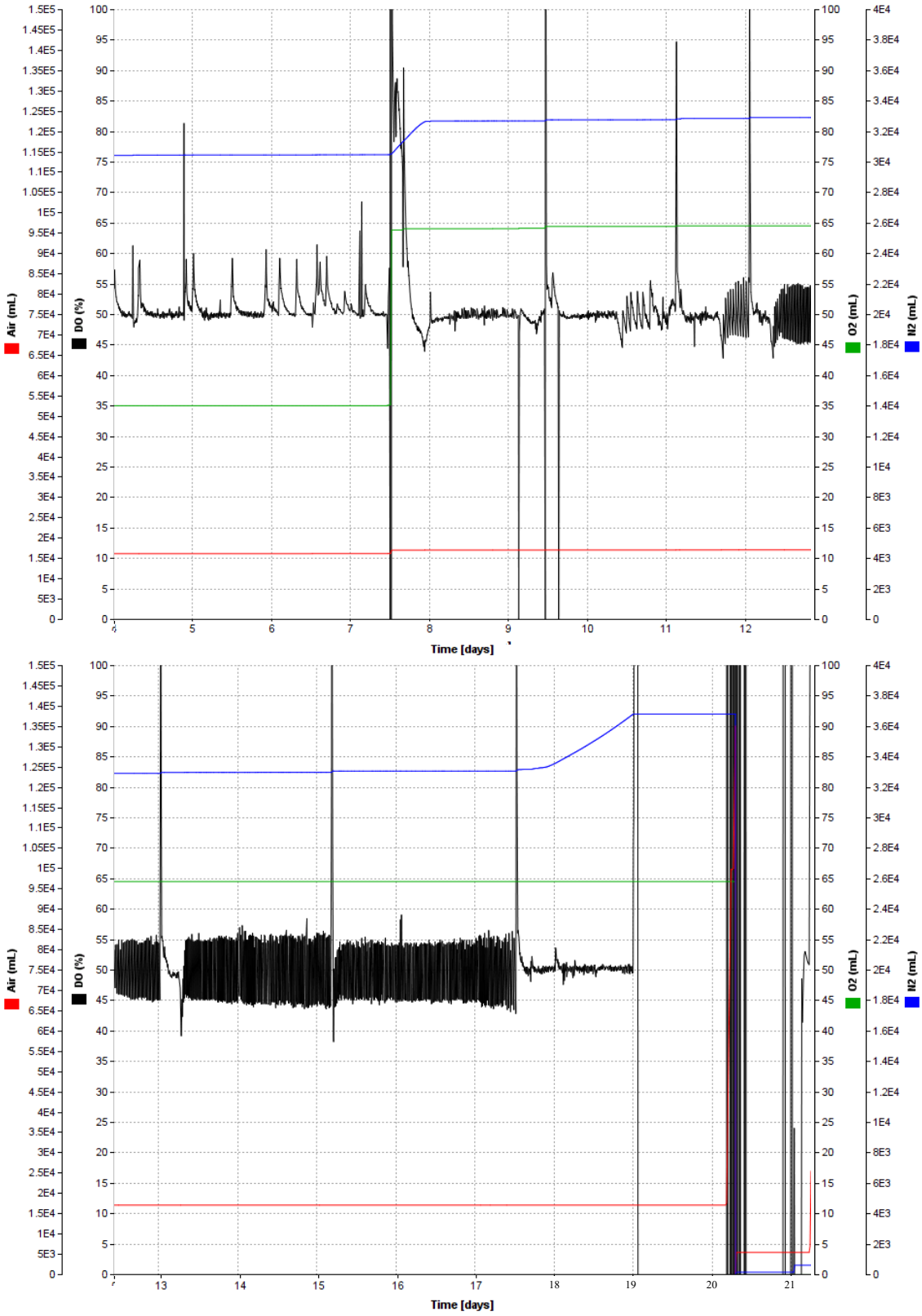


Figure E.3: Monitoring of DO (%), air (mL), O_2 (mL), and N_2 (mL) for day 4-21 during inoculation II with C192 in bioreactor. DO was upregulated with air (mL) and O_2 (mL). DO was downregulated with N_2 (mL).

E.3 Inoculation III

Monitoring of DO (%), air (mL), O_2 (mL), and N_2 during bioreactor cultivation of C192 in inoculation III is shown in Figure E.4.

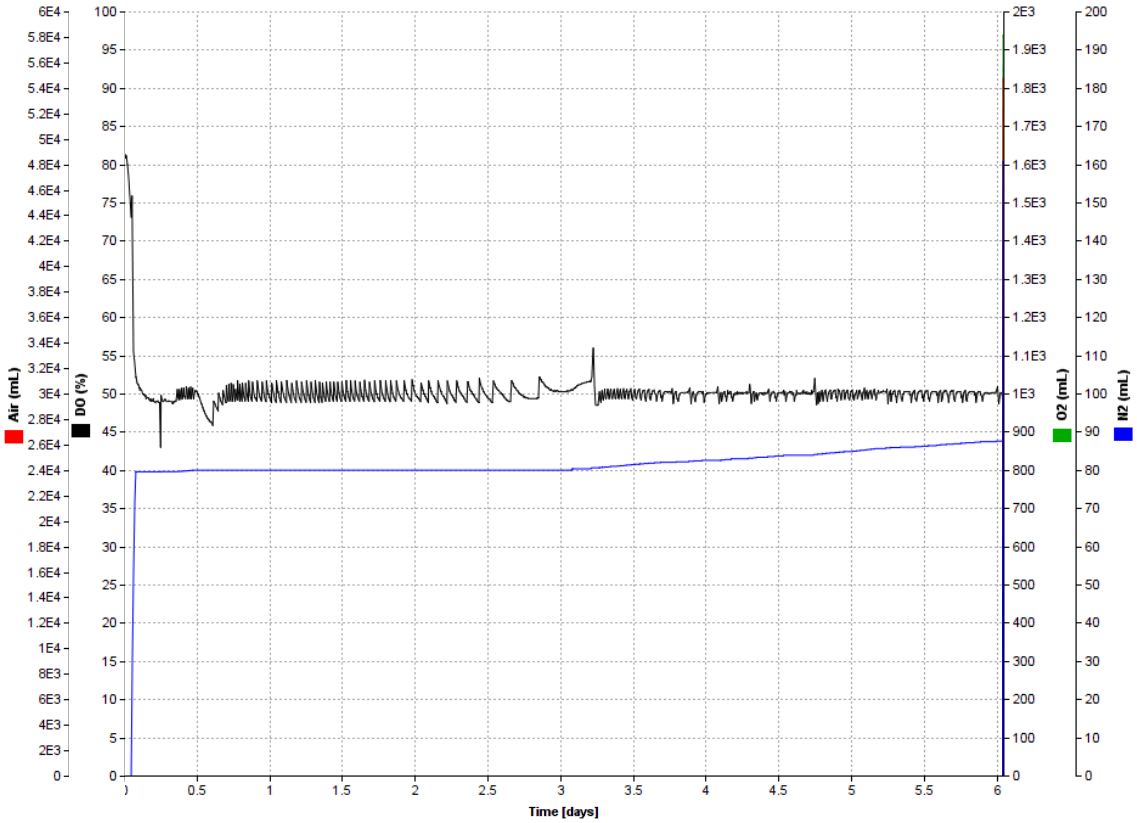


Figure E.4: Monitoring of DO (%), air (mL), O_2 (mL), and N_2 (mL) during inoculation III with C192 in bioreactor. DO was upregulated with air (mL) and O_2 (mL). DO was downregulated with N_2 (mL). The monitoring was on for 6 days.

E.4 Inoculation IV

Monitoring of DO (%), air (mL), O_2 (mL), and N_2 during bioreactor cultivation of C192 in inoculation IV is shown in Figure E.5.

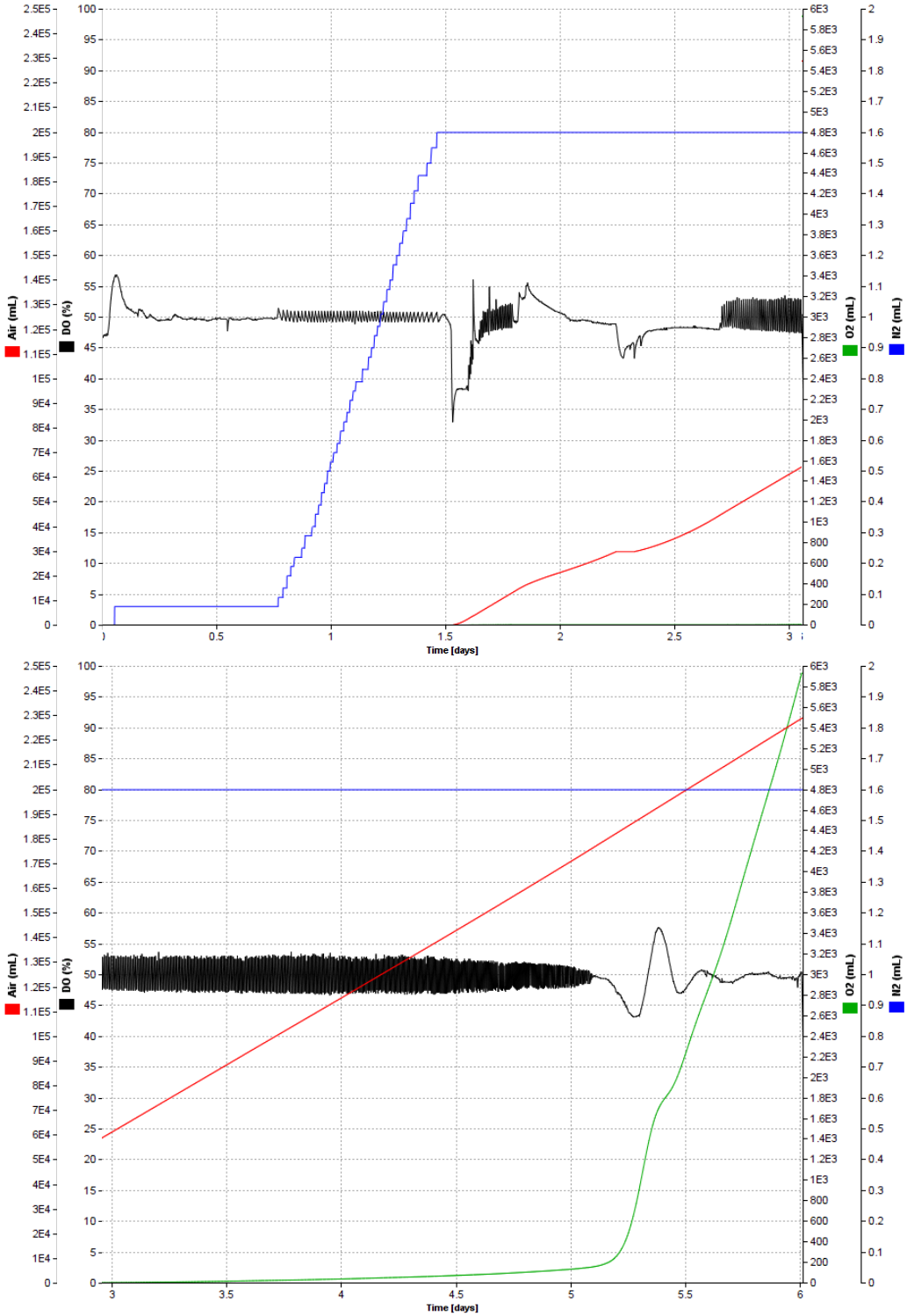


Figure E.5: Monitoring of DO (%), air (mL), O_2 (mL), and N_2 (mL) during inoculation IV with C192 in bioreactor. DO was upregulated with air (mL) and O_2 (mL). DO was downregulated with N_2 (mL). The monitoring was on for 6 days.

E.5 Inoculation V

Figure E.6 displays how DO (%) was regulated with air (mL), O_2 (mL), and N_2 (mL) during inoculation V of the bioreactor.

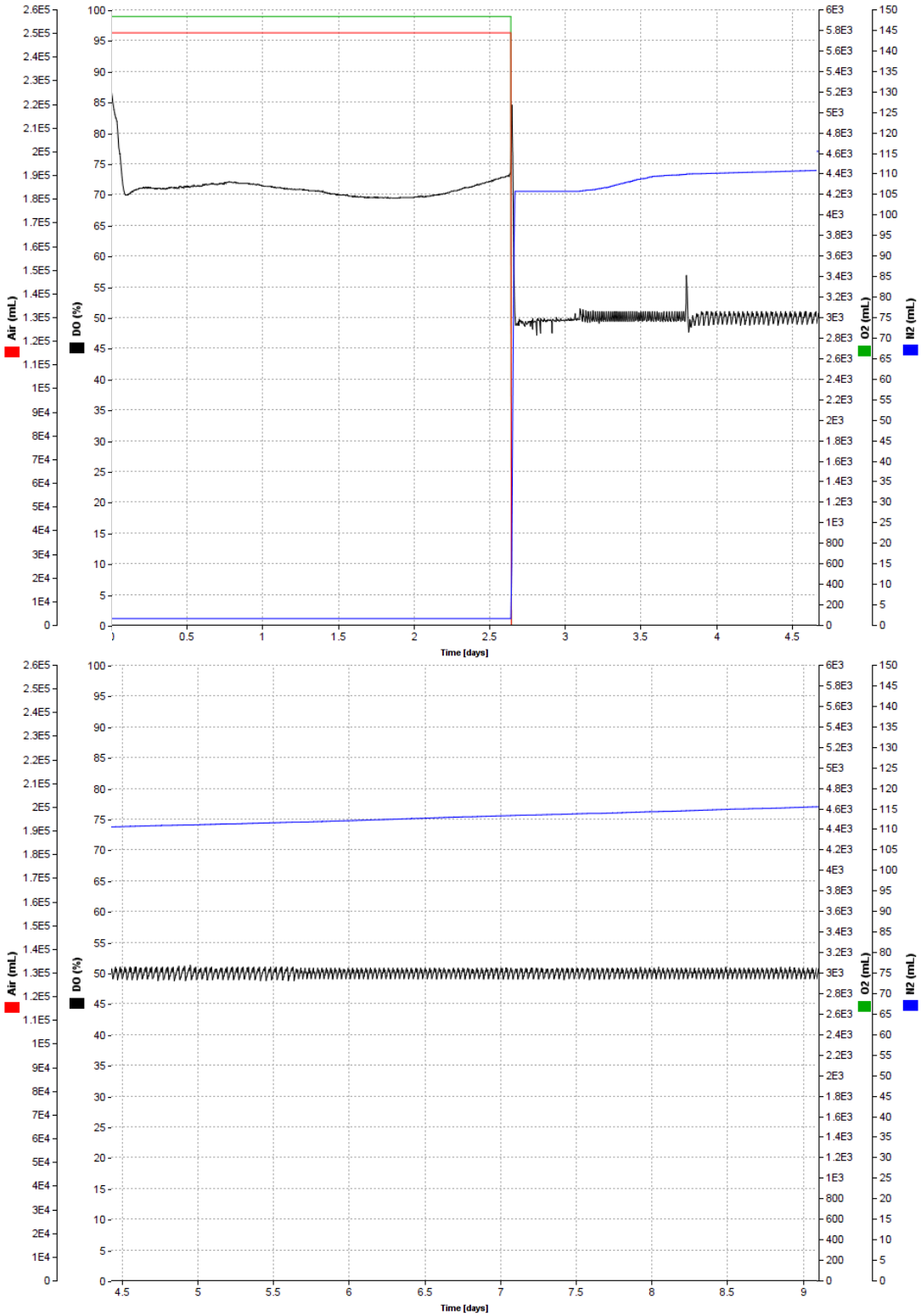


Figure E.6: Monitoring of DO (%), air (mL), O_2 (mL), and N_2 (mL) during inoculation V with C192 in bioreactor. DO was upregulated with air (mL) and O_2 (mL). DO was downregulated with N_2 (mL). The monitoring was on for 9 days.

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