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Development of an End-to-End Monoclonal Antibody Production Process

Dissertation submitted by

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in partial fulfilment of the requirements for the degree of

MRes in industrial Pharmaceutics

under the supervision of

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and

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at

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Aug 2024

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Declaration

This dissertation is submitted by the undersigned to University College Cork for examination for the degree of MRes in industrial Pharmaceutics. It has not been submitted as an exercise for a degree at any other university. I carried out all the work except where duly acknowledged. This manuscript was written by me with the aid of editorial advice from Dr Evin Allen & Dr Alex Zhdanov. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Student Name: Date:

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List of Abbreviations

AA – Amino Acids

ADC – Antibody Drug Conjugates

AP – Alkaline Phosphatase

BSA – Bovine Serum Albumin

BSE – Bovine Spongiform Encephalopathy

CD – Circular Dichroism

CE – Capillary Electrophoresis

CEX – Cation Exchange Chromatography

CHO – Chinese Hamster Ovary

cNISTmAb - cNational Institute of Standards and Technology Monoclonal antibody

CQA – Critical Quality Attributes

CSPD - *Tradename for chemiluminescent agent

ddH_2O – Double Distilled Water

DLS – Dynamic Light Scattering

DMSO – Dimethyl Sulfoxide

DO – Dissolved Oxygen

DSC – Differential Scanning Calorimetry

ELISA – Enzyme Linked Immunosorbent Assay

Fab – Fragment antigen binding domain

FBS – Fetal Bovine Serum

Fc – Fragment Crystallizable domain

FITC – Fluorescein Isothiocyanate

FPLC – Fast Protein Liquid Chromatography

FTIR – Fourier Transform Infrared Spectroscopy

HEK293 – Human Embryonic Kidney Epithelial cells

HIC – Hydrophobic Interaction Chromatography

HPLC – High-Performance Liquid Chromatography

HRP – Horseradish Peroxidase

HPEAC-PAD – High-Performance Anion-Exchange with Pulsed Amperometric Detection

IC50 – Inhibitory Concentration

IgG – Immunoglobulin G

kDa – Kilo Dalton

M – Molar

mAb – Monoclonal Antibody

MCB – Master Cell Bank

mM – millimolar

mOsm – Milliosmoles

MS – Mass spectrometry

NaCl – Sodium Chloride

nm – Nanometer

PBS – Phosphate Buffer Saline

PE – Phycoerythrin

Pen/Strep – Penicillin/Streptomycin

RCF – Relative Centrifugal force

RPLC – Reverse Phase Liquid Chromatography

SDS-PAGE – Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis

SEAP – Secreted Alkaline Phosphate

SEC – Size Exclusion Chromatography

SPR – Surface Plasmon Resonance

TFF – Tangential Flow Filtration

TNF Alpha – Tumor Necrosis Factor alpha

TSE – Transmissible Spongiform Encephalopathy

v/v – Volume/Volume

VCD – Viable Cell Density

WCB – Working Cell Bank

β – Beta

Abstract

Monoclonal antibodies (mAbs) represent the dominant modality of biological therapeutics currently marketed. The manufacture of monoclonal antibodies consists of a series of processing steps including upstream cell culture, downstream purification and formulation and a final step of drug product manufacture. In this thesis, a small-scale end-to-end production process was developed for two monoclonal antibodies namely an anti-TNF Alpha mAb and cNISTmAb whose cell lines were sourced from commercial and public bodies respectively.

A cell culture process for both cell lines was successfully developed including the creation of working cell banks (WCB). Titers were optimised, for both cell lines through a series of media, bioreactor and cell density modifications. In addition, the critical quality attributes of potency and purity were assessed via cell-based assays and SDS-PAGE respectively, that were designed and developed as part of this thesis. The potency of anti-TNF Alpha mAbs produced was assessed through two distinct in-vitro cell-based assays developed using HEK and L929 reporter cell lines and determined to be functional and more potent than a commercially available anti-TNF Alpha antibody The stability of cNISTmAb was also analysed across a range of temperature conditions from -20°C to 40°C after 50 days and also post-lyophilisation, with stability maintained at room temperature. Additional modifications of cNISTmAb were performed in an attempt to yield antibody fragments through pepsin digestion with partial success through pepsin digestion to yield F(ab')2 fragments. This work demonstrates the successful production of two monoclonal antibodies and fragments but also sets the stage for further process improvement and enhanced characterisation to fully define and analyse an end-to-end monoclonal antibody production system.

1. Introduction

The invention of acetylsalicylate, a synthetic drug in 1895 later patented as aspirin (by Bayer in 1900) marked the beginning of the modern pharmaceutical industry. There was a significant emphasis on the development of synthetic antibiotics throughout the 1900s for infectious diseases. The discovery of polypeptide hormones from animal organs including the pancreas and thyroid provided insight into disease pathophysiology and created a pathway to the treatment of metabolic diseases like diabetes and dwarfism. This led to the first animal derived commercially available insulin preparation Iletin, that was produced from bovine or porcine sources (Eli Lilly in 1923). In the early 1940s different polyclonal antibody products such as immunoglobulins derived from human plasma became available as treatments, subsequently antihemophilic factor VIII, a blood clotting factor was commercialized in 1968 (Berger, 2002). These therapeutics provided life-saving breakthroughs but suffered significant safety and production-scale limitations due to their animal/human source (Curran et al., 1984). Industrial scale production of peptides has significantly advanced since, with the synthesis of peptides in solution and by solid phase (Bray, 2003). In addition, recombinant protein therapeutics are widely available on the market due to the advances made in molecular biology, removing the need to source peptides and proteins from cadavers. Today, there is a wide range of therapeutic modalities on the market from small molecules to advanced therapeutic medicinal products (ATMPs) **Figure 1**.

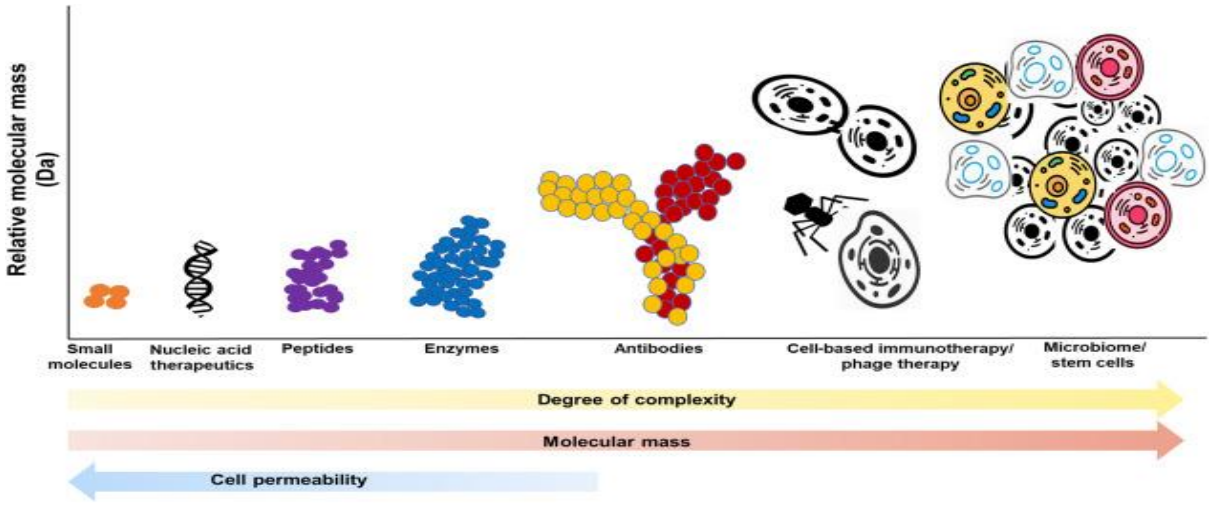


Figure 1 Molecular mass comparison between different Biologics (Chhabra, 2021)

1.1 Recombinant Therapeutic Proteins

Recombinant therapeutic proteins are subset of biopharmaceuticals which have been playing an ever-increasing role in the treatment of human diseases over the last forty years. These therapies have made great advances in the treatment and prevention of many rare and severe diseases including diabetes, cancer, anemia, autoimmune and inflammatory diseases (Lamanna et al., 2018)

There are several modalities of recombinant therapeutic protein, including vaccines, hormones, enzymes, monoclonal and polyclonal antibodies and fusion proteins. Over 370 pharmaceutical drugs products employing recombinant therapeutic proteins were approved to date for human therapeutic use in the European union (EU) and United States of America (USA). The most prevalent modality are monoclonal antibodies, which comprise over half (53%) of approvals (Walsh, 2018). Accordingly monoclonal antibodies (mAbs) are the focus of this thesis and introduction.

1.2 Antibodies

Antibodies are a group of glycoproteins that belongs to the immunoglobulin (Ig) superfamily, which are secreted by B cells found in the bone marrow and activated in lymphoid tissues to recognize and neutralize any foreign organisms or antigen (Edelman, 1970). Individual mature B cells produce a specific antibody that recognizes a specific antigen. There are 5 different types of antibodies in humans IgG, IgM, IgA, IgE, IgD (Justiz Vaillant et al., 2024) as shown in **Figure 2**. IgM has a molecular weight of 970 kDa, it is a pentamer and activates the complement system. IgE is a monomer and has a molecular weight of 188 kDa and will protect against parasites, it will bind to high affinity receptors on mast cells and also basophils. IgA antibodies appear in two molecular structures, monomeric form (serum) and dimeric structure (secretary) and have a molecular weight of 160 kDa and 385 kDa respectively. IgA is mostly secreted in saliva, tears, colostrum, intestinal, genital tract and respiratory secretions. IgD is a monomer and has a molecular weight of 184 kDa. IgG is a monomer and has a molecular weight of approximately 150 kDa.

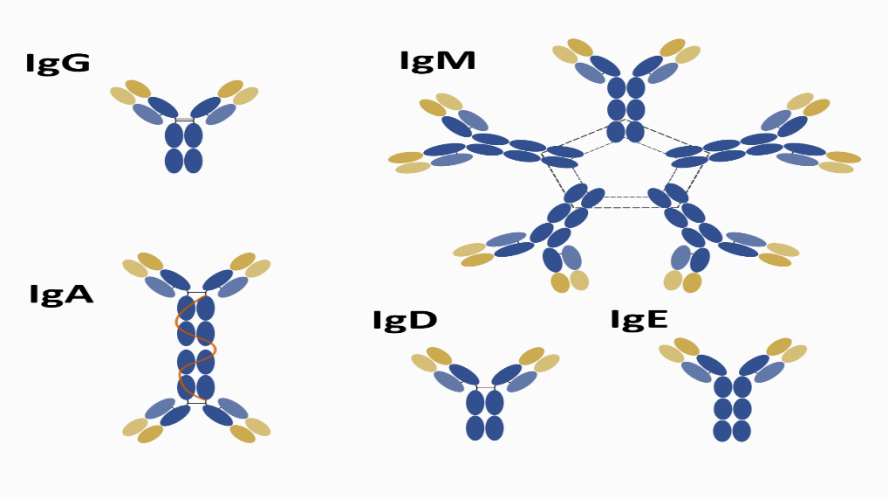


Figure 2 Antibody Types (Funfrock, 2020)

IgG is considered to be divalent which means it has two identical antigen binding sites, as per **Figure 3,** mostly IgG is secreted in the secondary immune response of an immunogen. There are 4 different classes of IgG, namely IgG1, IgG2, IgG3, IgG4. Around 65% of total IgG is comprised of IgG1 (Justiz Vaillant et al., 2024). IgG antibodies are comprised of two identical heavy polypeptide chains and two identical light polypeptide chains as illustrated in **Figure 3** and each of these antibodies are divided into different isotypes depending on the difference in their heavy chain (Buss et al., 2012). Every antibody contains two domains, Fab domain (the fragment antigen binding domain) one Fc domain (fragment crystallizable domain). The Fab domain is composed of antigen binding regions consisting of two hypervariable regions on each heavy and light chain. The Fc domain is responsible for the effector function, and is composed of two constant domains of heavy chain. Both these chains are connected by disulfide bonds and non-covalent forces (Vermeer and Norde, 2000)

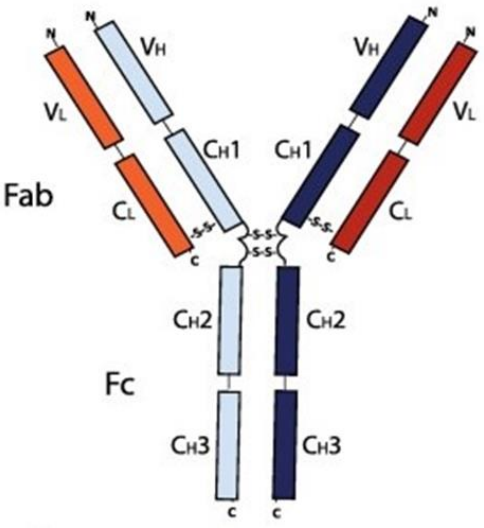


Figure 3: Illustration of an IgG monoclonal antibody structure including heavy and light chains (blue) and light chains (orange) (Shah et al., 2018).

1.2.1 Fc Effector Function

The Fc domain imparts structure and IgG isotype determination, but it also plays an important role in serving antibodies to initiate an effective immune response against a pathogen and orantigen. This Fc domain generates the link between the antibody and other parts of the immune system, including the innate immune response by binding to Fc receptors on other immune cells.

Several immune cells such as mast cells and B cells, express Fc receptors that bind to the Fc domain of an antigen-bound antibody and induce immune responses. These types of receptors are extremely specific to each isotype class or even subclass and are a critical part of the reason why different isotypes demonstrate different types of immune responses. All these receptors are named after the isotypes that they interact with, example IgG isotypes specifically bind to Fcγ receptors, likewise IgE binds to Fcγ receptors and so on. There are several different receptors, but all their downstream effects are categorized into three key effector functions as outlined in **Figure 4** (Leeson, n.d.)

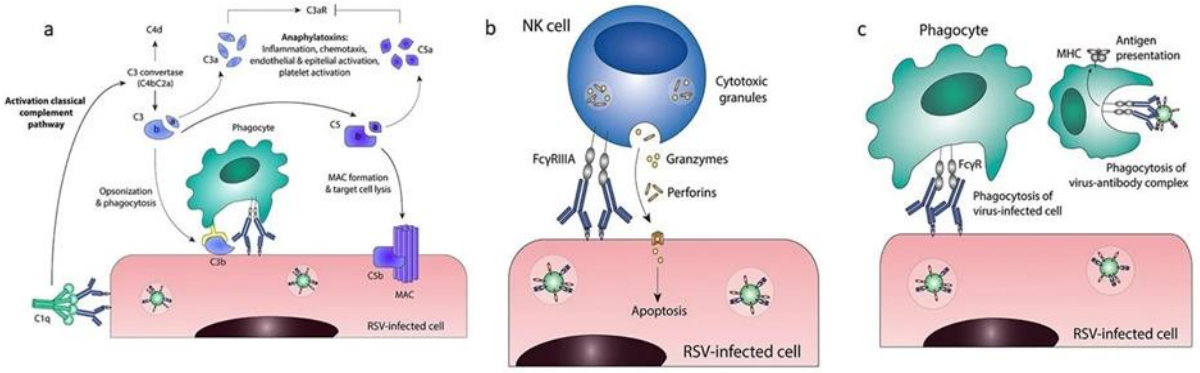


Figure 4: Fc effector functions in an immune response to a viral infection (a) antibody-dependent complement deposition (ADCD) (b) antibody dependent cellular cytotoxicity (ADCC) (c) antibody dependent cellular phagocytosis (ADCP) (Leeson, n.d.)

1.2.1.1 Antibody-dependent complement deposition (ADCD)

Antibodies can bind to its specific pathogen (antigen) and physically inhibit it from entering a cell, neutralization is a non-Fc-dependent effector function. However, antibodies are also able to specifically bind to a pathogen and indicate it as a target for other immune cells, in a process called as opsonization. In an antibody-dependent complement deposition (ADCD), opsonization of IgG and IgM antibodies on a pathogen activates the immune system's classical complement pathway, starting a cascade of events that result in the pores being opened in the pathogen's cell membrane (Dunkelberger and Song, 2010). The pathogen cells cannot survive when holes are formed their membranes and as a result the pathogens quickly lyse and die

1.2.1.2 Antibody dependent cellular cytotoxicity (ADCC)

Antibody dependent cellular cytotoxicity (ADCC) is a more direct method than opsonization of cell death. In ADCC, IgG antibodies bind to a pathogen and natural killer (NK) cells expressing Fcγ receptors binds to IgG Fc domain and releases cytotoxins that kill the target cell. If the pathogen is a large parasite, ADCC can be induced through a very similar interaction between IgE antibodies and eosinophils, where the eosinophils release cytotoxins via degranulation.

1.2.1.3 Antibody-dependent cellular phagocytosis (ADCP)

In antibody-dependent cellular phagocytosis (ADCP) Fcγ receptors on macrophages bind IgG antibodies via the Fc domain. The macrophages then engulf and digest the pathogen the antibodies are bound to in a process known as phagocytosis.

1.3 Monoclonal Antibodies

Monoclonal antibodies (mAbs) are a type of antibody which are produced by a single clone of a B cell or other mAb producing cell line and are specific to a single epitope on an antigen. In contrast, polyclonal antibodies while targeting the same antigen have varying epitopes as a result of the presence of multiple different clones. Compared to polyclonal antibodies, monoclonal antibodies are homogenous and monospecific in nature, and this makes mAbs an important tool in the development of therapies and diagnostics (Breedveld, 2000).

The first therapeutic mAb, Muromonab, was a mouse IgG2a and was created using hybridoma technology in 1986 and licensed in 1997 (Liu, 2014). Over the past decades numerous cell lines have developed as popular hosts expression systems of marketed recombinant protein products. These cell lines include murine myeloma lymphoblastoid cells (NS0), Chinese Hamster Ovary (CHO), Baby Hamster Kidney (BHK-21) and human embryonic kidney epithelial cells (HEK 293) (Kuystermans and Al-Rubeai, 2015). CHO cell lines are today the most popular mAb producing cell line.

1.3.1 Monoclonal Antibody Application

Therapeutic monoclonal antibodies are typically of the γ-immunoglobulin (IgG) isotype. They have been shown to significantly benefit the health care system by acting as efficient agents for the treatments of various potentially fatal diseases notably various types of cancer, diabetes mellitus and auto-immune diseases (Akash et al., 2015).

1.3.1.1 Immune mediated diseases

Monoclonal antibodies have revolutionized the treatment of auto-immune diseases and several mAbs have been launched in the past decades for the treatment of these conditions. mAbs can target different components of the immune system to suppress the excessive inflammatory responses that characterize autoimmune diseases. Some of the mechanism of mAbs to treat autoimmune disorders include blockade and depletion of T cells and/or B cells, inhibition of

the interaction between T cells and antigen-presenting cells, blockade of T and B cell recruitment, blockade of T-cell differentiation or activation and blockade of pro inflammatory cytokines. These antibodies have been used for the therapy of rheumatoid arthritis for more than a decade and also show efficacy in psoriatic arthritis, Crohn's diseases, ulcerative colitis, psoriasis and ankylosing spondylitis (Bruno et al., 2011). Such products have focused on inhibiting the inflammatory TNF-Alpha cascade as in **Figure 5.**

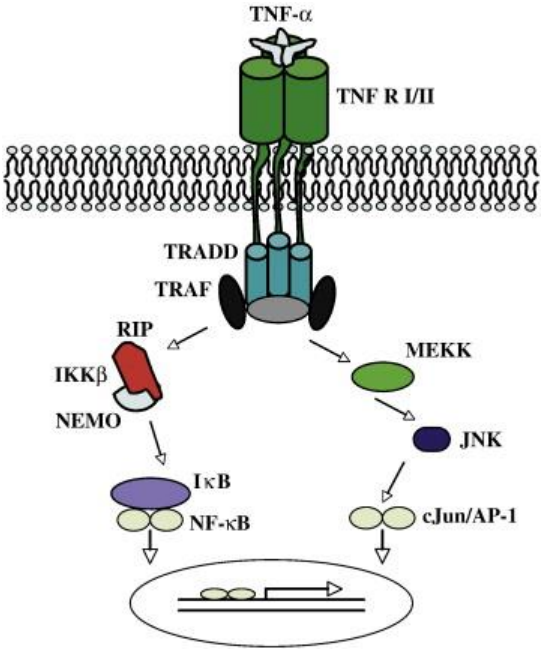


Figure 5: Illustration of an TNF alpha signaling cascade (Park and Bowers, 2010)

1.3.1.2 Oncology

A number of mAbs have been developed for the treatment of various neoplasia's, including both hematologic malignancies and solid tumors. One approach is the use of mAbs to target tumor antigens and kill cancer cells. However, the main approach is the use of therapeutic mAbs to target growth factor receptors that are overexpressed in tumor cells, such as members of the epidermal growth factor receptor (EGFR) family, including EGFR and Human Epidermal Receptors (HER). In these cases, mAbs block the receptors that in turn blocks ligand binding/signaling, which can decrease growth rate, induce apoptosis and sensitize

tumors to chemotherapy. Blockbuster antibodies in this area include Herceptin, Rituxan and Keytruda (Cruz and Kayser, 2019). They can also be used for the selective delivery of radioisotopes selectively to cancer cells (Scott et al., 2012).

1.3.1.3 Infectious diseases

The first effective treatment for infectious diseases was the administration of hyperimmune sera from immunized animals or human donors containing polyclonal antibodies. Although this approach was widely replaced with antibiotic treatment it still remains useful for the treatment of infectious diseases including those caused by cytomegalovirus, hepatitis A and B viruses among others (Flego et al., 2013). There are advantages of mAbs for the treatment of infectious diseases, over immune sera-derived preparations, such as low lot-to-lot variability, low risk of pathogen transmission, and no immunological complications associated with the use of heterologous sera. However, the development of mAbs against infectious diseases has been slower in comparison to their development for oncology and immune/ inflammatory diseases (Castelli et al., 2019). Several monoclonal antibody products were developed against the SARS-coV-2 virus during the recent pandemic including Sotrovimab demonstrating the applicability of this modality against infectious diseases.

1.4 Cell Culture for Monoclonal Antibody Production

Over the past few decades, the production of monoclonal antibodies has significantly increased in the biopharmaceutical industry, driven by the developments in biotechnology and molecular biology. Different types of cell systems and conditions are utilized today to produce monoclonal antibodies

The structure and function of recombinant protein therapeutics are integrally related to the production or expression system employed. Recombinant production of proteins initially started with the expression and purification in *Escherichia coli* a bacterial system. There were several successful recombinant proteins produced from *E. coli* such as insulin in 1982, that were of low complexity. However prokaryotic expression systems like *E. coli* lacked the ability to perform post-translation modifications such as glycosylation required for more complex proteins (Lee and Jeong, 2015). Thereafter, different host systems such as yeast were developed, however the post translational modification was still limited. Insects cell expression systems that employed baculovirus as a transfection vector were developed, with the PTMs of closer to homology to human proteins but not identical. In addition, the scalability was an issue due to baculovirus inhibiting secretion minimizing yields (Korn et al., 2020). In parallel, mammalian cell lines like CHO (Chinese hamster ovary) were established and could produce products that closely resembled the glycosylated human protein structures using recombinant DNA technology. These mammalian cells facilitate the production of more complex and large therapeutic proteins as a result of the ability to fold proteins intact and can perform complex post translational modifications like N-linked and O-linked glycosylation which are important for biological activity and also stability (Higel et al., 2016). Today protein engineering is a key tool in the development of recombinant proteins with enhanced performance in terms of protein stability, immunogenicity, yield and efficacy being possible due to changes in amino acid sequence introduced at protein design phase.

1.4.1 Prokaryotic systems

The possibility of expressing antibodies in large amounts in *E.coli* cells has attracted the attention of biotechnologists since the early days of genetic engineering (Fernández, 2004) due to robustness of these cells, high growth rate and short cell culture times. However, due to the inability to perform key PTMs as described above, they are only used to for producing smaller antibody fragments such as Fab and scFv. This expression system offers rapid and large yield production, but they lack the ability to perform post-translational modifications, required for efficacy, which is significant challenge when compared to mammalian system (Stech and Kubick, 2015).

1.4.2 Hybridoma Technology

Hybridoma technology, is a foundational technique for mAb production developed by Kohler and Milstein in 1975 (Köhler and Milstein, 1975). This method involves the fusing of immortal myeloma cells together with antibody-producing B-lymphocytes with the creation of hybrid cells efficient for unlimited growth and antibody secretion **Figure 6**. Each hybridoma cell expresses one specific mAb. This process takes advantage of a host animal's natural ability to make functional, highly specific and high affinity mAbs. To date, several mAbs are developed using this technology and are presently used for diagnosis, prevention and treatment of different diseases. Initially hybridoma technology was limited to murine antigens only but with advancement in this technology it become a well-established technology to develop mAbs against a range of different antigens and from different kind of species like rabbit (Kuo et al., 1985) humans (Andrabi et al., 2012) chickens, goats, sheep, cows, mice, guinea pigs, rats (Parray et al., 2020).

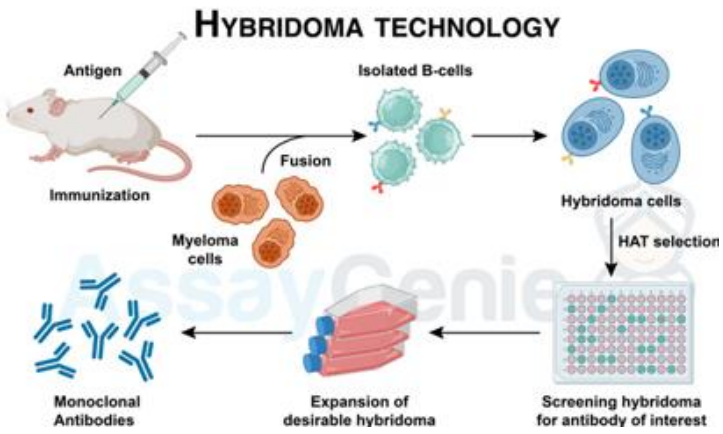


Figure 6: Hybridoma Technology: starting from immunization of rat to production of monoclonal antibodies ("Hybridoma Technology," n.d.)

1.4.3 Mammalian Cell System

Therapeutic antibodies are mainly produced in mammalian host cell lines including NS0 murine myeloma cells, PER.C6® human cells, and Chinese hamster ovary (CHO) cells (Yang et al., 2007). The selection of a given expression system is determined by productivity with acceptable quality of the product and the preferences of each individual companies which is often influenced by their historical experiences. Compared to NS0 and CHO cells, PER.C6® cells are relatively a new technology. Cells are derived from human embryonic retina cells that have been immortalized by transfecting the E1 genes from adenovirus 5 DNA. Like NS0 and CHO cells, PER.C6® cells can also proliferate indefinitely in suspension under serum free conditions. In comparison to CHO and NS0 cells, PER.C6 cells naturally produce proteins with human sequences and glycosylation patterns.

CHO cells are the predominant host cell used to produce therapeutics proteins today. About 70% of all recombinant proteins produced today are made in CHO cells including DUXB11, DG44 AND CHOK1 lineages. DUXB11 and DG44 cells don't have dihydrofolate reductase (DHFR) activity. While CHOK1 cells have endogenous DHFR activity (Li et al., 2010) as in **Figure 7. DHFR can catalyze the conversion of folic acid into tetrahydrofolate which is**

necessary for the biosynthetic pathways which produces glycine, purines and thymidylic acid (Cacciatore et al., 2010). The absence or dampening of DHFR activity allows for selection of clones with high copies of recombinant sequence added and ultimately more productive clones.

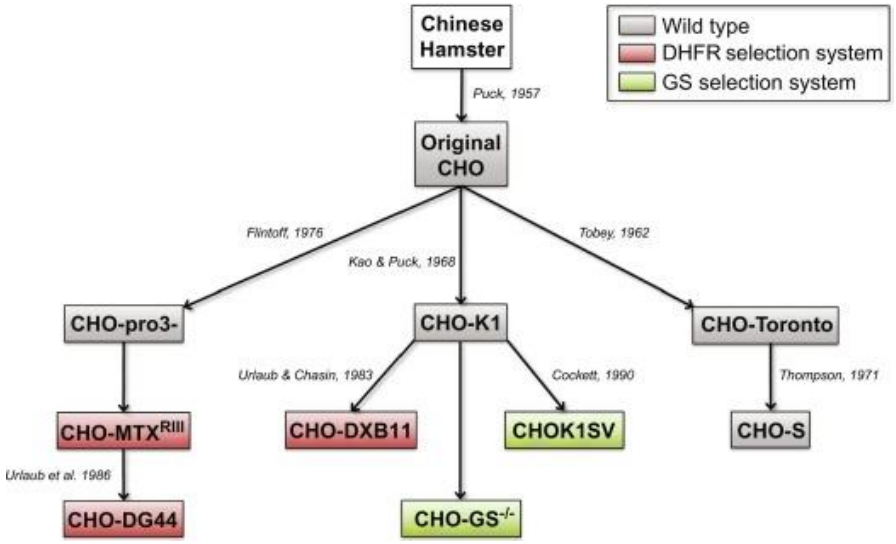


Figure 7. CHO Cell Lineages (Lindskog et al., 2018)

1.4.4 Cell Culture Parameters

Antibody production requires optimization of different conditions and parameters for the cell system to produce high yields, proper folding of mAbs and functional activity of the antibodies **Figure 8**. This includes cell line selection, culture conditions, genetic engineering, bioprocess optimization and downstream processing.

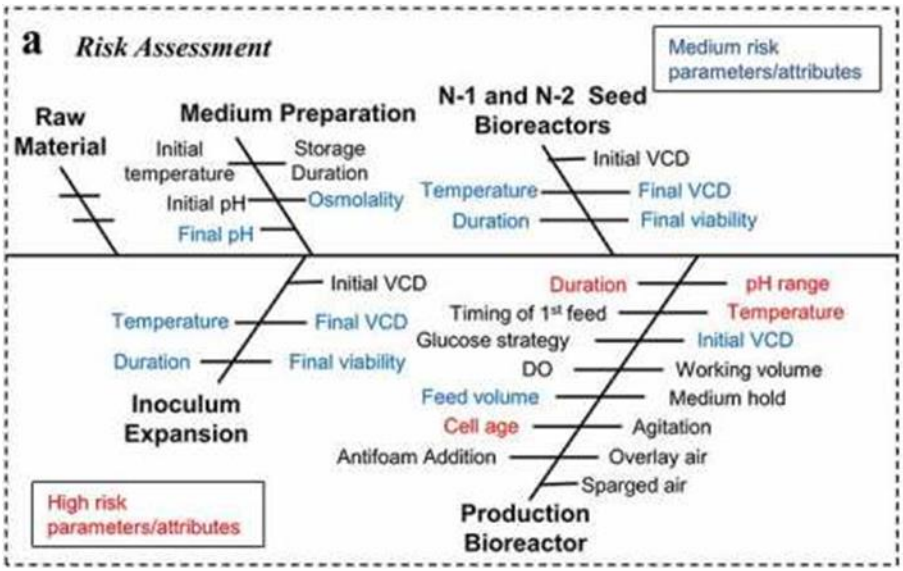


Figure 8. Fishbone diagram: Different cell conditions for antibody production (Xu et al., 2022)

A high level of overview of the mAb upstream production process is outlined here in **Figure 9.** The process consists of thawing a frozen vial from a working cell bank, followed by a series of expansion steps in vessels of increasing volume, until a final cell culture in the largest volume vessel. In this production bioreactor, conditions are programmed as such to drive cells towards protein expression and antibody production and not towards cell growth.

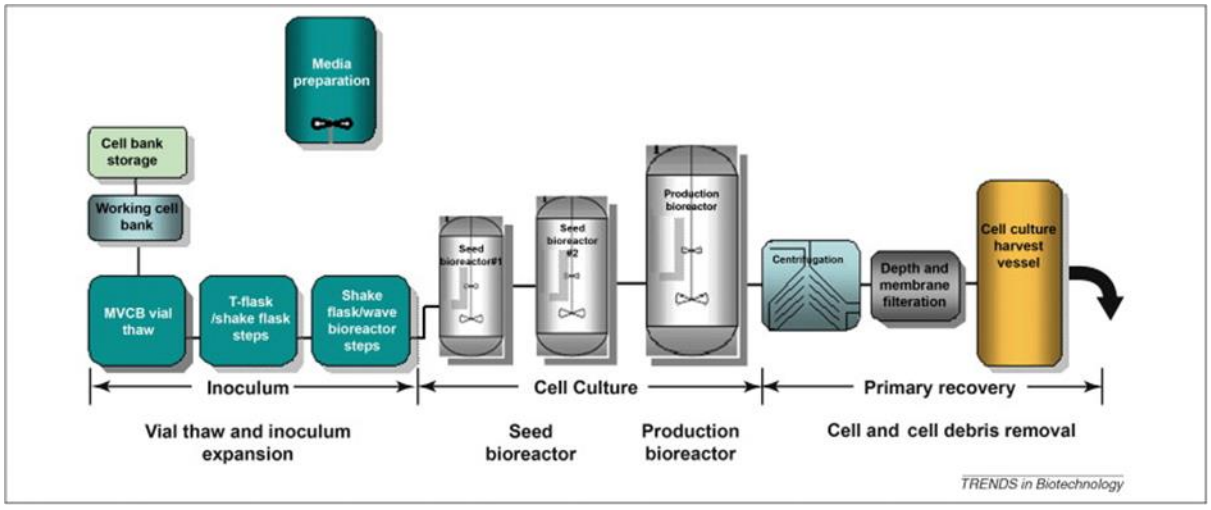


Figure 9. Monoclonal Antibody Upstream production process (Shukla and Thömmes, 2010)

1.4.4.1 Cell line selection

The creation of mAb producing cell clones is mainly based on a high-expressing vector and optimized selection strategies of stable cell clones (Li et al., 2010). Mammalian expression vector construction is used to express genes in human cells (Toporova et al., 2023). CHO cells are using widely to produce therapeutic antibodies because of their capability to perform complex post-translational modification and to increase the yield. The cell line selection is based on productivity and ability to maintain genetic stability when selection markers are removed. It is important that cells do not shed copies of inserted gene or mutate inserted gene when cultured and passaged over time.

1.4.4.2 Culture conditions

The effect of media composition and environmental factors are widely studied. For a higher yield and higher proliferation rate the media should contain all the essential amino acids, vitamins, minerals salts, fatty acids, Carbon, Nitrogen, Phosphate, trace elements, water and glucose. In most media formulations glucose functions as the primary energy source through the Kreb's cycle and as a carbon source. Amino acids are key components in cell culture media. The presence of amino acids plays an important role in influencing cell growth and increasing cell titers. In addition, certain amino acids at certain concentration may have protective effects for cells and also shown to be eliminate the negative effects from pCO2 and ammonium. Similarly, lipids are one of the major components of biological membranes and are also used as a source of energy and in signaling. Vitamins serve as co-enzymes, co-factors in signaling cascades or prosthetic groups as well as in enzyme inhibition and also activation. Because of the high reductive capacity vitamins are able to protect cells from oxidative radicals (Ritacco et al., 2018).

Traditionally FBS has been used to supplement media but due to the risk of adventitious agents such as BSE, TSE etc. FBS has been phased out of media composition for clinical and

commercial mAb manufacturing. A current trend is to use the serum-free and chemically defined media for reproducibility and regulatory compliance (Dangi et al., 2018) and also feed strategies in which nutrients are added in each specific intervals will also enhance the cell density and the growth.

Normally optimal cells growth is observed at a temperature of 37ºC, and optimum level of CO_2 (5%) leads to higher proliferation rate and higher yield through management of pH. Maintaining optimum pH and dissolved oxygen level is also critical quality attribute for increased cell metabolism and antibody production.

Cell media is typically replenished during mAb production to maximize the duration of antibody production through the maintenance of cell health. This processing strategy is known as fed-batch. in contrast to traditional batch culture where no bolus feed media was added to the basal media

1.4.5 Bioprocess Optimization

As the demand for mAbs has exploded over the years in addition to pressures to reduce the cost of goods from governments and healthcare payers, there has been a major industry effort to maximize production yields while minimizing costs.

1.4.5.1 Scale up strategies

Different types of scale up strategies can be introduced to increase the yield including changing bioreactors size, geometry and type. Scaling up from small flasks to large bioreactors like stirred tanks increase agitation, aeration, nutrient supply can maintain constant production levels (Zhang et al., 2022) Today, some companies are attempting to scale-out with single use technology in contrast to the traditional scale-up.

1.4.5.2 Monitoring and control

Real time monitoring using different types of sensors and automated process control systems for the constant monitoring of parameters like temperature, pH, dissolved oxygen level, glucose, other essential nutrients can help to maintain optimal condition of cell viability and proliferation for higher production rate, and this can also be helpful to detect any issues like contamination or apoptosis in the early stage of cell culture (Stech and Kubick, 2015).

1.4.5.3 Perfusion Culture

A perfusion culture can be defined as the culture in which the waste medium is removed continuously from a culture and the wasted medium is replenished with a fresh medium while retaining the cells in the reactor. This will ensure a high cell density and allow for the extension of the cell culture production phase. The advantage of perfusion cultures is that it only needs a smaller scale when compared to batch cultures to obtain a desired yield. (Kim et al., 2007)

1.5 Downstream Processing

Purification of antibodies can be achieved through a highly standardized system that comprises of centrifugation, microfiltration, protein affinity chromatography, ultrafiltration, virus inactivation, IEC, and hydrophobic interaction chromatography (Ramos de la Peña et al., 2019) The process of purification must be reliable and should predictably produce a product that is suitable for human use. The process should produce an acceptable yield while the impurities such as host cell protein, endotoxin, DNA, aggregates, adventitious and endogenous viruses and some other species must be eliminated (Liu et al., 2010). In addition, the mAb must be formulated in an appropriate buffer for long-term storage and possible freezing.

1.5.1 Harvesting the culture

The first step in the recovery of a monoclonal antibody from a cell culture is harvest. All the cells and cell debris must be removed after cell culture process. Two steps can be utilized to remove the debris (**Figure 10)**. In the majority of cases, cells excrete the recombinant protein extracellularly into the suspension media and the focus of harvest is cell removal. In certain scenarios, the cell may excrete cell intracellularly which requires lysis of cells to extract protein of interest. In this case, harvest is focused on retaining cells and removing cell culture suspension media.

1.5.1.1 Centrifugation

The culture is centrifuged at a normal rate removing all cell and debris to waster, leaving the supernatant with antibodies to be further processes.

1.5.1.2 Depth Filtration

Subsequently the supernatant is filtered using depth filters or microfiltration membranes to further clarify the supernatant Depending on specific company practices, centrifugation may be replaced by an initial depth filtration followed by a second depth filtration.

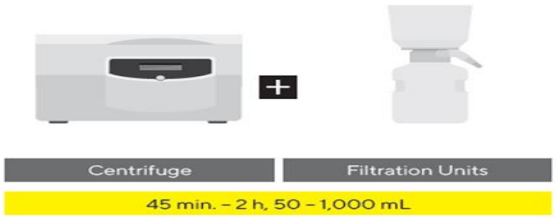


Figure 1: Standard clarification method

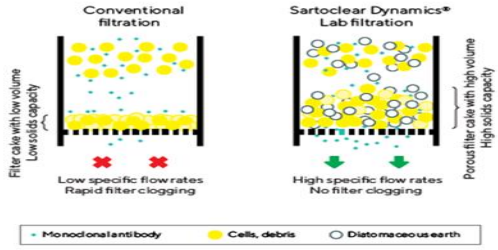


Figure 2: Principles of clarifying cell cultures using conventional filtration and Sartoclear Dynamics® Lab

Figure 10. Culture harvesting using centrifugation and filtration ("Rapid Mammalian Cell Harvest for Antibody Purification | Cell and Molecular Biology," n.d.)

1.5.2 Protein Affinity Chromatography

Affinity chromatography is the most widely used separation technique in biopharmaceutical industries, because of its high resolution (Cuatrecasas and Anfinsen, 1971). It exploits the unique interaction or affinity between protein A or protein G resins and antibodies. This high affinity is generally not found for other resin-protein interactions making mAb purification relatively straight forward. It is utilized due to its high specificity towards the Fc region of the antibodies. The most widely used two types of affinity chromatography are Protein A affinity chromatography and Protein G affinity chromatography **Figure 11**. In protein A affinity chromatography, the Protein A resin binds to the Fc region of an antibody particularly of IgG antibodies with high affinity. This allows for efficient capture from the supernatant that has been harvested (Zhang et al., 2022). Protein G affinity chromatography is very similar to Protein A with difference only in their binding profiles and also it can be used to purify certain subclasses of IgG antibodies that does not have a binding profile with Protein A. However, remains the most popular resin type use because of its high degree of purity and a larger rate of recovery in a single step (Fahrner et al., 2001).

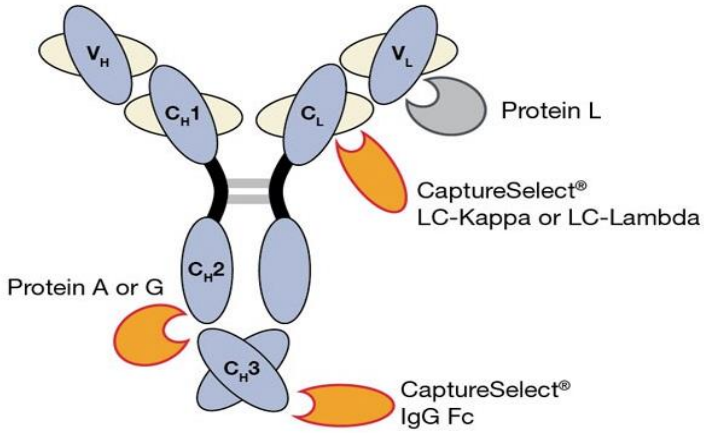


Figure 11. Protein A/G/L Binding Locations in Affinity Chromatography ("Affinity Columns - IE," n.d.)

1.5.2.1 Column Equilibration

Before every binding step the column will be washed and equilibrated with buffer to remove any proteins or contaminants that may have bound to the column from prior batches or column production.

1.5.2.2 Column Binding

Column binding is performed by using a biologically related binding agent as stationary phase through a selective and reversible binding that can be seen in different biological interactions like antigen and antibody, enzyme and substrate, or hormone and receptors. Different columns use these kinds of interaction to immobilize one pair of interacting agents into the column and this immobilizing agent is called affinity ligand (Wilchek, 1984).

The column binding works in a way that the sample as a complex mixture will pass through a column using an application buffer and the targeted molecules will bind to the column while other while other sample components get eluted (Rodriguez et al., 2020) as per **Figure 12**.

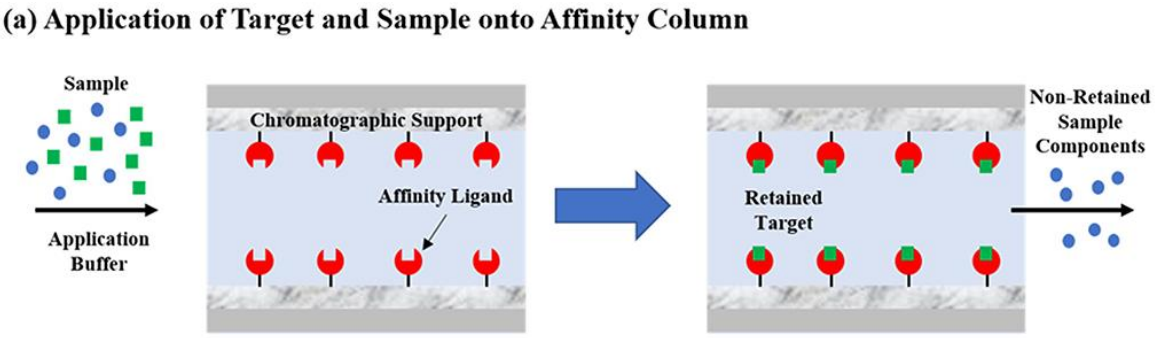


Figure 12. Sample application and binding, on a column (Rodriguez et al., 2020)

1.5.2.3 Low pH elution and Neutralization

Antibodies need to elute from the affinity column after the chromatography step. A low pH buffer (preferably pH 3-4) should be utilized for this process. Immediately before elution, the column is equilibrated with a buffer of pH 7.0-7.4 (in the case of mAb purification) to elute the unbound proteins from the column. After elution the antibodies will be in a low pH buffer, that can cause subsequent denaturation, and thus the solution must be neutralized to avoid degradation.

1.5.2.4 Additional Chromatography Steps

Additional chromatography steps are applied after affinity chromatography known as polishing steps, like Cation and Anion Exchange Chromatography (CEX) which further purify the antibodies with respect to their charge, Similarly Hydrophobic Interaction Chromatography (HIC) is employed for separating proteins according to their hydrophobicity (Birch and Racher, 2006) and in particular for removal of host cell lipases that can cause polysorbate degradation.

Afterwards to ensure complete viral clearance, a viral filtration step is employed to eliminate any potential viral contaminants, with filters with a pore size of 20nm utilized. These process these filters can block the smallest viruses and yet easily allow antibodies to pass through (Li, 2022).

1.5.3 Ultrafiltration & Diafiltration

As a final filtration step ultrafiltration and diafiltration is employed to produce a final formulation with appropriate buffer and excipients for long-term storage in the liquid or frozen states. Unlike sterile or dead-end filtration, TFF is used to remove buffer salts and other excipients and also to concentrate mAb to desired concentration.

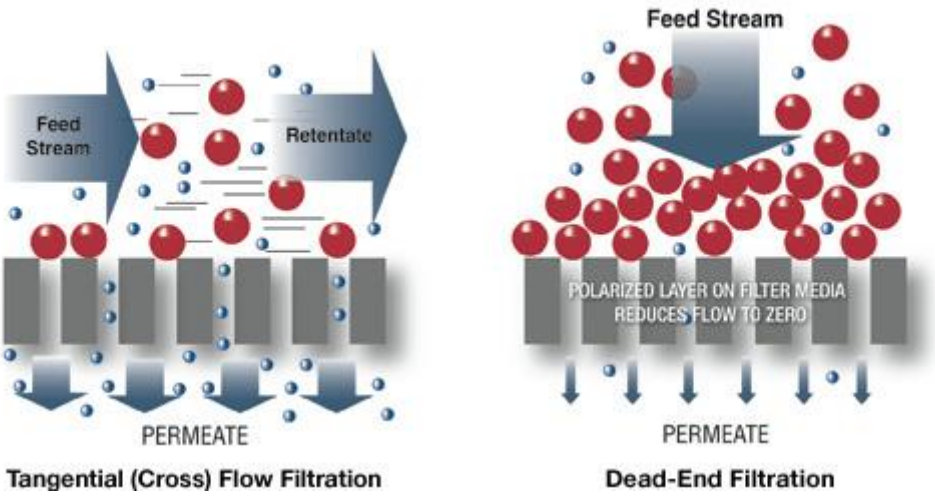


Figure 13. Comparison between Tangential Flow Filtration (TFF) and Dead-End Filtration

(Catalina, M., et al.2013) "

Ultrafiltration concentrates the antibody solution by removing solution and the small molecules (buffer, excipients) present in the solution while diafiltration is the process of exchanging the current buffer with a final formulation buffer by repeatedly adding and removing the final buffer with sample solution **Figure 13**. This process ensures the antibody solution will be formulated into final product formulation that includes all the preservatives, surfactants, pH adjusters, stabilizers etc. The final formulated antibody solution is filtered through a 0.2µm sterile filter to ensure a low bioburden solution before filling into containers for storage or any other purposes. Each step of the purification process is important to ensure and achieve maximum purity and output of mAbs (Dutta et al., 2015).

1.6 Monoclonal Antibody Analysis & Characterization

mAbs can display heterogeneity as a result of oxidation, fragmentation, disulfide bond mismatch, C-terminal lysine processing, deamidation, glycation, isomerization, glycosylation leading to a complex purity/impurity profile comprising of several molecular variants (Jakes et al., 2021). There are different analytical methods for analyzing monoclonal antibodies which will ensure the safety, quality, and efficacy of the final drug product of. The key techniques that are used for the analysis of different monoclonal antibodies include spectroscopic, chromatographic and electrochemical methods. The methods employing these principles cover the majority of different critical quality attributes (CQAs) of mAbs such as biological activity, concentration, purity, structural integrity, and identity **Figure 14**.

Product Variants	Process Impurities	Purity/Potency
Aggregation	Microbiological purity	Size-Exclusion Chromatography (SEC) purity
Conformation	Viral purity	Glycosylation profile
C-Terminal Lysine	DNA	Concentration
Deamidated Isoforms	HCP	pH
Disulfide Bonds	Protein A	Bioburden/Sterility
Fragmentation	Selective agent	Appearance
Glycation	Cell culture media components	Particle free (essentially)
Glycosylation	Purification buffer components	Product/Excipient Concentration
Oxidation		Osmolality
Thioether link		pH
		Pre-Filled Syringe / Device Functionality

Figure 14. Typical CQAs for a monoclonal Antibody Aggregate Analysis ("Process Validation in Biologics Development," n.d.)

1.6.1 Aggregation

Aggregation is one of the major product quality risks that surrounds mAb production. There are different analytical methods for purity and impurity analysis like size-Exclusion

Chromatography (SEC) which can determines the size and aggregation of antibodies by separating the molecules according to their size, classifying different monomers, dimers and high-order aggregates. The technique can separate and purify mAbs from all other heterogenic impurities that can cause immunogenic responses (Fekete et al., 2013). In addition, Capillary Electrophoresis (CE) -SDS will provide high-resolution separation of antibodies and its analogues (Gahoual et al., 2016) and has replaced traditional SDS-PAGE gels methods which were traditionally used to identify molecular weight of samples. All methods will identify the presence of high molecular weight structures such as aggregates but crucially will also detect low molecular weight structures such as antibody fragments.

1.6.2 Glycosylation & Post-Translational Modifications

Glycosylation is typically a critical quality attribute for mAbs, particularly during cell culture stage where PTMs such as glycosylation will occur. Glycosylation can be analyzed via a number of different techniques such as RPLC and MS. Different modes like Reverse phase liquid chromatography (RPLC) can be used to separate the antibodies using hydrophobicity, the technique has good resolution in evaluating protein variation that arises due to various post-translational modifications (Yan et al., 2007).

Glycosylation analysis can also be performed mainly using different types of chromatographic techniques like High-Performance Anion-Exchange with Pulsed Amperometric Detection (HPEAC-PAD) which can analyze glycan structures. This method helps to provide detailed profiles of both monosaccharide and oligosaccharide structures to quantify the glycan species (Rohrer et al., 1995). Similarly, Lectin Affinity Chromatography which helps analyze specific glycan structures, can analyze and isolate different glycosylated forms by specifically binding the lectins to certain sugar moieties (Cummings and Etzler, 2009).

1.6.3 Charge Variant Analysis

Charge variants can be formed due to different modifications like deamidation, glycation etc. Charge variants can be analyzed using different techniques such as isoelectric focusing which aids to separate the proteins according to the isoelectric point (pI). Likewise, ion-exchange chromatography is also performed to assess charge variants by separating the mAbs according to differences in the charge and to detect isoforms and also the presence of degradation (Du et al., 2012)

1.6.4 Potency & Biological Function

Biological functionality is a vital assessment of antibody products to confirm the anticipated *in vivo* effects when clinical trials and animal studies are not appropriate. Biological activity is required to be tested before product release in line with ICH guidelines. The bioassays used to demonstrate biological activity are typically carried out using three different methods The most straightforward method involves binding assays like Enzyme-Linked Immunosorbent Assay (ELISA) which can measure the binding activity of an antibody towards its targeted antigen and this assay can quantify the binding affinity and the specificity of the antibody (Flatman et al., 2007). Secondly, Surface Plasmon Resonance (SPR) which can measure and analyze real time binding interaction and can provide the kinetic data on the interaction like association and dissociation rates. The third approach is to measure the potency which will determines the product capability to produce the vital response using relevant biological models like cell-based assays. Normally, signal transductions assays, reporter gene, complement mediated cytotoxicity, cell proliferation are the specific means used to measure the antibody activity in cell-based assay. The principle to be used is dependent on the product in question and how the product is intended to function *in-vivo* and mechanism of action of drug product. For example, in the case of products where the pathophysiology of the disease to be treated includes a signaling cascade, that is modified by the drug product, molecules impacted by the

modification of the cascade downstream of drug products are typically quantified with a substrate that can be easily measured. Cellular assays are considered to more relevant and sensitive to measure the antibody activity compared to the binding assays. However, as cells are required to perform assay, significant variability and challenges can occur during method execution in addition to the significant time required to perform assay.

1.6.5 Higher Order Structure

The physical and chemical stability of mAbs can be analyzed by different techniques like Differential Scanning Calorimetry (DSC) which will measures the thermal stability and determine the melting temperatures and gives understanding about the thermal stability of the antibody under the influence of thermal stress (Li et al., n.d.). Similarly, Fourier Transform Infrared (FTIR) Spectroscopy detects the changes in the protein's secondary structures in various conditions and analyzes the chemical stability. Different analytical techniques can be used to confirm the structure and conformational studies like Circular Dichroism (CD) Spectroscopy that characterize the secondary and tertiary structures and provides information on the folding and the stability of the purified antibody.

1.7 Post-purification mAb Processing

In addition to whole, native mAbs, different forms of antibodies have and are being marketed including fragmented, pegylated and (small-molecule drug) conjugated antibodies Fragmentation and conjugation are two of the most used processing techniques for therapeutic and diagnostic applications. These modifications are intended to enhance specificity, functionality and efficacy of modified mAb and enhance clinical outcomes.

1.7.1 Fragmentation

Fragmentation is the process of cleaving a full-length antibody into smaller functional fragments by either chemically or enzymatically means (Vlasak and Ionescu, 2011). The antigen binding capability of the full-length original antibody will be retained in these fragments and can be used for various applications including the same clinical indication that the whole mAb was or is being used for. With their smaller size and lack of an Fc binding region, tissue penetration, immunogenicity and pharmacokinetic profiles will be enhanced. Usually, fragmentation is performed either with enzymatic digestion with papain or pepsin., Papain digestion will give two Fab fragments, and pepsin will give $F(ab)_2$ fragment likewise **Figure 15**, It should be noted that due to their smaller size and lack of complexity, expression of antibody fragments also in bacterial systems can also be used for fragment generation.

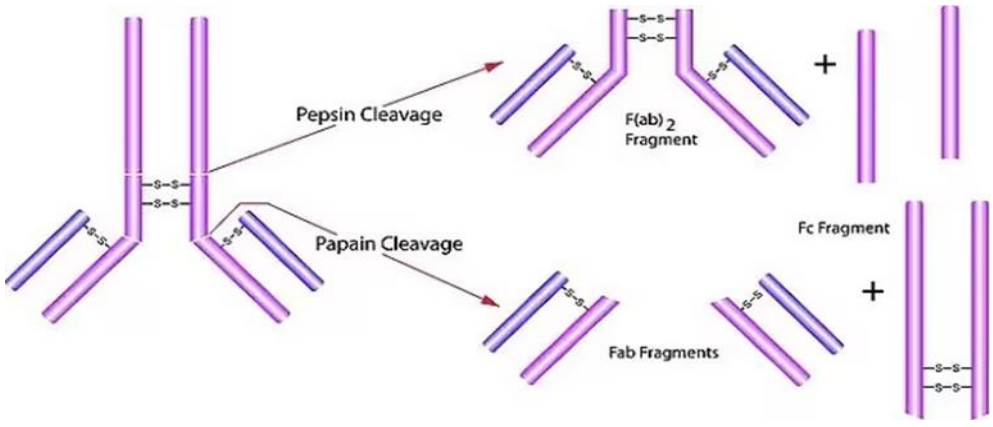


Figure 15: schematic illustration of antibody fragmentation with papain and pepsin ("Antibody Fragmentation with Pepsin Digestion," n.d.)

There are different types of antibody fragments such as Fab that contains one antigen-binding domain which includes the heavy and light chains of the whole mAb. Normally, Fab fragments are produced by enzymatic digestion using papain which will cleave the hinge region between Fab and Fc domain. This is thought to increase the efficacy of the antibody in therapeutic and diagnostic applications by increasing penetration into tissues. Fab fragments are one of the most successful fragments that have entered clinical development (Nelson, 2010).

The $F(ab)_2$ fragment contains two antigen binding domains, $F(ab)_2$ is produced by enzymatic digestion by pepsin **Figure 15**. Unlike papain, pepsin will cleave the antibody below the hinge region producing a divalent fragment and will retain the bivalent nature of the antibody

Single-Chain Variable Fragments (scFvs) are recombinant molecules which have antigen binding domains encoded with variable regions of light and heavy chains combined to form a single polypeptide (Nelson, 2010). Typically used in for gene therapies or as a targeting moiety in chimeric antigen receptor (CAR) T-cell therapies and because of their small size and easier to genetic manipulation they have been used in different diagnostic assays (Muñoz-López et al., 2022).

The Fragmented antibody purification process is carried out using Protein L affinity columns which is a universal binding ligand. In biotechnology protein L is noteworthy due to its ability to not only to bind whole immunoglobulins but also antibody fragments containing light chains such as single chain variable fragments (scFvs) and fragment antigen binding (Fabs). It differs from other affinity ligands like protein A and G and has a unique interaction with immunoglobulin light chains. A protein L column has up to 5B (binding) domains that will selectively bind kappa light chains of immunoglobulins unlike Protein A and G. It was determined that protein L binds to kappa subtypes 1, 3 and 4.

A further example to reduce the size of antibodies has been to miniaturize full sized mAbs by removing regions that are deemed to be non-essential or non-functional. These molecules are considered to be third generation molecules (Hu et al., 1996).

1.7.2 Conjugation

Conjugation is the process in which various molecules are attached to an antibody to enhance their therapeutic or diagnostic capabilities. Antibody modification using different conjugates can be achieved mainly in one of three ways; Natural: conjugation in the naturally occurring sites that exist in the native antibody structure, Non-Natural: modification that happens in non-naturally occurring sites by genetic manipulation on the backbone and some other unique antibody modifications that do not fall under both natural or non-natural neatly are called special. These special molecules include, fluorescent labels, radionuclides or different enzymes.

Antibody conjugated with a cytotoxic drug are called Antibody-Drug Conjugates (ADCs). ADCs have an enhanced localized effect utilizing the antibodies targeted delivery to provide specificity and the cytotoxic payload as an active agent. The conjugation will be stable in circulation but cleave and release the drug inside the targeted cells providing a localized effect to minimize systemic toxicity associated with the drug alone. ADCs are primarily used in cancer therapy to deliver cytotoxic drugs exactly into the cancer cells and will minimize every other off-target effect (Alves, 2019).

Antibodies that are conjugated to radionuclides are called Radioimmunoconjugates. Specially used for cancer treatments in radioimmunotherapy (RIT) and also used in radio immunoimaging for diagnostic purposes. These conjugates will help to deliver the radiation to cancer cells precisely while avoiding healthy tissues (Bourgeois et al., 2017).

There are number of other conjugates like Enzyme-Antibody conjugates, which are enzymes like Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) linked by forming stable covalent linkage to antibodies for the use in different immunoassays like ELISA, immunohistochemistry and also western blotting (Winston et al., 2001). Fluorescent Antibody conjugates which are antibodies that are labelled with different fluorescent dyes like FITC, PE, Alexa Flour and used in flow cytometry or immunofluorescence microscopy and can also be used in other fluorescence-based assays to quantify and visualize targeted antigens (Wells et al., 1966). Biotinylated Antibodies are antibodies that are conjugated with biotin mainly used in conjunction with streptavidin or avidin in different assays like ELISA, western blotting and immunoprecipitation because of the strong interaction between biotin and streptavidin.

1.8 Formulation, Fill-Finish and Storage

Proteins are generally unstable in liquid solution when stored for extended periods post purification. This requires careful re-formulation, storage at refrigerated temperatures or freezing between drugs substance and drug product manufacture steps.

1.8.1 Formulation

The drug substance/drug product formulation must consider the following factors (Gervasi et al., 2018).

- pH
- Osmolality
- Antibody stability
- Isoelectric point
- Processing risks such as freezing, thawing or lyophilization
- Glass Transition Temperature
- Oxidation
- Surfactant requirement

Freeze/thaw cycles and different transport and storage temperatures heavily impact stability, efficacy, and safety of antibodies. This requires the presence of cryoprotectants such as sucrose and trehalose that form amorphous glasses during freezing and freeze-drying. In addition, Formulations and excipients play a vital influence in maintaining the stability of antibodies through maintenance of pH. Common buffers include citrate, Tris, histidine or acetate buffers. excipients that will prevent oxidation like methionine may also be included when oxidative degradation is a known risk-factor.

Sucrose is also widely used a cryo and lyo protectant Sucrose as an excipient has an enhanced effect in the antibody stability, sucrose can increase the folding stability and can led to a slower aggregation by slower reduction of the monomer content and also it will decrease local flexibility in most of the mAb structures and formation of insoluble aggregates (Manikwar et al., 2013). Sucrose is also widely used a cryo and lyo protectant. Surfactants including

Polysorbate 80 or Polysorbate 20 are also known to play a key role in ensuring antibody stability through minimizing interfacial stress.

1.8.2 Freeze/Thaw & Storage

Various temperatures are used to store antibodies. Common temperatures used are 2-8ºC, -20ºC, and -80ºC. Most of the commercially available monoclonal antibodies are stored at 2-8ºC. It is considered to be as the most common storage temperature and is sufficient to maintain the stability of antibodies for at least two years, which is required for commercial usefulness. This storage temperature can slow down degradation like deamidation, oxidation and aggregation. Refrigerated storage is also advisable as it avoids freeze-thaw cycles that could lead to crystallization of buffer and non-buffer components, phase separation, ice formation redistribution of solutes and also pH fluctuation.

Freezing the antibodies at -20ºC or -80ºC are used as a long-term storage when compared to 2-8ºC. It will significantly slow down all the physical and chemical degradation and can achieve long-term stability. Degradation or damage caused from freeze thaw cycles in -20ºC can be reduced by the addition of cryoprotectants like the polyols sucrose or trehalose. Antibodies should be dispensed into aliquots in smaller volumes before freezing to avoid multiple freeze thaw cycles (Chang et al., 1996).

Monoclonal antibodies can also be stored at room temperature only for shorter periods and convenient for transport and immediate uses. Lyophilized and antibodies formulated with specific stabilizers can be kept at room temperature.

1.8.3 Freeze drying

Protein stability can be increased by the removal of water from the antibody formulations (Daugherty and Mrsny, 2006). A common method performed for the removal of water is freeze

drying (Lyophilization). It involves the freezing of the antibody solution and then decreasing the chamber pressure and temperature to sublimate the frozen water. The unfrozen water is then removed by secondary drying at raised temperatures, resulting in residual moisture contents of 1-3%. This ensures long-term stability even at room temperature when stored in a moisture free/protected environment. The protein containing unfrozen section containing cryo/lyo-protectants, proteins and other excipients must be maintained a glassy state in order to ensure long-term stability (Ma et al., 2020). The freeze-dried cake must be carefully reconstituted in order to prevent loss of activity. Normally freeze-dried antibodies are rehydrated using sterile water or saline.

1.9 Thesis Overview

The thesis experimental work will focus on the development of an end-to-end monoclonal antibody production process for two distinct cell lines, namely an anti-TNF Alpha mAb hybridoma cell line and a CHO cell line that produces a mAb against an unspecified antigen (cNISTmAb).

This CHO cell line is a high producing cell line relative to hybridoma cell lines and is made available for non-commercial research by the National Institute of Standards and Technology (NIST), to address the lack of publicly available CHO cell lines. CHO cell lines are generally expensive to develop and optimize and thus have remained internal to commercial organizations, hence the lack of availability. As part of the cNISTmAb cell line development with Merck, the antigen has not been disclosed which limits the use of bioassays such as potency and ELISA.

An anti-TNF alpha hybridoma mAb cell line will also be studied with a focus on cell culture optimization, purification and downstream processing development. This particular cell line has been selected as there are several commercially available anti-TNF Alpha products marketed including Humira, Remicade, Embrel that target this pro-inflammatory molecule.

As well as optimizing cell culture through media, bioreactor and processing changes, the thesis will focus on the development of characterization assays for the analysis of key CQAs such as potency and purity for both mAbs produced. These assays will support characterization of the individual unit operations and the final formulated drug product.

A final element of the thesis will assess the possibility of creating antibody fragments from produced whole antibodies through papain and pepsin, with the intent of understanding their stability and potency relative to the whole mAb.

2 Objectives & Hypothesis

Within the research unit where this thesis was conducted, no mAb production facilities currently exist. This requires the ongoing purchase of commercial antibody stocks for any formulation or processing research work requiring antibodies or limits research to the use of model proteins such as BSA, lysozyme etc., that are not representative of industrial proteins. It was in this context that this thesis was conducted with the following specific objectives and hypotheses.

The specific objectives of this thesis were as follows:

- Develop an end-to-end monoclonal antibody manufacturing process from cell culture to final formulation of drug product. using publicly available mAb cell lines to include hybridoma and CHO cell lines. A CHO cell line was selected to have a high titer, industrially relevant cell-line while hybridoma cell line was chosen as these cell lines are widely available in contrast to CHO cell lines.
- Develop bioassays and other characterization assays to assess the efficacy and structure of monoclonal antibody produced and examine the potential impact of enhanced productivity on product CQAs
- Determine the stability of monoclonal antibody produced at refrigerated and ambient temperatures in the liquid state as well as in the solid state when freeze-dried.

The hypotheses of this were as follows:

- A commercially available anti-TNF Alpha monoclonal antibody cell line could be cultured and mAb purified, with titers optimized based on modifying recommended cell culture conditions without impact to product potency and purity.

- Potency of anti-TNF Alpha mAb could be characterized with an in-vitro cell-based HEK293 & L929 potency assay.
- Anti-TNF Alpha mAb could be characterized in terms of aggregates, antigen binding using common bench techniques such as SDS-PAGE and ELISA and that downstream processing i.e. purification, TFF and formulation would not impact product CQAs
- A publicly available CHO cell line, cNISTmAb, targeting a non-disclosed antigen could be cultured and mAb purified, with titers optimized based on modifying recommended cell culture conditions.

3 Materials & Methods

3.1 Materials

Material	Grade	Supplier	Part Number	Application
Cell Culture				
Anti Human TNF Cell Line	ECACC	Merck	920306 3-1VL	Hybridoma Cell Line
cNISTmAb	Research Grade Test Material	NIST	10197	CHO Cell Line
RPMI 1640-1640 Medium	Cell Culture	Sigma Aldrich	R8758	Expansion Media
Fetal Bovine Serum (FBS) Heat Inactivated	Cell Culture	Sigma Aldrich	F9665-500ML	Cell Resuscitation & Media
Penicillin-Streptomycin	Cell Culture	Sigma Aldrich	P4333	Prevention of microbial contamination
HEPES SOLUTION 1 M, PH 7.0-7.6	Cell Culture	Sigma Aldrich	H0887-100ML	Media
MEM Amino Acids (50x) Solution	Cell Culture	Sigma Aldrich	M5550-100ML	Media
Glucose	Cell Culture	Sigma Aldrich	G7528-250G	Media
L-Glutamine Solution 200mm	Cell Culture	Sigma Aldrich	59202C-100ML	Media
Milli Disposable Hemocytometer	N/A	Millipore	MDH-2N1-50PK	Cell counting
Nutrient Mix F12 Ham	Cell Culture	Sigma Aldrich	N6658-500ML	Media
Dulbecco's Phosphate Buffered Saline	Cell Culture	Sigma Aldrich	D8537-500ML	Washing of Cells
Trypsin-EDTA Solution 0.25%	Cell culture	Sigma Aldrich	T4049-100ML	Detachment of adhered cells
EX-CELL® CD CHO	Cell Culture	Sigma Aldrich	14360C-1000ML	Media
Sodium Butyrate	≥98.5% (GC)	Sigma Aldrich	B5887-1G	Enhanced Cell production

Material	Grade	Supplier	Part Number	Application
HEK293 Cell Based Potency Assay				
HEK293 Cell Line	N/A	Internal	N/A	Reporter Cell Line
DMEM - High Glucose	Cell Culture	Sigma Aldrich	D6429-6X500ML	Media
Zeocin	Cell culture	Mechem Express	HY-K1053-1mL	Selection Marker
Puromycin	Cell culture	Mechem Express	HY-K1057-1mL	Selection Marker
Dulbecco's Phosphate Buffered Saline	Cell Culture	Sigma Aldrich	D8537-500ML	Washing of Cells
Trypsin-EDTA Solution 0.25%	Cell culture	Sigma Aldrich	T4049-100ML	Detachment of adhered cells
Collagen From Human Placenta	Cell culture	Sigma Aldrich	C5533-5MG	Cell adherence
Bovine Serum Albumin, Lyophilized	>96% Pure	Sigma Aldrich	A2153-50G	Increase antigen stability
Human-TNF Alpha	Research	Shenandoah	100-111-100ug	Antigen
Human anti-TNF Alpha IgG antibody	Research	BioSciences Limited (ThermoFisher)	MA5-23720	External Antibody
Phospha-Light™ SEAP Reporter Gene Assay System	Cell Culture	Applied Biosystems	T1015	SEAP Detection
L929 Cell Potency Assay				
L929 Cells, mouse C3H/An connective tisue	NCTC	Merck	85011425-1VL	Reporter Cell Line
Cell Titer-Glo® 2.0 Cell Viability Assay	N/A	Promega	G9241	ATP detection
Actinomycin D, From Streptomyces	≥95% (HPLC)	Sigma Aldrich	A4262-2MG	Cell cytotoxicity
Cell Banking				
Dimethyl Sulfoxide Hybri-Max Sterile	Cell culture	Sigma Aldrich	D2650-5X5ML	Cell Banking/ Cryopreservation

Material	Grade	Supplier	Part Number	Application
Purification, Dialysis & TFF				
NeoTrap Protein G FLPC Column	Research	Neo Biotech	NB-19-0074-1ml	Affinity Chromatography
NeoTrap Protein G FLPC Column	Research	Neo Biotech	NB-19-0074-5mL	Affinity Chromatography
Sodium Phosphate Monobasic Dihydrate	Reagent	Sigma Aldrich	71505-1KG	Buffer A
Sodium Phosphate Dibasic Dihydrate	Reagent	Sigma Aldrich	71643-1KG	Buffer A
Glycine	>= 99% (HPLC)	Sigma Aldrich	T6066-1KG	Buffer B
Hydrochloric Acid 36%	Suprapur	Sigma Aldrich	1151861000	Buffer B
Tris-HCL	N/A	Internal	N/A	Buffer C
Ethanol	N/A	Internal	N/A	Column Storage & Wash
Tube-O-Dialyzer (Tm) Mini Dialysis System (4 KDa MWCO)	N/A	Geno Technology	Z742631-20EA	Dialysis
Sucrose	99%	Thermo Scientific Chemicals	A15583.0C	Drug Product Formulation
L-Histidine	98%	Thermo Scientific Chemicals:	166155000	Drug Product Formulation
Polysorbate 80	Chemical	Thermo Scientific Alfa Aesar	L13315.AP	Drug Product Formulation
Sodium Hydroxide	Reagent Grade, >=98%	Sigma Aldrich	S5881-1KG	TFF Membrane Storage
Pepsin Digestion				
Sodium Citrate	Cell Culture	Internal	N/A	Digestion Buffer
Pepsin	Cell Culture	Acros Organics	12357123	Digestion enzyme

Material	Grade	Supplier	Part Number	Application
Protein L Column	N/A	Cytiva	15149945	$F(ab')_2$ Affinity column
Trizma - Base	Cell Culture	Sigma Aldrich	T6066-1KG	Digestion quenching
ELISA				
Rabbit Anti-Mouse IgG Antibody, HRP Conjugate	Reagent	Sigma Aldrich	AP160P	Secondary Antibody
O-Phenylenediamine Dihydrochloride	Reagent	Sigma Aldrich	P9187-5SET	HRP Detection
Sodium Bicarbonate	Reagent	Sigma Aldrich	S5761-500G	Antigen Binding
Sodium Carbonate, Bioultra, Anhydrous	Reagent	Sigma Aldrich	71345-250G	Antigen Binding
Tween 20	Reagent	Sigma Aldrich	P1379-500ML	Plate washing
Milk	N/A	Internal	N/A	Blocking agent
SDS-PAGE				
ExpressPlus™ PAGE Gel,10×8, 4-20%, 10 wells	Reagent	Genscript	M42010	Gel
Tris-MOPS-SDS	Reagent	Genscript	M00138	Running Buffer
4X LDS	Reagent	Genscript	M00676-10	Sample Buffer
SDS-PAGE Ladder	Reagent	ThermoFisher Scientific	26619	Ladder

Equipment	Make	Model
Centrifuge	Thermo Scientific	Heraeus Megafuge 8
Vacuum Pump	Thermo Scientific	Aspiration Advantage System with 4L polypropylene
Incubator	Thermo Scientific	VIOS 160i CO2 SS IR 230V LK
Biosafety Cabinet	Thermo Scientific	MSC-Advantage Class II BSC
Microscope		
Purification Skid	Cytiva	AKTA START
UV Plate Reader	BMG	Spectro Star Nano
Visible Plate Reader	Revvity	Victor
SDS-PAGE Chamber & Power Pack	Bio-Rad	Mini-Protean Tetra Cell
UV Protein Concentration	Thermofisher Scientific	Nanodrop
Gel Scanner	Thermofisher Scientific	iBright 1500
TFF	Pall	Minimate EVO Tangential Flow Filtration (TFF) System

Consumable	Make	Model
T-25 Flask (non-adherent)	Sarstedt	83.3910.502
T-25 Flask (adherent)	Sarstedt	83.3910.002
T-75 Flask (non-adherent)	Sarstedt	83.3911.502
T-75 Flask (adherent)	Sarstedt	83.3911.002
T-175 Flask (non-adherent)	Sarstedt	83.3912.502
T-175 Flask (adherent)	Sarstedt	83.3912.002
Baffled flask, GL 45, with 4 bottom baffles, complete with membrane screw cap, 500ML	Duran	11736278
Flask, conical, baffled, membrane screwcap, 1000mL	Pyrex	15272816
S-Pak® Membrane Filter (0.22 Micron)	Millipore	GSWG047S6
Filtropur V100, Vacuum filtration unit, 1,000 ml, PES, 0.22 µm	Sarstedt	83.3942.001
10K Minimate Tangential Flow Filtration (TFF) capsule with Omega membrane	Pall	17194761

3.2 Methods

3.2.1 Cell Culture

3.2.1.1 Anti-TNF Alpha Hybridoma Cell Culture:

Cell Thawing:

Cells were removed from liquid nitrogen and placed in a beaker containing water at 37°C until vial contents had fully thawed. Once thawed, cells were transferred to a 50ml conical tube containing 20ml of RPMI 1640 media, 1% Penicillin & streptomycin, 1% HEPES buffer and FBS at a final concentration of 20% v/v in a biosafety cabinet under aseptic conditions. The 50 ml tube was then centrifuged at 0.1 RCF for 5 minutes using a centrifuge. The supernatant was aspirated using a vacuum pump to waste and the cell pellet was resuspended with 10ml of the above-mentioned media and transferred to a T-25 cell culture flask and cultured as suspension cells. The T25 culture flask and contents were then incubated at 37°C and 5% CO_2 for 3 days in an incubator.

Cell expansion:

After three days, the cell culture was aspirated from culture flask and placed in a 50ml conical tube and centrifuged at 0.1 RCF for 5 minutes. The supernatant was aspirated using vacuum pump to waste and the cell pellet was resuspended in 5ml of RPMI 1640 media containing 1% penicillin & streptomycin, 1% HEPES buffer and FBS at a final concentration of 10% v/v. This media is hereafter described as standard expansion media. The resuspended pellet was next transferred to a T75 cell culture flask which contained 20ml of expansion media. The T75 culture flask was then transferred to the incubator and incubated at 37°C, 5% CO_2 for 2 days. The previous expansion step was replicated for several iterations to achieve 4 T-175 cell culture flasks with a final volume of cell culture of 150ml per culture flask, e.g. 600ml in total. The expansion steps were performed approximately every three days based on cell confluence,

media appearance and cell density. Cell confluence and media appearance were analyzed visually as RPMI 1640-1640 contained phenol red that changes from red to yellow when media is consumed indicating a need to change and replenish media. Cell density was analyzed visually under microscope and quantitatively using a hemocytometer.

Cell Production:

Cell culture was aspirated from four T-175 cell culture flasks into 50ml conical tubes and centrifuged at 0.1 RCF for 8 minutes. Cell supernatant was aspirated to waste using vacuum pump and cell pellet was resuspended in 10ml of RPMI 1640, 1% FBS, 1% HEPES, 1% penicillin & streptomycin and 2% Amino Acid. This media composition is hereafter described as standard production basal media. The resuspended cell pellet was transferred into 4 T-175 cell culture flasks containing 140ml of standard production media and incubated at 37°C, 5% CO2 for 10 days. A standard production feed media of 10mM glucose, 1% glutamine (v/v), 1% amino acids (v/v) and 1% HEPES (v/v) was added to the cell culture every 2 days to cell culture i.e., days 2, 4, 6 & 8.

Cell culture glucose concentrations were analyzed every 2 days during production using a glucometer and test strips. 10mM Glucose was added to cell culture when glucose levels were found to be below 5mM. When glucose levels were above 5mM, glucose was removed from standard production feed media.

Harvest:

After 10 days of production cell culture, all media was aspirated into 50ml conical tubes and 50 ml conical tubes were centrifuged at 0.1 RCF for 10 minutes. The supernatant was collected, and the cell pellet was disposed of. The supernatant was then filtered using a 0.22-micron filter prior to purification step and stored at 4ºC.

3.2.1.2 cNISTmAb Cell Culture:

Cell Thawing:

For cNISTmAb cell culture, cells were removed from liquid nitrogen and placed in a beaker containing water at 37°C until vial contents had fully thawed. Once thawed, cells were transferred to a 50ml conical tube containing 20ml of Nutrient Mixture F-12 Ham media containing 1% HEPES (v/v), 1% penicillin/streptomycin (v/v) and FBS at a final concentration of 10% (v/v). The 50 ml tube was then centrifuged at 0.1 RCF for 5 minutes using a centrifuge. The supernatant was aspirated using a vacuum pump to waste and the cell pellet was resuspended with 10ml of the standard expansion media and transferred to a T-25 cell culture flask and cultured as adherent cells. The T25 culture flask and contents were then incubated at 37°C and 5% CO_2 for 3 days in an incubator.

Cell expansion:

After three days, the cell culture media was aspirated from culture flask and disposed of, 10ml of Dulbecco's PBS was then added to the flask to wash cells and then aspirated and disposed of. 1ml of Trypsin was added to the flask and placed in the incubator for 1 minute to detach adhered cells. After 1 minute the flask was removed from incubator and 5ml of Nutrient Mixture F-12 Ham media containing 1% HEPES (v/v), 1% penicillin/streptomycin (v/v) and FBS at a final concentration of 10% (v/v) was added to the flask containing detached cells and the cell suspension was mixed well for 2 minutes via pipette. This media is hereafter described as standard expansion media. The cell suspension was then transferred to the new T-75 flask which contained 20ml of the above-mentioned media (Nutrient Mixture F-12 Ham media, 10% FBS, 1% HEPES (v/v), 1% penicillin/streptomycin). The T75 culture flask was then transferred to incubator and incubated at 37°C, 5% CO_2 for 3 days. The previous expansion step was replicated for several iterations to achieve twelve T-75 cell culture flasks with a final volume

of cell culture of 25ml per culture flask. The expansion steps were performed approximately every three days based on cell confluence, media appearance and cell density. Cell confluence and media appearance was analyzed visually while, cell density was analyzed visually under microscope and quantitatively using a hemocytometer.

Cell Production:

Cell culture media was aspirated from twelve T-75 cell culture flasks and disposed of. 10ml of Phosphate Buffer Saline was added to each flask and aspirated it. 1ml of Trypsin was added and placed in the incubator for 1 minute. After 1 minute the flask was taken out and 5ml of standard expansion media was added to detached cells and mixed well for 2 minutes. The cell suspension from each flask was then completely transferred into 50ml conical tubes and centrifuged at 0.1 RCF for 8 minutes Cell supernatant was aspirated to waste using via vacuum pump and cell pellet was resuspended in 5ml of CD-CHO Fusion media containing 1% of HEPES buffer, 1% Pen/Strep and 2mM Glutamine. This media is hereafter referred to as standard production basal media. The resuspended cell pellet was transferred into four 500ml cell culture flasks containing 150ml of standard production basal media and incubated at 37C, 5% CO_2 in a shaker set-up at 120 rpm for 10 days as a suspension culture. Cell culture glucose concentrations were analyzed every 2 days during production using a glucometer and test strips. 10mM Glucose was added to cell culture when glucose levels were found to be below 20 mM as a feed media.

Harvest:

After 10 days of production cell culture, all media is aspirated into 50ml conical tubes and 50 ml conical tubes are centrifuged at 0.1 RCF for 10 minutes. The supernatant was collected, and the cell pellet was disposed. The supernatant was then filtered using a 0.22-micron filter prior to purification step and stored at 4°C.

3.2.2 Purification

For both Anti-TNF Alpha mAb and cNISTmAb, purification was performed using FPLC. After harvesting, the conditioned medium was connected to the FPLC machine. A 1ml protein G hi-trap affinity column was used to bind and purify Anti-TNF Alpha antibodies. A 5 ml protein G hi-trap affinity column was used to bind and purify cNISTmAb. Three buffers were used for the purification. Binding buffer which helped the antibodies to bind into the column. 20mM sodium phosphate buffer at a pH of 7.0 was used as the binding buffer. It was prepared using monosodium phosphate and disodium phosphate and the pH was adjusted using sodium Hydroxide or hydrochloric acid and a pH meter. A 0.1M Glycine solution at pH 2.7 was used as the elution buffer. A 1M Tris-HCL solution at a pH of 9.0 was used as a neutralization buffer which neutralized the pH of the eluted antibodies. All the buffers and conditioned medium were equilibrated to room temperature and degassed using vacuum filtration through a 0.2µm pore membrane for 10-15minutes prior to use. The neutralization buffer was added to Eppendorf tubes prior to the start of purification. The eluted antibodies were collected into these Eppendorf tubes that contain neutralization buffer to prevent denaturation. The eluted antibodies were then stored at 4ºC until further use or reformulation.

A flow rate of 1ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was observed via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, with successive 1ml elution fractions captured and a total of 15 fractions collected.

3.2.3 Dialysis & TFF

Purified Anti-TNF Alpha antibodies were formulated into a final formulation buffer of Sucrose, L-histidine, polysorbate 80 buffer to minimize degradation. The buffer was prepared to final concentrations of 10% sucrose, 0.01% polysorbate 80 and 10mM L-histidine at a pH of 6.0. Dialysis was performed overnight using Tube-O-Dialyzer tubes using the above-mentioned buffer. (the tubes were kept in an inverted floating position in the buffer using floaters and the buffer was stirring in the lowest speed of the stirrer at 4ºC).

Purified cNISTmAb were Dia-filtered using Cytiva Minimate, EVO Tangential Flow Filtration. Diafiltration was done using the above-mentioned formulation buffer (sucrose, L-histidine, polysorbate buffer). The purified antibodies were first concentrated into the membranes in the system with the purification buffer filtered from the system. 500ml of final formulation buffer was added and again concentrated and this process was repeated again, and the concentrated antibody solution was collected and stored at 4ºC.

3.2.4 Protein concentration

Concentration of the dialyzed and TFF samples were measured using Nanodrop. The final formulation buffer consisting of sucrose, L-histidine and polysorbate 80 was used as the blank for the measurement. 2 microliters of analyte were placed on the cell in the pedestal position with MilliQ water used to clean pedestal before and after analysis.

3.2.5 ELISA

In-vitro antibody binding was assessed via indirect ELISA using human TNF Alpha as antigen. Antigen was formulated in a bicarbonate buffer before coating. 96-well round bottom plates were coated with antigen at various concentrations from 4 µg/ml to 0.0039 µg/ml through a series dilution and plate was left overnight at 37°C. The plate was washed with PBS before blocking with 200 µl of 5% milk in PBS/Tween 20 for 40 minutes before being removed. The primary antibody (Anti-TNF Alpha) generated via internal cell culture studies or purchased

externally was added (100µl) at a dilution of 1:1000 from starting antibody stocks. The antibody stock for internal anti-TNF Alpha was 0.6 mg/ml while the external anti-TNF Alpha antibody stock was 0.5 mg/ml. The primary antibody was left for 45 minutes at room temperature before being washed with PBS/Tween 20. The secondary antibody at a dilution of 1:2,000 (Rabbit Anti-Mouse IgG Antibody, HRP Conjugate) was then incubated with primary antibody for 30 minutes at room temperature before being washed with PBS/Tween 20. OPD substrate was then prepared in 20ml of ddH20 and 100µl of OPD substrate was then added to each well and incubated at room temperature for 10 minutes. The plate was then covered to prevent ingress of light and read at 492nm using Viktor plate reader.

3.2.5 SDS-PAGE

A 10 well ExpressPlus™ PAGE Gel, 10x8, 4-20%, gel was used to perform the SDS-PAGE under reducing, denaturing conditions. Sample protein concentration was initially analyzed via Nanodrop (Section 2.4), and samples were diluted with 1X loading buffer containing SDS and β-mercaptoethanol to achieve a final sample concentration of 0.2mg/ml. Samples were then heated to 95°C for 5 minutes and left overnight at -20°C. 20µl of sample was added to each well (4µg of antibody per well). SDS-PAGE running buffer was prepared using Running Buffer powder dissolved in ddH2O. A pre-stained protein ladder was used in the gel as molecular weight standard. A power pack was Employed with a constant voltage of 50V supplied to the gel for 20 minutes. The voltage was increased to 100V, and samples allowed to run for another 2 hours. The gel was then taken out from the cast and placed in a petri dish which contained Coomassie blue stain (Quick Coomassie stain) and kept at 4ºC for overnight.

The gel was taken out and washed with ddH2O 3 times and the gel image was captured using an imaging system (iBright 1500, Thermofisher Scientific). Quantitative analysis of band intensity was not performed.

3.2.6 Cell Based Potency Assays

3.2.6.1 HEK 293 Cell Assay

Cell Thawing:

HEK293 cells were removed from liquid nitrogen and placed in a beaker containing water at 37°C until vial contents had fully thawed. Once thawed, cells were transferred to a 50ml conical tube containing 20ml of DMEM, 10% FBS, 1% HEPES, 1% Penicillin/Streptomycin, 0.1% Zeocin and 0.02% Puromycin. This will be hereafter described as standard expansion media for HEK 293 Cells. The 50 ml tube was then centrifuged at 0.1 RCF for 5 minutes using a centrifuge. The supernatant was aspirated using a vacuum pump to waste and the cell pellet was resuspended with 10ml of the above-mentioned media (DMEM, 10% FBS, 1% HEPES, 1% Penicillin/Streptomycin, Zeocin and Puromycin and transferred to a T-25 cell culture flask and cultured as adherent cells. The T25 culture flask and contents were then incubated at 37°C and 5% CO_2 for 3 days in an incubator.

Cell expansion:

After three days, the cell culture media was aspirated from culture flask and disposed of, 10ml of Phosphate Buffer Saline was added to the flask and aspirated it. 1ml of Trypsin was added and placed in the incubator for 1minute. After 1 minute the flask was taken out and 5ml of HEK 293 standard expansion media was added to the detached cells and mixed for 2 minutes. The cell suspension was then completely transferred to a new T-75 flask which contained 20ml of above-mentioned standard expansion media, total volume of 25ml. The T75 culture flask was then transferred to incubator and incubated at 37ºC, 5% CO_2 for 3 days.

Cell Seeding:

After 3 days, the cell culture media was aspirated from the flask and disposed of. 10ml of Dulbecco's PBS was added to the flask and aspirated to waste. 1ml of Trypsin was added and

placed in the incubator for 1 minute. After 1 minute the flask was taken out and 5ml of standard expansion media was added to the detached cells and mixed for 2 minutes. The cell suspension was then transferred into a 15 ml conical tube. The cell density was counted with a hemocytometer. 25ml of standard expansion media was added to a 50ml conical tube. Approximately 3 million cells were added to this 25ml of standard expansion media. Previously collagen coated 6 well plate was prepared by adding 4ml of collagen into each well and incubated for 24hrs. The cells in the 25ml media were then transferred into this collagen coated 6 well plate with each well containing 4 ml of cells (approximately 0.5million cells per well) with the well plate incubated at 37ºC, 5% CO_2 for 2 days.

Cell Stimulation:

After two days, the seeded well plate was taken out and the media was aspirated from all wells. 4ml of DMEM, 1% HEPES, 1% Penicillin/Streptomycin media was added to each well, in two additions (1ml and 3ml to avoid cells drying out) and kept in the incubator for approximately 15 minutes. Conditioned media was prepared using DMEM, 1% HEPES, 1% Penicillin/Streptomycin with BSA, hTNFα and anti-TNFα antibodies (externally sourced or from cell culture experiments) as per **Table 1.**

	1st well	2nd well	3rd well	4th well	5th well	6th well
BSA	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml
h TNFα	2ng/ml	2ng/ml	2ng/ml	2ng/ml	2ng/ml	0
anti-TNFα mAb	A ng/ml	B ng/ml	C ng/ml	D ng/ml	0	0

Table 1 Plate Layout for HEK293 Cell Assay

The 6-well plate was taken out of the incubator and the 4ml of existing media was aspirated from the first well and 2ml of corresponding conditioned media was added to the first well and the process was repeated for all 6 wells. The well plate was then incubated at 37ºC, 5% CO_2 for 24hrs.

The antigen concentration was kept constant at 2ng/ml with BSA concentration also being kept constant at 50µg/ml throughout all experiments. The antibody concentration in wells 1-4 varied as per objective of individual experiment. Wells 5-6 were always maintained as negative controls. See above well plate design in **Table 1**.

Cell Analysis:

After 24hrs, the stimulated well plate was taken out and the supernatants were collected. The supernatant was analyzed for the presence of SEAP (Secreted Alkaline Phosphate). A commercially available SEAP kit was purchased to conduct this analysis. The dilution buffer was diluted from 5X to 1X using ddH2O. 50µl of this dilution buffer was added into different 1.5ml Eppendorf tubes. 50µl from the different supernatants collected from stimulated cells was added into each of these tubes. These samples with dilution buffer were heated at 65ºC for 30 minutes and subsequently cooled on ice to room temperature and centrifuged at a low speed (0.9 rpm) for 1 minute. Reaction buffer was prepared by mixing CSPD substrate and reaction buffer diluent from the kit in 1:20 respectively. 50µl from Assay buffer from the kit was added to a white 96 well plate. 50 µl of the cooled down sample was then added to this assay buffer and incubated for 5 minutes. 50 µl of reaction buffer was then added to this mixture and incubated for 20minutes. The luminescence was recorded using Viktor plate reader at a wavelength of 492nm.

3.2.6.2 L929 Cell Assay

Cell Thawing:

L929 cells were removed from liquid nitrogen and placed in a beaker containing water at 37°C until vial contents had fully thawed. Once thawed, cells were transferred to a 50ml conical tube containing 20ml of DMEM, 10% FBS, 1% HEPES, 1% Penicillin/Streptomycin. This will be

hereafter described as standard expansion media for L929 Cells. The 50 ml tube was then centrifuged at 0.1 RCF for 5 minutes using a centrifuge. The supernatant was aspirated using a vacuum pump to waste and the cell pellet was resuspended with 10ml of the above-mentioned media (DMEM, 10% FBS, 1% HEPES, 1% Penicillin/Streptomycin) and transferred to a T-25 cell culture flask and cultured as adherent cells. The T25 culture flask and contents were then incubated at 37°C and 5% CO_2 for 3 days in an incubator.

Cell expansion:

After three days, the cell culture media was aspirated from culture flask and disposed of, 10ml of Phosphate Buffer Saline was added to the flask and aspirated it. 1ml of Trypsin was added and placed in the incubator for 1minute. After 1 minute the flask was taken out and 5ml of L929 standard expansion media was added to the detached cells and mixed for 2 minutes. The cell suspension was then completely transferred to a new T-75 flask which contained 20ml of above-mentioned standard expansion media, total volume of 25ml. The T75 culture flask was then transferred to incubator and incubated at 37ºC, 5% CO_2 for 3 days.

Cell Seeding:

After 3 days, the cells culture media was aspirated from the flask and wasted. 10ml of Phosphate Buffer Saline was added to the flask and aspirated it. 1ml of Trypsin was added and placed in the incubator for 1minute. After 1 minute the flask was taken out and 10ml of standard expansion media was added to the detached cells and mixed for 2 minutes. The cell suspension was then transferred into a 15 ml conical tube. The cell was counted with a hemocytometer. 5ml of standard expansion media was taken in a 15ml conical tube. Approximately 25 thousand cells are added to this 5ml of media. The cells in the 5ml media were then transferred into 24 wells of a 96 well plate, each well containing 200µl of cells (approximately 10 thousand cells per well). And the well plate was incubated at 37ºC, 5% CO_2 for 2 days.

Cell Stimulation:

After two days, the seeded well plate was taken out and the media was aspirated from all wells. 200µl of DMEM media with 1% HEPES and penicillin/streptomycin was added to each well and kept in the incubator. Conditioned media was prepared using DMEM with BSA, hTNFα as per **Table 2**. The 200µl of media was then removed by pipette followed by the rapid addition of 100µl of conditioned medium

	1st well	2nd well	3rd well	4th well	5th well	6th well
BSA	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml
h TNFα	2ng/ml	1ng/ml	0.5ng/ml	0.25ng/ml	0.125ng/ml	0

Table 2 Plate Layout for L929 Cell Assay

The well plate was taken out of the incubator and aspirated the media from one set of wells (e.g., 4 replicas of 1st well) and the corresponding conditioned media was added to the corresponding wells vertically down the plate. This was repeated for remining wells 2-6. The well plate was then incubated at 37ºC, 5% CO_2 for 48hrs.

The BSA concentration kept constant at 50µg/ml throughout all experiments. The antigen concentration in wells 1-5 varied as per objective of individual experiment. Well 6 was always maintained as negative controls. See above well plate design.

Cell Analysis:

After 48hrs, the stimulated well plate was taken out and 100µl of Promega ATP kit was added into each well (same to the volume of media present in the well). The contents of the well plate were mixed for 2 minutes in an orbital shaker using Viktor plate reader, shaking function. The 96 well plate was incubated at room temperature for 5 minutes. After incubation the content on

each well was transferred into an opaque 96 well plate for measurement. The luminescence was recorded using Viktor plate reader at 492nm.

3.2.6.2.1 Treated with Actinomycin D

The assay was replicated with the addition of Actinomycin D as a stimulant.

Conditioned media was prepared using DMEM with BSA, hTNFα and Actinomycin D as per **Table 3**. Each well contained 100µl of treated medium. And 4 replicas of each well were used to get 24 wells. ATP levels were detected as per the above methodology.

	1st well	2nd well	3rd well	4th well	5th well	6th well
BSA	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml
h TNFα	0	1ng/ml	0.5ng/ml	0.25ng/ml	0.125ng/ml	0
Actinomycin D	0.5µg/ml	0.5µg/ml	0.5µg/ml	0.5µg/ml	0.5µg/ml	0

Table 3 Plate Layout for L929 Cell Assay

3.2.6.2.2 Potency of anti-TNFα

Conditioned media was prepared using DMEM with BSA, hTNF Alpha, Actinomycin D and anti- TNF Alpha monoclonal antibody produced from cell culture experiments as per **Table 4.** Each well contained 100µl of treated medium. And 4 replicas of each well were used to get 24 wells. ATP levels were detected as per the above methodology.

	1st well	2nd well	3rd well	4th well	5th well	6th well
BSA	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml
h TNFα	1ng/ml	1ng/ml	1ng/ml	1ng/ml	1ng/ml	1ng/ml
Actinomycin D	0.5µg/ml	0.5µg/ml	0.5µg/ml	0.5µg/ml	0.5µg/ml	0.5µg/ml
anti-TNFα Abs (0.9mg/ml)	0	0	517.2ng/ml	172.4ng/ml	51.72ng/ml	17.24ng/ml

Table 4 Plate Layout for L929 Cell Assay

3.2.7 Pepsin Digestion

cNISTmAb samples were processed with pepsin to generate antibody fragments e.g. $F(ab)_2$. A 0.1M sodium citrate solution at pH 4.0 was used as the digestion buffer. The purified and formulated cNISTmAb samples were dialyzed overnight against the digestion buffer (0.1M sodium citrate). Pepsin was prepared in the digestion buffer at a concentration of 10mg/ml (with 10mg of pepsin was dissolved in 1ml of digestion buffer). 120µg (12µl) of this dissolved pepsin was added into the dialyzed cNISTmAb sample. Pepsin was added in a proportion of 6% (w/w) with the antibody (if processing 10mg of antibody add 60µg pepsin) and incubated at 37ºC for 2 hours. The pepsin reaction was quenched by titrating pepsin treated samples against 2M Tris Base to a final pH of 8.0. The pepsin treated samples were dialyzed with 20mM PBS at a pH of 7.0 overnight. The dialyzed Abs were collected and purified using FPLC machine using a 1ml protein L Hi-trap column and Protein G NeoTrap affinity columns in series with protein L column first in the series.

3.2.8 Freeze Drying

Lyophilized samples were prepared using a laboratory-scale freeze-dryer from VirTis Advantage (USA) as described by (Giannachi et al., 2024) Sample aliquots of 2 mL were transferred into 10 mL lyophilization vials and placed on the lyophilizer shelf, which was held at 5 °C for 30 min. Samples were frozen by cooling the shelves to −40°C at a rate of 0.5 °C/min. After the samples were held at this temperature for 3.5 h, primary drying was started by reducing chamber pressure to 50 mTorr and continued by increasing the shelf temperature to −30°C for 62 h. Secondary drying was initiated by increasing shelf temperature to 35°C at a rate of 0.5 °C/min, while maintaining chamber pressure at 50 mTorr for 6 h.

3.2.9 Stability Studies

Stability studies were performed with formulated cNISTmAb produced from CHO cells in batches 1 and 2, that had been pooled. 100µl of cNISTmAb were aliquoted at a concentration

of 2.44mg/ml into Eppendorf tubes and were kept at different temperatures for pre-defined timepoints as per **Table 5**. Upon reaching desired timepoints samples were removed and stored at -80ºC until testing. One aliquot was kept at -80ºC as a control from the start of stability study i.e. Time 0.

Temperature	Time points					
-20ºC	4 days	1 week	2 weeks	3 weeks	1 month	50 days
+4ºC	4 days	1 week	2 weeks	3 weeks	1 month	50 days
+25ºC	4 days	1 week	2 weeks	3 weeks	1 month	50 days
+40ºC	4 days	1 week	2 weeks	3 weeks	1 month	50 days

Table 5 Stability Study Protocol

Three aliquots were freeze dried and kept at -20ºC, +4ºC and at room temperature for 50 days. All the samples were thawed after 50 days and analyzed using SDS-PAGE with comparison to the control frozen at -80ºC, at time zero.

4. Results

4.1 Production of Anti-TNF alpha mAb

4.1.1 Anti-TNF alpha mAb Cell Culture Overview

The cell line used to produce the Anti-TNF alpha mAb was a mammalian NS0 murine hybridoma cell line specifically with reactivity against human anti-TNF-alpha. This strongly neutralizing antibody is directed against both natural and recombinant human TNF alpha. The produced antibody has an IgG1 isotype.

The vendor recommended culture media was RPMI 1640, 2mM glutamine, 10% FBS. However, the standard cell culture expansion steps were carried out using RPMI 1640 1640 culture medium contains 2mM of Glutamine, 10% (v/v) of FBS, 1% (v/v) of HEPES and 1% (v/v) of penicillin streptomycin. Cell culture expansion was carried out in T-flasks of different size from T-25 to T-175 specially designed for suspension cells and was in an environment of 5% CO2 and 37ºC. Cells were viable and 100% proliferated after 2 days of each step in the expansion.

For antibody production the cells were then transferred into T-175 flasks or conical flasks containing a standard production basal media or variations of this media composition. A standard production feed media was also supplied at regular intervals, glucose concentration was monitored for later batches.

A summary of the cell culture experiments performed is detailed in **Table 6** describing the initial cell culture and establishment of a working cell bank through to yield optimization experiments focusing on different media and bioreactor strategies

Table 6 Anti-TNF Alpha mAb batch descriptions

Batch	Purpose	Vial Thaw or Split	Expansion Scale	Production Media	Production Scale	Analysis Performed
	Establishment of working cell bank	MCB Vial Thaw	N/A	N/A	N/A	N/A
1	Initial cell culture to generate baseline	Split from MCB vial	**600ml** 4 x 150ml in T175 Flasks 10 days	Standard production basal media Standard production feed media *No Glucose monitoring*	**600ml** 4 x 150ml in T175 Flasks 10 days	Nanodrop Potency assay ELISA
2	Assess cell density increase impact on yield	WCB Vial Thaw	**600ml** 4 x 150ml in T175 Flasks 15 days	Standard production basal media Standard production feed media *Glucose monitoring*	**750ml** 4 x 187.5ml in T175 Flasks 10 days	Nanodrop Potency assay SDS-PAGE
3	Production Media optimization study for higher yield	WCB Vial Thaw	**600ml** 4 x 150ml in T175 Flasks 15 days	8 Production basal media assessed Standard production feed Media** *Glucose monitoring*	**800ml** 8x 100ml in T175 Flask for each study	N/A*
4	Production Media optimization study for higher yield	Split from Batch 3	**600ml** 4 x 150ml in T175 Flasks 15 days	8 Production basal media assessed Standard production feed Media** *Glucose monitoring*	**800ml** 8x 100ml in T175 Flask for each study	Nanodrop
5	Bioreactor optimization for higher yield	WCB Vial Thaw	**600ml** 4 x 150ml in T175 Flasks 15 days	Standard production basal media Standard production feed media *Glucose monitoring*	**600ml** 4 x 150ml in conical Flasks 10 days	Nanodrop

**Microbial contamination due to extended hold times resulted in batch being disposed.*

***For certain media compositions, glucose was removed from standard production feed media when present above 5mMol*

4.1.2 Generation of working cell bank

A WCB was generated from a MCB vial purchased from Merck. Cells previously frozen in DMSO, shipped on dry-ice were thawed, centrifuged, and resuspended in resuscitation media of RPMI 1640, 20% FBS, HEPES buffer and penicillin/streptomycin and cells were cultured for 2 days at 37°C and 5% CO2 before being expanded in a T-75 flask containing the standard expansion media. The FBS concentration in media was then reduced to 10% for subsequent passages, as cell viability has increased after initial expansion. This cell culture continued to be split until the 7th passage when high cell viability was established and then the cell working bank was generated. Cells were frozen down under two conditions (1) with 10% DMSO and 90% FBS (2) with 10% DMSO and 15% FBS and 75% RPMI-1640 the above media with FBS at a concentration of 10%. There were 15 vials of each stock created during this activity. Each vial contained 700µl of cells at a concentration of 2.5x10^6 and was banked in liquid nitrogen tank for long term storage. All batches manufactured as part of this thesis were produced from this working cell bank.

4.1.3 Batch 1

Production of Hybridoma monoclonal antibody

Cell Culture

During the development of the working cell bank, a split was created at 7th passage (T75 flask) that was further expanded to a final scale of 600ml contained in four T-175 flasks designed for suspension cell culture as per F**igure 16**. The expansion step took 10 days to complete once split was created.

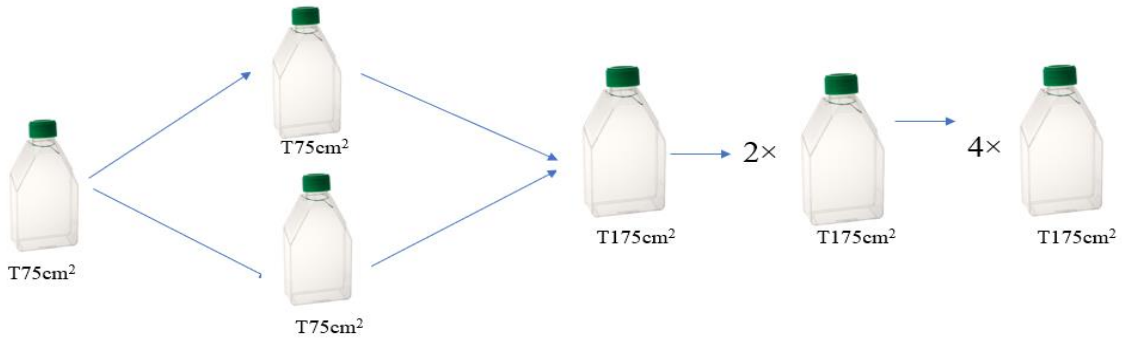


Figure 16. Cell Culture Expansion Process

For the production, the media was then changed to standard production basal media, with FBS concentration reduced to 1% (v/v) to minimize the animal component impurities in the final product. The production step consisted of a 10-day culture period without glucose analysis, however cells were supplemented with standard production feed media (glucose, amino acids, glutamine and HEPES). Glutamine was added as the amino acid media purchased did not include glutamine.

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 550ml of conditioned media was purified using FPLC with a 1ml protein G column, with a 20mg binding capacity. A flow rate of 1ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was measured via in-line protein concentration analysis via UV analysis at 280nm on purification system, with successive 1ml elution fractions captured and a total of 15 fractions collected.

The in-process data from purification process and specifically the elution step is presented in F**igure 17**. The elution step consists of the introduction of Buffer A (Phosphate buffer pH 7.0) to elute unbound proteins to waste, a concurrent drop off in conductivity and UV is observed

from the high initial conductivity and UV of the conditioned media containing antibody. After 15 minutes, Buffer B (0.1M Glycine HCL) is introduced to elute bound proteins and a large peak is observed via UV for fractions 5 & 6 demonstrating antibody elution. After antibody elution, the protein concentration becomes negligible, and conductivity increases as only buffer is present. The purification then ends with the opening of waste valve and the column is equilibrated with buffer B followed by a final equilibration with buffer A.

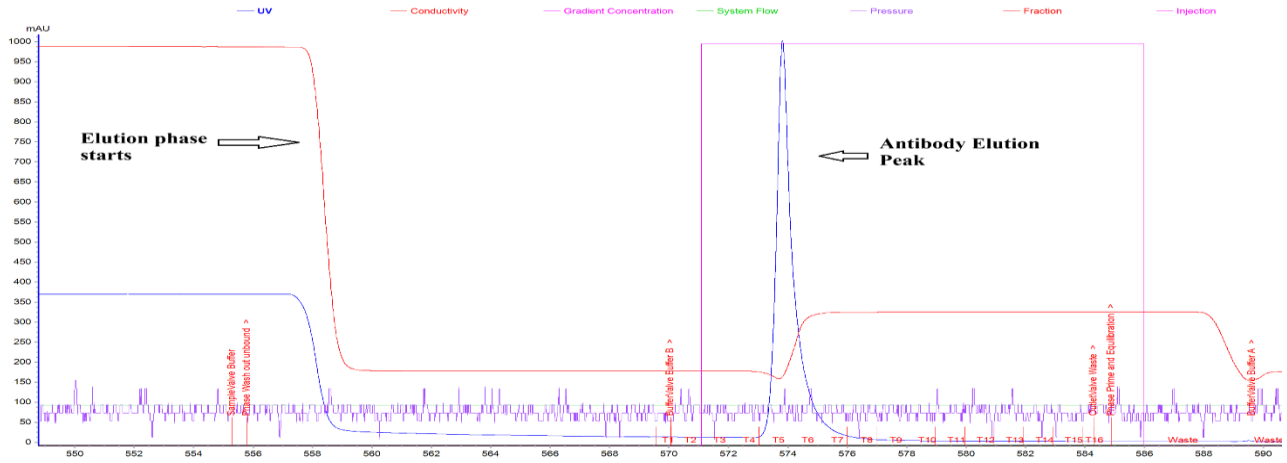


Figure 17. Online Attribute analysis from FPLC of Batch 1 demonstrating elution phase, and reporting UV (Blue) conductivity (Red), Gradient concentration (Pink), Pressure (Violet), Fraction number (T1-T15)

A total of 15 fractions were collected which contained monoclonal antibodies in 1ml of elution buffer (Buffer B) and 200µl of neutralization buffer (Buffer C). The monoclonal antibody concentration of collected fractions was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions 4 and 5 as per F**igure 18**. These fractions were then pooled, and protein analysis conducted via nanodrop.

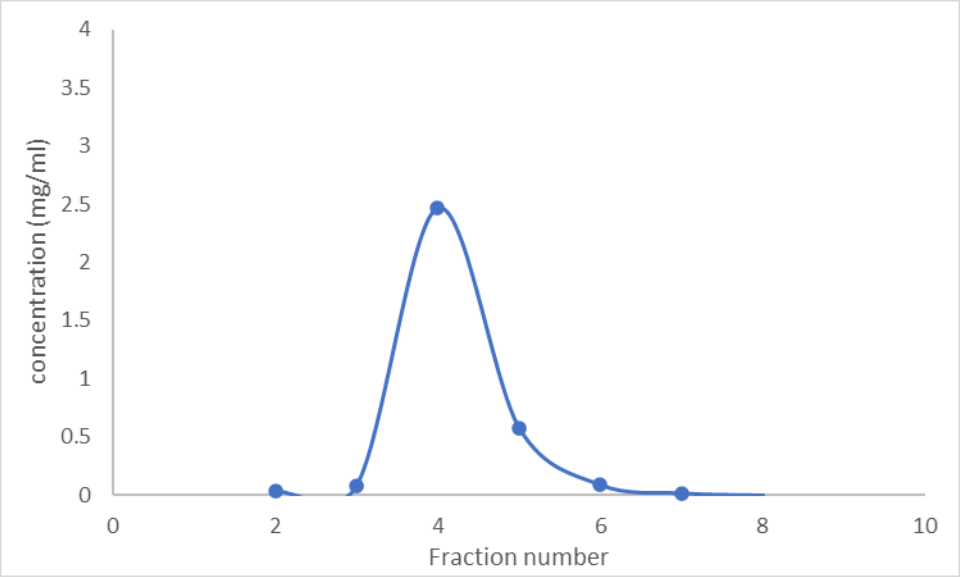


Figure 18. offline protein concentration analysis of Batch 1. 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 1ml column. 15 fractions were collected, only 8 fractions were analyzed for offline protein concentration by UV at 280nm

The purified solution was neutralized by buffer C with a pH of 9.0 and a protein concentration of 1.52mg/ml was measured with a volume of 2.4ml collected during elution with a total yield of 3.66mg of antibody. This corresponded to a titer of 0.007mg/ml in the bioreactor during production step. The full 600ml of conditioned media was not purified as purification of all the conditioned media would result in air entrapment in the column and backpressure in the system that could damage the column and result in protein degradation, impurities, and low yields.

Dialysis

Dialysis of the purified antibody solution was performed with a sucrose, histidine and polysorbate 80 based final formulation with a pH of 6.0. Dialysis was performed overnight with a low agitation speed using a stirrer bar and magnetic mixer. The final protein concentration post-dialysis was 1.18mg/ml with a final volume of 2.4ml recovered. This represents a loss of antibody during dialysis of 22.4%. This final formulated monoclonal antibody solution was then stored at 2-8°C until further usage.

Antibody Characterization:

Antibody Potency

Potency & Comparison to external antibody

A subsequent assay was performed successfully that measured SEAP levels in the presence of increasing antibody concentrations. A second potency assay was executed using commercially available antibody in place of antibody produced in batch 1 cell culture production. This second assay was performed to compare the potency of commercial antibody against internally made antibody and to verify assay performance. The combined data is presented below in **Figure 19**.

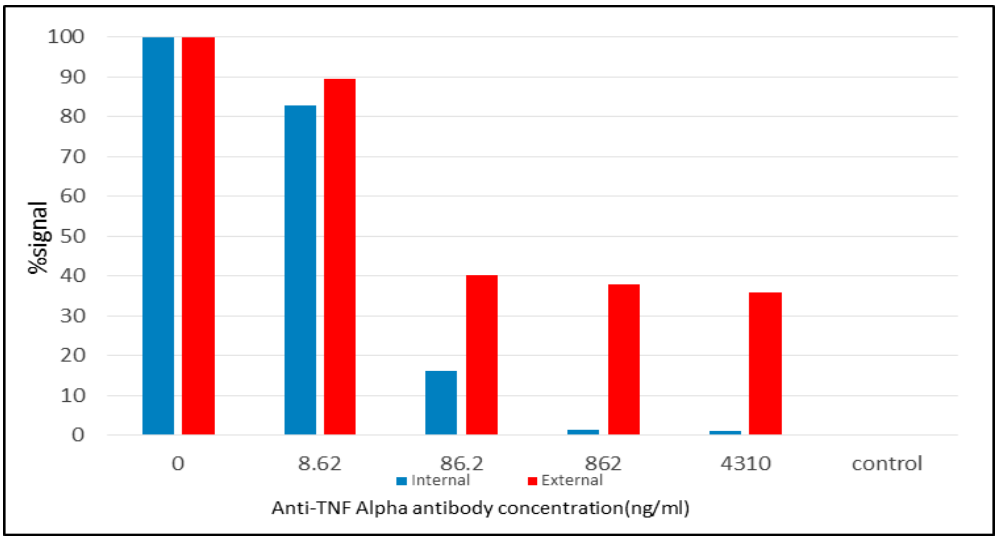


Figure 19. comparing internal (Blue)and external (Red) Anti-TNF alpha mAb potency, the internal mAb was produced in batch 1 while the external mAb was purchased from vendor, increasing amounts of anti-TNF alpha mAb were added with signal measured to determine mAb potency via HEK293 potency assay with SEAP readout as measured by UV at 490nm. A negative control was included that did not contain neither antibody nor antigen and is recorded as "control".

A positive control not containing anti-TNF alpha antibody (0 ng/ml) provided 100% signal with increasing antibody concentrations resulting in decreasing signal as expected. At a concentration of 86.2 ng/ml or a ratio of 5:1 antibody to antigen a large decrease in signal was

observed for both internal and external antibodies, with 100% potency observed for internal antibody at a concentration 862ng/ml. At a concentration of 86.2ng/ml, the external antibody did not show an increase in potency above 60% despite exponentially increasing antibody concentrations. Both assays had negative controls that did not contain antigen or antibody, this negative control did not produce a signal as expected demonstrating the expected functioning of this assay.

IC50 Determination

Analysis of the initial potency assay of internally produced anti-TNF alpha antibody indicated that the inhibitory concentration (IC) was in the region of a molar ratio of 1:1 and 1:30 antigen to antibody. A subsequent potency assay was conducted that determined IC50 of internal antibody. Antibody concentrations of 17.24 to 517.2ng/ml were tested (**Figure 20**) and the IC50 was determined to be 26.65ng/ml **(Figure 21).**

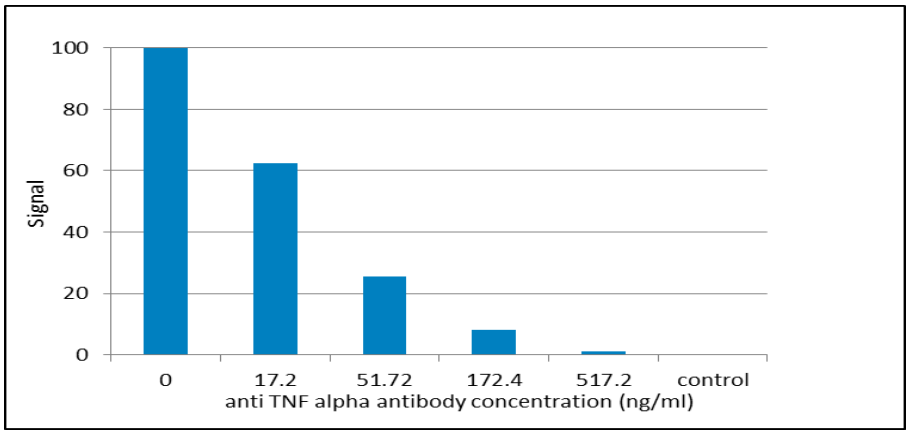


Figure 20. IC 50 Analysis of Anti-TNF alpha mAb. Increasing amounts of anti-TNF alpha mAb were added with signal measured to determine mAb IC50 via HEK293 potency assay with SEAP readout as measured by UV at 490nm. A negative control was included that did not contain neither antibody nor antigen and is recorded as "control".

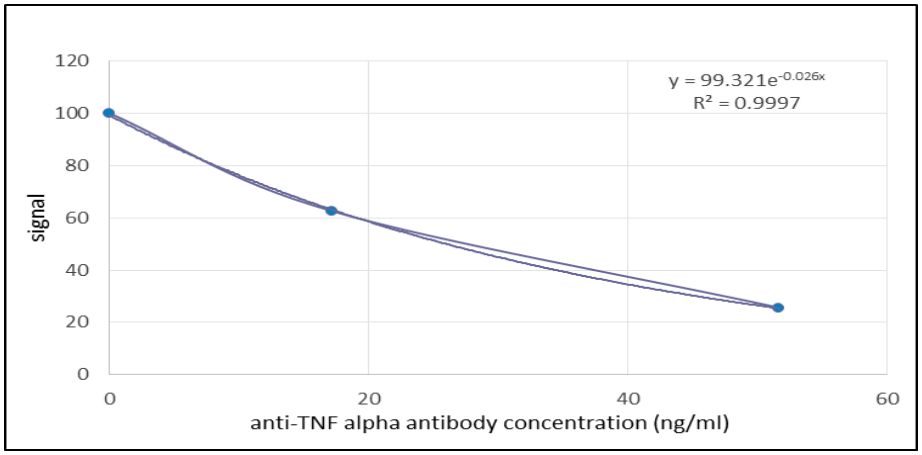


Figure 21. IC 50 Analysis of Anti-TNF alpha mAb. The IC 50 data was plotted with an exponential fit in addition to trendline being added.

ELISA Analysis

The binding efficacy of the antibody was also determined via ELISA and a comparison between internal and external antibodies performed. Antigen was added to wells at various concentrations between 0 to 4ug/ml and a standard concentration of anti-TNF Alpha mAb was added (0.6mg/ml for internal and 0.5mg/ml external mAb). While both antibodies demonstrated binding to antigen, the internal antibody demonstrated higher binding than external/commercial antibody, even allowing for differences in concentration **Figure 22**.

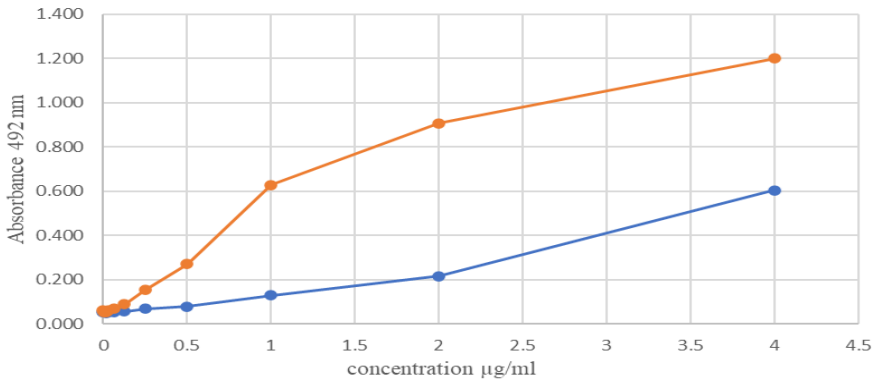


Figure 22. Internal (Red)and External (Blue)Anti-TNF alpha mAb binding efficacy analysis with ELISA The internal mAb was produced in batch 1 while the external mAb was purchased from vendor,

Impurity Analysis

Because of the presence of FBS in the media, the purified antibody was expected to contain some protein impurities in the form of antibodies. This impurity content was analyzed by purifying the expansion media only (RPMI 1640 containing 1% FBS) alone. For this the 550ml (same quantity of cell culture media) media was prepared by using 1% of FBS and kept for 10 days (same time for the cell culture production). The media was then purified using FPLC with a 1ml protein G column. A flow rate of 1ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was measured via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, with successive 1ml elution fractions captured and a total of 15 fractions collected.

The in-process data from purification process and specifically the elution step is presented in **Figure 23**. After 15 minutes of elution using buffer A, Buffer B (0.1M Glycine HCL) was introduced to elute bound proteins and a large peak is observed via UV for fractions 5 & 6 demonstrating antibody elution. After antibody elution, the protein concentration becomes negligible, and conductivity increases as only buffer is present.

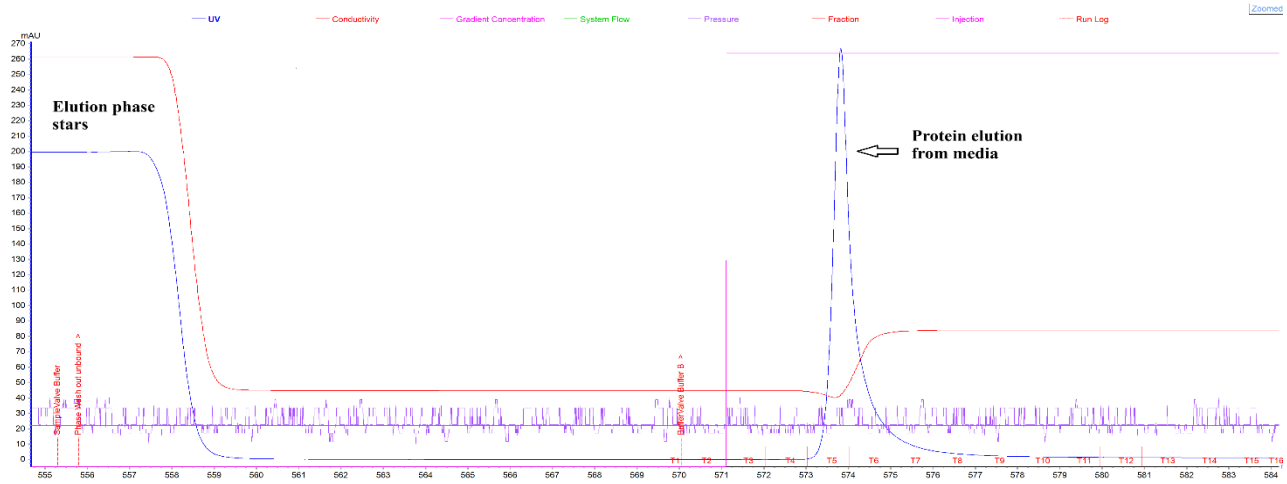


Figure 23. online Attribute analysis from FPLC demonstrating elution phase, and reporting UV (Blue) conductivity (Red), Gradient concentration (Pink), Pressure (Violet), Fraction number (T1-T15)

The Protein concentration of collected fractions was measured offline via nanodrop analysis and protein was observed in fractions 4 and 5 as per **Figure 24**. These fractions were then pooled, and protein analysis conducted via nanodrop

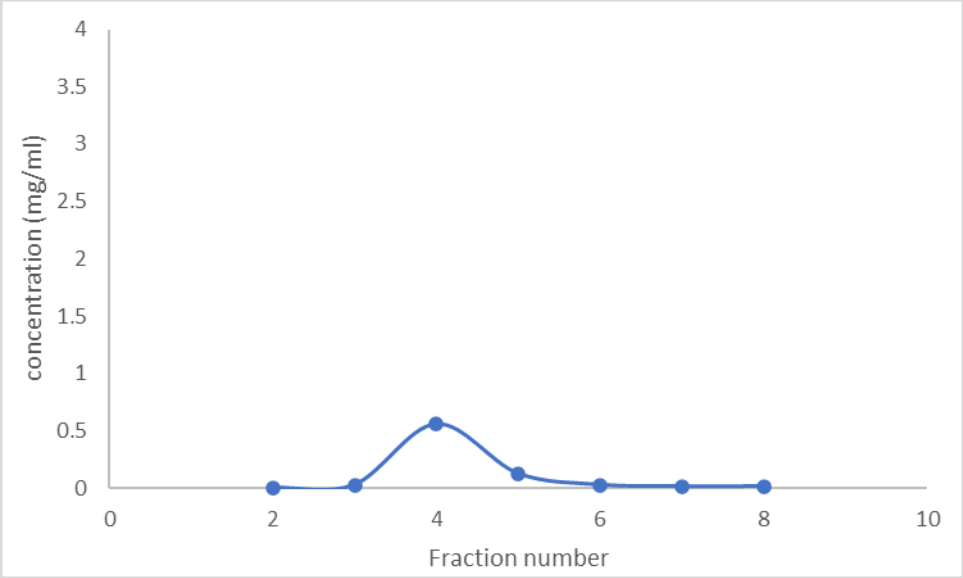


Figure 24. offline protein concentration analysis. 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 1ml column. 15 fractions were collected, only 8 fractions were analyzed for offline protein concentration by UV at 280nm

The purified solution was neutralized by a buffer of pH of 9.0 and protein concentration of 0.34mg/ml was measured with a volume of 2.4ml collected during elution with a total yield of 0.82mg of protein was found from the media in the presence of 1% FBS. Comparing this with antibody purified using cell culture (1.52mg/ml - 2.4ml total volume collected), the impurity percentage was found to be 22.5% and the pure anti TNF alpha percentage in the purified and dialyzed solution was found to be 77.5% and the final concentration of pure anti-TNF Alpha excluding the impurity after dialysis was found to be 0.9mg/ml

4.1.4 Batch 2

This batch was cultured using increased density of cells in larger volume in the expansion step with the aim of increasing yield.

Production of Hybridoma monoclonal antibody

Cell Culture

A sample vial was removed from the working cell bank previously generated in batch one and these cells previously frozen in DMSO were thawed, centrifuged, and resuspended in RPMI 1640, 20% FBS, 1%HEPES buffer and 1%Pen/Strep and cells cultured for 2 days at 37°C and 5% CO2 before being expanded into a T-75 flask containing the standard expansion media. This T-75 flask was initially expanded to a final scale of 4 T175 flasks as per Batch 1. In order to increase the cell density prior to production, cells were passaged again in T-175 flasks for three more days resulting in a total expansion time of 15 days (**Figure 25)**.

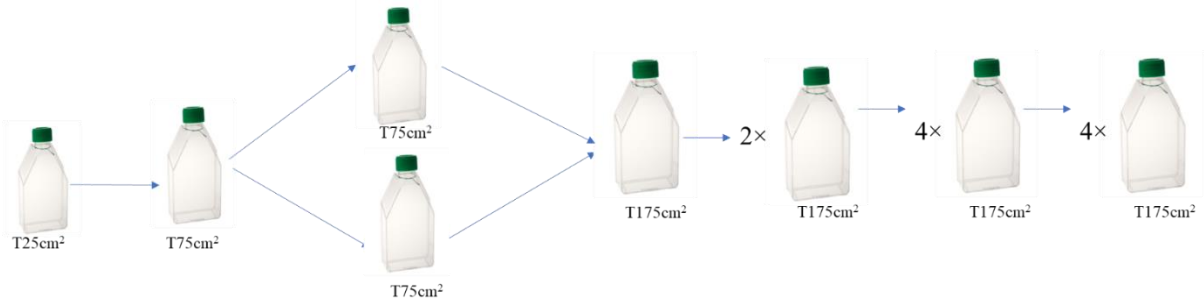


Figure 25. Cell Culture expansion process for Batch 2

Further for the production step, the standard production basal media volume was increased to 750ml to allow for increased cell media demand. The production step again consisted of a 10-day culture period with glucose analysis on alternating days, the cells were supplemented with standard production feed media.

Cell density was measured throughout the production phase and glucose concentrations were also measured (Table 7). Glucose was removed from production feed media on days 4 and 8 as glucose concentrations remained above 5mM **(Table 7)**.

Days	Cell Density (per ml)	Glucose Concentration
0		11mM
2	2.786 x10^6	0.9mM
4	3.130 x10^6	6.9mM
6	5.712 x10^6	1.8mM
8	6.365 x10^6	5.3mM

Table 7 Batch 2 Production Phase Cell Culture Cell Density and Glucose Concentration analysis during 10-day production. The 11mM value on Day 0 was not measured and is a calculated value.

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 700ml of conditioned media was purified using FPLC with a 1ml protein G column.

The monoclonal antibody concentration of collected fractions was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions 4 and 5 as per F**igure 26**. These fractions were then pooled, and protein analysis conducted via nanodrop.

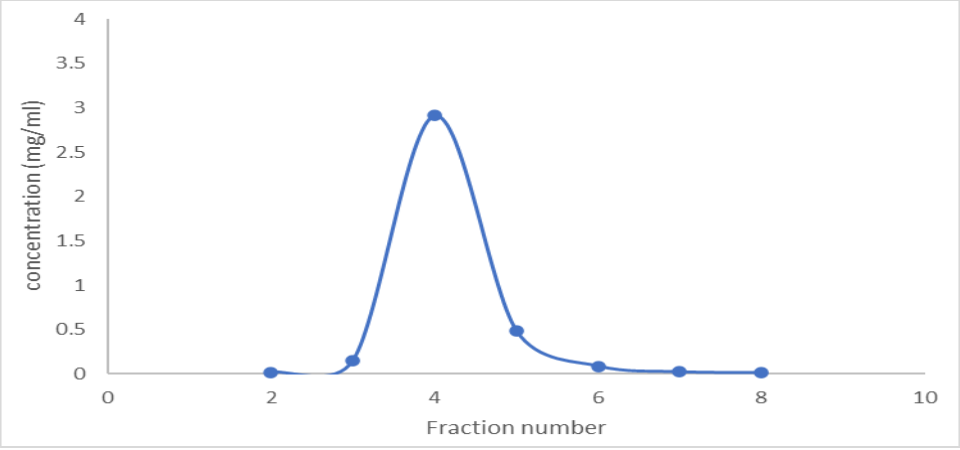


Figure 26. offline protein concentration analysis of Batch 2. 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 1ml column. 15 fractions were collected, only 8 fractions were analyzed for offline protein concentration by UV at 280nm

The purified solution was neutralized by a buffer of pH of 9.0 and protein concentration of 1.7mg/ml was measured with a volume of 2.4ml collected during elution with a total yield of 4.08mg of antibody. This corresponded to a titer of 0.006mg/ml in bioreactor during production step. The full 750ml of conditioned media was not purified as purification of all the conditioned media would result in air entrapment in the column and backpressure in the system that could damage the column and result in protein degradation, impurities, and low yields.

Dialysis

Dialysis of the purified antibody solution was performed with a sucrose, histidine and polysorbate 80 based final formulation with a pH of 6.0. Dialysis was performed overnight with a low agitation speed using a stirrer bar and magnetic mixer. The final protein concentration post-dialysis was 1.46mg/ml with a final volume of 2.4ml recovered. This represents a minor loss of antibody during dialysis of 14%. This final formulated monoclonal antibody solution was then stored at 2-8C until further usage.

Antibody Characterization:

Potency

The purified and formulated antibody potency was characterized using an alternative potency assay employing L929 reporter cells with a readout of ATP levels determining antibody potency.

The development of this assay was performed using purified and formulated antibody from batch 2 and antibody was found to demonstrate potency. The results are presented in **section 4.4**.

SDS-PAGE Analysis

The purified and formulated anti-TNF alpha mAb was analyzed using SDS-PAGE under reducing, denaturing conditions. Approximately 4µg per sample was added to each well/lane.

The analysis was performed in a 10 well gel and 4 replicates of the same sample were analyzed through it **(Figure 27).** The disulfide bonds in the antibody were reduced using β-mercaptoethanol generating two bands; 2 heavy chains with a band at ≥50 kDa and 2 light chains with a band at ≤25 kDa were observed in the gel. Some possible trimers or larger aggregates were analyzed from this gel with a band width of approximately between 250 kDa and 130 kDa.

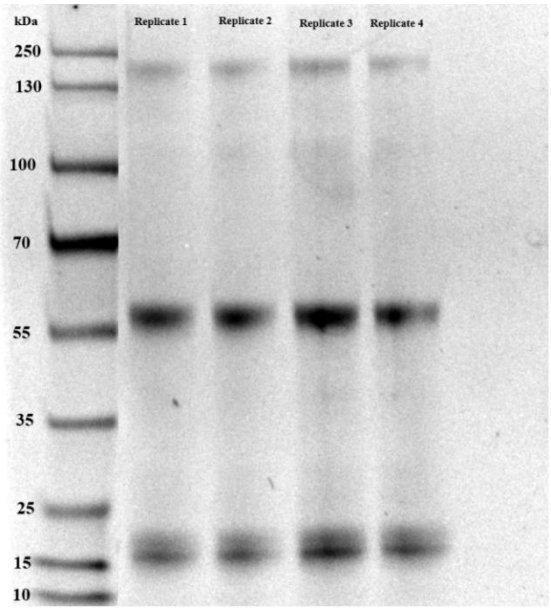


Figure 27. SDS-Page of Batch 2 formulated anti TNF alpha mAb. Replicates analyzed under reducing, denaturing conditions in lanes 2-5 and molecular weight marker ladder in lane 1.

4.1.5 Batch 3

This batch was performed to optimize culture media, using 7 alternative production basal media compositions to increase the total yield from the culture in comparison to standard production basal media.

Production of Hybridoma monoclonal antibody

Cell Culture

A sample vial was removed from the working cell bank generated in batch one, thawed, cultured and eventually expanded to a final scale of 600ml contained in four T-175 flasks as standard. The total expansion time was 15 days as per Batch 2 (**Figure.28)**.

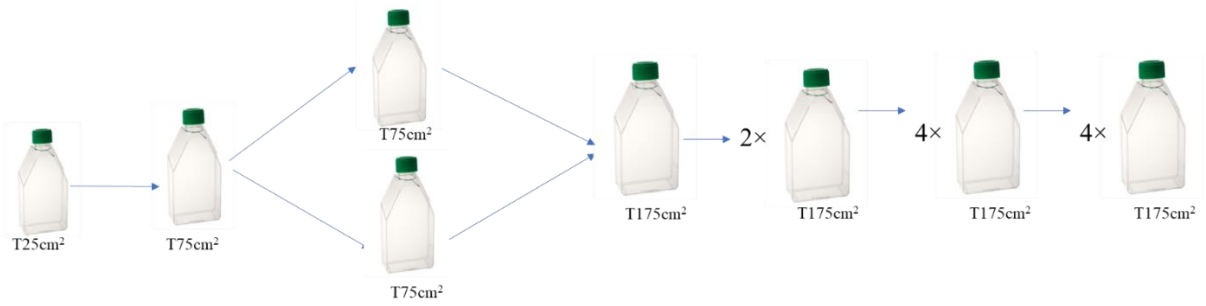


Figure 28. Cell Culture Expansion for Batch 3

Cell density was measured from 1xT175 flask to the end of the expansion phase **(Table 8)**

Expansion step	Cell Density (per ml)
1xT-175	4.310 x10^6
2xT-175	5.600 x10^6
4xT-175	8.880 x10^6
4xT-175	14.210 x10^6

Table 8 Batch 3 Expansion Phase Cell Culture Cell Density

After 3 days of final expansion step, cells were collected from the four T-175 flasks, combined into a single tube before being divided into 8 different tubes equally. Cells were then transferred

to 8 different T-175 flasks each containing a distinct basal production media. All media contained RPMI 1640 media, 1% HEPES buffer, 1% Pen/strep as constant.

The first flask contained the standard production basal media and served as a positive control; the second flask did not contain amino acids to understand the effect of amino acids in the media. The third flask contained standard production basal media with the initial addition of 33 mM of (NaCl) sodium chloride to increase osmotic pressure followed by a second addition of 37mM of NaCl after 6 hours to achieve a final osmolarity of 440 mOsm. The fourth flask contained the standard production basal media with an addition of sodium butyrate (1mM) as a source of short chained fatty acids (SCFA).

The fifth flask contained standard production basal media, without FBS, i.e. without animal derived components. The sixth flask was also deficient in FBS but was supplemented with an initial addition of 33 mM of NaCl to increase osmotic pressure followed by a second addition of 37mM of NaCl after 6 hours to achieve a final osmolarity of 440 mOsm. The 7^{th} flask was similar to flask four with the addition of sodium butyrate but without FBS. The 8^{th} flask employed a CHO cell culture media, Excell CD-CHO fusion media, in place of RPMI-1640. The 8^{th} flask also contained 1% HEPES, 1% Penicillin/Streptomycin and 2mM glutamine.

Each of these flasks contained a final volume of 100ml of media. The production step consisted of a 10-day culture period with glucose analysis on days 2,4, 6 and 8. Cells were supplemented with standard production feed media including glucose at days 2, 4, 6 & 8 with the exception of cell cultures with glucose levels above 5mM. For flask two, to ensure the absence of amino acids in the cell culture, amino acids were not part of feed media provided to the 2^{nd} Flask. A summary of the media compositions and their intended function and hypothesis are detailed in **Table 9**

Flask	Change To standard production media	Function/Hypothesis
1	No change	Positive control/Standard
2	-AA	To understand the effect of AA
3	+NaCl	To induce osmotic pressure
4	+ Sodium Butyrate	To increase titer
5	0% FBS	Reduce animal component
6	0% FBS + NaCl	Reduced animal component and to increase titer by inducing osmotic pressure
7	0% FBS + Sodium Butyrate	Reduced animal component and increased titer
8	Excell CD-CHO fusion media	Different media optimization

Table 9 Batch 3 Cell Culture Media Compositions

Cell density was measured on the 4th and 6th days of the production phase **(Table 10)**

	Cell Density (per ml)	
Day	4	6
Flask 1	2.650 x10^6	3.140 x10^6
Flask 2	2.340 x10^6	2.890 x10^6
Flask 3	1.870 x10^6	2.100 x10^6
Flask 4	1.410 x10^6	1.980 x10^6
Flask 5	0.921 x10^6	0.987 x10^6
Flask 6	0.210 x10^6	0.225 x10^6
Flask 7	0.304 x10^6	0.367 x10^6
Flask 8	2.530 x10^6	3.650 x10^6

Table 10 Batch 3 Production Phase Cell Culture Cell Density during 4th and 6th days of 10-day production.

Glucose concentrations were also measured throughout the production phase **(Table 11)**. Glucose was removed from the production feed media on the 4th and 8th day of the first three flasks as glucose concentrations remained above 5mM. On the 4th flask after 6th day the cell density was found to be very low hence the glucose was removed from the feed media. On the 5th flask after 6th day the glucose was found to be very low and was added on 6th day. On the 6th flask, the glucose was not added as the cell density and concentration was average; similarly, on the 7th flask the glucose was removed from the feed media as it was high. The initial glucose concentration of the media in the 8th flask was 30mM and the glucose was added when the measured glucose concentration was below 20mM.

	Glucose concentration (mM)				
Day	0	2	4	6	8
Flask 1	11	0.8	5.4	1.1	4.8
Flask 2	11	1.2	6.3	1.7	6.7
Flask 3	11	1.8	7.8	2.1	6.9
Flask 4	11	7.1	5.4	3.9	2.2
Flask 5	11	6.7	3.6	1.8	7.8
Flask 6	11	6.2	5.6	3.7	1.9
Flask 7	11	8.8	8	7.1	6.4
Flask 8	30	19.2	19	18.8	19.3

Table 11 Batch 3 Production Phase Glucose Concentration analysis during 10-day production. The 11mM value on Day 0 was not measured and is a calculated value

After harvest and centrifugation, the supernatant was filtered and then stored at 4ºC. At this time, an equipment fault with AKTA Purification system was detected with a faulty valve resulting in leakage and loss of pressure. This required harvested media to be stored for over one month due to a delay in repair. The stored medias were then discarded due to contamination in the media.

4.1.6 Batch 4

This batch was performed to repeat batch 3 which was discarded due to contamination

Production of Hybridoma monoclonal antibody

Cell Culture

The cell culture for batch 4 was performed identically as per batch 3, including expansion, production and media compositions. The sole difference was that split from batch 3 was used to expand batch 4.

Cell density was measured from 1xT175 flask to the end of the expansion phase **(Table 12)**

Expansion step	Cell Density (per ml)
1xT-175	3.398 x10^6
2xT-175	5.688 x10^6
4xT-175	9.976 x10^6
4xT-175	15.630 x10^6

Table 12 Batch 4 Expansion Phase Cell Culture Cell Density

Similarly, the cell density was measured on the 4th and 6th day of the production phase **(Table 13)**

	Cell density (per ml)	
Days	4	6
Flask 1	2.580 x10^6	3.210 x10^6
Flask 2	2.555 x10^6	3.100 x10^6
Flask 3	1.705 x10^6	1.750 x10^6
Flask 4	1.445 x10^6	1.557 x10^6
Flask 5	0.970 x10^6	1.101 x10^6
Flask 6	0.301 x10^6	0.328 x10^6
Flask 7	0.402 x10^6	0.431 x10^6
Flask 8	2.980 x10^6	4.100 x10^6

Table 13 Batch 4 Production Phase Cell Culture Cell Density during 4th and 6th days of 10-day production.

Glucose concentrations were also measured throughout the production phase **(Table 14)**. Glucose was removed from the production feed media on the 4th and 8th day of the first three

flasks as glucose concentrations remained above 5mM. On the 4th flask after 6th day the cell density was found out to be very low hence the glucose was removed from the feed media. On the 5th flask after 6th day the glucose was found to be very low and was added on 6th day. On the 6th flask, the glucose was not added as the cell density and concentration was average; similarly, on the 7th flask the glucose was removed from the feed media as it was high. The initial concentration of the media in the 8th flask was 30mM and the glucose was added when the measured glucose concentration was below 20mM.

	Glucose concentration (mM)				
Day	0	2	4	6	8
Flask 1	11	0.9	4.9	0.7	5.6
Flask 2	11	1.4	6.7	1.9	5.8
Flask 3	11	1.7	6.9	3.1	7.1
Flask 4	11	7.6	6.1	5.5	2.6
Flask 5	11	5.1	3.9	2.1	7.9
Flask 6	11	6.4	5.2	3.4	1.8
Flask 7	11	8.4	7.6	6.1	5.5
Flask 8	30	18.7	19.1	19.2	19

Table 14 Batch 4 Production Phase Glucose Concentration analysis during 10-day production. The 11mM value on Day 0 was not measured and is a calculated value

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 90ml from each of eight different conditioned medias was purified using FPLC with a 1ml protein G column. Antibody elution of all eight conditions was observed via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, with successive 1ml elution fractions captured and a total of 15 fractions collected.

The monoclonal antibody concentration of collected fractions was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions 4 and 5 as per F**igure 29**.

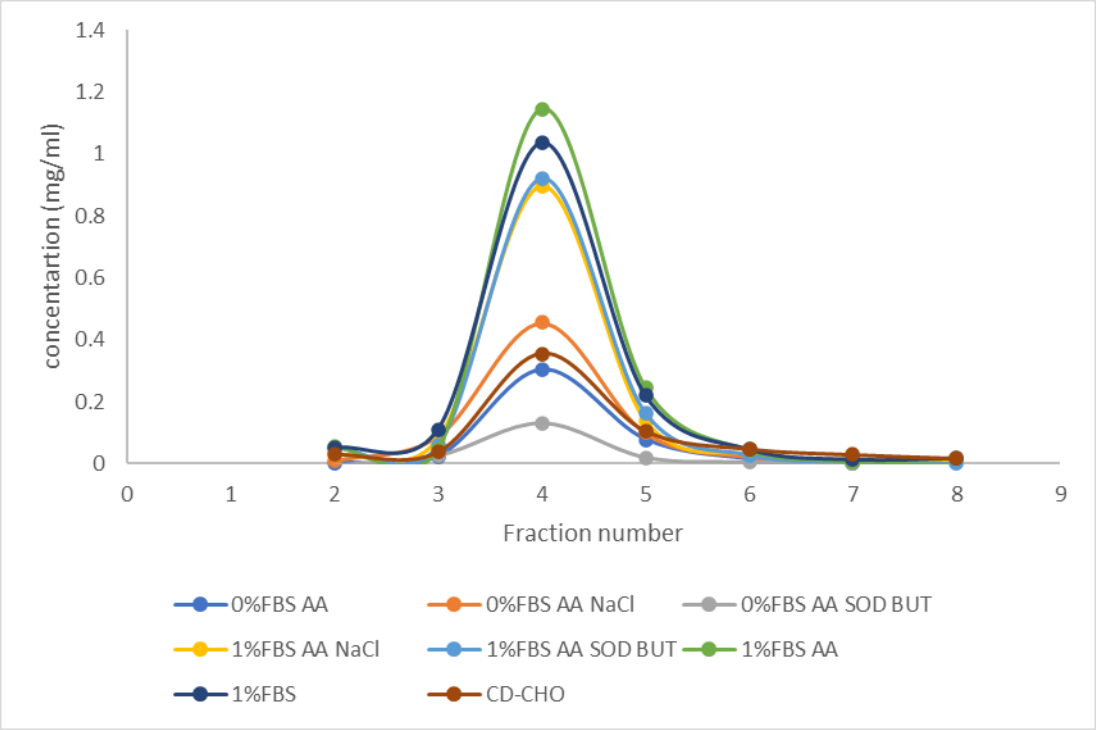


Figure 29. offline protein concentration analysis of Batch 4 with 8 different media optimization studies 1.2 ml aliquots or fractions were collected during purification for each study when eluting mAb from 1ml column. 15 fractions were collected, only 8 fractions were analyzed for offline protein concentration by UV at 280nm

The full 100ml of conditioned media was not purified as purification of all the conditioned media would result in air entrapment in the column and backpressure in the system that could damage the column and result in protein degradation, impurities, and low yields.

4.1.7 Batch 5

This batch was cultured using increased density of cells in and the production step was carried out in 500ml conical cell culture shake flask with vented caps to optimize the bioreactor used for the production.

Production of Hybridoma monoclonal antibody

Cell Culture

A sample vial was removed from the working cell bank which generated in batch one and these cells previously frozen in DMSO were thawed, centrifuged, and resuspended in RPMI 1640, 20% FBS, HEPES buffer and Pen/Strep and cells cultured for 2 days at 37C and 5% CO2 before being expanded into a T-75 flask containing the above media with exception of a 10% FBS solution in place of 20% as cell viability has increased after initial expansion. This T-75 flask was further expanded into two T-75 flasks to increase the cell density, and this was further expanded to a final scale of 600ml contained in 4 T-175 green cap (suspension) flasks. In order to increase the cell density furthermore, the media in these 4 T-175 flasks were changed two times in 4 days with an interval of two days as per **Figure 30**. The expansion time was 15 days.

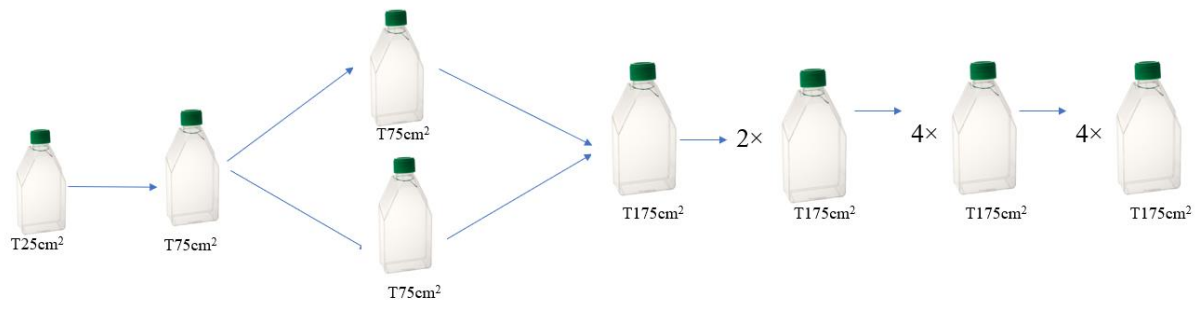


Figure 30. Cell Culture Expansion process of Batch 5

For the production step the media was then changed to above media with exception of a 1% FBS solution in place of 10% in 4 500ml conical cell culture shake flask with vented caps for

oxygen exchange with 150ml in each flask. The production step consisted of a 10-day culture period with glucose analysis on alternating days, the cells were supplemented with a standard production feed media.

Cell density was measured throughout the production phase and glucose concentrations were also measured **(Table 15)**. Glucose was removed from production feed media on days 4 and 8 as glucose concentrations remained above 5mM.

Days	Cell Density (per ml)	Glucose Concentration
0		11mM
2	2.693 x10^6	2.3mM
4	3.103 x10^6	7.9mM
6	3.427 x10^6	2.1mM
8	3.718 x10^6	6.3mM

Table 15 Batch 5 Production Phase Cell Culture Cell Density and Glucose Concentration analysis during 10-day production. The 11mM value on Day 0 was not measured and is a calculated value.

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 550ml of conditioned media was purified using FPLC with a 1ml protein G column. A flow rate of 1ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was observed via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, with successive 1ml elution fractions captured and a total of 15 fractions collected.

The monoclonal antibody concentration of collected fractions was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions 3,4,5 and 6 as per F**igure 31**. These fractions were then pooled, and protein analysis conducted via nanodrop.

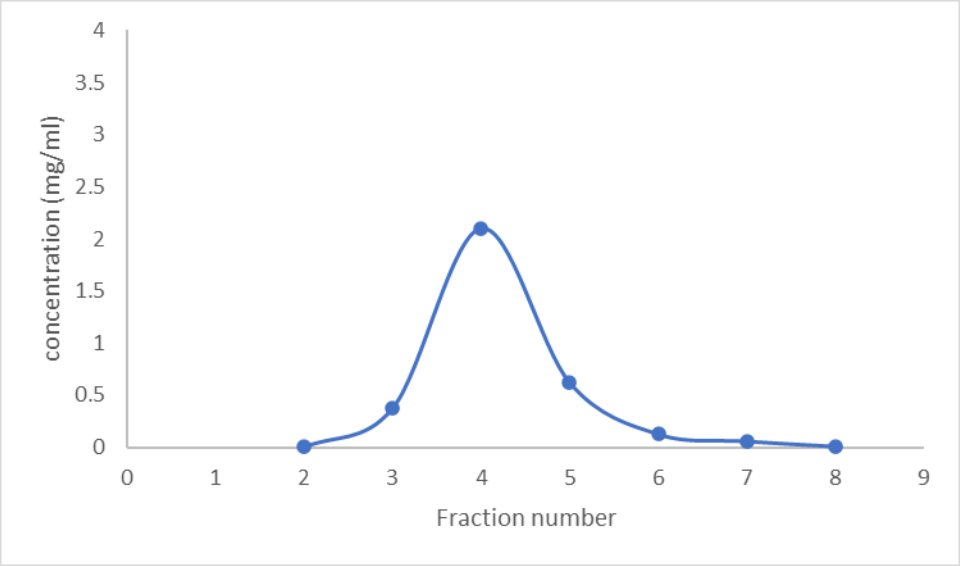


Figure 31. offline protein concentration analysis of Batch 5 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 1ml column. 15 fractions were collected, only 8 fractions were analyzed for offline protein concentration by UV at 280nm

The purified solution was neutralized by a buffer of pH of 9.0 and protein concentration of 0.8mg/ml was measured with a volume of 4.8ml collected during elution with a total yield of 3.84mg of antibody. This corresponded to a titer of 0.007mg/ml in bioreactor during production step. The full 600ml of conditioned media was not purified as purification of all the conditioned media would result in air entrapment in the column and backpressure in the system that could damage the column and result in protein degradation, impurities, and low yields.

Dialysis

Dialysis of the purified antibody solution was performed with a sucrose, histidine and polysorbate 80 based final formulation with a pH of 6.0. Dialysis was performed overnight with a low agitation speed using a stirrer bar and magnetic mixer. The final protein concentration post-dialysis was 0.72mg/ml with a final volume of 4.8ml recovered. This represents a minor loss of antibody during dialysis of 10%. This final formulated monoclonal antibody solution was then stored at 2-8ºC until further usage.

4.1.8 Anti-TNF Alpha mAb production Summary

Batch	Concentration (mg/ml) including impurity	Concentration (mg/ml) excluding impurity*	Yield (mg) **	Titer (mg/ml media in production)	% Loss after dialysis	Final Concentration (mg/ml) excluding impurities
1	1.520	1.18	3.66	0.007	22.4	0.9
2	1.700	1.25	4.08	0.006	14	1.1
3	N/A	N/A	N/A	N/A	N/A	N/A
4-1	0.695	0.67	1.67	0.02	N/A	N/A
4-2	0.628	0.60	1.51	0.015	N/A	N/A
4-3	0.519	0.50	1.25	0.012	N/A	N/A
4-4	0.543	0.52	1.30	0.013	N/A	N/A
4-5	0.191	0.191	0.46	0.005	N/A	N/A
4-6	0.277	0.277	0.66	0.007	N/A	N/A
4-7	0.131	0.131	0.16	0.002	N/A	N/A
4-8	0.230	0.230	0.55	0.006	N/A	N/A
5	0.800	0.62	3.84	0.007	10	0.56

Table 16 Hybridoma cell culture titers. *The impurities (IgG from FBS) were analyzed during batch 1 using 550 ml of media with FBS then this value was extrapolated with all other volumes
**Total volume purified was 2,4ml on all batches other than batch 5(4.8ml)

4.2 CHO Cell Culture cNISTmAb

The cell line employed was a CHO K1 subtype lineage which produced an IgG1 antibody hereafter called cNISTmAb. This cell line has been made publicly available by the National Institute of Standards & Technology (NIST) in collaboration with Merck for non-commercial research. The cell culture expansion was carried out in nutrient mixture HAM-F12 media that contained 10% of Fetal Bovine serum (FBS) v/v, 1% of HEPES v/v and 1% of penicillin streptomycin v/v for all batches. Cells were expanded initially as adherent cells. Cell culture expansion was carried out in T flasks of different sizes from T-25 to T-75 specially designed for adherent cells as per **Figure 32**, in an environment of 5% CO_2 and 37ºC. Cells viability was confirmed, and cell cultures had completely proliferated after 2 days of each step in the expansion process.

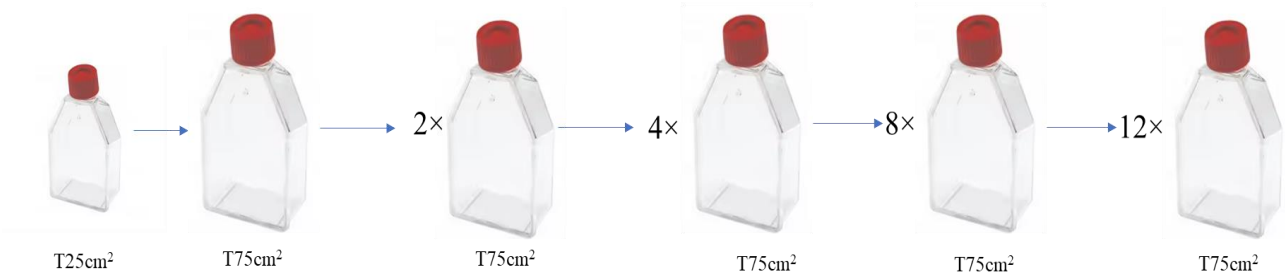


Figure 32. cNISTmAb Cell culture expansion process (Batches 2&3)

For antibody production, cells were then transferred into 4 different 500ml conical flask contain a basal media of EX-CELL CD CHO fusion media containing 1% of Glutamine v/v, 1% of HEPES v/v and 1% of penicillin streptomycin v/v. Cells were cultured as suspension cells in an environment of 5% CO2 and 37ºC.

Table 17. cNISTmAb Batches

Batch	Purpose	Vial Thaw or Split	Expansion Scale	Production Media	Production Scale	Analysis Performed
	Initial cell culture and starting a working cell bank	MCB Vial Thaw	N/A	N/A	N/A	
1	Antibody production using standard media	Split from MCB	**600ml** 4 x 150ml in Conical Flasks **18 days expansion**	Standard Basal media Standard Feed Media *No glucose analysis*	**1000ml** - 4 x 125ml in 0.5L conical flasks – 2 x 250ml in 1L conical flasks **5 Days production**	Nanodrop
2	Antibody production using standard media	Split from Batch 1	**360ml** 12 x T75 Flasks **12 days expansion**	Standard Basal media Standard Feed Media *Glucose Analysis*	**600ml** 4 x 150ml in 0.5L conical flasks **10 Days production**	Nanodrop
3	Study with two different cell culture system for higher yield	Split from Batch 2	**360ml** 12 x T75 Flasks **12 days expansion**	Standard Basal media Standard Feed Media *No glucose analysis*	**160ml** 1x 0.5L Conical flask **10 Days production**	Nanodrop
				Standard Expansion Media (w/o FBS) Standard Feed Media *No glucose analysis*	**160ml** Media 4x 40ml in T75 flask **10 Days production**	

4.2.1 Generation of working cell bank

NIST CHO cell line working cell bank was generated from master cell bank vial supplied by National Institute of Standards & Technology (NIST). Cells previously frozen in DMSO were thawed, centrifuged, and resuspended in Nutrient mixture HAM-F12 media, 10% FBS, 1% HEPES buffer and 1% Pen/Strep and cells cultured for 2 days at 37C and 5% CO2 before being expanded into a T-75 flask containing the above media. This cell culture continued to be split until passage 7 until high cell viability was established and then cell working bank was generated. Cells were frozen down with 10% DMSO and the above media with FBS at a concentration of 10%. There were 15 vials created during this activity. Each vial contained 700µl of cells at a concentration of 2.5x10^6 with 10% DMSO and 15% FBS and 75% the above media with FBS at a concentration of 10% and was banked in liquid nitrogen tank for long term storage. All batches manufactured as part of this thesis were produced from this working cell bank

4.2.2 Batch 1

Cell Culture

A split was created during the generation of working cell bank, from 7th passage, and that was further expanded as adherent cells in Nutrient mixture HAM-F12 media containing 10% FBS, 1% HEPES buffer and 1% Pen/Strep to a scale of 3 T75 flasks. This media is hereafter referred to as standard expansion media. This was followed by expansion into a single 500ml conical flask and grown as suspension cell culture with standard expansion media to a final scale of 600ml contained in four 500ml conical cell culture shake flasks with vented caps for oxygen exchange with 150ml cell culture suspension in each flask as per **Figure 33**. The expansion cell culture was 18 days in total, it was anticipated that each expansion step would be 2 days in duration, however the expansion steps in conical flasks demonstrated low initial density and each expansion step was extended to 4 days.

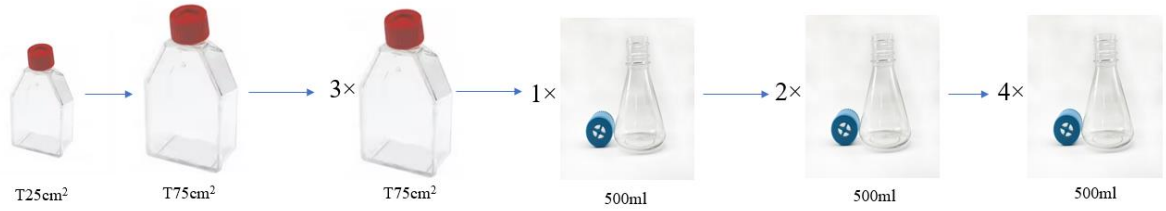


Figure 33. Batch 1 cNISTmAb Cell culture Expansion

Further for the production step the media was then changed to Excell CD-CHO Fusion media containing 1% of HEPES buffer, Pen/Strep and Glutamine to a final scale of 1000ml contained in four 500ml conical cell culture shake flask with vented caps for oxygen exchange with 125ml in each flask and two 1000ml conical cell culture shake flask with vented caps with 250ml in each flask. This media composition is hereafter referred to as standard production basal media. The production step consisted of a 5-day culture period without glucose or cell density analysis, however cells were supplemented with glucose on days 2 and 4.

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 950ml of conditioned media was purified using FPLC with a 5ml protein G column, with a capacity of 100 mg of IgG. A flow rate of 2ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was measured via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, with successive 1ml elution fractions captured and a total of 30 fractions collected.

The monoclonal antibody concentration of collected fractions was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions from 7 to 16 as per **Figure 34**. The purified solution was neutralized by a buffer of pH of 9.0 (buffer C).

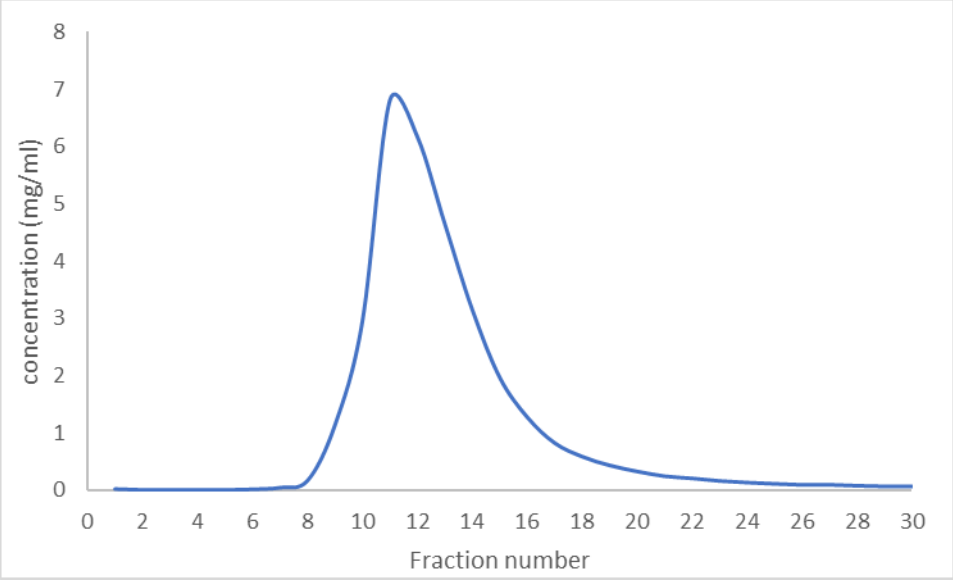


Figure 34. offline protein concentration analysis of Batch 1 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 5ml column. 30 fractions were collected and analyzed for offline protein concentration by UV at 280nm

On storage of individual aliquots overnight, protein precipitation was observed in the purification buffer. These aliquots were kept at 4°C and pooled with Batch 2 after being individually centrifuged to remove precipitates.

4.2.3 Batch 2

Production of NIST monoclonal antibody

Cell Culture

A split was created during Batch 1 (3 x T75 flask step) and that was further expanded as adherent cells in the standard expansion media to a final scale of 12 T-75 flasks (**Figure 35**). The expansion step took 12 days in total.

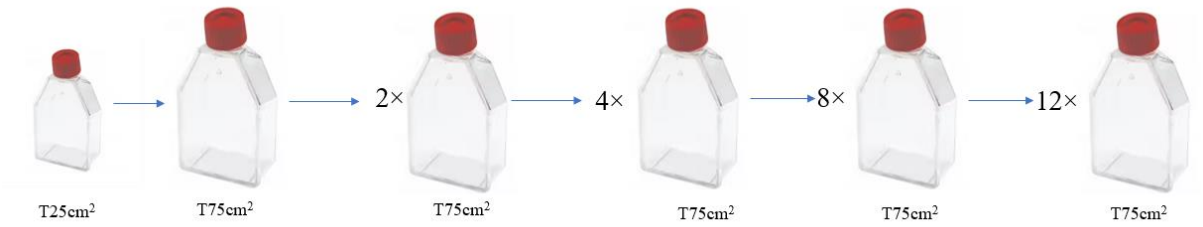


Figure 35. Batch 2 cNISTmAb Cell culture Expansion

Further for the production step the media was then changed to standard production basal media and cells were transferred to a final scale of 600ml contained in four 500ml conical cell culture shake flask with vented caps for oxygen exchange with 150ml in each. The production step consisted of a 10-day culture period with glucose and cell density analysis **(Table 18)**, the cells were supplemented with glucose on alternating days e.g. days 2, 4, 6 and 8. The starting glucose concentration for standard production basal media was 30 mM and 10mM glucose was added when concentration values of less than 20mM were observed.

Days	Cell Density (per ml)	Glucose Concentration
0		30mM
2	3.195 x10^6	16.7mM
4	4.126 x10^6	19.2mM
6	6.986 x10^6	17.3mM
8	9.518 x10^6	15.2mM

Table 18. Batch 2 Production Phase Cell Culture Cell Density and Glucose Concentration analysis during 10-day production. The 30mM value on Day 0 was not measured and is a calculated value.

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 550ml of conditioned media was purified using FPLC with a 5ml protein G column. A flow rate of 2ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was measured via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, Due to a faulty valve within the AKTA purification system, there was a leakage which resulted in an elution of only 400µl of antibody solution in each Eppendorf tube with a total of 30 tubes collected. The expected elution volume per Eppendorf tube was anticipated to be 1000µl, indicating a 60% loss in antibody recovery.

The monoclonal antibody concentration of collected fractions was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions from 9 to 20 as per **Figure 36**. The purified solution was neutralized by a buffer of pH of 9.0.

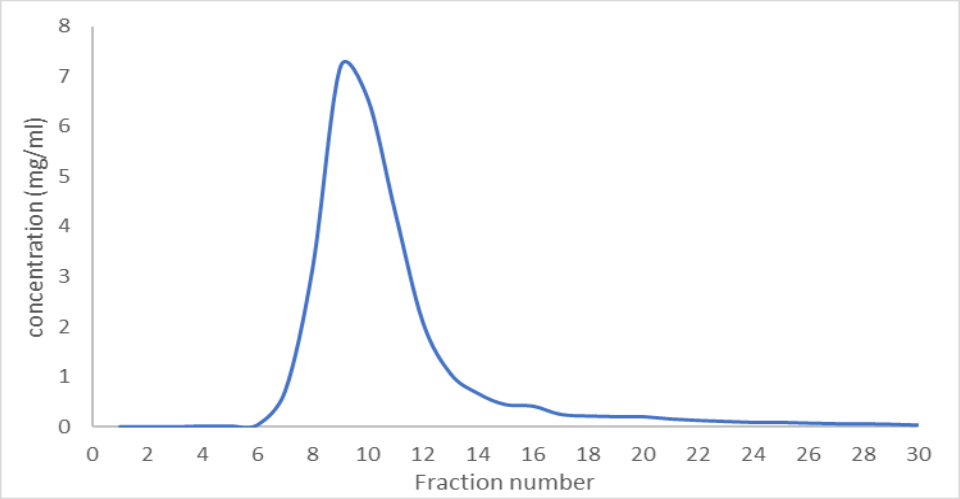


Figure 36. Offline protein concentration analysis of Batch 2 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 5ml column. 30 fractions were collected and analyzed for offline protein concentration by UV at 280nm

Antibody containing fractions from both batch 1 &2 were pooled and protein concentration of 3.9mg/ml was measured with a volume of 18ml collected during elution with a total yield of 70.2mg of antibody. This corresponded to a titer of 0.05mg/ml in the bioreactor during the production step.

Tangential flow filtration (TFF)

Ultrafiltration/diafiltration of the purified antibody solution was performed with a sucrose, histidine and polysorbate 80 based final formulation with a pH of 6.0. Ultrafiltration/diafiltration was performed on Cytiva Minimate at a flow rate of 6ml/min on a 10k membrane. The final protein concentration post-TFF was 2.44mg/ml with a final volume of 28ml recovered. This represents a minor loss of antibody during TFF of 4%. This final formulated monoclonal antibody solution was then stored at 2-8C until further usage. It was noted that a minimum volume of 10ml was required to perform TFF in order to prevent large volume losses in tubing during tangential filtration.

SDS-PAGE Analysis

The purified and formulated anti-TNF alpha was analyzed using SDS-PAGE under reducing, denaturing conditions. Approximately 4µg per sample was added to each well/lane. The analysis was performed in a 10 well gel and 4 replicates of the same sample were analyzed through it (**Figure 37)**. The disulfide bonds in the antibody were reduced using β-mercaptoethanol generated two bands, 2 heavy chains with a band width of 50 for each and 2 light chains with a band width of 25 for each were observed in the gel.

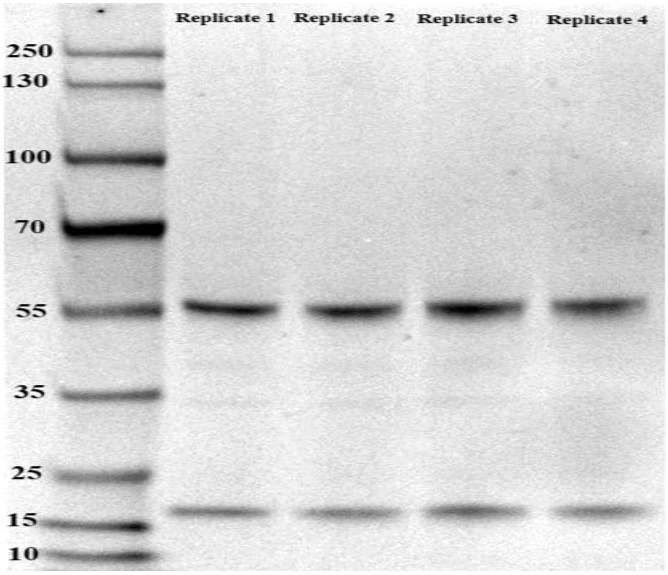


Figure 37. SDS-Page of formulated cNISTmAb. Replicates analyzed under reducing, denaturing conditions in lanes 2-5 and molecular weight marker ladder in lane 1.

4.1.3 Batch 3

This batch was performed to optimize culture type by both adherent and suspension to increase the total yield from the culture.

Production of NIST monoclonal antibody

Cell Culture

A split was created during batch 2 (12 x T-75 flask step) and that was further expanded as adherent cells in the above-mentioned media to a final scale of 12 T-75 (**Figure 38)**.

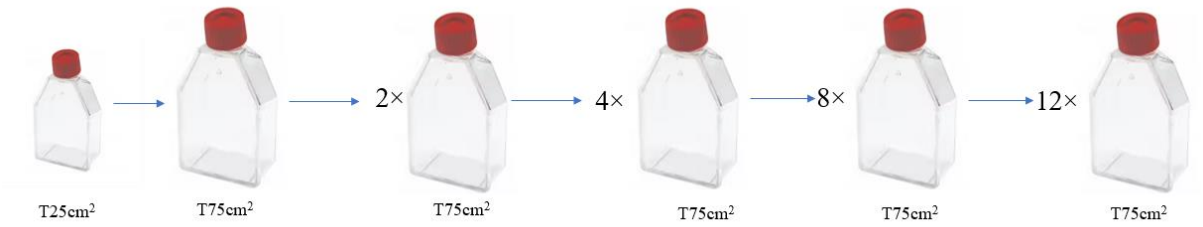


Figure 38. Batch 3 cNISTmAb Cell culture Expansion

Further for the production step the media was then changed to standard production basal media to a final scale of a 160ml contained in a 500ml conical cell culture shake flask with vented caps for oxygen exchange for suspension type and 4 T-75 flasks containing standard expansion media each containing 40ml of media to a total of 160ml for adherent type. The production step consisted of a 10-day culture period without glucose analysis due to a shortage in test strips, the cells were supplemented glucose on alternating days e.g. days 2, 4, 6 and 8.

Purification

After harvest and centrifugation, the supernatant was filtered and then approximately 140ml of both conditioned medias was purified using FPLC with a 1ml protein G column. A flow rate of 1ml/min was selected, with a maximum pressure of 0.3 psi employed and purification was run overnight. Antibody elution was measured via in-line protein concentration analysis via UV analysis at 280nm on Akta Purification system, with successive 1ml elution fractions captured and a total of 15 fractions collected.

The monoclonal antibody concentration of collected fractions from both experiments was measured offline via nanodrop analysis and monoclonal antibody elution was observed in fractions in 3,4 and 5 as per **Figure 39**.

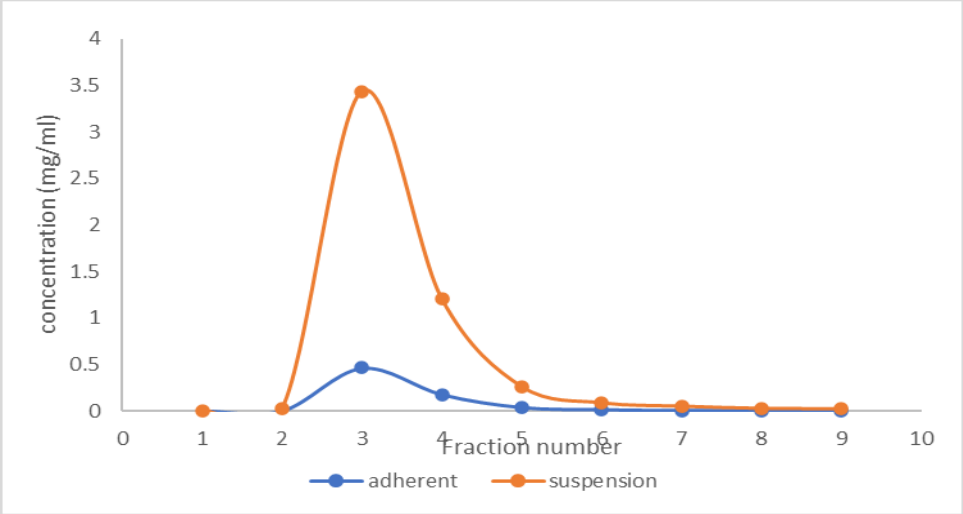


Figure 39. Offline protein concentration analysis of batch 3 with comparison of two different cell culture types. 1.2 ml aliquots or fractions were collected during purification for both study when eluting mAb from 1ml column. 15 fractions were collected, only 9 fractions were analyzed for offline protein concentration by UV at 280nm

The purified solution was neutralized by a buffer of pH of 9.0 and protein concentration of 1.63mg/ml was measured with a volume of 3.6ml collected during elution with a total yield of 5.87mg of antibody for suspension type culture. This corresponded to a titer of 0.04mg/ml in bioreactor during production step. And in the case of adherent type culture a protein concentration of 0.22mg/ml was measured with a volume of 3.6ml collected during elution with a total yield of 0.792mg. This corresponded to a titer of 0.005mg/ml in bioreactor during production step

Tangential Flow Filtration

No TFF was performed on Batch 3 for either the suspension or adherent subcultures due to low titers and volumes for adherent and suspension subcultures respectively.

4.1.4 CHO Cell mAb Titers

Batch	Concentration (mg/ml)	Yield (mg)	Titer (mg/ml media in production)	% Loss after TFF	Final Concentration (mg/ml)	Final Volume (ml)
1	3.9	70.2	0.05	4%	2.44	28
2						
3A	1.63	5.87	0.04	N/A	N/A	N/A
3B	0.22	0.792	0.005	N/A	N/A	N/A

Table 19. CHO cell culture titers

When comparing Batch 1 and Batch 2, the total purified volume has lowered from batch 1 to batch 2, but the total antibody produced was found to be increased in batch 2 after a 60% loss of the antibody due to FPLC equipment malfunction. This was because of the cell density difference in the expansion stage of both batches. For batch 2 the cell density at the expansion stage was higher than batch 1 (cell density was observed visually for both batches) as it was expanded as adherent cells and not suspension cells

4.1.5 NIST mAb Post Purification Processing

Pepsin Digestion

Purified and formulated cNISTmAb from batch 1 and batch 2 that had been pooled were processed by digesting with pepsin, an endopeptidase, to generate antibody fragments e.g. $F(ab')_2$. Sodium citrate at a pH of 4.0 was used as the digestion buffer. cNISTmAb was digested with pepsin for two hours followed by an in-series purification with protein L & protein G columns. While both columns will have affinity for digested and undigested mAb, protein L columns will have higher affinity for $F(ab')_2$ fragments.

The columns were connected to the system in series, protein L column being the first one and protein G the next. Different columns were used to collect all antibodies from the solution even if they are not digested. The undigested antibodies which don't attach to the protein L gets trapped in the protein G column.

The purification was conducted to eliminate pepsin from the solution, as pepsin doesn't have affinity for Protein L or Protein G columns. This is important as pepsin has a molecular weight of 34-37 kDa which is similar to antibody fragments and antibody light chain molecular weights. For the elution step in the purification the protein G column was disconnected from the system at first and elution takes place only for protein L column, after this the protein L column was disconnected and protein G column was connected back into the system and eluted.

The antibody concentration of eluted fraction from both columns were measured offline via nanodrop analysis and the antibody was observed in elution 4 and 5 as per **Figure 40** and pooled together for protein L and no antibody was observed in the fractions from protein G column as per F**igure 41**

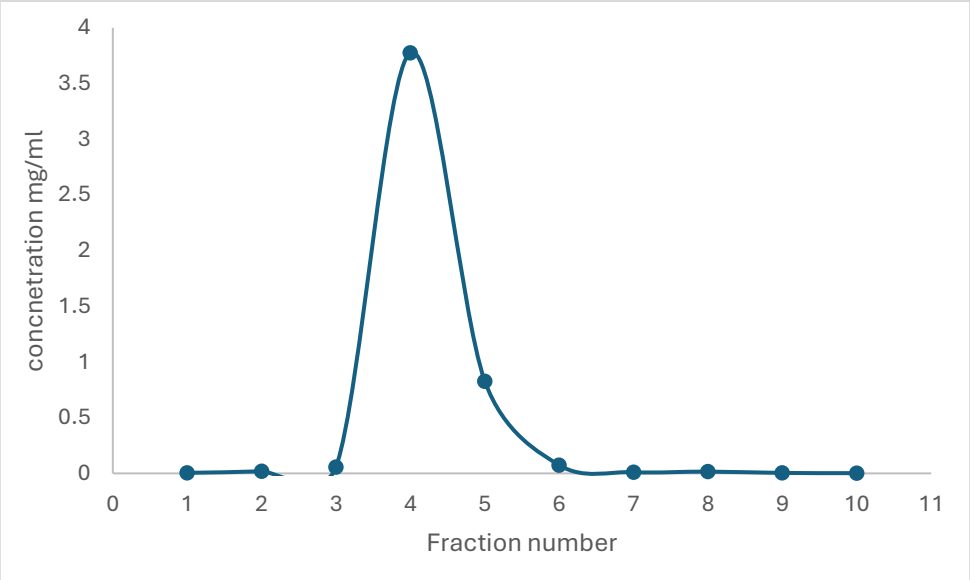


Figure 40. Offline protein concentration analysis of samples from protein L column 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 1ml column. 15 fractions were collected, only 10 fractions were analyzed for offline protein concentration by UV at 280nm

The purified solution was neutralized by a buffer of pH of 9.0 and protein concentration of 2.305mg/ml was measured with a volume of 2.4ml collected during elution with a total yield of 5.532mg of digested antibody.

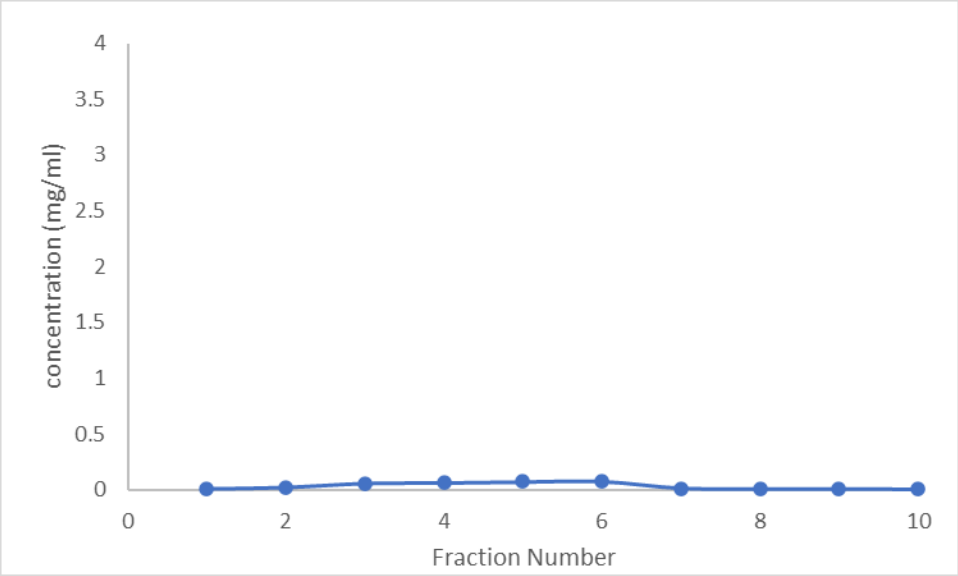


Figure 41. Offline protein concentration analysis of samples from protein G column 1.2 ml aliquots or fractions were collected during purification when eluting mAb from 1ml column. 15 fractions were collected, only 10 fractions were analyzed for offline protein concentration by UV at 280nm

Dialysis

Dialysis of the purified antibody solution was performed with a sucrose, histidine and polysorbate 80 based final formulation with a pH of 6.0. Dialysis was performed overnight with a low agitation speed using a stirrer bar and magnetic mixer. The final protein concentration post-dialysis was 2.05mg/ml with a final volume of 2.4ml recovered. This represents a minor loss of antibody during dialysis of 11%. This final formulated monoclonal antibody solution was then stored at 2-8ºC until further usage.

SDS-PAGE

The purified antibody fragments from protein L column were then analyzed using SDS-PAGE analysis under reducing, denaturing conditions. Approximately 4µg per sample was added to each well/lane. The analysis was performed in a 10 well gel and 4 replicates of control was used in the gel and 4 replicates of the sample after digestion were used and analyzed through it **(Figure 42).** The control consisted of reduced and denatured whole antibody undigested by enzyme.

For the control sample, bands were observed at ~55kDa and ~25kDa indicative of antibody heavy and light chains respectively under reducing conditions. For the digested samples, bands were again observed at ~55kDa and ~25kDa however the bands demonstrated changes in intensity compared to control samples. The band at ~25 kDa were of high intensity relative to control bands but also relative to digested sample bands at ~55 kDa indicating digestion of whole antibody and generation of $F(ab')_2$ fragments.

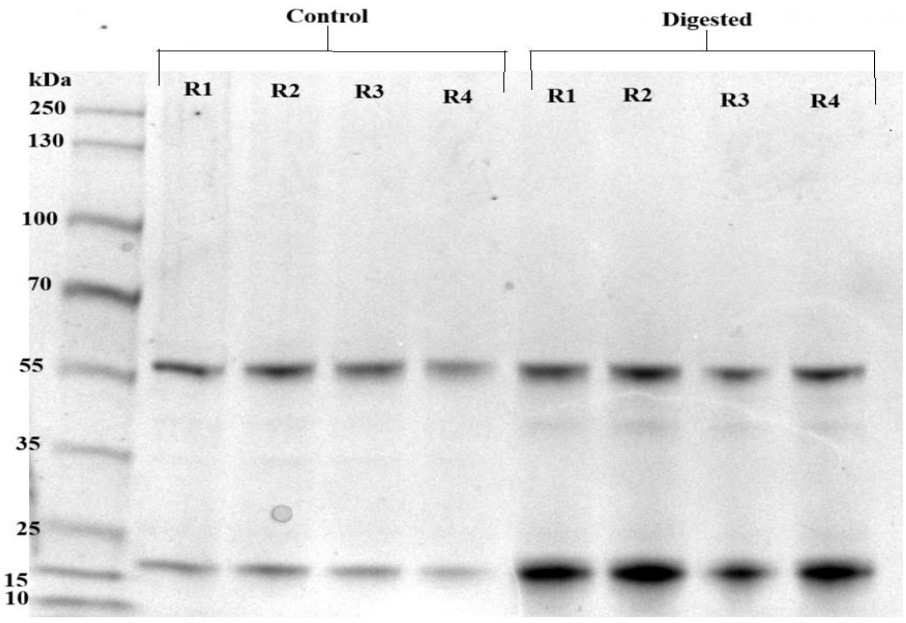


Figure 42. SDS-PAGE of pepsin treated cNISTmAb under reducing, denaturing conditions. Control (untreated cNISTmAb replicates are in lanes 2-5), while treated and digested cNISTmAb is present in Lanes 6-9 (replicates) and molecular weight marker ladder in lane 1

4.3 STABILITY

Characterization of mAb samples for both anti-TNF alpha mAb and cNISTmAb demonstrated integral protein production via SDS-PAGE and potency assays (anti-TNF alpha mAb only) conducted shortly after final formulation. However, longer term stability was not initially assessed and thus a specific stability study was conducted to determine cNISTmAb stability. cNISTmAb stability was only assessed due to large stocks available compared to anti-TNF alpha mAb.

Different temperature and different time points were considered to analyze the stability of the purified and formulated cNISTmAb. The cNISTmAb was stored at -80ºC was set as a control and compared with all other samples. The samples were set at each temperature and on different time points the samples the removed from that temperature and stored at -80ºC for analysis. Four different temperatures were selected -20ºC, +4ºC, +25ºC, +40ºC and different time points starting from 4 days to 50 days were selected.

4.3.1 cNISTmAb stability at -20ºC

Six aliquots of 100µl of formulated antibody were stored in -20ºC freezer and at each time point starting from 4 days ,1 week, 2 weeks, 3weeks, 1month, up-to 50 days the corresponding aliquot was taken out and stored at -80ºC until the end of the stability study. All samples were then analyzed together to remove any analysis variation**.**

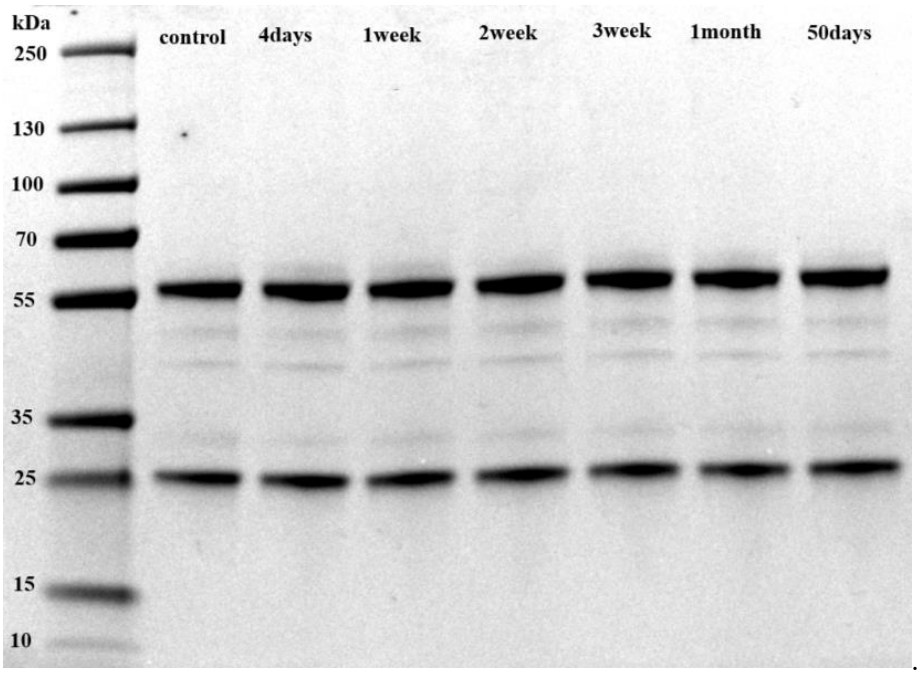


Figure 43. SDS-PAGE of cNISTmAb stored at -20ºC for stability studies under reducing, denaturing conditions. Control, which was stored in -80ºC was in lane 2, while cNISTmAb stored at -20ºC and removed at different time points were in lanes 3-8 and molecular weight marker ladder in lane 1.

After 50 days the aliquots in -80ºC were analyzed using SDS-PAGE under reducing, denaturing conditions **(Figure 43)**. Aliquot stored at -80ºC at start of stability study as taken as control and the sample removed from each timepoints were added to the gel from 4 days to 50days. Both heavy chain and light chain at a band width of 55kDa and 25 kDa respectively can be observed in every sample, no difference in the structure is observed in each time points when compared to control. A possibility of aggregates and low weight fragments can be observed in between heavy chain and light chain even in the control as the control samples were stored at 4°C for 3 months after purification and in time no difference was observed in these aggregates and low

weight fragments up-to 50 days. This indicates the formulated cNISTmAb were stable at -20ºC up-to 50 days and can be freeze-thawed.

4.3.2 cNISTmAb stability at 4ºC

Six aliquots from the formulated antibody were stored at 4ºC and at each time point from 4 days ,1 week, 2 weeks, 3weeks, 1month, up-to 50days the corresponding aliquot was taken out and stored at -80ºC until final analysis. All samples were then analyzed together to remove any analysis variation

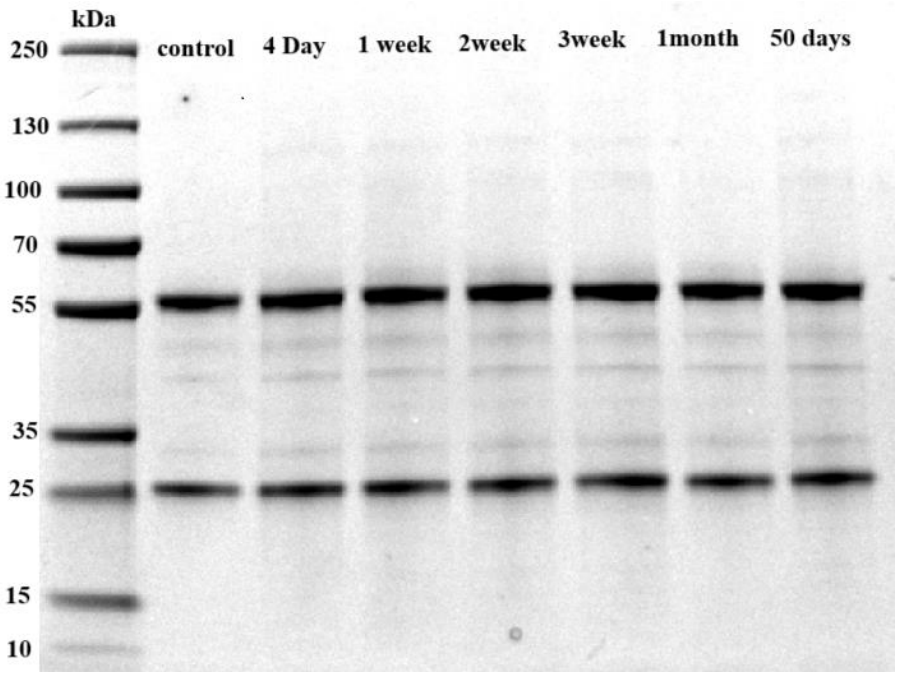


Figure 44. SDS-PAGE of cNISTmAb stored at +4ºC for stability studies under reducing, denaturing conditions. Control, which was stored in -80ºC was in lane 2, while cNISTmAb stored at +4ºC and removed at different time points were in lanes 3-8 and molecular weight marker ladder in lane 1.

After 50 days the aliquots in -80ºC were analyzed using SDS-PAGE under reducing, denaturing conditions **(Figure 44)**. Both heavy chain and light chain at a band width of 55kDa and 25 kDa respectively can be observed in every sample. As in -20ºC there was a possibility of aggregates and low weight fragments can also be observed at 4°C in between heavy chain and light chain even in the control. in time, no difference was observed in these aggregates and low weight

fragments up-to 50 days. This indicates the formulated cNISTmAb was stable at 4ºC up-to 50 days. A faint possibility of dimer formation can be observed after 2 weeks at ~100kDa

4.3.3 cNISTmAb stability at 25ºC

Six aliquots from the purified and formulated antibody were stored at 25ºC in a temperature controlled unit and at each time point from 4 days ,1 week, 2 weeks, 3weeks, 1month, up-to 50days the corresponding aliquot was taken out and stored at -80ºC.

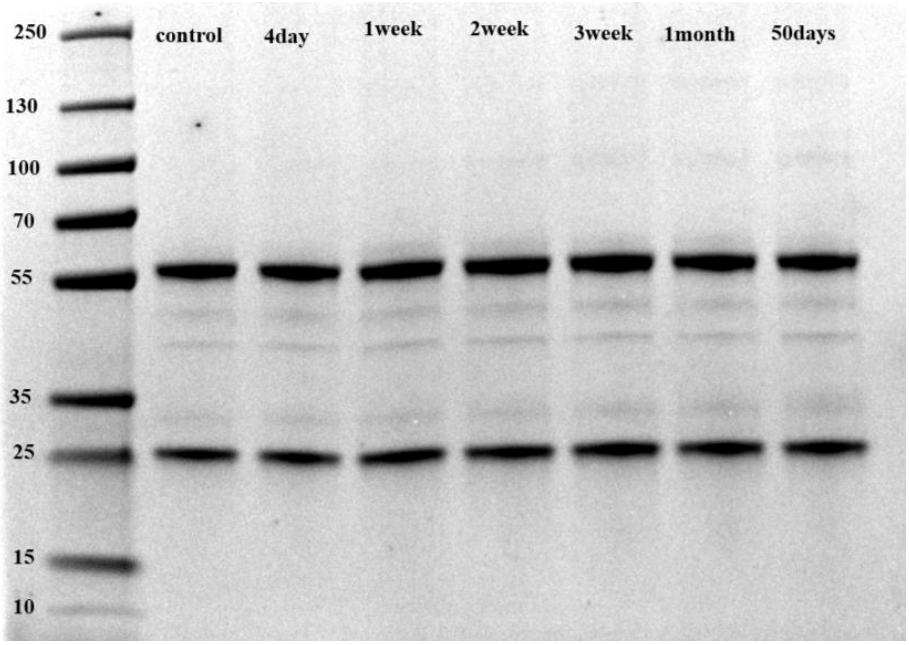


Figure 45. SDS-PAGE of cNISTmAb stored at +25ºC for stability studies under reducing, denaturing conditions. Control, which was stored in -80ºC was in lane 2, while cNISTmAb stored at +25ºC and removed at different time points were in lanes 3-8 and molecular weight marker ladder in lane 1.

After 50 days the aliquots in -80ºC were analysed using SDS-PAGE under reducing, denaturing conditions **(Figure 45).** Both heavy chain and light chain at a band width of 50kDa and 25 kDa respectively can be observed in every sample. Possibility of aggregates and low weight fragments can be observed as seen in -20ºC and 4ºC and there were no changes after 50 days to the structure. There is a faint band suggestive of dimer and trimer formation can also be observed after 2 weeks at ~100 and ~150 kDa respectively.

4.3.4 cNISTmAb stability at 40ºC

Six aliquots from the purified and formulated antibody were stored at 40ºC in a incubator and at each time points from 4 days ,1 week, 2 weeks, 3weeks, 1month, up-to 50days the corresponding aliquot was taken out and stored at -80ºC

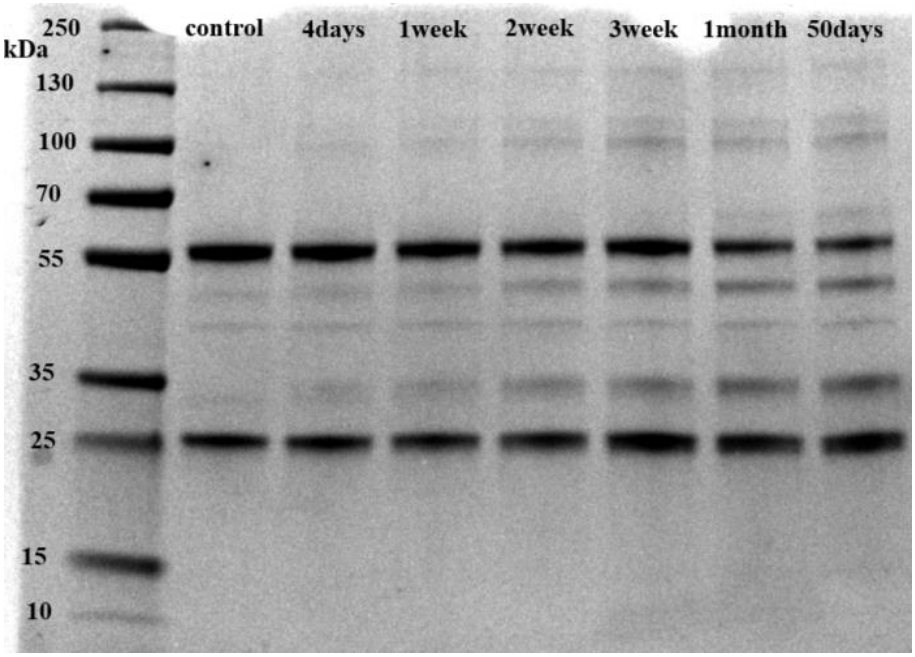


Figure 46. SDS-PAGE of cNISTmAb stored at +40ºC for stability studies under reducing, denaturing conditions. Control, which was stored in -80ºC was in lane 2, while cNISTmAb stored at +40ºC and removed at different time points were in lanes 3-8 and molecular weight marker ladder in lane 1.

After 50 days the aliquots in -80ºC were analyzed using SDS-PAGE under reducing, denaturing conditions **(Figure 46)**. Both heavy chain and light chain at a band width of 50kDa and 25 kDa respectively can be observed in every sample. After 2 weeks the heavy chains start to degrade forming a new band at ~35kDa and also the light chain had an increased quantity of the degraded sample. The dimer and trimer formation has increased from 2weeks implies the antibody is not stable from 2 weeks in a temperature of 40ºC.

4.3.5 cNISTmAb stability post lyophilization

Monoclonal antibodies and biologics more generally are often formulated and processed as lyophilized drug products to ensure long-term stability. In this experiment, formulated cNISTmAb was freeze dried in three vials using a platform drying cycle recipe. These three vials were stored at -20°C, 4°C and room temperature for 50 days before being reconstituted with purified water and being analyzed via SDS-PAGE **(Figure 47).** The samples were run in both reducing, denaturing conditions as well as non-reducing, denaturing conditions.

All freeze-dried samples demonstrated equivalent bands to control (cNISTmAb stored at -80°C on day 0) regardless of temperature and of processing.

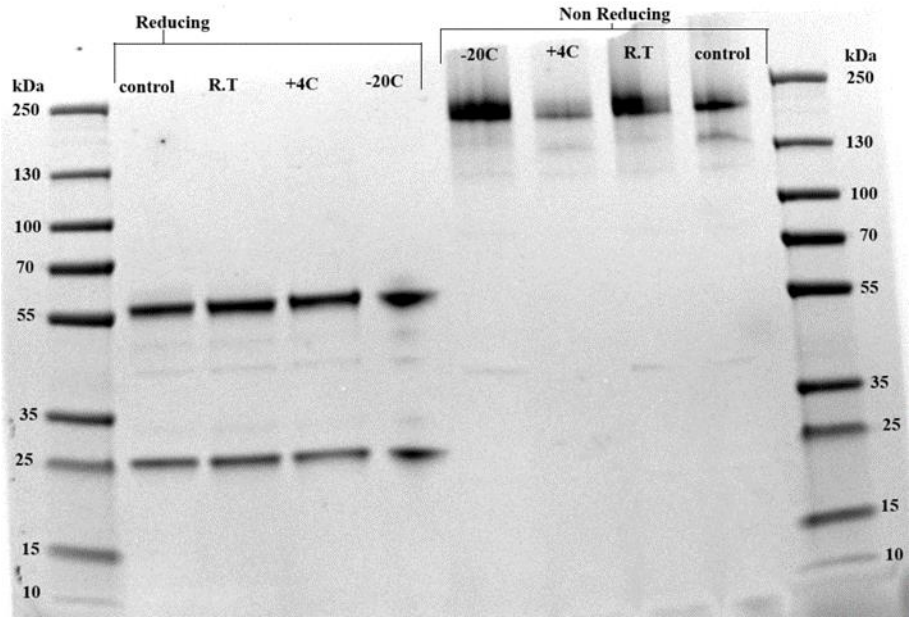


Figure 47. SDS-PAGE of lyophilized cNISTmAb for stability studies under reducing, denaturing condition and non- reducing denaturing conditions. Control, which was stored in -80ºC was in lane 2 and 9 while cNISTmAb stored at 3 different temperatures Room temperature, +4ºC, -20ºC in lanes 3, 4and 5 (reducing condition) and in lanes 6, 7, and 8 (non-reducing condition) and molecular weight marker ladder in lanes 1 and 10

4.4 Cell-based Potency Assay Method Development

Biological function and activity are critical quality attributes for all biological medicines and are required to be assessed as part of process development activities as well as during routine quality control analysis as part of batch release process.

Clinical trials in humans followed by pre-clinical studies in animal models serve as the gold standard in assessing biological function and activity. However, the use of such studies for process development and batch release is unfeasible, unethical and impractical and *in-vitro* bioassays are used as a surrogate to demonstrate *in vivo* function and activity.

Two bioassays were developed to characterize the anti-TNF alpha mAb potency including two cell-based potency assays. The cell-based potency assays employed HEK293 cells as well as L929 cells.

No bioassay characterization was performed for the cNISTmAb as the antigen for this mAb is not provided by the vendor.

4.4.1 HEK293 Potency Assay

Formulated anti-TNF alpha mAb solutions were analyzed via a HEK293 cell-based assay to determine *in vitro* antibody potency. The assay determines antibody potency indirectly via detection of Secreted alkaline phosphatase (SEAP), when HEK-293 cells are stimulated by TNF-alpha, a signaling cascade is initiated leading to the production of SEAP that can be measured via luminescence at 492nm **(Figure 48)**. The presence of anti-TNF Alpha antibody leads to decreased SEAP levels and lower luminescence signal.

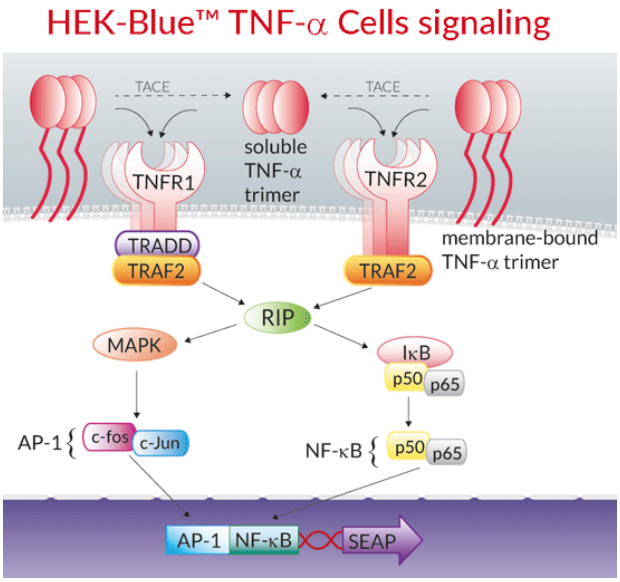


Figure 48. HEK293 Cell Base Anti-TNF Alpha Potency assay mechanism

(https://www.invivogen.com/hek-blue-tnfa)

4.4.1.1 Assay Development

The HEK-293 potency assay is a commercially available reporter cell line and was developed according to vendor technical documentation. The monoclonal antibody concentrations used in this assay were calculated based on the molar ratio of antibody to antigen, with TNF-Alpha having a molecular weight of 17.4 kDa and the anti-TNF Alpha antibody having a molecular weight of 150 kDa. This molecular weight ratio of antigen to antibody in this case was 1: 8.62.

The TNF-Alpha concentration was fixed at 2ng/ml with a starting concentration of 8.62ng/ml which represents a molar ratio of antigen to antibody of 1:0.5. The antibody concentration was increased 10, 100 and 500-fold to ascertain antibody potency resulting in antigen: antibody ratios of 1:5, 1:50 and 1:250 respectively.

Bovine serum albumin was added to the assay at a fixed concentration of 50µg/ml to stabilize antigen.

4.4.1.2 Method Development

An initial assay was performed that demonstrated unexpected results and variability in percentage signal response **(Figure 49).** A 100% signal response was assigned to samples that did not contain antibody (e.g. 0mg/ml) with negative controls of absence of TNF-Alpha & anti-TNF alpha mAb or absence of TNF-alpha.

While standard and negative controls worked as expected, the signal response did not initially decrease with increasing antibody concentration as anticipated. While a decrease in signal was observed between the three antibody concentrations assessed, the signal response for 86.2ng/ml was found to be at ~100%. This indicated either an error in this sample or in the control. On review, pipetting error was assigned as probable root cause, and the results of this run were discounted.

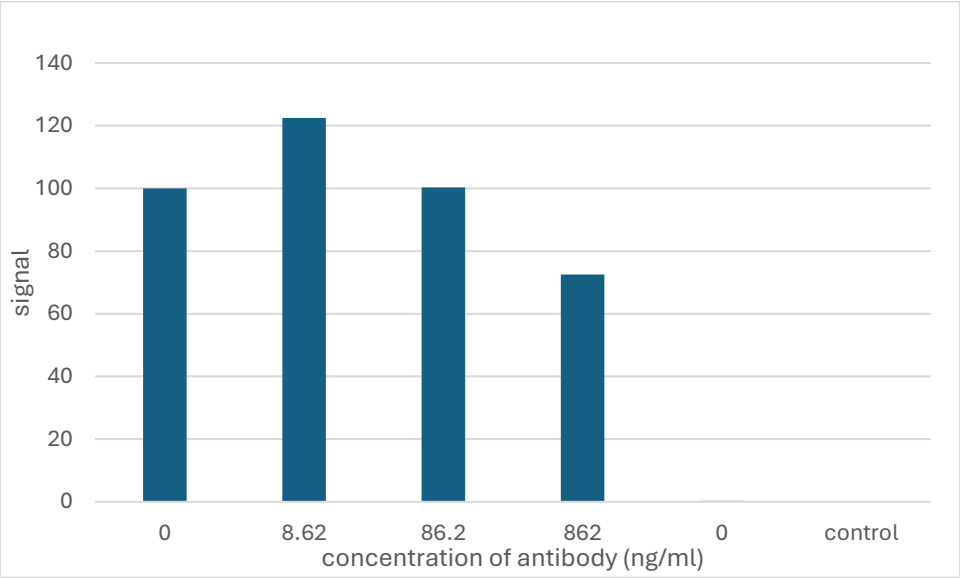


Figure 49. Anti-TNF alpha mAb potency showing variability in results in first run Increasing amounts of anti-TNF alpha mAb were added with signal measured to determine mAb potency via HEK293 potency assay with SEAP readout as measured by UV at 490nm. A negative control was included that did not contain neither antibody nor antigen and is recorded as "control".

A second run was performed with improved results and a decreasing signal response with increasing antibody response **(Figure 50)**. These results were generated with anti-TNF alpha mAb produced in Batch 1 (**section 4.1.3**). This assay was considered developed and used to further characterize anti-TNF alpha mAb produced in Batch 1 in terms of IC50 value and in comparison, to a commercially available anti-TNF alpha mAb (**section 4.1.3**).

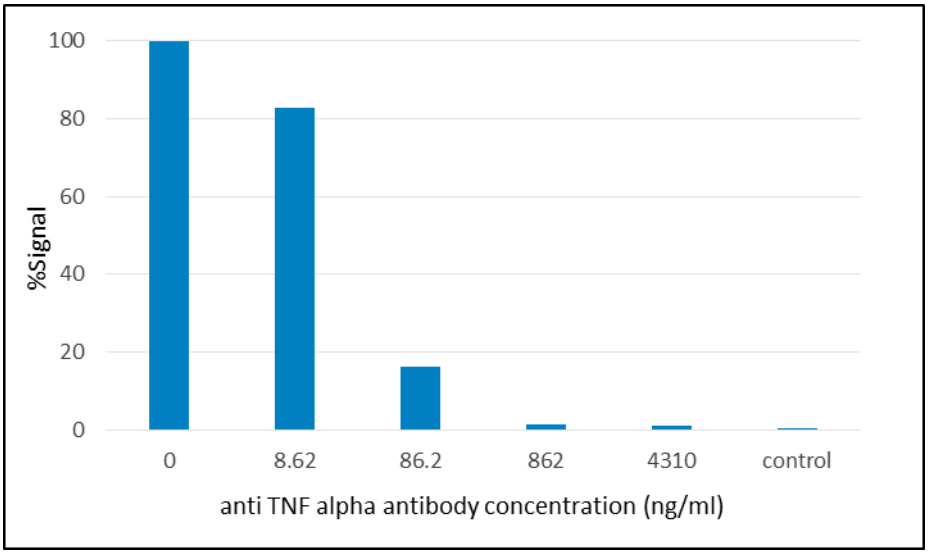


Figure 50. Batch 1 Anti-TNF alpha mAb potency. Increasing amounts of anti-TNF alpha mAb were added with signal measured to determine mAb potency via HEK293 potency assay with SEAP readout as measured by UV at 490nm. A negative control was included that did not contain neither antibody nor antigen and is recorded as "control".

4.4.2 Anti-TNF Alpha Potency Assay Method Development

The formulated anti-TNF alpha mAb sample was also analyzed via a second cell-based potency assay. An L929 cell-based was used that determines antibody potency indirectly via detection of total ATP (Adenosine triphosphate) content from the viable cells in the culture. The assays functions by cell lysing and generation of luminescent signal at 492nm proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture. This assay relies on the properties of a proprietary thermostable luciferase which will generates a stable glow type luminescent signal **(Figure 51).**

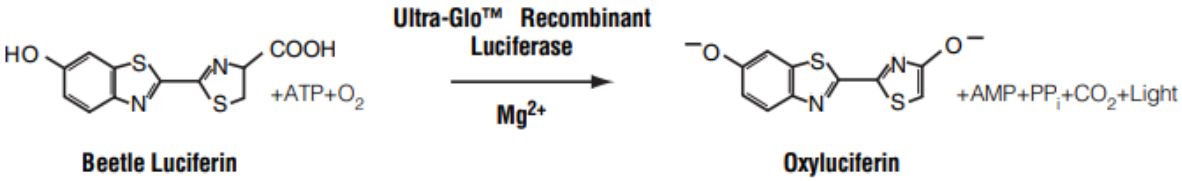


Figure 51. L929 Cell Base Anti-TNF Alpha Potency assay mechanism (https://ch.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-glo-luminescent-cell-viability-assay-protocol.pdf)

The assay was developed in L929 cells in 3 different stages with cell stimulation in the presence of TNF-alpha alone, TNF-Alpha and actinomycin D and finally with TNF-Alpha, actinomycin D and anti-TNF alpha mAb

4.4.2.1 TNF-Alpha stimulation

In this stage the cultured L929cells were treated with human TNF-Alpha to find the reactivity towards the cells. The assay was performed with a negative control without the presence of TNF-alpha and different concentrations of TNF-alpha were used, from 2ng/ml to 0.125ng/ml in a serial dilution as in **Figure 52.** The assay was performed so that six conditions were analyzed, including the negative control in 6 wells of a 96 well plate. These 6 wells were then replicated, producing a total of four replicates, with a total of 24 wells in the same 96 well plate.

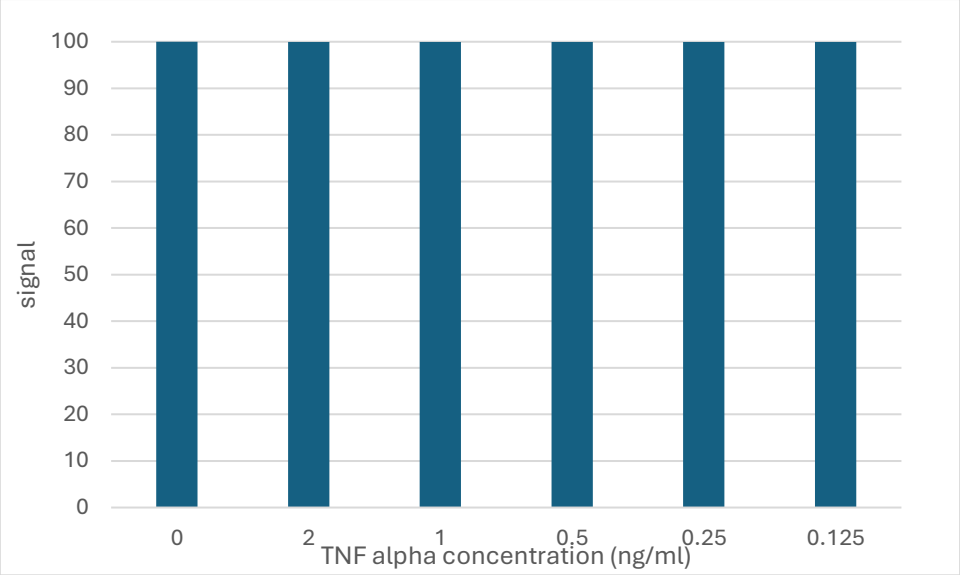


Figure 52. TNF alpha treated L929 cell assay. Decreasing amounts of TNF alpha antigen were added with signal measured to determine effect of TNF alpha towards the L929 cells with total ATP content as a readout and measured by UV at 490nm. A control was included that did not contain TNF alpha antigen

A control not containing human TNF alpha (0 ng/ml) provided 100% signal and with decreasing antigen concentrations the signal stays the same demonstrating that the human TNF Alpha did not have any effect on the cell viability.

4.4.2.2 Stimulation with TNF ALPHA & Actinomycin-D

The second stage of assay development was treating the cells with TNF alpha and Actinomycin D, a cytotoxic drug, to determine the effect of TNF alpha in the presence of Actinomycin d drug. The assay was performed using a negative control without the presence of both TNF Alpha and Actinomycin D, a control containing just Actinomycin D and an increasing concentration of TNF-Alpha from 0.125ng/ml to 1ng/ml was applied in a serial dilution in the presence of Actinomycin D drug. The concentration of Actinomycin D drug was set to a constant of 0.5µg/ml. The assay was performed so that six conditions were analyzed, including the negative control in 6 wells of a 96 well plate. These 6 wells were then replicated, producing

a total of four replicates, with a total of 24 wells in the same 96 well plate. And this assay was triplicated (**Figure 53).**

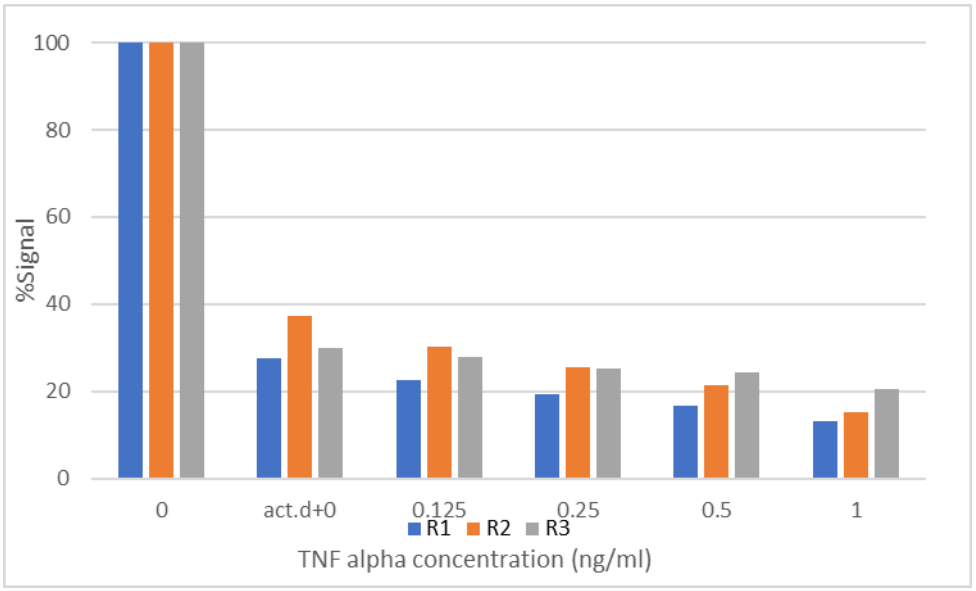


Figure 53. L929 cells treated with TNF alpha, and Actinomycin D Decreasing amounts of TNF alpha antigen and constant amount of Actinomycin D drug were added with signal measured to determine effect of TNF alpha and Actinomycin D drug towards the L929 cells with total ATP content as a readout and measured by UV at 490nm. A negative control was included that did not contain TNF alpha antigen and Actinomycin D drug. R1, R2 and R3 were the three replicates of the assay.

A control not containing human TNF alpha (0 ng/ml) and Actinomycin D drug provided 100% signal and in the presence of Actinomycin D alone approximately 70% of total signal was reduced and with increasing concentration of TNF-Alpha the signal increases furthermore and at a TNF-alpha concentration the 1ng/ml an 80% of total signal was decreased. Showing the effect of TNF alpha to the cells.

4.4.2.3 Stimulation with TNF Alpha, Actinomycin-D & Antibody

The third stage of the assay was developed to determine the potency of the formulated Anti-TNF alpha mAb. A negative control was used in duplicate consisting of a TNF alpha and Actinomycin D in the absence of anti-TNF Alpha antibody. Concentration of TNF Alpha was set at 1 ng/ml and concentration of Actinomycin D drug was also set to a constant at 0.5µg/ml in all wells. The formulated antibody was added in a decreasing concentration from 517.2ng/ml to 17.2ng/ml. The antibody concentrations were selected from the concentration used on the potency assay with HEK 293 cells from the first batch used to find the IC50 value. The assay was performed so that six conditions were analyzed, including the negative control in 6 wells of a 96 well plate. These 6 wells were then replicated, producing a total of four replicates, with a total of 24 wells in the same 96 well plate. And this assay was duplicated (**Figure 54.**

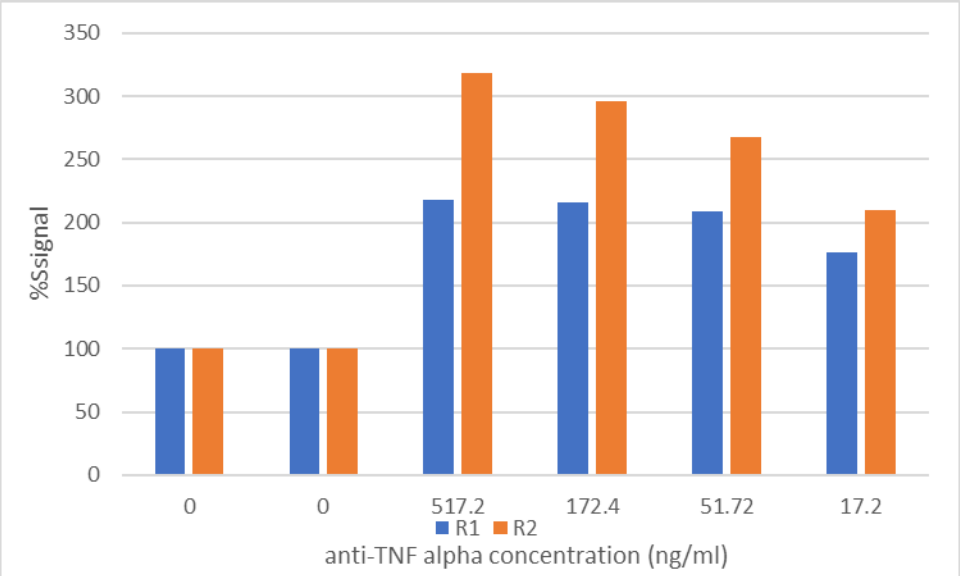


Figure 54. Anti-TNF alpha mAb potency assay. Decreasing amounts of anti-TNF alpha mAb and constant amount of TNF alpha and Actinomycin D drug were added with signal measured to determine mAb potency via L929 potency assay with total ATP content as a readout and measured by UV at 490nm. Two negative controls (duplicates) were included that contain TNF alpha antigen and Actinomycin D drug. R1 and R2 were the two replicates of the assay.

The controls that contain human TNF alpha (0 ng/ml) and Actinomycin D drug provided signal and in the presence of 517.2ng/ml the total signal from the cells increased by 2 times and with decreasing concentration of antibody the total signal is also decreased when compared to the largest concentration of antibodies. In here the antibodies are minimizing the effect of TNF alpha from the treatment by interacting with them and increasing the total signal of the cells by increasing the viability. A total of four replicates were performed, but the last two assays failed due to an issue with plate reader. After stimulation the reagent was added to the cells and the plate was put into read the absorbance. The reader repeatedly aborted the assay and the run was stopped with no data generation. As the assay was time sensitive, it was not possible to re-read plates with alternative reader.

5.Discussion

Two monoclonal antibodies were successfully produced, purified, formulated and characterized at lab scale as part of this research project over a 9-month period. However, despite this initial success in developing an end-to-end monoclonal antibody production process for two distinct cell lines (hybridoma & CHO), there are a number of limitations to the data presented here. In particular, due to time constraints many of the studies performed represent a sole data point which limits the conclusions that can be extrapolated from the corresponding results. Additionally, the equipment, financial and analytical limitations that existed here in comparison to the biopharma industry should be noted; where advanced technology for process monitoring exist, expensive chemically derived media is widely available, and an array of characterization techniques are available to process development teams. However, the development of the two mAb production processes, an antibody fragment, two potency assays for anti-TNF alpha mAb and characterization of cNISTmAb stability across a number of temperature conditions over 50 days represents a solid foundation for further research.

Cell culture in the biopharmaceutical sector has transitioned away from high yielding, rapid production of low complexity recombinant proteins through *E. coli* fermentation towards lengthy, expensive cell culture of high-producing CHO cells producing complex recombinant proteins, mainly monoclonal antibodies. It is in this backdrop, that this thesis focused on monoclonal antibody production and in particular a CHO cell line, however the sourcing of said CHO cell lines was challenging as outside of therapeutic applications, few other applications exist for these cell lines which greatly restricts their availability outside of multinational biopharma companies who do not share such cell lines. This resulted in the use of cNISTmAb as the CHO cell line in this research. A hybridoma cell line producing a mAb

targeting human TNF-Alpha was also used in this thesis as it was readily available from commercial vendors and the antigen, TNF-Alpha, is a widely targeted antigen ensuring the availability of reagents and reference antibodies that could act as comparators.

Cell culture as a process, while performed for decades, is still not a fully understood process particularly for mammalian cells where productivity in the order of >10g/L is required while ensuring high levels of precision around glycosylation and aggregation. A further complication is the requirement to develop cell-based assays to confirm potency, which is in itself a separate a cell-culture process. Here, four cell culture operations were developed with two required for antibody production (CHO, Hybridoma) and two for potency evaluation (HEK293, L929).

The major areas of study in early-stage process development outside of cell line engineering are definition of culture parameters, media optimization and scale-up to pilot scale. Accordingly, initial cell culture experiments for CHO & Hybridoma cell lines were exclusively focused on resuscitating cells, creating working cell banks and determining the functionality of both cell lines. WCBs were successfully generated for both cell lines after a number of passages that generated healthy cells as determined by microscopic analysis. In addition, antibodies were recovered after protein A purification and dialysis allowing for the development of potency assays. These initially studies confirmed that both cell lines were functional and could be used for further studies. Upon analysis of mAb concentrations it was found that both cell lines had disappointing titers in respect of literature values for both types of cell lines. Evidently, hybridoma cell lines are low producing cell lines, however that said the titers observed were lower than expected and required optimization.

The first strategy employed was to increase density, this was achieved through prolonging the expansion phase from 10 days to 15 days. This allowed for a greater volume of

cells to be cultured during production phase. However, the strategy did not yield any immediate improvement in titers in Batch 2, that said titers in Batch 4 were tripled from 0.006mg/ml to 0.02mg/ml. While not all other factors were constant, the production volume in bioreactor was different between batch 2 and 4, preventing definitive conclusions being drawn, the increased cell density can be seen as positive and in-line with industry norms where production bioreactors titers are being maximized. A confounding factor may have been the cell density counts, while cell density was measured it should be noted that cell counting did not discriminate between viable and non-viable cell density. This may have played a significant factor in the low titers observed as many of the cells may have been non-viable and underscores why viable cell density is an in-process control.

Upon analysis of cell growth curves during production for both CHO & Hybridoma cell lines, it could be seen that cell growth continued during the production phase which indicates that conditions in production bioreactor supported growth but ultimately may have been suboptimal in terms of production. During the production phase, it would be envisaged that cells would be in a stationary phase where cell growth matches cell death and cell machinery is focused on protein production.

While cell density was maximized for the hybridoma cell line to 15 days, to generate increased cell density to maximum operationally relevant timeline, the impact of media had not been assessed. Media plays a critical role in the cell culture process from both a technical and regulatory perspective. In terms of CHO cell culture due to the significant financial opportunity, significant research has and is being undertaken to optimize media formulations for CHO cell culture, resulting in chemically defined media that supports high cell densities during expansion and production while also meeting the regulatory best practice of avoiding animal derived components and their inherent risk of adventitious agent contamination. There is a wide variety of commercially available CHO specific media that is sterilized and ready to

use. The sole drawback of such a media choice is the prohibitive cost in an academic environment where the cost can be ten-fold that of non-chemically defined media. For hybridoma cell culture, no such optimized media exist with media compositions being predominantly FBS based.

It was in this context that, that the media optimization studies in Batches 3 & 4 were performed for hybridoma cell line. The cell line supplier recommended an FBS based media composition, however due to the presence of IgG in FBS as an impurity, a major driver was to reduce or remove FBS from production media. While FBS could be reduced from 10% in the expansion phase to 1% in production phase, the phasing out of FBS could not be achieved without significant impact to cell counts and crucially titers. For all cell cultures that lacked FBS, a 60-90% reduction in antibody titers was observed **(Table 16).**

Cell growth was impacted with all cell cultures lacking FBS demonstrating titers below $1x10^{\wedge 6}$ and concomitantly low glucose consumption. Neither the presence of sodium chloride nor sodium butyrate improved titers when FBS was not part of standard basal production media composition. This confirms the presence of FBS to be an integral factor in hybridoma culture.

The standard basal production media composition lacking amino acids was assessed to understand role of amino acids. There was no significant change in cell growth with standard conditions, as the cell density increased in the production phase and the metabolism was also analyzed by glucose monitoring in each stage showing similar trend towards the standard production media, albeit with slightly lower glucose consumption. The titer from this was found to be decreased (0.015mg/ml) when comparing to the control (Table 16).

The effect of NaCl in the media (Lin et al., 1999) for enhanced mAb production as a result of enhanced osmotic pressure was also explored. When compared to standard production media the cell density was found to be lower in each stage of production phase and the glucose

concentration follows a similar trend but with a lower consumption rate, as expected. The titer from this was further decreased when comparing with control at 0.012mg/ml.

Sodium butyrate was added to increase the mAb production (Cherlet and Marc, 2000) according to the literature the sodium butyrate has an inhibitory effect on cells and a stimulating effect on protein synthesis. From the cell density analysis, the number of cells were further decreased when compared to the sodium chloride cell culture but still higher than media lacking FBS. The metabolism of the cells was lowered to a high level with a less consumption of Glucose. In contrast with cell density and glucose consumption, the titer was slightly increased compared to the sodium chloride cell culture (0.013mg/ml) but only approximately 66% of control.

A chemically defined media optimized for CHO cells was also assessed. As the culture progressed in the production phase, the cell density in each stage was found to increase compared to control and the total glucose consumption was also decreased in each stage and glucose was added as feed media to keep the optimum concentration at all time points. But the titer from this culture was found to decreased (0.006mg/ml). This indicates that this chemically defined media was not suitable for hybridoma culture. This data also demonstrates the differences that occur in media functionality in terms of cell growth and production and the need for production specific media

As optimized CHO cell media already exists, there was no media optimization studies performed for CHO cell line in production phase. As the expansion phase is media intensive, the vendor recommended expansion media (chemically defined media) was replaced with HAM F12 media, which in of itself is specialized for CHO cell culture. While HAM F12 is specifically developed for CHO cell culture, further studies for CHO cell culture for this cell line could look at changes in expansion media to more expensive, characterized media and

assess CHO chemical defined media from multiple suppliers (GIBCO, Avantor etc.) and not just the EX CELL CD CHO media supplied by Thermo Fisher scientific used exclusively in these studies.

The final parameter that was chosen to be investigated was cell culture mode and vessel. Ultimately, to generate large antibody yields, suspension cell culture must be used as is widely accepted and practiced in the biopharma industry. While microcarrier systems, shaker bottles and flasks can be used, there applications are limited to modalities such as vaccine and ATMPs. Thus, both cell lines were initially cultured during expansion and production phases as suspension cell cultures with the aim of performing production in conical flasks at a volume of 1L (containing 250ml of cell culture). Hybridoma cells responded well to growth in suspension in T-flasks while CHO cells demonstrated poor cell health when observed microscopically as a suspension culture in a conical flask (Batch 1), but positively when cultured as adherent cells (Batch 2). It was envisaged that CHO cells could be grown immediately as a suspension culture that would allow for higher cell densities and greater volumes, evidently this is not possible. The strategy of adherent cell culture during expansion with suspension cell culture during production was further confirmed in Batch 3, with adherent-adherent cell culture mode resulting in low titers.

While hybridoma cell culture initially was easier to culture compared to CHO cells in terms of defining cell culture mode (suspension or adherent), the scale up from T-flasks to conical flasks was not successfully performed with titers ~65% lower than reference conditions (Batch 5, Table 16). Additional work is required to understand potential issues in terms of gas-transfer, media consumption, shear, surface area to volume etc. In contrast, CHO cells were healthy and performed well when grown in conical flasks when compared with the poor growth of hybridoma cells. This strong growth in conical flasks is as expected with industry norms where cells are cultured in large, stirred tank bioreactors.

The increase in hybridoma cell culture titers from batch 2 to batch 4 (Table 16) may be partially attributed to a lower media volume of 100ml used in T-175 flask in comparison to Batch 1 & Batch 2 that contained 150ml and 187.5ml respectively allowing for enhanced gas diffusion. This finding in addition to the poor scale-up of hybridoma production into conical flasks demonstrates the challenges in terms of optimizing process parameters and scale-up. Advanced process controls such as dissolved oxygen, shear analysis, pH and Temperature measurement may provide data to inform future processing strategies.

The purification of all batches was performed using same buffers with same concentration, with little processing issues observed demonstrating the successful purification of antibody via protein A affinity chromatography. FPLC Equipment related issues demonstrated the requirement for minimizing the hold time between harvest, sterile filtration and purification. While all efforts were undertaken to minimize microbial contamination, the process is performed in non-GMP conditions and not in a cleanroom environment. This explains the microbial contamination that became an issue after 30 days of harvest being held and the rejection of Batch 3 for hybridoma cells.

A sucrose, histidine and polysorbate 80 based formulation buffer was used as final drug substance/drug product formulation, sucrose does not crystalize when lyophilizing or freezing while histidine will reduce aggregation of antibodies in sample solution and polysorbate 80 will stabilize the aqueous solution. The purified anti-TNF alpha from batch 1, Batch 2 and Batch 5 were dialyzed and formulated into a different buffer as the purification buffer has the potential to degrade and aggregate the antibodies due to the low pH. Dialysis was done using Tube-O-Dialyzer tubes. After dialysis the samples were collected, and protein concentration analysis was performed. On every batch, a percentage of antibody loss was observed. In batch 1 a loss of 22.4% and in the second batch the percentage loss was decreased to 14% and in the

fifth batch the percentage loss was further decreased to 10%. The decreasing rate of antibody loss are encouraging and potentially reflect improved understanding and experience.

In addition, as FBS is known to contain some IgG antibodies (Jiskoot et al., 1991) and despite the FBS concentration being decreased to 1% in the production step of cell culture process, impurity analysis of the FBS lot used determined 22% of the IgG antibody recovered in Batch 1 purification was impurity emanating from FBS. Future work may look at purification of FBS through Protein A/G columns before use, to ensure FBS contains minimal IgG impurity. As a result of low titers, the volumes dialyzed were small and could not be performed using the available TFF equipment. A potential next step would be to replace dialysis of hybridoma solution with TFF.

Similar to anti-TNF Alpha mAb studies, no issue was observed with the purification process itself as such, e.g. no pressure spikes, no overloading of column compared to theoretical binding capacity. However, equipment related issues persisted with a leaking valve contributing to approximately 60% of cNISTmAb being lost for Batch 2. This issue was remediated but identifies the need to ensure correct equipment function throughout operations.

Of note, it was visually observed that cNISTmAb was unstable post-purification with precipitation observed. This is in contrast to anti-TNF alpha mAb and may be potentially due to higher concentration of mAb present or possibly the lower stability of cNISTmAb in purification buffer. The purified cNISTmAb from initial two batches which were pooled together and formulated using TFF into a different buffer as the purification buffer will further degrade and aggregate the antibodies. After TFF a 4% loss of antibody was observed. This is a much more positive loss than dialysis. While the TFF process was used as functionally, there is further merit in optimizing TFF steps and using to manage final concentration as well as final formulation i.e. as a true UFDF system.

Despite the low yields and relatively high impurity levels, it was found that the anti-TNF alpha mAb demonstrated high potency compared to a commercially available anti-TNF Alpha mAb (Section 4.1.3 Potency & Comparison to external antibody Figure 19). An IC50 value for this batch was also determined and found to be 26.65ng/ml (Section 4.1.3 IC50 Determination, Figures 20 and 21). This relative increase in potency was also observed in the ELISA assay, that demonstrated consistently higher binding profile across a range of antibody concentrations compared to the same commercially available anti-TNF alpha mAb (Section 4.1.3 ELISA Analysis Figure 22). This potency characterization was confirmed again by L929 cell-based potency assay (Section 4.4.2.3 Figure 54).

Size by molecular weight was analyzed using SDS-PAGE under reducing and denaturing conditions. The sample after dialysis was kept at -20°C and the analysis was conducted after 5 months. The analysis shows possible dimer or trimer formation in the sample, but the majority of sample was found to be as expected with characteristic bands at 50 and 25 kDa for heavy and light chains respectively. Further analysis such as Size Exclusion Chromatography via HPLC is required to better understand high molecular weight species.

The characterisation of cNISTmAb was limited to molecular weight analysis via SDs-PAGE. The pooled purified antibodies from batch 1 and batch 2 were analysed using SDS-PAGE under reducing and denaturing conditions with two different bands observed as expected e.g. heavy and light chains. A possible degradation in between heavy band and light band was observed as faint bands, these bands were also observed on stability study strongly suggesting that they are degradation products. As the sample for the SDS PAGE analysis was stored at 4°C for 3 months after purification, this may explain the presence of these bands e.g. not true time

zero samples. Further analysis is required to elucidate the nature of these bands as discussed below.

Specific stability studies were performed for cNISTmAb to analyse long-term stability. Different storage conditions, -20°C, 4°C, 25°C and 40°C were assessed during this stability study for up to 50 days. Different time points were also analysed throughout the study. The samples for each temperature and each time points were then analysed through SDS-PAGE under reducing and denaturing conditions. Light band can be observed at 45, 40 and 30 kDa can be observed even at the control (time zero) for every sample indicating minor degradation and these light bands were found to be increasing over time particularly at stress conditions of 40°C. This indicates the starting material itself may have been degraded through processing conditions or by age. At 40°C the samples were found to be degrading and aggregating with respect to time as expected. For the accelerated stability analysis samples at 25°C were analysed. A light band suggesting possible dimer formation can be observed from 3 weeks up-to 50 days. When comparing samples kept at temperatures -20°C and 4°C with 25°C and 40°C there was no aggregation or degradation other than the degradation in starting material. Further analysis like size exclusion chromatography or charge variant analysis or structural analysis like mass spectrometry for different species reorganizations should be performed for an extended result. In addition, the time of stability should be extended for samples at 4°C.

The characterization of both mAbs was limited due to time constraints and available techniques. CQAs such as particulates, charge variants, glycosylation, higher order structure require analysis to fully understand the ability of the production process to produce intact mAbs of acceptable product quality. Stability studies should be continued for cNISTmAb, preferably with true time zero material and lyophilized material at all storage conditions and stability studies initiated with TNF-Alpha

The potency of the formulated mAbs were analyzed by cell-based potency assay using two reporter cell lines. The development of the cell-based potency assays was relatively straightforward for HEK293 assay and followed a commercially available assay. Method development did not require additional modification outside of remediating analyst pipetting errors. The assay worked as expected with all controls responding as anticipated and a decrease in signal with increasing antibody concentration. Further work could be performed to validate this potency assay as per work performed with L929 potency assay. In addition, this potency assay was more responsive than L929 assay and required less precision in fluid handling (6-well plate for HEK293 compared to 96 well plate for L929 assay).

In contrast, the development of the L929 assay was challenging and required greater development but was partially qualified. The assay was developed in different stages. The cells were stimulated with TNF alpha and Actinomycin D that showed a decreasing signal with increasing TNF alpha concentration as expected. The potency was analysed by adding different concentration of formulated antibodies showing the total signal to be increased and by decreasing antibodies the signal was decreased. The assay was partially qualified by performing two duplicate runs. The increased difficulties in method development i.e. need to use actinomycin D reflect the somewhat general nature of the assay i.e. ATP measurement as a function of cell viability, in comparison to the cell signalling cascade specific to TNF-Alpha. While the final assay was only performed successfully twice, the root cause has been established for assay failures, and it is likely that a third run would also be successful when functional plate reader is employed. The formulated cNISTmAb was processed by pepsin digestion and the digested sample was purified to remove the pepsin from the sample. Two types of affinity columns were used for purification as the protein L and protein G in a series, undigested samples which are unattached to protein L will attach to protein G. The nanodrop analysis of protein G showed no presence of antibodies indicating that all antibodies digested

or undigested present had been bound to protein L column. In hindsight, attachment of protein G column first may have been more appropriate as digested $F(ab)_2$ fragments would have lower affinity for protein G and may be separated from undigested sample. In the set-up used, the eluted sample may theoretically contain both digested and undigested antibody.

SDS-PAGE was performed under reducing and denaturing condition for further analysis of the pepsin treated antibodies. From SDS-PAGE the light chain band has increased indicating digestion, as the digestion will cleave every disulphide bond the molecular weight has decreased showing the decrease in the band width. The presence of heavy chains also observed in the gel indicates the digestion is not complete. Further analysis like mass spectrometry and or size exclusion chromatography should be performed to confirm digestion. SDS-PAGE should also be performed under non-reducing conditions to confirm $F(ab)_2$ size as under reducing conditions $F(ab)_2$ will be dissociated. From a methodological approach, while having a molecular weight of 34-37 kDa and not observed in the gels, the non-affinity of pepsin for protein G / Protein L columns should be verified. The incomplete digestion demonstrates further work is required which may include modification of enzyme concentration, duration of digestion and antibody concentration. In addition, the recovery of mAb also requires study as total recovery rates were low suggesting loss in purification, dialysis and digestion steps.

6.Conclusion

In summary, this research project developed monoclonal antibody production processes from two different mammalian cell lines with the partial characterisation of both mAbs.

For anti-TNF Alpha mAb, a number of different titer optimisation strategies were assessed as the first batch showed low productivity. Evidently with all the optimizing strategies the hybridoma cells were found to be low producing cells. Additional work is required here to further analyse the low yields and associated root causes, with viable cell density and media strategies of particular interest.

Similarly, the cNISTmAb cell culture process was partially optimised as the expansion strategies for batch 1 was found to be poor having a low proliferation rate. Batch 2 rectified this issue and increased the productivity, and this was found to be successful in term of mAb production. However, further work is required to fully optimise the cell culture step in terms of viable cell density, media optimisation and scaling up production volumes using the available wave bag technology within the research unit.

While cell culture was performed through a fed-batch approach with manual sampling and visual observation, further work should consider the use of process analytical technology in terms of glucose, pH, dissolved oxygen and CO_2 levels. This may provide additional information critical to optimising cell health, metabolism and ultimately cell productivity.

Importantly, this work also assessed antibody fragment production with initial studies exploring pepsin digestion to yield $F(ab)_2$ fragments. While providing an initial foundation in antibody fragment products, significant work remains in terms of characterising and optimising the pepsin digestion up-to and including characterising potency in comparison to whole,

undigested native mAb. Once implemented, papain digestion of whole mAbs could be considered as a next step.

Drug product formulation assessment was performed with a lyophilised formulation of cNISTmAb successfully manufactured and demonstrated to be stable post lyophilisation and on stability. Further work is required here to understand the long-term stability at stress condition of 40°C compared to liquid formulation. Potential formulation changes should be assessed for both liquid and lyophilised formulations based on this assessment.

Two different cell-based bioassays were developed for Anti-TNF alpha antibody to demonstrate potency and found out to be successful and for further analysis SDS-PAGE and ELISA were performed. Potency assay for cNISTmAb were not performed as the antigen against it was not provided by the vendor. SDS-PAGE analysis was performed on this mAb to confirm integral protein production.

A comprehensive characterization of formulated antibodies is essential to understand process and formulation impacts on mAb CQAs. Further work is required here to assess glycosylation, aggregation, charge variant, particulates, higher order structure amongst other attributes.

7. References

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