CLONING AND EXPRESSION OF A BIOSIMILAR CHIMERIC MONOCLONAL ANTIBODY DIRECTED AGAINST THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR AND ITS PRODUCTION IN CHO CELLS FOR TREATMENT OF COLO-RECTAL CANCER

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BY

HATIM MAQBULHUSEN MOTIWALA

DEPARTMENT OF MICROBIOLOGY AND BIOTECHNOLOGY CENTRE
FACULTY OF SCIENCE
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
VADODARA-390002, GUJARAT, INDIA

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Dedicated to my loving parents

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Acknowledgement

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-Sincerely,
Hatim

LIST OF ABBREVIATIONS

3D	Three Dimensional
Ab	Antibody
A CN	Acetonitrile
ADCC	Antibody-dependent Cellular Cytotoxicity
ADCP	Antibody-dependent Cellular Phagocytosis
Ala	Alanine
APRT	Adenine Phosphoribosyl transferase
APS	Ammnium Per Sulfate
Asn	Asparagine
Asp	Aspartic Acid
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
B-Cells	Bursa of Fabricus Cell
BGH	Bovine Growth Hormone
BHK cells	Baby Hamster Kidney cells
BLA	Biologics License Application
Bp	Base pair
BSA	Bovine Serum Albumin
CaCl2	Calcium Chloride
Cat #	Catalogue Number
CD	Chemically defined
CDC	Complement-dependent Cytotoxicity
CDR	Complementarity Determining Regions
CE	Capillary Electrophoresis
CEX	Cation Exchange
cGMP	current-Good Manufacturing Practice
CHO	Chinese Hamster Ovary cells
CMV	Cytomegalo Virus
CO_2	Carbon Dioxide
Conc	Concentration
Corr	Corrected
CpB	Carboxypeptidase B
CQA	Critical Quality Attribute
CSI	Clone Select Image Software
C-terminal	Carboxy Terminal End of Protein
CTP	Cytosine triphosphate
CV	column volume
CZE	Capillary Zone Electrophoresis
Da	Daltons
DHFR	Dihydro folate reductase

DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothretol
ECACC	European Collection of Cell Culture
EDTA	Ethylenediamine Tetra Acetic Acid
E_18	Project code given internally during cell line development work
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMEA	European Medicines Agency
EPO	Erythropoeitin
ERAD	Endoplasmic Reticulum Associated Degradation
ESI	Electron Spray Ionization
Fab	Fragment , antigen binding
FACS	Fluorescent Activated Cell Sorter
FBS	Fetal Bovine Serum
Fc	Fragment, crystalizable
FcR	Fc receptors
FDA	Food and Drug Administration
Fg	femtogram
FITC	Fluorescein Isothiocyanate
Fuc	Fucose
Fv	Fragment Variable
Fv	Fragment, variable
G0F	Glycan with no galactose but with fucose
G1F	Glycan with one galactose and fucose
G2	Glycan with two galactose
Gal	Galactose
GFP	Green Fluorescent Protein
GlcNAc	N-acetylGlucoseamine
Gly	Glycine
GS	Glutamine synthetase
GTP	Guanosine triphosphate
HAMA	Human Anti-mouse antibody
HAT	Hypoxanthine-adenine thymidine
HC / H1	Heavy Chain of anti-EGFR chain
HCl	Hydrochloric Acid
HCP	Host Cell Protein
HEK cells	Human Embryonic Kidney cells
HER	Human Epidermal Growth Factor Receptor
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
HIV	Human Immunodeficiency Virus

HPLC	High Performance Liquid Chromatography
HRP	Horse Raddish Peroxidase
HT	Hypoxanthine Thymidine
ICH	International Conference on Harmonization
IEF	Iso-electric focusing
IgG	Immunoglobin G
IL	Interleukin
IPA	Iso Propyl Acohol
IVCC/IVCD	Integral Viable Cell Count or Density
Kb	Kilo base pair
L	Liter
LB	Luria-Bertanii
LC / L1	Light Chain of anti-EGFR Mab
LC-MS/MS	Liquid Chromatography Mass Spectromety[2]
LD	Limiting Dilution
Lys	Lysine
LysC	Endoproteinase C
mAb	Monoclonal Antibody
MALDI	Matrix-assisted Laser Desorption/ionization
Man	Mannose
MAPK	Mitogen Activated Protein Kinase
MCB	Master Cell Bank
MCS	Multiple Cloning Site
MCT	Micro Centrifuge Tube
MEM	Minimal Essential Media (Earle's)
MES	2-(N-morpholino)ethanesulfonic acid
Met	Methionine
Mfg.	Manufacturer
Mg	Milligram
MilliQ water	Deionized water generated from MilliPore system
Min	Minutes
MOA	Mechanism of Action
MS	Mass Spectrometry
MSX	Methionine Sulfoxamine
MTX	Methotrexate
mV	milli volts
NA	Not Applicable
Na2S04	Sodium Sulfate
NANA	N-acetylneuramic acid
NDA	New Drug Application
NeuAc	N-acetylneuraminic acid (Sialic acid)
NGNA	N-glycolylneuramic Acid
NK cells	Natural Killer Cells

NSCLC	Non-small Cell Lung Cancer
N-terminal	Amino Terminal End of protein
OD	Optical Density
OPD	o-phenylenediamine
PA	Protein-A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCB	Primary Cell Bank
Pcd	picogram / cell / day
PCR	Polymerase chain Reaction
PER C6	Human Retinal cells
Pfu	*Pyrococcus furiosus*
PITC	Phenyl Isothiocyanate
PLA	Parallel Line Assay
PNGase F	Peptide: N-Glycosidase F
PQA	Product Quality Attribute
PTH	Phenyl Thiohydantoin
PTM	Post Translational Modification
PVDF	Polyvinylidene fluoride
QA	Quality Attribute
qP	Specific Productivity
R&D	Research and Development
RBC	Red Blood Cell
RCB	Research Cell Bank
RE	Restriction Enzymes
RMP	Reference Medicinal Product
RNA	Ribonucleic Acid
RNAi	Interferring Ribonucleic Acid
RP	Reverse Phase
RPMI Media	Rosewell Park Memorial Institure Media
RSD	Relative Standard Deviation
Rt	Retention Time
RT	Room Temperature
S8	Project code give during cell line development and further
SEB	Subsequent Entry Biologics
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
SF	Shake Flask
siRNA	small interferring Ribonucleic Acid
SP2/0	Myeloma Cell Line
Std	Standard
SV40	Simiam Vacuolating Virus 40

SY	Soyatone Yeast Extract
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween 20
TC	Tissue Culture
TCA	Trichloro Acetic Acid
TCF	Tissue Culture Flask
TEMED	N,N,N',N'-Tetramethylethylenediamine
Temp	Temperature
TGF	Transforming Growth Factor
Thr	Threonine
TK	Tyrosine Kinase
TMB	3, 3',5,5'-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
Tof	Time-of-flight
tPA	Tissue Plasminogen Activator
TTP	Thymidine triphosphate
UPS	Ubiquitine Proteosome System
US-FDA	United States - Food and Drug Administration
UV	Ultra violet
VCC / VCD	Viable Cell Count / Density
WB	Western Blot
WCB	Working Cell Bank
WCX	Weak Cation Excahnge
WFI	Water For Injection
XGPRT	Xanthine-guanine phosphoribosyl transferase

LIST OF SYMBOLS

µg	Microgram
µL	Micro Liter
µm	Micrometer
µM	Micro molar
^{0}C	Degree Celsius
α	Alpha
A^0	Angstrom
β	Beta
Fg	Femto gram
γ	Gamma
G	Gram
Λ	Lambda
L	Liter
M	Molar
mM	milli Molar
Ms	milli Siemens
mV	milli Volts
N	Normal
N	number of samples / replicates
Ng	Nanogram
nM	nano Molar
Nm	Nanometer
v/v	volume by volume

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CHAPTER 1

INTRODUCTION

1.1. Overview of Biosimilars

'Biopharmaceuticals' or 'Biologics' are defined as products manufactured by/from living organisms (involving bioprocessing) for the therapeutic use. This includes proteins (e.g. growth factors, antibodies), nucleic acids (DNA, RNA or antisense oligonucleotides) and living organisms like virus or bacteria (e.g. vaccines) or their toxins. This does not typically include products such as synthetic peptides or natural products chemically extracted from dead tissues (e.g., heparin, collagen). Since the approval of *Escherichia coli*-derived recombinant human insulin (Humulin) in 1982 and Chinese Hamster Ovary (CHO)-derived tissue plasminogen activator (tPA, Activase®) in 1986, recombinant protein therapeutics have revolutionized modern medicine (Munro, *et al* 2010). Biopharmaceuticals are produced from microbial cells (recombinant *E.coli*, yeast cells), mammalian cell lines (transformed stable cells), plant cell culture and moss plants in photobioreactors of various configurations. Protein therapeutics are rapidly growing, with more molecules getting added every year, which includes cytokines, serum proteins and monoclonal antibodies for human clinical use (Butler 2005). The total sale of biologics in the year 2010 has grown by 17% to US$ 107.7 billion versus US$ 91.78 billion in year 2009 (LaMerie 2010) this has potential to reach around US$ 200 billion by 2015 considering the fact that there are more than 400 biologic drugs in late stage clinical development (Munro, *et al* 2010). Together with vaccines and gene therapy products, the biopharmaceuticals represent half of the FDA-approved therapeutics in the pipeline. Therefore, therapeutic mAbs and derivatives have a considerable share in the market amongst pharmaceuticals.

Therapeutic Monoclonal antibodies (mAbs) are now the second largest category of biopharmaceutical products in development and are predominantly manufactured by mammalian cells in culture. These are well accepted class of therapeutics especially in the fields of oncology, immunology, and organ transplant. It has been predicted that oncology would be the largest therapy segment by 2016 in terms of % worldwide market share and % sales growth as depicted in Figure 1.1

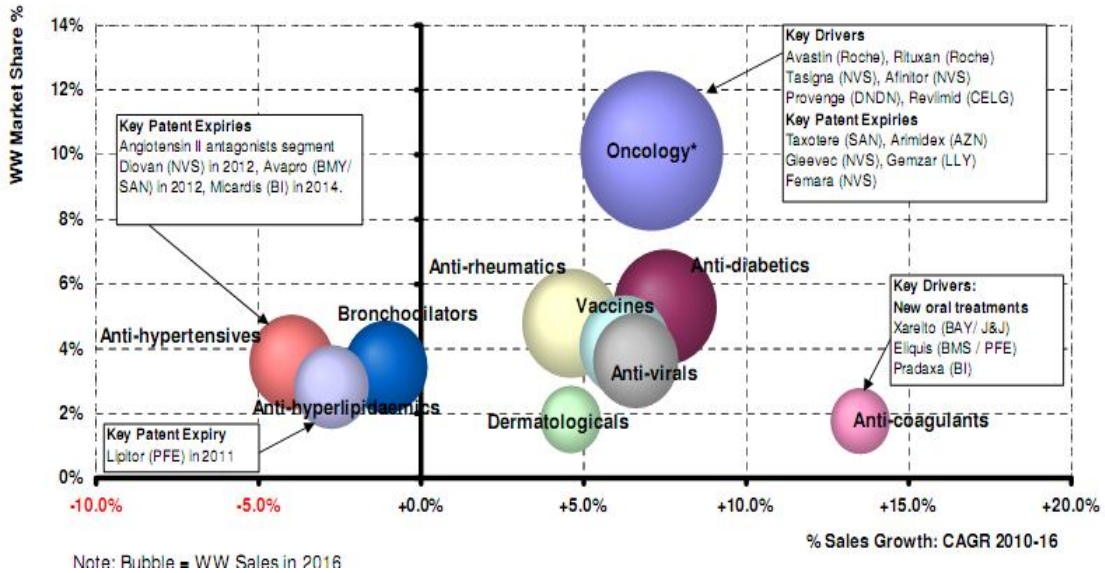


Figure 1.1 Analysis of top 10 therapy area in 2016, market share and sales growth (2010-16).
Ref.: (www.evaluatepharma.com).

The quality of life for patients has improved a lot by the use of the mAb targeted therapy with fewer side effects than the traditional cytotoxic drugs (Chu and Chartrain 2008). Till the end of year 2011 approximately 137 biopharmaceutical products are approved out of which around 32 mAbs or mAb fragments were approved by US-FDA. There were 12 full approvals for biopharmaceutical products in 2011 only. All of the qualifying products were approved as biologics (BLAs), except for 1 drug (NDA). Only 4 of the 12 (33%) approvals were for recombinant proteins, a relatively low number and percentage. This included 3 monoclonal antibody-based products (http://www.biopharma.com/approvals_2011.html).

The currently registered mAbs for human use are produced at commercial scale through mammalian cells that have been genetically engineered to over-produce the mAb of interest. One exception is the use of the bacterium *E. coli* in the production of mAb fragments. The production of mAbs at industrial scale is an integrated approach that encompasses many technically complex and lengthy steps. Typical cycle times for these multiple steps usually range between 16-24 months (Carroll and Al-Rubeai 2004).

"Biologics", considered one of the fastest growing sectors of the pharmaceutical industry, has introduced many new treatments to life-threatening and rare illness. The first generation of biopharmaceutical products manufactured using recombinant

technologies was launched in the 1980s, and they are now on the way to patent expiration. As a result, research-based and generic pharmaceutical companies alike are pursing the opportunity to develop "generic" substitutes for original biologics, herein referred to as Biosimilars (Sekhon and Saluja 2011). "Biosimilars" or "follow-on-biologics" are terms used in Europe and US respectively to describe officially-approved subsequent version of "innovator biopharmaceutical product" made by a different sponsor following patent and exclusivity expiry of the innovator's product. Biosimilars are also referred as Subsequent Entry Biologics (SEB) in Canada and "Biocomparables" in Mexico and, in this thesis "biosimilars". Reference to the innovator's product is an integral component of the approval process. The biologics due to the fact that it is derived from the living cell exhibit high molecule complexity and are very sensitive to even minor changes in the manufacturing process, which is commonly not seen in the small-molecule drugs. Major points of differences between generic, biologic and biosimilar drugs are tabulated in Table 1.1

Process	Biologic	Biosimilar	Generic
Manufacturing	• Produced by biological process in cell lines • Sensitive to production process changes-expensive and specialized production facilities • Reproducibility difficult to establish	• Produced by biological process in host cell lines • Sensitive to production process changes-expensive and specialized production facilities • Reproducibility difficult to establish	• Produced by using chemical synthesis • Less sensitive to production process changes • Reproducibility easy to establish
Clinical Development	• Extensive clinical studies, including Phase I-III	• Extensive clinical studies, including Phase I-III	• Often only Phase I studies
Regulation	• Needs to demonstrate "comparability" • Regulatory pathway defined by Europe (EMEA) • Currently no automatic substitution intended	• Needs to demonstrate "similarity" • Regulatory pathway defined by Europe (EMEA) • Currently no Automatic substitution intended	• Needs to show bioequivalence • Abbreviated registration procedures in Europe and US • Automatic substitution allowed

Table 1.1 Comparison of biologic, biosimilar and generic drugs.

Ref. Sekhon and Saluja 2011

Follow-on manufacturers or biosimilar developers donot have access to the Innovator's molecule clone nor original cell bank, nor they have access to the exact fermentation and purification process, nor to the active drug substance. They do have access to the commercialized innovator's drug product to be used as a reference molecule. Because no two recombinant cell lines developed independently can be considered identical, biopharmaceuticals are not fully copied. Structural differences, presence of impurities and/or breakdown products in the final formulation can have serious health implications.

The European Medicinal Agency (EMEA) has recognized this fact, which has resulted in the establishment of the term "biosimilar" in recognition that, whilst biosimilar products are similar to the innovator product, they are not exactly the same. The changes occurring in the molecule due to minor changes in cell processivity and environment in which the biosimilar is been produced has created a concern and doubt for the drug regulatory authorities whether copies of biologics could perform similar to the original branded version of the product. Generally for any pharmaceutical product, development guidelines are published by different drug regulatory authorities like India, Europe, US, Japan, etc. to ensure the quality of the product going to patients. However, because of the complexity of biologics US FDA has yet not came up with guidelines. This stems mainly from the difficulty in providing sufficient analytics to completely define something that is essentially 'acceptably heterogenous' Thus, developing guidelines to ensure safety and efficacy while providing a simplified approval pathway has been challenging (Munro 2010).

Biosimilar Drug Development is a multi-stage process requiring careful selection of the best candidate clone, process, materials and methods through out the development with the main objective of maintaining close similarity to the "Innovator's Drug Product". These stages are shown in Figure 1.2 and the stages worked during this project are explained under individual chapters of this thesis.

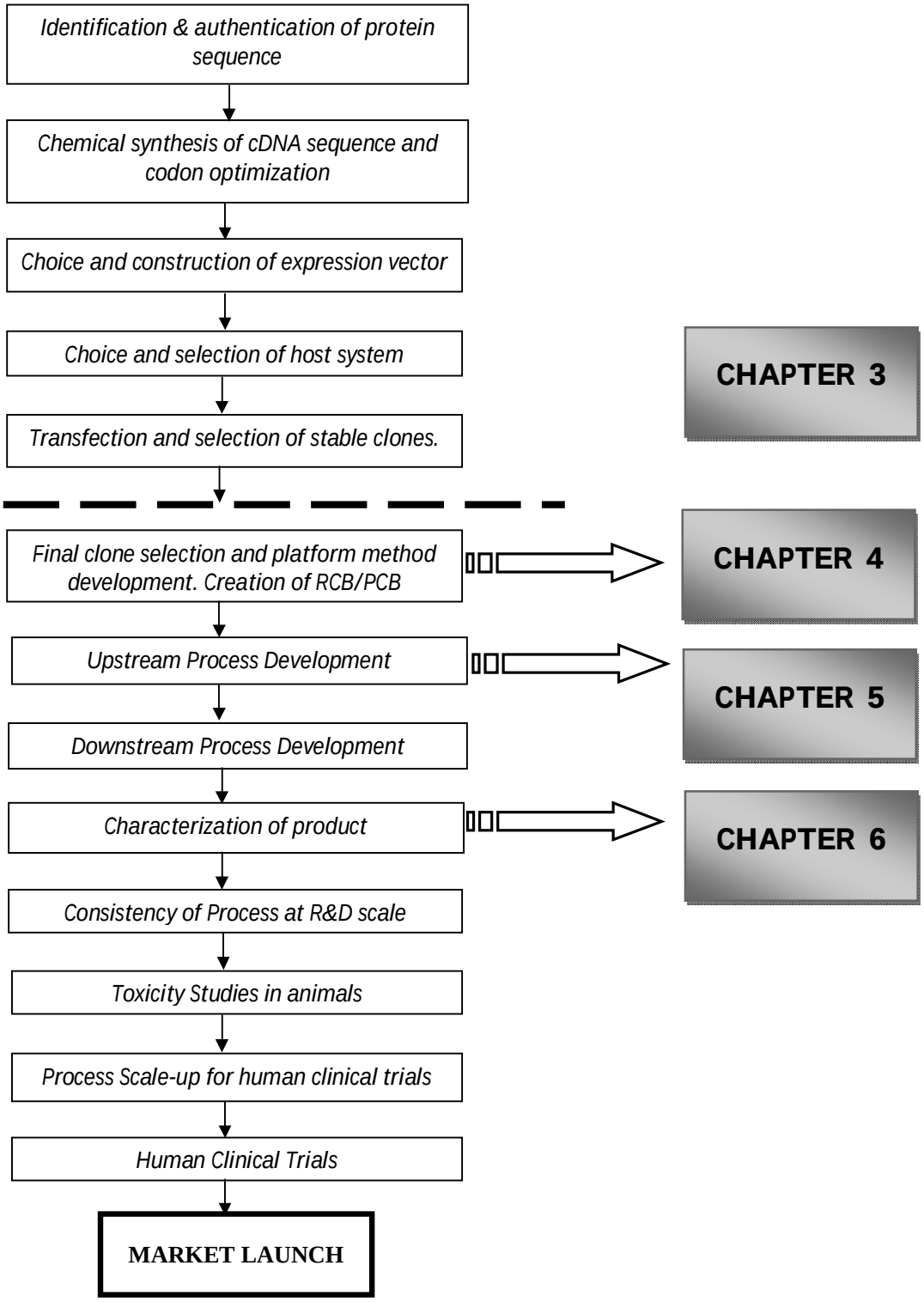


Figure 1.2 General process flow chart for biosimilar product development.

The work carried during this work is highlighted.

Manufacturing processes for the production of therapeutic recombinant proteins generally involve a complex series of steps each having a dramatic impact on downstream protein quality and, ultimately, the safety and efficacy of the final product.

An indepth understanding of how formulation and process factors affect product quality and bioactivity is required to be studied and designed to yield "well-characterized biologics" or "defined biologics" (Henry 1996). This requires advanced analytical methodologies to unambiguously confirm protein sequence, structure, and bioactivity. Typically the functional areas depicted in Figure 1.3 come into play during the development of a biopharmaceutical product to achieve quality product (Srebalus Barnes and Lim 2007).

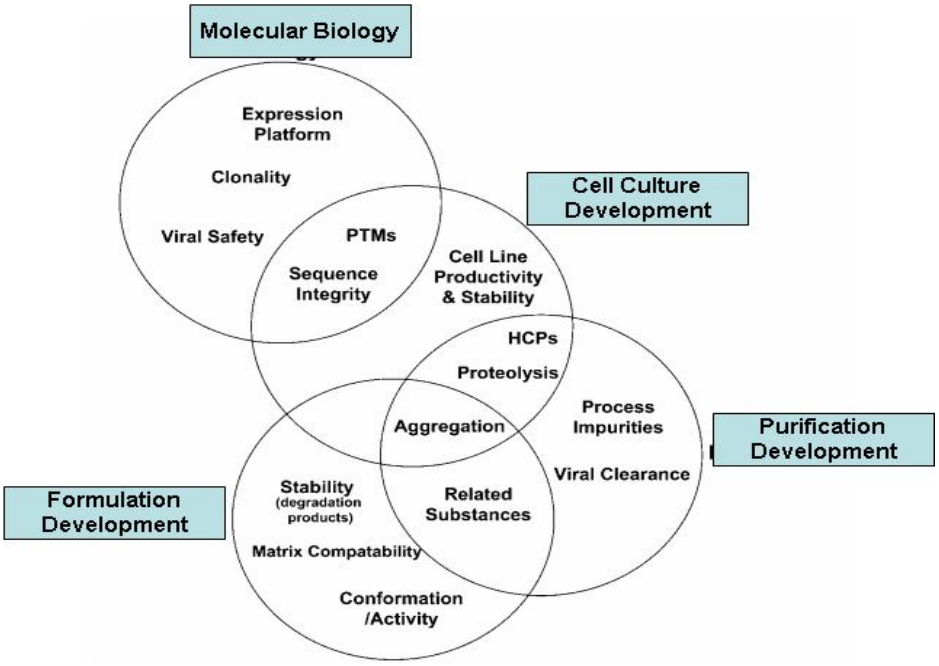


Figure 1.3 Interplay of various functional areas during biopharmaceutical development.
The process or product quality attributes are generally addressed by each group. Attributes in overlap regions represent product quality or process robustness concerns that are common to multiple development groups. PTMs = post-translational modifications and HCPs = host cell proteins.

1.2. Biologics versus Small-molecule drugs

The major difference between biologics and small molecule arises from the way they are manufactured. Chemical methods are generally used to produce small-molecule drugs whereas biological products are synthesized usually by cells or living organisms. This difference in origin leads to difference in structure, composition, manufacturing methods and equipment, intellectual property, formulation, handling, dosing, regulation and marketing (Marshall, *et al* 2003). The differences are listed in Table 1.2 as adopted from (Sekhon and Saluja 2011).

Comparison Property	Small Molecule Drugs	Biologics Drugs
Product-related differences	• Produced by chemical synthesis • Low molecular weight, 100 - 1000 Da • Stable • Single entity, high chemical purity, purity standards well established • Administered through different routes of administration • Rapidly enters systemic circulation through blood capillaries • Distribution to any combination of organ/tissue • Often specific toxicity • Often non-antigenic	• Biotechnologically produced by host cell lines • High molecular weight, 10,000-150,000 Da • Complex physiochemical properties • Sensitive to heat and shear (aggregation) • Heterogeneous mixture, broad specifications which may change during development, difficult to standardize • Usually administered parenterally • Larger molecule primarily reach circulation via lymphatic system, subject to proteolysis during interstitial and lymphatic transit • Distribution usually limited to plasma and/or extra-cellular fluid • Mostly receptor mediated toxicity • Usually antigenic
Manufacturing Differences	• Completely characterized by analytical methods • Easy to purify • Contamination can be generally avoided, is easily detectable and removable • Not affected by slight changes in production process and environment	• Difficult to characterize • Lengthy and complex purification process • High possibility of contamination, detection is harder and removal is often impossible • Highly susceptible to slight changes in production process and environment

Table 1.2 Distinguishing features of Small-molecule drug and Innovator's Biologics

When compared on size matter biologics are 100 to 1000 times larger possessing several hundred amino acids (average molecular weight of 150 per amino acid). In contrast, small-molecule drugs are far smaller, i.e., molecular weight <1000, self-contained, generally organic or inorganic molecules, that are, usually, chemically synthesized (Revers and Furczon 2010a). Biologics been bigger molecule, the greater number of atoms makes up its structure and hence greater is the complexity (Figure 1.4). Biologics attend complicated 3D structure when the primary (amino acid sequence) gets arranged in secondary (α-helix and β-pleated sheet) structures, which results in tertiary structure to be biologically active (Crommelin, *et al* 2003). In some biologics, stable associations of tertiary structures of individual proteins form a quaternary structure. These molecules, during synthesis by cells, often further modified by post-translational modifications such as glycosylation or sialylation, which may be crucial for biological activity (Revers and Furczon 2010b).

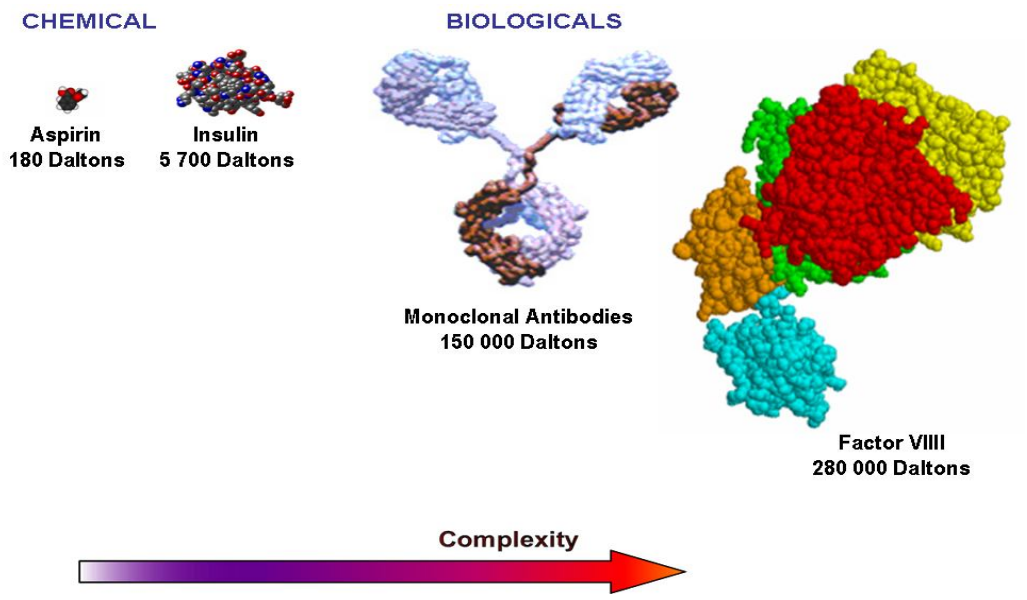


Figure 1.4 Biologics versus small molecule drug.

For small molecule-drug, aspirin is taken as example while for biologics a low molecular weight molecule, insulin and high molecular weight molecule, antibody is depicted.

Although there is a tremendous advancement in the development of novel techniques for characterizing structural and physicochemical properties, the inherent complexity of biologics means that the picture will be incomplete even if all available methods are used. Practically to characterize biologics fully is impossible and may vary with different manufacturing processes (Goldsmith, *et al* 2007). Moreover, biologics

production methods are also more complex, involving several steps and subject to variations affecting the biological and clinical properties of the drug substance. In contrast, traditional small-molecule drugs have a well-defined and stable chemical structure, which can be completely characterized by analytical methods (Crommelin, *et al* 2005) in fact fewer analytical methods than used for biologics. The most critical difference between biologics and small-molecule drug is their immunogenecity. Antibody response is induced by almost all therapeutic proteins, irrespective of whether these proteins are (partly) non-human or completely human homologs, because of microheterogenity exists from different processes. At times these proteins can induce severe side effects by neutralizing endogenous counterparts or homologs (Schellekens H, *et al* 2010). The above described complexity of biologics post critical commercial challenges compared with small-molecule drugs – the most important of which pertains to developing a viable pricing, distribution and reimbursement model that is intrinsically geared to the special characteristics of biologics and the expectations of a diverse customer population. Due to the fact that, biologics now comprise about 33% of the medicines approved, understanding whether their biological differences translate into understanding of safety, economics and anticipated public health improvements. Biologics overall differ from the small-molecule drugs in their innate biology, production, and dosing regimes, leading to a major differences in product development, regulatory approval, distribution, and commercial paths. Thus, although they have the same medical goal – to treat disease – small-molecule and biologics therapeutics differ substantially in ways that might affect innovation, safety, costs, clinical adoption, patient access and pricing (Sekhon and Saluja 2011).

1.3. Production Platforms

The earliest technology of microbial fermentation is used till date for the manufacturing of many less complex, recombinant proteins, such as industrial enzymes, and few cytokines at a fraction of the cost of mammalian cell-derived biologics. Mammalian cell line engineering has traditionally had only modest success; however, the recent advent of new molecular and 'omics' tools should change this equation by enabling the field of rational cell engineering to mature. Highly optimized host cell lines would provide an efficient platform for maximal low cost biosimilar production. Pharmaceutical and biotechnology industries are eyeing on the growing demand for biologics and the end-of-

patent protection for many existing treatments. The focus for biosimilar equivalent will be speed and/or cost. With the emergence of the new blockbuster biologics, research is widely spread in the areas of developing new expression and host cell technologies in order to get higher and higher productivity so as to reduce manufacturing cost and hence cost to the patient. These new technologies may also create an avenue for the creation of biosuperiors (or biobetters), which represent enhanced versions of the innovator product. However, till today the best-practice for industry during early cell line development still relies heavily on traditional amplification systems in combination with immortalized mammalian cell lines such as CHO, SP2/0 and NS0 cell (Wurm 2004). A novel mammalian cell line such as human retinoblast cells (PERC6®) is now being explored in order to achieve higher productivity and quality (Paul, *et al* 2001). In addition, alternative eukaryotic hosts such as glyco-engineered yeast (Wildt and Gerngross 2005) and insect cells (Coxx and Holister 2009), or even transgenic plants and animals are being explored. These new expression systems will likely catalyze the progression of the biosimilar market in future.

Although many immortalized mammalian cell lines can be potentially used, since they were in use at the time the first mAbs were developed for clinical and commercial applications; as of today, with the exception of the two mAbs produced by hybridoma technology, the production of all currently commercialized therapeutic mAbs is achieved in mammalian cells of mouse (NS0 and SP2/0 cell lines) or Chinese Hamster Ovary (CHO) cell origin. A few selected commercialized mAbs and their respective production platform is listed in Table 1.3:

CHO Cells	**NS0**	**SP2/0**	**Murine Hybridoma**
Avastin, Campath, Herceptin, Humira, Raptiva, Rituxan, Vectibix, Xolair, Zevalin, Campath, Prolia, Actemra, Adcetris, Yervoy	Mylotarg, Soliris, Synagis, Tysabri, Zenapax, Benlysta, Arzerra,	Erbitux, Remicade, Reopro, Ilaris, Simponi	Bexaar, Orthoclone Simulect,

Table 1.3 Commercialized mAbs Production cell platforms

1.3.1. NS0 and SP2/0 Cell Lines

These cell lines originated from mouse plasmacytoma cells that have undergone several steps of cloning and selection to yield immortalized non-IgG secreting B cells (Kohler and Milstein 1976; and Kohler, *et al* 1976). NS0 and SP2/0 are manipulated using molecular biology techniques so that they produce mAbs for human use at industrial scale. These cells can be cultivated in serum containing or serum-free media and are reasonably amenable to scale-up in large bioreactors (upto 20,000L scale). However, there are some drawbacks that complicate their cultivation. Unlike most mammalian cell lines, NS0 cells require addition of cholesterol, which is usually derived from serum (Sato, *et al* 1984 and 1987). The use of NS0 cells are undesired on a technical and regulatory front as requirement of cholesterol for growth which is of animal origin is essential. Cholesterol is not soluble in protein-free medium as it requires the use of carriers such as cyclodextrins to enhance cholesterol "solubility" (Gorfien, *et al* 2000 and Walowitz 2003). A lot of research has gone in to develop cholesterol independent NS0 cell lines which could be used industrially (Hartman, *et al* 2007) but, little progress is made to establish commercial production of mAbs using NS0 cholesterol independent cells. These modified NS0 lineage can offer definite advantage of simplicity and use at industrial scale in future once cholesterol independency is addressed.

One of the most discussed aspects in biologics is the glycosylation of produced molecule because it varies from cell lines to cell lines and process to process, but reasonably it is similar in the CHO, NS0 and SP2/0 cell lines. The point of concern is regarding glycosylation pattern distribution ratio (G0F, G1F and G2F) of the IgGs produced by NS0 and SP2/0 which is not similar to that of circulating human IgGs. In addition, these cells produce small amounts of murine-like glycans such as the addition of an extra-galactose (α-Gal) to the terminal galactose and the insertion of N-glycolyneuraminic acid (NGNA) in place of N-AcetylNeuraminic Acid (NANA) (Raju 2003), which have the potential to trigger an immune response. Because of these minor changes in glycosylation (e.g. NGNA) some clinical adverse events and anaphylactic shock, have been reported for mAbs (such as Cetuximab) produced by cultivation of SP2/0 cells (Chung, *et al* 2008). However, there is consistency in the glycoforms observed in mAbs expressed by NS0 and SP2/0 cells because of which they have been used for some products. The difference in the glycosylation pattern is depicted in Figure 1.5 (Beck, *et al* 2008).

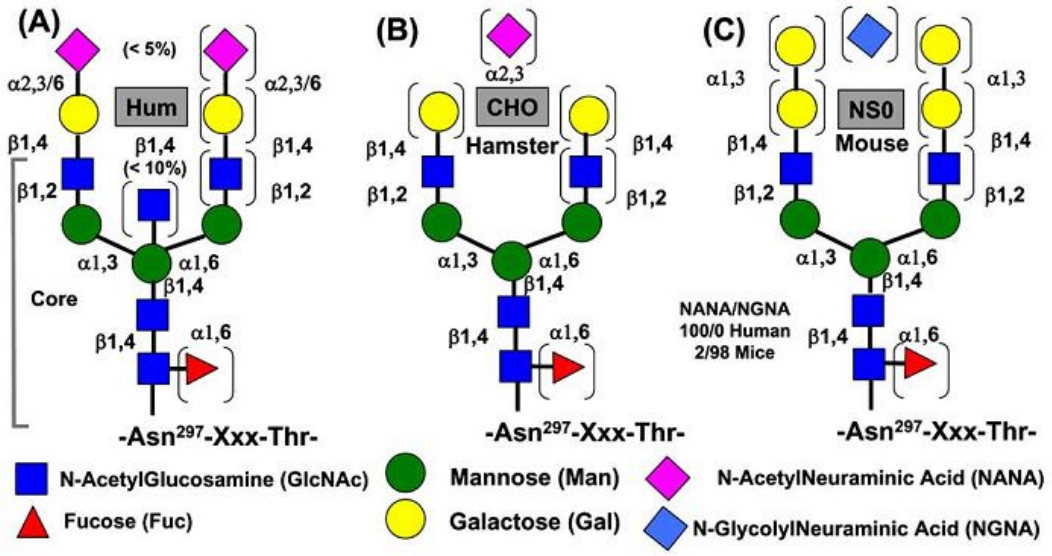
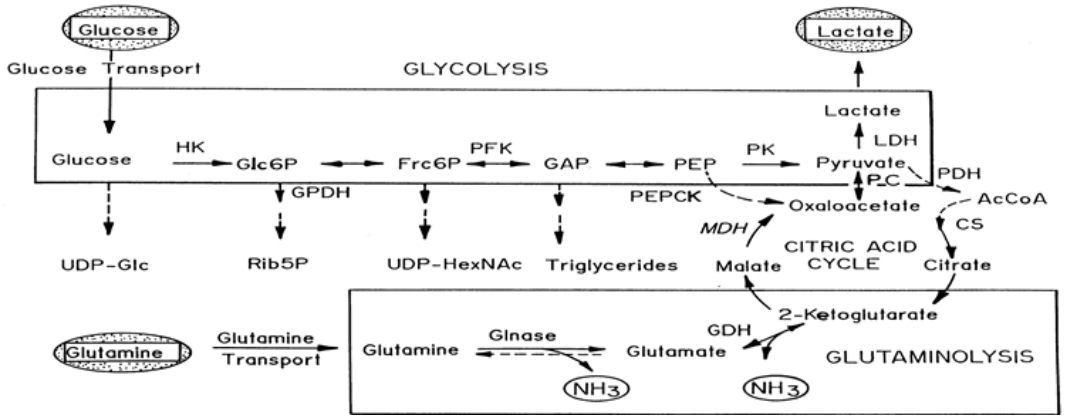


Figure 1.5 Structure and nomenclature of the carbohydrate moieties typically observed in recombinant monoclonal IgG molecules expressed in different cell lines.

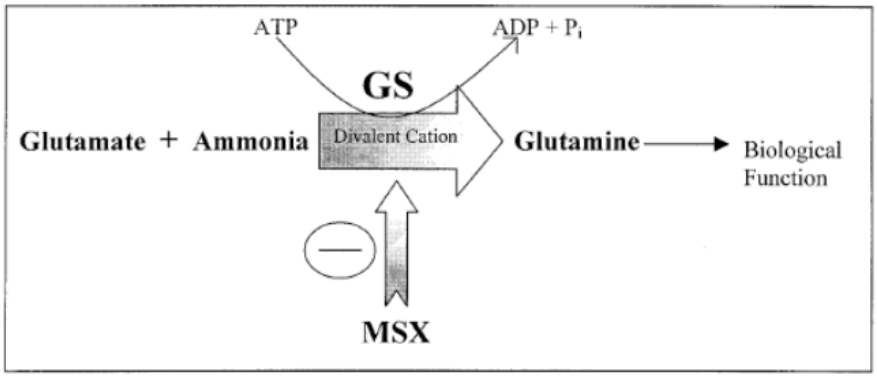
In pursuit of developing cell line expressing mAb *trans*-gene, the host cell generally is rendered metabolically deficient or sensitive to the antibiotics are employed. The overall philosophy is to use cells with a deficient metabolic background; while the genetic element containing the mAb coding region carries a gene that once expressed will complement the metabolic deficiency. Various mode of transfection are used to deliver one or several copies of the genes carried on the plasmid which will integrate into one or several chromosomes of the recipient cells where they will be transcribed and translated. Post-transfection and during clone selection, a selective pressure aimed at favoring those cells that have incorporated the gene coding for the correction of the metabolic deficiency or resistance to the antibiotics is applied in order to favor the growth of those cells that have integrated the foreign DNA (Chu and Chartrain 2008). Several variations on this strategy are described below.

A popular NS0 cell lineage is the GS-NS0 commercialized by Lonza Biologics. The Glutamine Synthetase (GS) is also used while using SP2/0 and CHO cells and is also used in this work plan. The role of glutamine and its generation from the glutamate available in the cell culture media is described in Figure 1.6:

A)



B)



C)

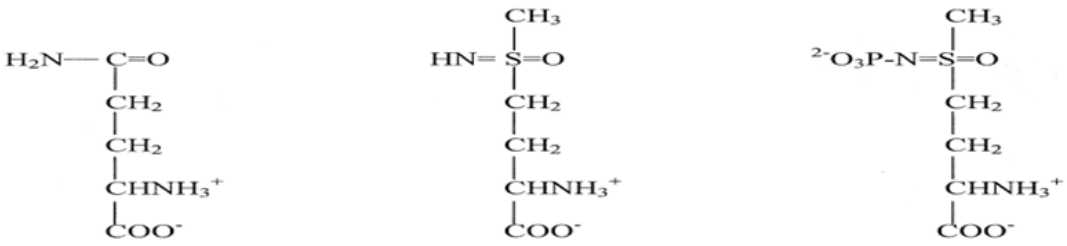


Figure 1.6 **Glutamine synthesis pathway**

A) Role and entry point of glutamine in the biochemical pathway

B) Conversion of glutamate to glutamine by glutamine synthetase enzyme

C) Structure of glutamine and glutamine analogue, methionine sulfoximide (MSX)

NS0 cells have very low levels of the endogenous glutamine synthetase (GS), and require exogenous glutamine in order to grow. It is only when transfected with a copy of the GS gene that the cells can grow in the absence of glutamine (Barnes, *et al* 2000).

A vector, designed to carry both the genes (heavy and light chains) coding for the mAb to be expressed and for GS, once integrated into the genome will allow the expression of mAb and GS genes and thereby enabling selection of producing clones. In order to select the cells with integrated foreign DNA in high expressing region of chromosome and to reject the non-expressing cells or cells without foreign gene integrated in a region of high expression; the cells are cultivated in a medium devoid of glutamine and in the presence of methionine sulfoximide (MSX), an inhibitor of GS. Only those clones that produce large amount of GS will survive. Correlatively, these clones are likely to also produce large amounts of the mAb. Interestingly this approach seems to yield high producing clones that have integrated low copy number (less than five) of the *trans* gene (Bebbington, *et al* 1992). This GS system is also used in SP2/0 cells for high-level expression of mAb genes. In this study also GS was used with SP2/0 and CHO cells.

Another method of selection relies on the fact that SP2/0 and NS0 cells are deficient in Hypoxanthine-Guanine Phosphoribosyl Transferase (HGPRT). This negative genetic trait can be exploited by inserting a copy of a microbial gene (from *E.coli*) coding for the synthesis of XGPRT, an enzyme that can substitute for HGPRT. Only the cells that have integrated the XGPRT gene will be able to use the nucleotide synthesis salvage pathway. When cultivating the transfectants in a selective medium containing aminopterin which inhibits the *de novo* nucleotide synthesis pathway, only those cells that have incorporated the XGPRT gene will grow. Since the gene coding the mAb of interest is co-located with the XGPRT gene on the plasmid, the probability of co-integration is high, and transfectants expressing high levels of IgG are likely to be selected by using this method (Mulligan and Berg 1981).

SP2/0 cells were used for the production of therapeutic proteins like EPO where the glycosylation is required for the *in-vivo* biological activity of protein. High level of expression was observed after transfection without gene amplification procedures. In addition, the purified recombinant hEPO showed sufficient *in-vitro* biological activity but the *in-vivo* activity was very low which could be due to the fact of low molar concentration of terminal sialic acid (Sugaya, *et al* 1997). Hence for any heavily glycosylated protein SP2/0 may not be suitable for expression and production because of difference in the carbohydrate composition and structure.

1.3.2. CHO Cells

Chinese Hamsters (scientific name, *Cricetulus griseus*) belong to a family of rodents that are native to the deserts of northern China and Mongolia. Since 1919 they are used in life-saving biomedical research and were first used as laboratory specimen replacing mice for typing pneumonia cocci (Box). Chinese hamsters are known as carriers of the deadly parasite *Leishmania* spp. which cause Kala-azar (also known as black fever of leishmaniasis), because of this fact in early 1920s, they gained reputation as valuable tools in epidemiological research. In 1948, they were literally smuggled into the U.S. by Dr. C.H. Hu and Dr. Robert Watson (Jayapal, *et al* 2007). Subsequent efforts at domestication by Dr. George Yerganian and others in the mid-20th century led to the development of spontaneous hereditary diseases due to inbreeding, spurring research interest in hamster genetics. Chinese hamsters were found to be useful models in study of radiation cytogenetics, metabolic studies and tissue culture because of the low chromosome number (2n=22) (Deaven and Petersen 1973). In 1957, Dr. Theodore T. Puck of the Department of Medicine at the University of Colorado received Chinese hamster from Dr. Yerganian laboratory at Boston Cancer Research Foundation for investigating the usefulness of various cells in somatic genetics. At this time Dr. Puck isolated an ovary from a female Chinese hamster and established the cells in culture plates (Puck, *et al* 1958). The ovary cells thus, isolated was observed to be proline auxotrophs and have been spontaneously immortalized during their successive transfers (Kao and Puck 1967 and 1968) and functionally hemizygous for many genes primarily due to gene inactivation (Chasin and Urlaub 1975; Simon, *et al* 1982). These cells are easily grown in either monolayer or suspension culture may be synchronized by a variety of techniques (Petersen, *et al* 1969; Enger and Tobey 1972). CHO cells have been extensively in studies for mutagenesis in mammalian cells (Kao and Puck 1969), cellular radio-sensitivity (Walters and Petersen 1968), linkage relationships in cell hybrids (Kao and Puck 1970), metabolism of macromolecules (Gurley and Hardin 1968; Zapisek, *et al*. 1969) so much so, that they have been termed as the mammalian equivalent of the model bacterium *E. coli.* Lately, mutants deficient in the dihydrotetrafolate enzyme (DHFR) and adenine phosphoribosyl transferase (APRT) were isolated (Taylor, *et al* 1977; Urlaub and Chasin 1980). In addition, mutants defective in transcription-RNA Polymerase II (Chan, *et al* 1972), thymidine mutants (Adair and Carver 1979) translation and machineries for certain amino acid-Asparagine (Goldfarb, *et al* 1977; Wayne and Stanner 1979) was also isolated. The DHFR enzyme is widely used in the biopharmaceutical industry for the selection of clones. This enzyme is required for the

synthesis of reduced tetrahydrofolate, a cofactor required in the synthesis of DNA precursors. Two lineages, DG44 and DUKX-B11 which are CHO-*dhfr* minus are available and used at commercial scale. These lines were developed in Urlaub and Chasin's Lab at Columbia University (Urlaub and Chasin 1980; Urlaub, *et al* 1983; Urlaub, *et al* 1986).

DHFR is a small monomeric enzyme that catalyzes the conversion of folic acid, a common vitamin, to tetrahydrofolate (THF). The latter is a cofactor carrier for one-carbon moieties required in various biosynthetic reactions, particularly synthesis of glycine, purine and thymidine.

This selection strategy is similar to that of the GS system described previously except here the inhibitor of DHFR, methotrexate (MTX) is used. Since, DHFR deficient cells are triple auxotrophs for glycine, hypoxanthine (a purine derivative) and thymidine, introduction of heterologous genes into cells can be accomplished by co-transfection with a functional copy of the DHFR gene, which obviates the need for these nutrients. The expression plasmid containing mAb *trans* genes along with the DHFR gene is transfected and cells which have integrated this cassette stably will only grow and produce mAb of interest in presence of MTX (Kaufman, *et al* 1985). With the increase in concentration of MTX, the *trans* gene copy number increases and hence the selection of high producing clones increases (Kaufman and Sharp 1982). The DHFR selection strategy can be enhanced with the use of resistance to aminogalactoside antibiotics such as neomycin and kanamycin conferred by an added resistance gene on the plasmid (Sautter and Enekel 2005). However, using DHFR selection strategy often leads to genetic instability of the recombinant cell line.

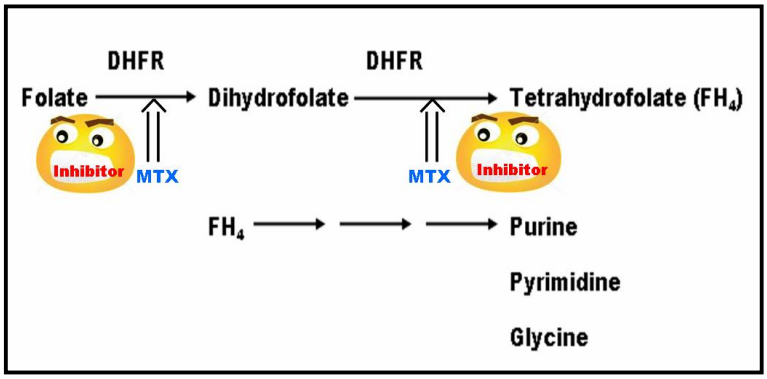


Figure 1.7 Biochemical reaction catalyze by DHFR.

The yellow color cartoon represents inhibition of reaction by addition of an inhibitor, MTX.

The parental CHO-K1 cells possess low GS activity hence the system described for NS0 and SP2/0 cell line development could also be used for CHO-K1. The flow of steps starts with transfection of cell line with plasmid bearing *trans* genes for mAb and GS gene followed by cultivation of cells in absence of glutamine and in the presence of MSX allows growth of cells with stable integration of plasmid. Since the expression of GS is present at a very low level, this system requires the presence of MSX during the cell expansion in order to keep a sufficient genetic pressure to prevent deletion of the foreign DNA (Bebbington, *et al* 1992).

These two strategies allow rapid selection of high producing clones. Due to the fact that CHO cells are robust in terms of growing either in adherent or suspension form, they can rapidly adapt to serum and protein-free chemically defined medium in large scale bioreactors. In contrast to NS0, CHO cells do not require cholesterol and tend to remain viable for a longer period of time. This ease of genetic selection coupled with their robust and scaleable growth at an industrial level have resulted in the CHO cell line being used in more than half of the registered mAb production processes (Chu and Chartrain 2008).

CHO cells being of rodent origin, the glycosylation pattern distribution ratio (G0F, G1F, G2F) of mAbs do not completely match with the circulating human IgG_1 (Figure 1.5). In addition, CHO cells produces a small amounts of non-human like glycan patterns, such as α2-3 linked sialic acid residues that have the potential to be immunogenic (Raju 2003). But, these forms are present in very low proportions and mAbs produced from CHO cells have shown remarkable safe profiles in the clinic (Roshos, *et al* 2004).

The choice of host cells for protein expression should be judiciously done and has a direct impact on product characteristics and maximum attainable yields. Protein folding and post-translational modifications conferred by the hosts dictate the pharmacokinetics and pharmacodynamic properties, and hence their solubility, stability, biological activity and residence time in humans. Product safety is another key aspect that must be considered in choosing host cells. The production host must not allow the propagation of any adventitious pathogenic agents that may eventually find their way into humans. From an industrial perspective, the ability to adapt and grow cells in suspension instead

of adherent cultures is highly desirable as it allows volumetric scalability and use of large stirred-tank bioreactors. Finally, the host cells must be amenable to genetic modifications allowing easy introduction of foreign DNA and expression of large amounts of desired protein. CHO cells are now there in industry for twenty five years and experience with it has demonstrated that, to a large extent, they possess many of these characteristics (Jayapal, *et al* 2007). CHO cells have a proven tract record for producing number of recombinant proteins and mAbs with glycoforms that are both compatible and bioactive in humans. One of the early concerns in recombinant protein production was that cultured mammalian cells were presumably derived through perturbation of oncogenes, and thus, can proliferate without the effects of senescence. However, CHO cells have been proven safe, with the value of products being generated considerably outweighing any associated risks.

Considerable development work has been done in downstream purification processes for CHO cell products which resulted in purified product of interest with not more than picogram levels of contaminating CHO DNA per dose of the product (Wurm 2005). A study carried out by Wiebe and group, who tested 44 human pathogenic viruses, majority of them, including HIV, influenza, polio, herpes and measles do not replicate in CHO cells (Wiebe, *et al* 1989). CHO cells are quite adaptable and convenient for genetic manipulation and can grow to very high densities in suspension cultures that are readily scaled to >10,000L bioreactors. From a regulatory point of view, CHO cells have gone under extensive testing and safety data accumulated to satisfy the needs. The most recently published CHO-K1 genome sequence will make the regulatory hurdle easier (Wang, *et al* 2011).

In this study GS selection system was used tried to develop recombinant CHO and SP2/0 cell line expressing anti-EGFR mAb.

1.3.3. Cell Lines to look in future

The scope of this project was to produce mAb using mammalian cell lines. Although CHO cells are the most prevalent for producing glycoprotein therapeutics, other cell lines such as human embryonic kidney cells (HEK) (Durocher, *et al* 2007), baby hamster kidney cells (BHK) and human retinal cells (PER.C6®) (Jones, *et al* 2003; Wurm 2004; Petricciani and Sheets 2008) are being developed as high producing cell lines. HEK cells are generally used for transient expression of protein at high levels, however,

advancement has lead to establishment of stable cell lines particularly for blood factors and neuronal proteins. At industrial scale it is used for production of blood factors and Activated Protein C (APC). These proteins possess post-translational modifications such as presence of unusual amino acids, including γ-carboxyglutamic acid and β-hydroxyaspartic acid which are generally not processed by CHO cells (Ehrlich, *et al*. 1989). BHK cells are used generally for vaccine production and not very famous in the biosimilar industry. The PerC6® cell line appears to be most advanced in its usage and acceptance. PerC6® are human retina cells that were immortalized by the use of the early gene E1 of Adenovirus (Cramer, *et al* 1998). This cell line offers the potential for human like glycosylation pattern with the added advantage of a lack of undesired murine glycan. This cell line is cultivated to very high densities at large scale and that they are capable of supporting elevated recombinant protein yields of upto 10 g/L of a mAb (Jones, *et al* 2003; Yallop, *et al* 2005). With this expression yield and glycan structure close to humans it has gained focus in the recent time. However, as of today, no mAb produced from PER.C6 has been licensed. Unlike well established cell lines such as CHO, NS0 and SP2/0 that have been used for the production of several commercial mAbs, new and previously unregistered cell lines are likely to face higher regulatory scrutiny. This is especially true on the topic of associated viruses, and in the clearance of residual genetic material.

1.4. Clone Selection Approach

The cells after cultivation under selection pressure are rapidly subjected to cloning for which various methods are available. Cloning of cells to get a homogeneous cell population which will have consistent and predictable growth, in addition most importantly it is essential for ensuring that the produced mAb molecule will have high homogeneity. Clonality is therefore a requirement emphasized by regulatory authority. The most widely used, simple and popular method is the use of limiting dilution where one or less than one cell is dispensed per culture well. The cells are allowed to grow under the desired selective conditions until confluence is observed. This is achieved by classical dilution and estimation of cell population based on viable cell counts. This method is labor and time intensive. Recently the use of automated cell sorting equipments (Flow cytometer, ClonePix, etc.) are in vogue to make the single cell isolation and selection simpler and time saving. This method is simple and predictable, but is more expensive and less labor intensive. Several technologies have evolved with

the aim to directly identify potential high producing cell in addition to simple sorting. One approach has been to estimate the secretion potential of a cell by entrapping it in a gel. After an incubation period, the secreted entrapped mAb is detected using a fluorescent-labeled antibody. The entire gel drop, containing cells and their respective captured secreted product, are sorted by the flow cytometer according to their surrogate level of mAb production, thereby potentially achieving a rapid selection (Browne and Al-Rubeai 2007; Caroll and Al-Rubeai 2004; Bohm, *et al* 2004). Other approach to estimate the expression of mAb is to detect intra-cellular co-expression of DHFR (Yosida 2001) which is linked with the mAb genes. However, this will not give the true picture of the secretion efficiency of the cells (DeMaria, *et al* 2007).

The production potential of the cells could be better captured by the methods which rely on the cultivation of the cells on a soft agar surface as in ClonePix method, which retains the antibody secreted in the cell vicinity, and detected by using labeled antibody. The high expressing clones then can be directly picked and cultivated for further evaluation. The potential advantage of this technique over the FACS based methods is that it subjects the cells to high shear stress; it offers a more cell-friendly environment and captures antibody secretion over a longer period of time, thereby increasing accuracy of detection (Burke and Mann 2006; Hanania, *et al* 2005).

Cell Clonality could be ensured by microscopic observation of each well during the incubation period. The clonal populations were further cultivated in individual larrge cell culture vessels to detect the potential of expressing mAb. The high producer clones were then tested for the product quality (eg. glycan distribution, correct amino acid sequence, etc.). The best producing clones are selected for laboratory scale cultivation under defined conditions (Kunert, *et al* 2004). The high expressing clones giving desired quality product were further re-cloned either by limiting dilution or by sorting the cells using a flow cytometer or any other automated method.

Following second round of cloning, the best producing clones (usually 3-5) are selected for clone stability studies. Since the mammalian cell genome is very dynamic which tend to reject, modify or relocate integrated foreign DNA, it is important to ensure that the clone selected will produce the mAb of interest for a period of time desired based on the total production cycle time calculated theoretically keeping in mind the doubling

time of the cells. Clone stability is not only measured in terms of production levels but also on the biochemical attributes of the mAb such as sequence integrity and fidelity in the glycosylation pattern (Berthold 1993). The clone stability is determined by continuous cultivation of cells for extended number of generations under conditions that mimic process conditions as closely as possible and generations beyond the targeted number in manufacturing process. Over this growth period reduction in the amount of mAb expressed is commonly observed as well as changes in some of the biochemical characteristics of the molecule may be observed (Barnes, *et al* 2004; Strutzenberger, *et al* 1999; Barnes, *et al* 2003; Barnes, *et al* 2001; Barnes, *et al* 2006). The most important parameter in selection of the candidate clone is maintenance of biochemical profile of the molecule, as quality attributes are essential to the safety and potency of the mAb. Once these steps are completed, the selected clone is preserved in a set of cell banks that upon testing and qualification (purity, lack of adventitious agents, etc.) will be used for commercial production of the therapeutic mAb.

These procedures are lengthy, require a highly specialized and competent workforce, expensive equipment and therefore weigh heavily on the overall product development cycle time, as these activities are performed on the critical path. This is where the advantage of integrating novel technologies can be of great impact since they have a high probability of reducing the extent and duration of the screening activities.

1.5. Antibody Structure and Function

The antibody therapeutics ("Magic Bullets") could be developed to selective target disease was first hypothesized by Paul Erhlich in 1906. This hypothesis became practical with the development of hybridoma technology by Kohler and Milstein (Kohler and Milstein 1975). A short brief about the antibody structure and function is described below before gaining understanding the therapeutic mAbs structure and function.

1.5.1. Structural Characteristics

Typically an antibody (Immunoglobulin) are heterodimeric protein molecule formed by linking two molecules of light chain (green color in Figure 1.8) and two molecules of heavy chain (pink color in Figure 1.8) with the help of a disulfide bond. The Y-shaped structure of antibody is formed by inter- and intra-molecular disulfide bonds (yellow in Figure 1.8). Antibody (Ab) is also known as immunoglobulin as it is a globular protein

with immune function. Ab is produced by B-cells as humoral immune response to the foreign antigen such as bacteria or viruses entering the body thereby neutralizing the foreign antigen. The total molecular weight of Ig is ~150 kDa; light chain of ~25 kDa and heavy chain of ~50 kDa size. The light chain comprises of around 214 amino acids and heavy chain of 446 amino acids. The antigen binding site is formed as a pocket by six hypervariable loops of three each from light chain and heavy chain supported by a highly conserved β-sheet framework where the antigen molecule binds.

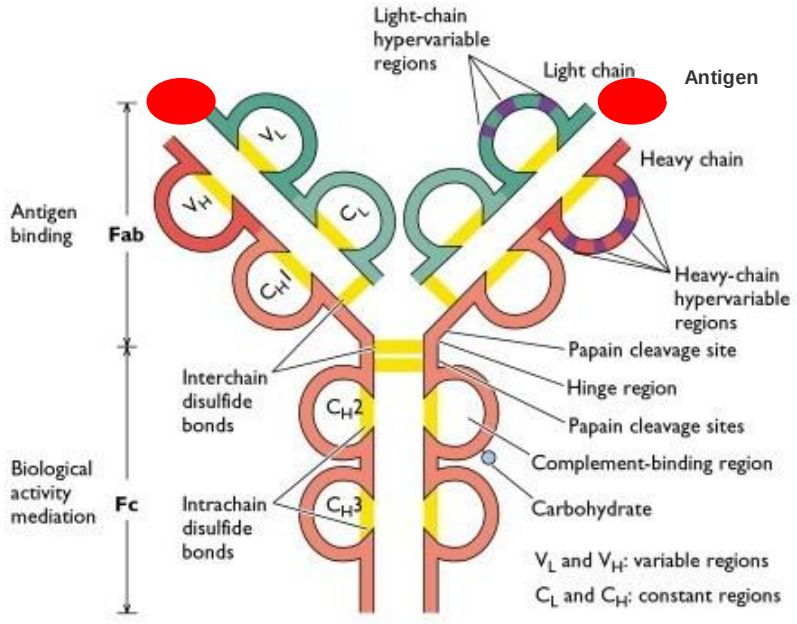


Figure 1.8 Typical Structure of antibody molecule bound to antigen (red circle).

Antibodies are glycoprotein molecules with one or two carbohydrate moieties (light blue in Figure 1.8) added to amino acid residue (generally Asn). In IgG there are two disulfide bonds at the hinge region giving stability to the molecule. In addition the hinge region gives flexibility to the entire structure when an antigen molecule binds to the antibody. The hinge region is prone to papain digestion resulting in two halves, a Fab (*fragment, antigen binding*) and a Fc (*fragment, crystalization*) The antibody naturally exists in different forms depending on the number of antibody molecules linked to form a dimer (IgA), four IgG (telofish IgM) or mammalian IgM.

1.5.2. Biological Function of antibody

An antibody molecule performs various functions namely virus and toxin neutralization, neutralization of microbes (bacteria, virus and toxins), immuno-modulation in the body, antibody dependent cellular cytotoxicity and complement dependent cytotoxicity to kill the target cells, opsonization of antigens, generation of oxidants, and anti-inflammatory response. A general outline on the functions performed by Ab molecule in animal body is depicted in Figure 1.9 however; there is interplay of many more components to elicit the immune response and clearance of antigen which are out of the scope of this work.

Each type of antibody can be produced as a circulating molecule or as a stationary molecule on the surface of the B cells. Both the direct antigen binding portion and constant Fc regions of antibodies contribute to their biological activity.

The direct antigen binding portions involves highly specific recognition of and binding to the antigen, mediated through the variable domain that is a common hallmark of antibodies. The constant domains of the Fc region associates with complements as well as immune effector cells to mediate antibody-dependent cell mediate cytotoxicity (ADCC), phagocytosis, release of inflammatory cytokines, and perhaps more significantly, with Fc receptors (FcRs) present on phagocytes and other immune cells (Figure 1.9).

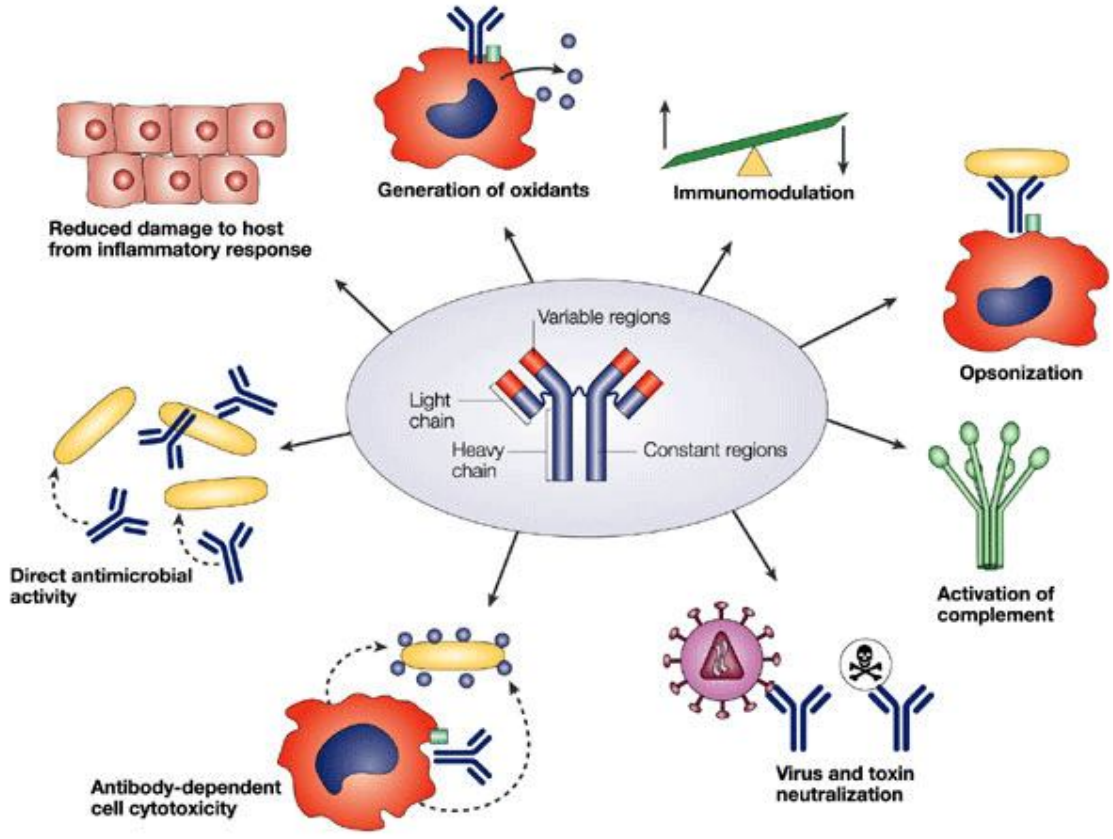


Figure 1.9 Different biological functions of antibodies.

Toxin and virus neutralization, complement activation and direct antimicrobial functions such as the generation of oxidants are independent of other components of the host immune system, whereas antibody-dependent cellular cytotoxicity and opsonization depend on the host cells and mediators. (adapted from Casadevall *et al* 2004)

1.6. Therapeuic Monoclonal Antibody

1.6.1. Structural Characteristics and Classification

The most abundant form of antibody found is IgG and as of today all immunogenec mAbs are IgG (Chu and Chartrain 2008). The typical structure of Ig is described above. The mAbs which are used for therapeutic purpose impose specificity to epitope on antigen which is dictated by the amino acid sequence at Complementarity Determining Region (CDR) present in the variable domains of both light chain and heavy chain. The amino acid sequence at the hyper-variable region forms a loop structure which tightly interacts with the antigen. IgG possess glycosylation at each heavy chain which adds to the complexity of the molecule through a conserved N-linked glycosylation site

generally at Asn_{297} (Jefferis 2005) except in Cetuximab (molecule in discussion) where this site is at Asn_{299}.

The mAb production through hybridoma technology presents some serious drawbacks due to the fact that they are generated from non-human host. Since the coding region of the mAb directly originates from the immunized animal without any subsequent genetic manipulations, the mAb produced is of mouse origin in its amino acid sequence, and upon repeated injections an immune response from the human recipient (referred to as Human Anti-Mouse Antibody or HAMA). Further improvement and continuous improvement by using genetic manipulations were done to replace the murine amino acid sequences with human sequences to the effect that the HAMA response gets minimized.

The human anti-human antibody (HAHA) response is a normal part of the human adaptive immune system. Humanized and fully human monoclonal antibodies contain greater or lesser numbers of effector and regulatory T-cell epitopes just as natural antibodies do (De Groot and Martin 2009). Engineered antibodies which, by chance, contain a relatively large number of effector epitopes and a relatively smaller number of regulatory T-cell epitopes are likely to induce HAHA responses in human subjects. In this case, there is no process of B cell editing, competition, or apoptosis to help reprogram the deleterious immune response.

Based on these improvement, the recombinant therapeutic mAbs are classified as murine (100% murine sequence), chimeric (murine 40-30% + human 60-70%), humanized (murine 5-10% + human 95-90%) and fully human (Figure 1.10). The naming of a mAb molecule also follows a particular format as illustrated in Table 1.4. As depicted in Figure 1.10, the immunogenecity decreases as the molecule developed are more human like.

In addition to the full mAb molecule, antibody fragments are also used for therapeutic applications.

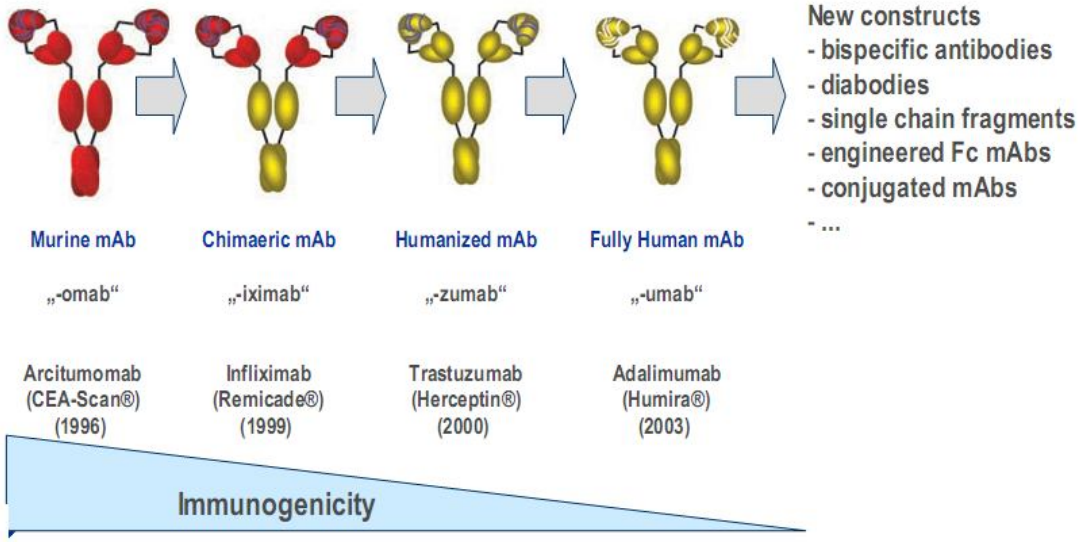


Figure 1.10 **Nomenclature of mAbs.**

Red are portions originating from murine while the yellow one is human origin.

Prefix	Target			Antibody Source		Suffix	Some Examples
Variable	Non-tumor Target	Viral	*-vir-*	*-u-*	Human	*-mab*	Pali-vi-zu-mab (humanized antiviral Mab)
		Bacterial	*-bac-*				
		Immune	*-lim-*	*-o-*	Murine		Ada-lim-u-mab (human Mab against immune disease target)
		Infectious lesions	*-les-*				
		Antifungal	*-fung-*	*-a-*	Rat		E-fung-u-mab (human antifungal Mab)
		Cardiovascular	*-ci(r)-*				
		Neurologic	*-ne(r)-*	*-e-*	Hamster		Bapi-neu-zu-mab (humanized Mab against neurobiology target)
		Interleukins	*-kin-*				
		Musculoskeletal	*-mul-*	*-i-*	Primate		Uste-kin-u-mab (human anticytokine Mab)
		Bone	*-os-*				
		Toxin as target	*-toxa-*	*-xi-*	Chimeric		
	Tumor target	Colon	*-col-*	*-zu-*	Humanized		Den-os-u-mab (human antibone target Mab
		Melanoma	*-mel-*				
		Mammary	*-mar-*				Ab-ci-xi-mab (chimeric Mab against CV target)
		Testis	*-got-*	*-axo-*	Rat/murine hybrid		
		Ovary	*-gov-*				Ore-gov-o-mab (murine Mab for ovarian cancer)
		Prostate	*-pr(o)-*	*-xizu-*	Chimeric + humanized		
		Miscellaneous	*-tu(m)-*				Adeca-tum-u-mab (human antibody against miscellaneous tumor target)

Table 1.4 Naming of mAb molecule

The antibodies are also classified based on the method of action (MOA) as described in detail by Jiang, *et al* 2011. On the basis of their putative MOA, most therapeutic antibodies (including Fc fusion proteins) can generally be classified into three categories, from which their potential for Fc functionality can be ranked (Figure 1.11).

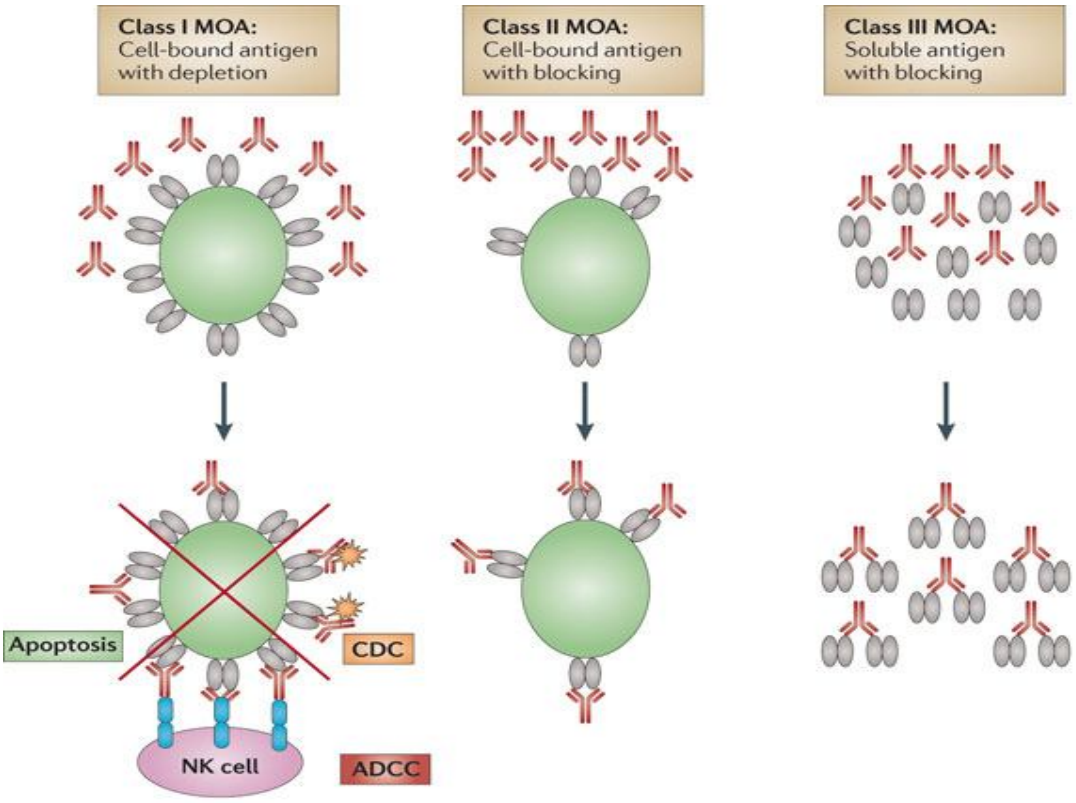


Figure 1.11 Classification of therapeutic antibodies based on MOA.

Class I antibodies recognize and bind to cell-bound antigens and the complex remains stable. The Fc effector functions are generally part of the mechanism of action (MOA) for antibodies in this class. Examples of Class I molecules include rituximab (Rituxan/Mabthera) and alemtuzumab (Campath). Class II antibodies also recognize and bind to cell-bound antigens but their proposed MOA does not involve Fc effector functions. Examples of Class II molecules include basiliximab (Simulect; Novartis) and cetuximab (Erbitux). Class III antibodies bind to and neutralize soluble antigens, and their MOA often involves blocking the soluble ligand from binding to its cognate receptor. Examples of Class III antibodies include bevacizumab (Avastin; Genentech/Roche) and palivizumab (Synagis; MedImmune). ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; NK, natural killer.

Excluding Fab fragments and antibody-drug conjugates (ADCs), there are currently 29 antibodies and Fc fusion proteins approved by the US-FDA for therapeutic purposes. Based on FDA-approved label information (available at the Drgs@FDA website) and the guidelines described below, the proposed classficiation and effector function potential of these molecules are summarized in Table 1.5:

Therapeutic Ab Type	CLASS I: Cell-bound antigen stable upon Ab binding. MOA involving Fc effector function (ADCC, CDC, ADCP)	CLASS II: cell-bound antigen. MOA not involving Fc effector function	CLASS III: soluble antigen. MOA not involving Fc effetor function (blocking)
IgG_1 and IgG_3	High	Moderate	Low
IgG_1 and IgG_3 with Fc mutations to enhance Fc functionality	High	Not applicable	Not applicable
Afucosylated IgG_1	High	Not applicable	Not applicable
IgG_1 and IgG_3 with Fc mutations to reduce Fc functionality, or aglycosylated IgG_1 and IgG_3	Not applicable	Low	Low
IgG_2 and IgG_4; IgG_2 and IgG_4 with Fc mutations to reduce Fc functionality, or aglycosuylated IgG_2 and IgG_4	Not applicable	Low	Low

Table 1.5 Ranking of Fc Effector function potentials for therapeutic Abs

Ab, Antibody; ADCC, antibody-dependent cell mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; MOA, mechanism of action. (Adapted from Jiang, *et al* 2011)

Class I: Class I therapeutic antibodies recognize cell surface antigens and are designed to kill target cells through Fc-mediated effector functions, including antibody- dependent cell-mediate cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP) and Fc-dependent apoptosis (Figure 1.11) Class I antibodies can be of the IgG1 or IgG3 subclass, and they can either be unmodified, or modified through the introduction of Fc amino acid mutations and / or glycoengineering to enhance their effector functions. One common characteristic of this class of antibodies is their reliance on Fc-mediated functionality to achieve cytotoxicity or apoptotic responses as the main focus of their MOA (beyond simple antigen binding). These therapeutic antibodies are designed to have a high effector function potential (Table 1.5), e.g, Rituximab, Alemtuzumab, etc.

Class II: Class II therapeutic antibodies also recognize cell-bound antigens; however, they are not designed to kill antigen-expressing target cells. Thus, effector functions are not part of their MOA (Figure 1.11). The main MOA of this class of antibodies is to block ligand-receptor interactions, and in other cases, to act as agonists.

The Class II category may include antibodies with moderate or low effector functions potential. Because Fc-mediated function is not part of the intended MOA of Class II Abs. IgG2 or IgG4 isotypes (which have a lower propensity to elicit effector functions) are often selected. However, some human polymorphisms can be an exception to these general observations. Unmodified IgG1 or IgG3 isotype within this class are considered to have a moderate effector function poteintial because they contain an Fc region that is capable of binding to FcγRs. However, modulation of effector function poteintial should be considered based on the target biology, the introduction of mutations into the Fc region, or by altering the glycoform pattern (Table 1.5), Cetuximab, etc.

Class III: Class III therapeutic Abs are designed to bind soluble antigens and their MOA often involves blocking to its receptor. For this class of IgG1 or IgG3 Abs, it may be helpful to investigate whether there are membrane-bound forms of the ligand on any cell types. If this is the case, the Ab, may need to be classified as a Class II Ab with moderate effector function potential because there may be some safety concerns associated with binding to a cell-bound ligand. IgG2 and IgG4 Abs (or IgG1 and IgG3 Abs with modifications to minimize effector functions) are in the 'low potential' category (Table 1.5).

ADCs: ADCs are constructed by chemically conjugating one or more cytotoxic small-molecule drugs or radioisotopes to an antibody that binds to the desired cell surgace antigen target. The primary function of Abs used to prepare ADCs is to guide the highly toxic conjugated drugs specifically to tumor cells, in order to minimize potential systemic toxicity. In case where there is evidence that the Ab alone (before conjugation) has effector functions, the evaluation strategy appropriate for either Class I or II molecules should be considered.

1.6.2. Biological Function

The Class I type of Ab whereby Fc mediates ADCC and/or CDC activity of mAb. This is described in detail below as the mAb under discussion; Cetuximab is classified as Class I type.

The direct antigen binding portions involves highly specific recognition of and binding to the antigen, mediated through the variable domain that is a common hallmark of antibodies. The constant domains of the Fc region associates with complements as well as immune effector cells to mediate ADCC, phagocytosis, release of inflammatory cytokines, and perhaps more significantly, with Fc receptors (FcRs) present on phagocytes and other immune cells.

Majority of the therapeutic mAb molecules developed exert potent biological functions through multiple mechanisms, involving both non-immune and immune-mediated functions.

1.6.2.1. Non-immune Mediated Function

The tumor cells require certain specific growth factors for growth. The unconjugated mAbs inhibits binding of these growth factors thereby leading to the apoptosis of tumor cells. The receptor specific antibody (eg. Anti-EGFR) inhibit signaling cascade by binding to receptor thereby preventing the growth factor (EGF) to bind leading to cell apoptosis (Figure 1.12).

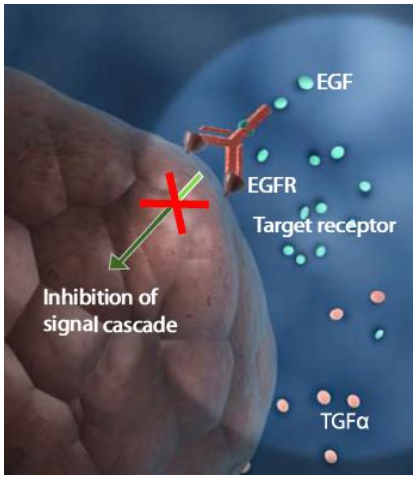


Figure 1.12 Non-immune Mediated Function of mAb.

Epidermal Growth Factor (EGF) and Transforming Growth Factor alpha (TGFα) are ligand for Epidermal Growth Factor Receptor (EGFR) required for cellular function like cell growth and aniogenesis.

1.6.2.2. Immune Mediated Function

As the majority of therapeutic MAbs, chimeric, humanized or fully human, possess an Fc region of the IgG_1 subclass, they can induce effector functions against pathogens and tumor cells following their interactions with the complement component C1q and / or with receptors for Fc region (FcγR).

- Complement Dependent Cytotoxicity (CDC) Function:

The formation of the antibody-antigen complexes results conformation change in the Fc region of the mAb due to torque happening at hinge region which exposes the binding site on for the C1q protein (C1q is one of three subcomponents of complement C1). This binding triggers a proteolytic cascade to activate complement, see Figure 1.13. This can lead to the formation of membrane attack complex that kills the target cell by disrupting its cell membrane. Alternatively, tumor cell bound C1q can bind to complement receptors on effector cells (macrophages, NK cells, neutrophils) leading to cell-mediated tumor-cell lysis or phagocytosis depending on tumor type of effector cell. Subsequent tumor-cell killing can occur in by complement-mediated cellular lysis (cell-independent) or complement-mediated phagocytosis (cell-dependent) manner.

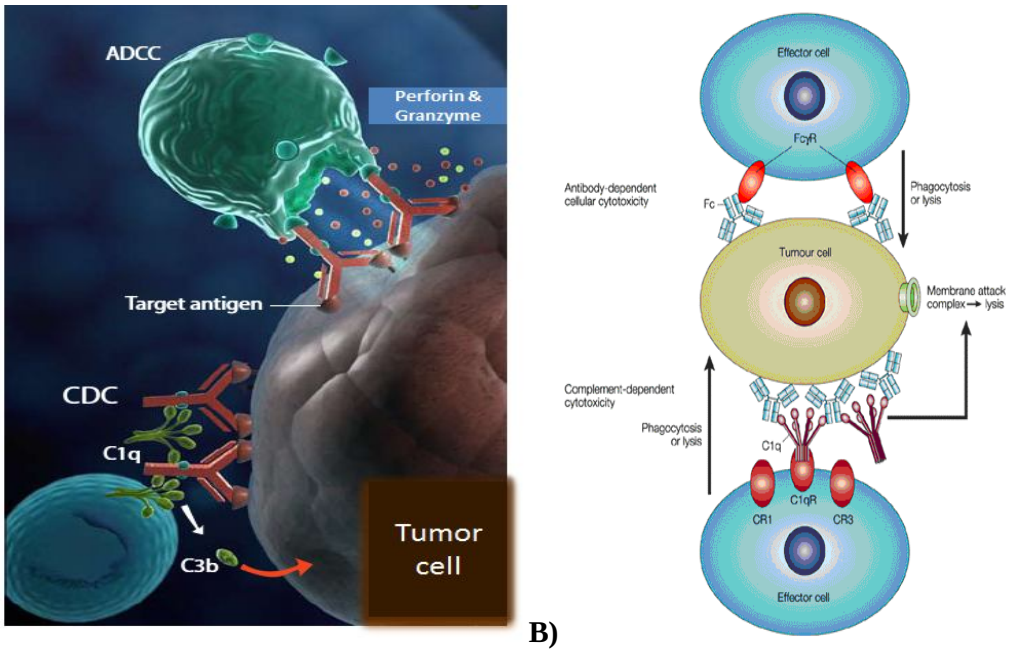


Figure 1.13 Immune Mediated Function of mAb

Binding of mAb with the tumor cell triggering CDC and ADCC activity. (FigA adapted from the Merck ADCC fact sheet and Fig B adapted from Carter P. 2001)

- Antibody Dependent Cellular Cytotoxicity (ADCC) Function:

ADCC is a well-known immune effector mechanism in which antibodies, bound to antigens on target cells, recruit immune effector cells (eg. Natural Killer Cells) of the innate immunity to direct the killing of the target cells (Figure 1.13) Natural ADCC is relevant in protection against various bacterial, parasitic and viral infections and is considered to correlate to the immune protection in HIV (Ahmad, *et al* 2001). ADCC occurs in both malignant and non-malignant conditions. The ADCC process requires the simultaneously presence of three components:

a) Tumor cells expressing target antigen on its surface
b) Therapeutic antigen-specific MAb
c) FcγR-bearing effector cells (Natural killer cell, monocytes/ macrophage neutrophils or dendritic cells).

Antibody coated tumor/target cells can be killed by FcγR-bearing cells, such as NK cells, in ADCC (Steplewski, *et al* 1983). NK cells express the activation receptor FcγRIIIa (CD16) on their surface, which recognize and bind to the Fc domains of IgG1 and IgG3 subclasses. The binding cross-links FcγR on the effector cells (NK cells). Consequently, the NK cells become activated and rapidly kill the tumor/target cells by the release of cytoplasmic granules containing perforin and granzymes (Figure 1.13). FcγRIIb is an inhibitory receptor expressed on B-cells and macrophages (but not NK cells) that co-ligates to FcγRIII activation receptors, leading to inhibition of FcγRIII signaling (Clynes, *et al* 2000) thus inhibiting effector cell activation.

The list of therapeutic mAbs have grown to around 30 and is growing at considerable rate with new and new target been identified.

The therapeutic mAbs and Ab fragments are listed in Table 1.6

Generic Name	Brand name	Originator Company	Approval date	Antibody Type	Isotype	Target	Production Host	Indication/s
Muromonab-CD3	Orthoclone OKT3	Johnson & Johnson	1986	Murine	IgG2a	T cell CD3 Receptor	Hybridoma	Transplant rejection
Abciximab	ReoPro	Centocor / Lily	1994	Chimeric	IgG1	inhibition of glycoprotein IIb/IIIa	SP2/0	Cardiovascular disease
Daclizumab	Zenapax*	PDL/Roche	1997	Humanized	IgG1	IL-2Rα receptor (CD25)	NS0	Transplant rejection
Rituximab	Rituxan, Mabthera	Genentech / BiogenIdec	1997	Chimeric	IgG1	CD20	CHO	Non-Hodgkin's Lymphoma, Chronic Lymphocytic Leukaemia, Rheumatoid Arthritis, Wegener's granulomatosis, Microscopic Polyangiitis
Basiliximab	Simulect	Novartis	1998	Chimeric	IgG	IL-2Rα receptor (CD25)	Hybridoma	Transplant rejection
Infliximab	Remicade	Centocor / Scherring	1998	Chimeric	IgG1	inhibition of TNF-α signaling	SP2/0	Several autoimmune disorders: Crohn’s Disease, Psoriatic Arthritis, Rheumatoid Arthritis, Ankylosing Spondilytis

Generic Name	Brand name	Originator Company	Approval date	Antibody Type	Isotype	Target	Production Host	Indication/s
Palivizumab	Synagis	MedImmune / Astraeneca	1998	Humanized	IgG1	an epitope of the RSV F protein	CHO	Respiratory Syncytial Virus
Trastuzumab	Herceptin	Genentech	1998	Humanized	IgG1	ErbB2	CHO	Breast cancer
Gemtuzumab	Mylotarg	Wyeth	2000	Humanized	IgG4	CD33	CHO	Acute myelogenous leukemia (with calicheamicin)
Alemtuzumab	Campath	Millenium / Genzyme	2001	Humanized	IgG1	CD52	Hybridoma	Chronic lymphocytic leukemia
Efalizumab	Raptiva*	Genentech Serono	2002	Humanized	IgG1	CD11a	CHO	Psoriasis
Adalimumab	Humira	Abbott	2002	Human	IgG1	inhibition of TNF-α signaling	CHO	Several auto-immune disorders
Ibritumomab tiuxetan	Zevalin	BiogenIdec	2002	Chimeric	IgG1	CD20	CHO	Non-Hodgkin lymphoma (with yttrium-90 or indium-111)
Tositumomab	Bexxar	GlaxoSmth Kline	2003	Murine	IgG2a	CD20	CHO	Non-Hodgkin lymphoma

Generic Name	Brand name	Originator Company	Approval date	Antibody Type	Isotype	Target	Production Host	Indication/s
Cetuximab	Erbitux	Imclone / Merck-Serono/BMS	2004	Chimeric	IgG1	epidermal growth factor receptor	SP2/0	Colorectal cancer, Head and neck cancer
Bevacizumab	Avastin	Genentech / Roche	2004	Humanizd	IgG1	Vascular endothelial growth factor (VEGF)	CHO	Colorectal cancer, Age related macular degeneration (off-label)
Omalizumab	Xolair	Genentech / Novartis	2004	Humanized	IgG1	Immunoglobulin E (IgE)	CHO	Mainly allergy-related asthma
Natalizumab	Tysabri	BioenIdec	2006	Humanized	IgG4κ	alpha-4 (α4) integrin,	NS0	Multiple sclerosis and Crohn's disease
Ranibizumab	Lucentis	Genentech / Novartis	2006	Humanized Fab	IgG1	Vascular endothelial growth factor A (VEGF-A)	*E.coli*	Macular degeneration
Panitumumab	Vectibix	Amgen	2006	Human (transgenic mice)	IgG2	epidermal growth factor receptor	CHO	Colorectal cancer
Eculizumab	Soliris	Alexion	2007	Humanized	IgG2/4	Complement system protein C5	NS0	Paroxysmal nocturnal hemoglobinuria

Generic Name	Brand name	Originator Company	Approval date	Antibody Type	Isotype	Target	Production Host	Indication/s
Certolizumab pegol[19]	Cimzia	UCB	2008	Humanized Fab	IgG2a	inhibition of TNF-α signaling	*E.coli*	Crohn's disease
Canakinumab	Ilaris	Novartis	2009	Human	IgG1	IL-1β	SP2/0	Cryopyrin-associated periodic syndromes (CAPS)
Ofatumumab	Arzerra	GlaxoSmith Kline	2009	Human	IgG1	CD20	NS0	Chronic lymphocytic leukemia
Golimumab	Simponi	Jansen	2009	Human	IgG1	TNF-alpha inihibitor	SP2/0	Rheumatoid arthritis, Psoriatic arthritis, and Ankylosing spondylitis
Catumaxomab	Removab	Fresenius Biotech / Trion Pharma	2009	Human trifunctional	IgG2a-Human; and IgG2b-murine	Human-EpCAM and Human CD3	Quadroma	Malignant Ascites
Denosumab	Prolia , Xgeva	Amgen	2010	Human	IgG2	RANK Ligand inhibitor	CHO	Postmenopausal osteoporosis , Solid tumor`s bony metasteses
Tocilizumab (or Atlizumab)	Actemra and RoActemra	Hoffman-La Roche / Chugai	2010	Humanized	IgG1	Anti- IL-6R	CHO	Rheumatoid arthritis

Generic Name	Brand name	Originator Company	Approval date	Antibody Type	Isotype	Target	Production Host	Indication/s
Belimumab	Benlysta	GlaxoSmith Kline	2011	Human	IgG1λ	Inhibition of B- cell activating factor	NS0	Systemic lupus erythematosus
Brentuximab vedotin	Adcetris	Seattle Genetics / Takeda	2011	Chimeric	IgG1	CD30	CHO (Ab conjugate)	Anaplastic large cell lymphoma (ALCL) & Hodgkin lymphoma
Ipilimumab (MDX-101)	Yervoy	Bristol-Myers Squibb	2011	Human	IgG1	blocks CTLA-4	CHO	Melanoma

Table 1.6 Therapeutic mAb and antibody fragments approved by US FDA.

* are molecules withdrawn from market.

approved in Europe but not yet in US.

In the present scope of work Anti-EGFR mAb was taken as a molecule to study its expression in Chinese Hamster Ovary (CHO) cells and compare the product quality with the product expressed in SP/0 cells by the originator company with Brand Name Erbitux® (Cetuximab).

1.7. Epidermal Growth Factor Receptor Biology

Substantial progress has been made over the last several decades in understanding specific cellular, molecular and genetic mechanisms that contribute to cancer growth and progression. Amongst several target to hit to control cancer progression, one most promising anti-cancer treatment identified is inhibition of Epidermal Growth Factor Receptor (EGFR).

The EGFR family consists of four transmembrane receptors, including EGFR (HER1/*erb*B-1), HER2 (*erb*B2/neu), HER3 (*erb*B3) and HER4 (*erb*B4) (Yarden 2001). EGFR is a 170 kDa transmembrane protein comprising an 1186 amino acid peptide chain (Schlessinger 1988) and is composed of three domains, extracellular ligand-binding domain, a trans-membrane lipophilic region and an intracellular protein tyrosine kinase domain (Figure 1.14:). The extracellular binding domain on the cell membrane is connected to intracellular machinery that possesses the tyrosine kinase activity via a single hydrophobic membrane (Carpenter and Cohen 1990).

Seven genetically distinct ligands – EGF, transforming growth factor-α (TGF-α), heparin-binding EGF, amphiregulin, betacellulin, epiregulin and neuregulin G2β – have been shown to be capable of binding with EGFR (Wantanbe, *et al* 1994; Toyoda, *et al* 1997; Wells 1999). Binding of ligand to erbB / EGFR receptor triggers dimerization of neighboring receptors, formation of receptor homodimers and / or heterodimers, and internalization (Yarden 2001; Wiley 2003). This in turn activates the intrinsic receptor tyrosine kinase domain. Protein phosphorylation and dephosphorylation, catalyzed by protein kinases (such as tyrosine kinase) and protein phosphatases respectively, represent fundamental biochemical events for subsequent intracellular signal transduction (Qu 2002). Autophosphorylation of tyrosine residues within the C-terminal tail of EGFR in the cytoplasm (Cohen 2003) following EGFR tyrosine kinase activation initiates a cascade of intracellular signaling pathways (Carpenter and Cohen 1990). The receptor

tyrosine kinase signal can be terminated by endocytosis of the phosphorylated receptor-ligand complex (Yarden 2001).

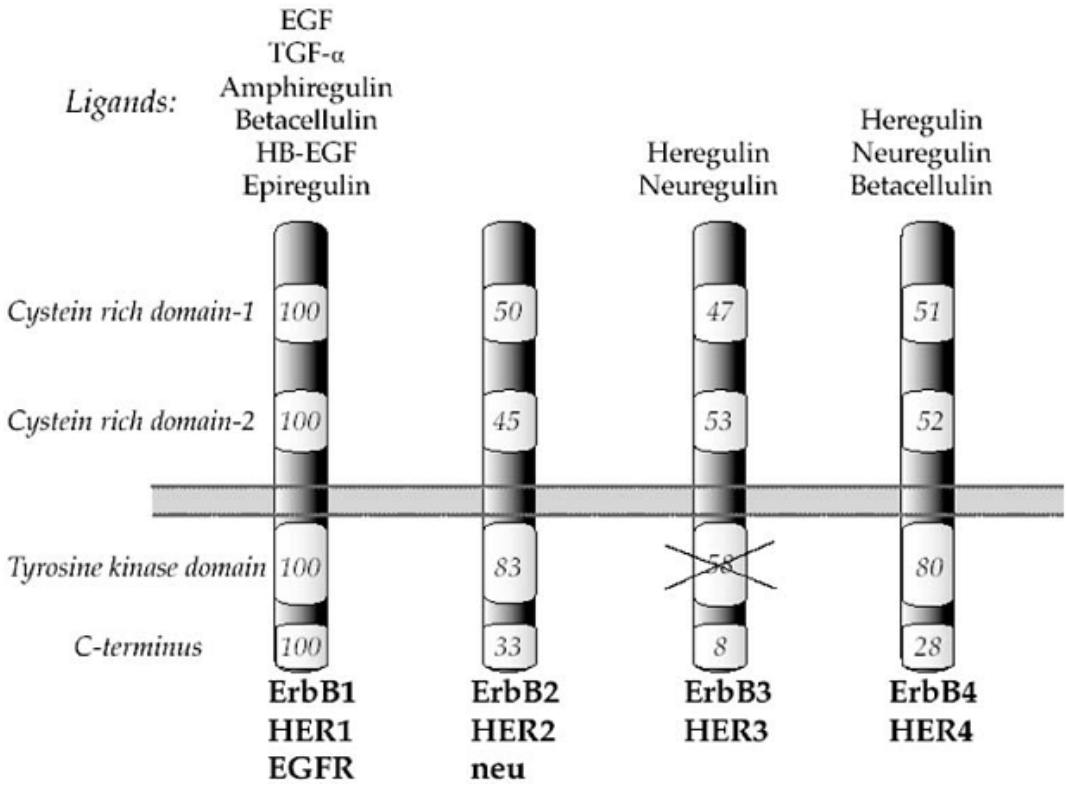


Figure 1.14 Schematic representation of the four erbB family member and their ligand.
Numbers denote the degree (expressed as a percentage0 of homology relative to ErbB1/EGFR. With the exception of the kinase-deficient ErbB3, there exists a high degree of homology in the tyrosine kinase domain (Harari, *et al* 2003)

The EGFR downstream intracellular signal transduction pathways include components of the Ras/mitogen activated protein kinase (MAPK), phosphatidyl inositol 3-kinase, signal transducer and activator of transcription, downstream protein kinase C and phospholipase D pathways (Wells 1999, Carpenter 2000, Grant, *et al* 2002) Some of these pathways serve to attenuate receptor signaling (Wells A., 1999). The interaction of EGFR system and the Ras/MAPK cascade, one of the major signaling routes (Alroy and Yarden 1997), and its downstream effects on the cell-cycle machinery is depicted in lucid manner in Figure 1.15. A comprehensive description of ErbB signaling pathways was described by Yarden and Sliwkowski (2001). The integrated biological responses to EGFR signaling are pleiotropic which include mitogeneiss, apoptosis, altered cellular motility, protein secretion and differentiation or dedifferentiation (Wells 1999).

Activation of the EGFR under abnormal circumstance stimulates tumor growth and progression, including the promotion of proliferation, angiogenesis, invasion, metastasis and inhibition of apoptosis (Salomon, *et al* 1995; Wells 1999; Woodburn 1999). EGFR is widely expressed by many cell types, including those of epithelial and mesenchymal lineages (Wells 1999). However, there is variability regarding the reported incidence of overexpression or dysregulation of EGFR in human malignancies (Nicholson, *et al* 2001; Arteaga, 2002). For example, some reports describe EGFR expression as being dysregulated in at least 50% of human epithelial tumors (Aaronson, 1991; Earp, *et al* 2003; Grunwald and Hidalgo 2003), while more conservative estimates suggest that one-third of epithelial malignancies express high-levels of EGFR (Mendelsohn 2001). This variability may be partially attributable to a lack of standardization in quantitation methodology, using either using simple immunohistochemistry or more sophisticated fluorescence *in situ* hybridization techniques. Overall, it does appear that many of our most common human epithelial cancers richly express the EGFR.

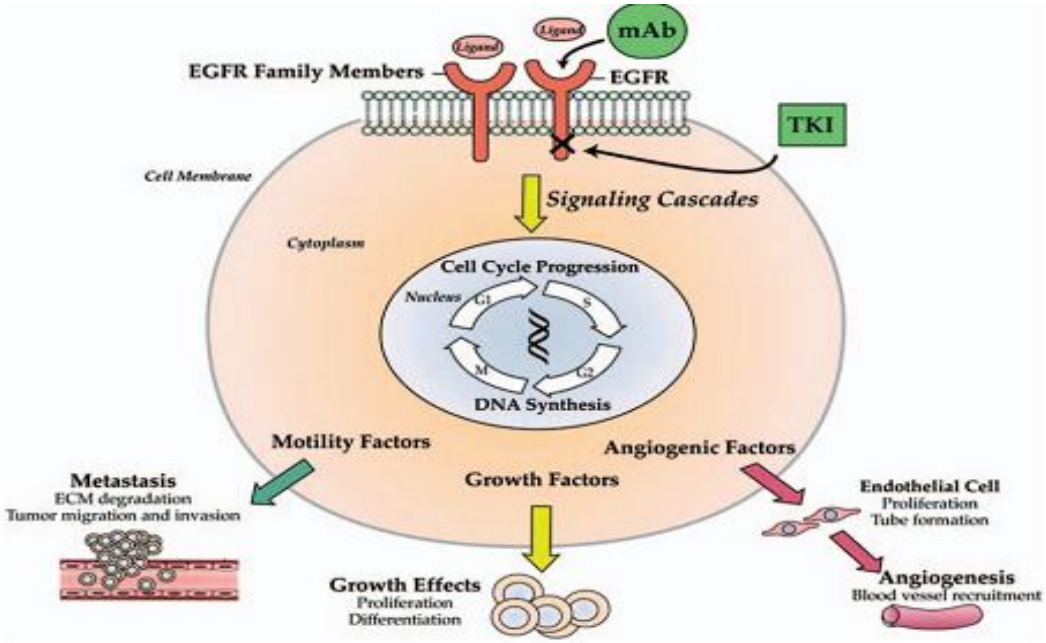


Figure 1.15 Simplified representation of EGFR pathways.

The potential downstream cellular and tissue effects of EGFR signaling inhibition are highlighted. The action site for EGFR inhibitors is depicted for mAbs and TKI (Harari and Huang 2001) TKI, Tyrosine Kinase I

In colorectal and, head and neck cancer, the vast majority of tumors are strongly EGFR-positive (Mendelsohn 2001). In addition to this studies have shown that EGFR overexpression in bladder, brain, breast, cervical, uterine, colon, esophageal, glioma, non-small cell lung cancer (NSCLC), ovarian, pancreatic and renal cell cancers (Nicholson, *et al* 2001; Herbst 2002, Mendelsohn and Baselga 2003).

Various labs are involved in the development of molecules targeted to EGFR, but the most promising work came from the M.D. Anderson Cancer Centre where Dr. John Mendelsohn developed chimeric monoclonal antibody directed against EGFR. This mAb was further developed by ImClone/Bristol Myer's Squibb Institute for human use. mAbs directed against EGFR have the following mechanisms of action: (i) extra-cellular binding; (ii) internalization of receptor-antibody complexes; (iii) inhibition of EGFR signaling pathways; and (iv) potential stimulation of an immunological response.

Several mAbs are in various stages of clinical development as listed in Table 1.7:

Generic / Trade Name	**Type**	**Institution**
Cetuximab / Erbitux (Approved)	Chimeric IgG1	ImClone/Bristol Myer's Squibb / Merck KGaA
Panitumumab/Vectibix (Approved)	Fully human IgG2	Abgenix / Amgen
-	Humanized IgG1	EMD Pharma/Merck KGaA
HuMab-Mouse	Humanized, bispecific: EGFR/FcRγ1	Medarex.Merck KGaA
Nimotuzumab/ TheraCIM (Approved)	Humanized	YM Biosciences / CIM
-	Anti-EGFR VIII	Ludwig Institute

Table 1.7 List of anti-EGFR mAbs in development

The focus of this work is on the development of anti-EGFR mAb, Cetuximab, hence its structure and functions are described below.

1.8. Cetuximab: An Anti-EGFR Monoclonal Antibody

The generic name of chimeric anti-EGFR mAb is Cetuximab market by Imclone / Bristol Myers Squibb in US and European market which in other countries marketed by Merck-Serono with a brand name Erbitux®. Cetuximab is an IgG1 human/mouse chimeric monoclonal antibody (mAb) with kappa light chain that is specific against the epidermal growth factor receptor (EGFR). In February 2004, the U.S. Food and Drug Association (FDA) approved Cetuximab for use in patients with metastatic colorectal cancer (Ciardiello, *et al* 2005). European-wide approval followed in June 2004. In March 2006, FDA again approved Cetuximab for the treatment of head and neck cancer (Conley 2006). In India the approval came in 2007. Cetuximab is now approved in more than 40 countries, and thousands of patients have been treated with this drug. The efficacy and safety of this drug have been proved to be excellent in a series of clinical trials that included patients with not only metastatic colorectal cancer but also advanced cancers such as head and neck cancer (Zhou, *et al* 2007). But, a high prevalence of hypersensitivity reactions has been reported which is correlated with IgE antibodies levels in the patients. The mechanism to hypersensitive reactions was found to be associated with the presence of galactose-α1, 3-galactose processed on the terminal N-linked glycosylation on Fab region. This is due to the fact that Cetuximab is produced in murine SP2/0 cell line, which expresses α1, 3-galactosyltransferase (Platts-Mills, *et al* 2008). In a vision to get reduced hypersensitivity reaction, the Cetuximab is cloned in CHO cells which is do not have α1,3-galactosyltransferase but, α2,3-galactosyltransferase and it do not generate IgE hypersensitive reaction.

Target	**mAb**	**Type**	**Indications**	**Selected Side Effects**
EGFR	Panitumumab	Fully Human	Monotherapy for EGFDR-positive metastatic colorectal carcinoma with non-mutated (wild-type) KRAS after failure of conventional chemotherapy	Infusion reactions Skin rashes in most patients (90%) Diarrhoea (60%), nausea and vomiting Hypomagnesaemia (2%)

	Cetuximab	Chimeric	EGFR-positive metastatic colorectal cancer Squamous cell carcinoma of head and neck	Severe infusion reactions IgE aginst oligosaccharide and HAMA Urticaria and dermatological toxicity Bronchospasm and pulmonary toxicity Hypomagnesaemia
	Trastuzumab	Humanized	ERBB2-positive breast carcinoma	Hypersensitivity and infusion reactions Cardiotoxicity with anthracyclines Skin reactions Pulmonary toxicity Hypomagnesaemia

Table 1.8 Side Effects of different anti-EFGR mAbs. (Adapted from Hansel, *et al* 2010)

Cetuximab is produced in the SP2/0 mammalian (murine myeloma) cell line. The molecular weight is approximately 152 kDa. It has potentially 3 N-linked glycosylation sites at Asn_{43} of the light chain and at Asn_{88} and Asn_{299} of the heavy chain. This is a unique IgG1 molecule amongst all the therapeutic IgGs available as of today in the sense that it possesses two N-linked glycosylation sites on the heavy chain (Figure 1.16The N-linked glycosylation in the framework-3 of the variable (Fv) region is at site **Asn_{88}**-Asp-Thr-Ala. The other site is within the CH2 domain at Asn_{299}-X-Ser/Thr of the heavy chain which is generally at Asn_{299} for IgG1. In contrast, the glycosylation site at Asn_{43} is not glycosylated (Platts-Mills, *et al* 2008). As depicted in a schematic diagram of Cetuximab in Figure 1.16, these oligosaccharides located at the two sites are a complex mixture of different glycans, making them challenging for structural characterization (Zhou, *et al* 2007).

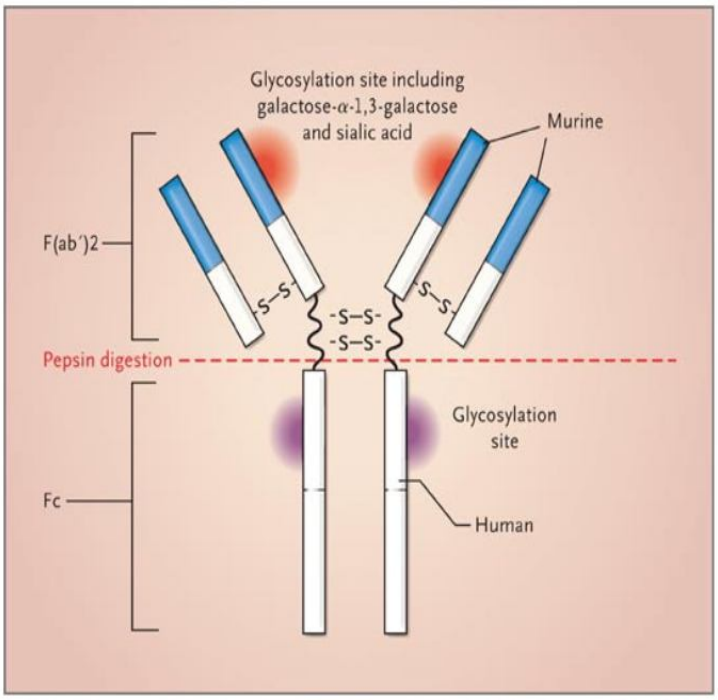


Figure 1.16 A typical structure of anti-EGFR mAb.

The shadowed red areas represents the Asn88 linked and purple area represents Asn_{299} N-linked glycosylation site unique to this antibody.

A typical structure of N-linked glycan structure at Asn_{297}/ Asn_{299} is represented in Figure 1.17. The number attributes only sugars that have been observed in the electron density. The sugars in bold belong to the oligosaccharide core which is found in all naturally occurring glycoforms, the addition of the other sugar residues is variable. The biantennary mannose residues Man5 and Man8 that are glycosidically linked to Man4 form α (1-6) and α (1-3) arms, respectively. In case of the Asn_{88} of Cetuximab available similar structure is reported except that at the end of α (1-3) arm a instead of Gal-NeuAc, Gal-NGNA is observed. Human cells cannot synthesize a terminal Gal-α1-3Gal motif (known as alpha-Gal) on N-glycans. As a consequence, they express antibodies against this structure (Galili 2004). Second, unlike other mammals, humans cannot biosynthesize the sialic acid Neu5Gc because the human gene *CMAH*, encoding Cytidine monophasphate-*N*-acetylneuraminic acid hydroxylase (CMAH), the enzyme responsible for producing CMP-Neu5Gc from CMP-N-acetylneuraminic acid (CMP-Neu5Ac), is irreversibly mutated (Varki 2007 and Ghaderi, *et al* 2010).

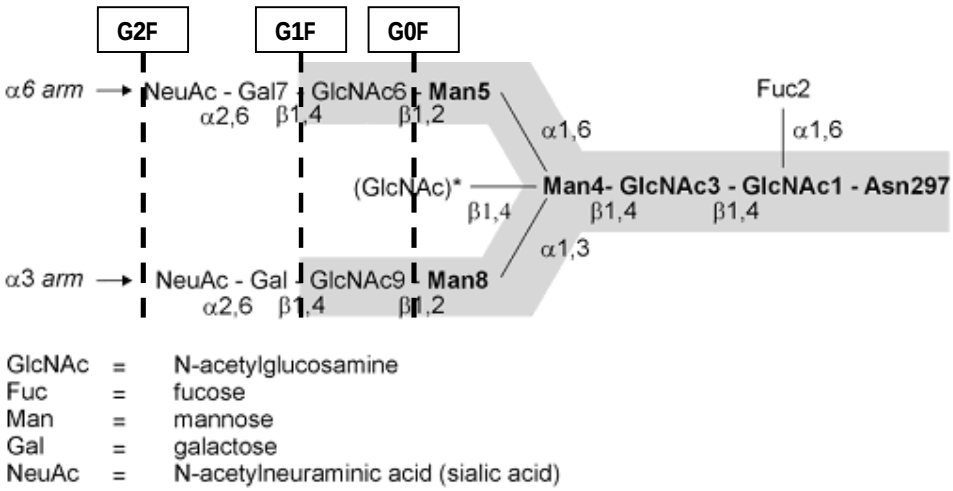


Figure 1.17 The carbohydrate sequence attached to Asn_{297} of human IgG1-Fc.

The detailed sugar molecule linked to core residue is represented Numbers attributes only sugars that have been observed under electron density. The biantennary mannose residues Man5 and Man8 that are glycosidically linked to Man4 form the α(1-6) and α(1-3) arms, respectively. The forms without terminal galactose (G0F), with one galactose on each arm (G1F) and two galactose on both the arms (G2F) are represented. Adapted from Sondermann, *et al* 2003

Traditionally, cytotoxic therapies are used to cure cancer however, these therapies do not discriminate between tumor and host cells, and hence considerable interest was generated in 1990s in developing new agents to improve the outcome of patients with solid tumors. Furthermore, the cytotoxic agents are generally effective against rapidly dividing neoplasms. In addition, tumor resistance may develop. The lack of specificity and limited efficacy of traditional cytotoxic agents has led to the rational design and development of targeted therapies that aim to differentiate between malignant and nonmalignant cells, thereby producing a higher therapeutic index and less toxicity than conventional therapies (Ciardiello and Tortora 2001). In order to develop target specific agents, it is necessary to identify the aberrant biochemical and molecular pathways that distinguish malignant cells from nonmalignant cells. The cell membrane receptors control generally controls the intracellular signal transduction pathways regulating cell proliferation and apoptosis, angiogenesis, adhesion, and motility which decides the growth and progression of non-malignant as well as tumor cells (Ciardiello and Tortora 2001). One such cell membrane receptor is the epidermal growth factor receptor (EGFR), which has been shown to play an important role in the growth and survival of many solid tumors.

Pathways involved in EGFR signal transduction have been proposed as possible anticancer targets, and agents to specifically target the EGFR have been developed.

Cetuximab is an antineoplastic factor used for the following indications:

a. Refractory metastatic colorectal cancer

b. Squamous cell carcinoma of Head and Neck Cancer

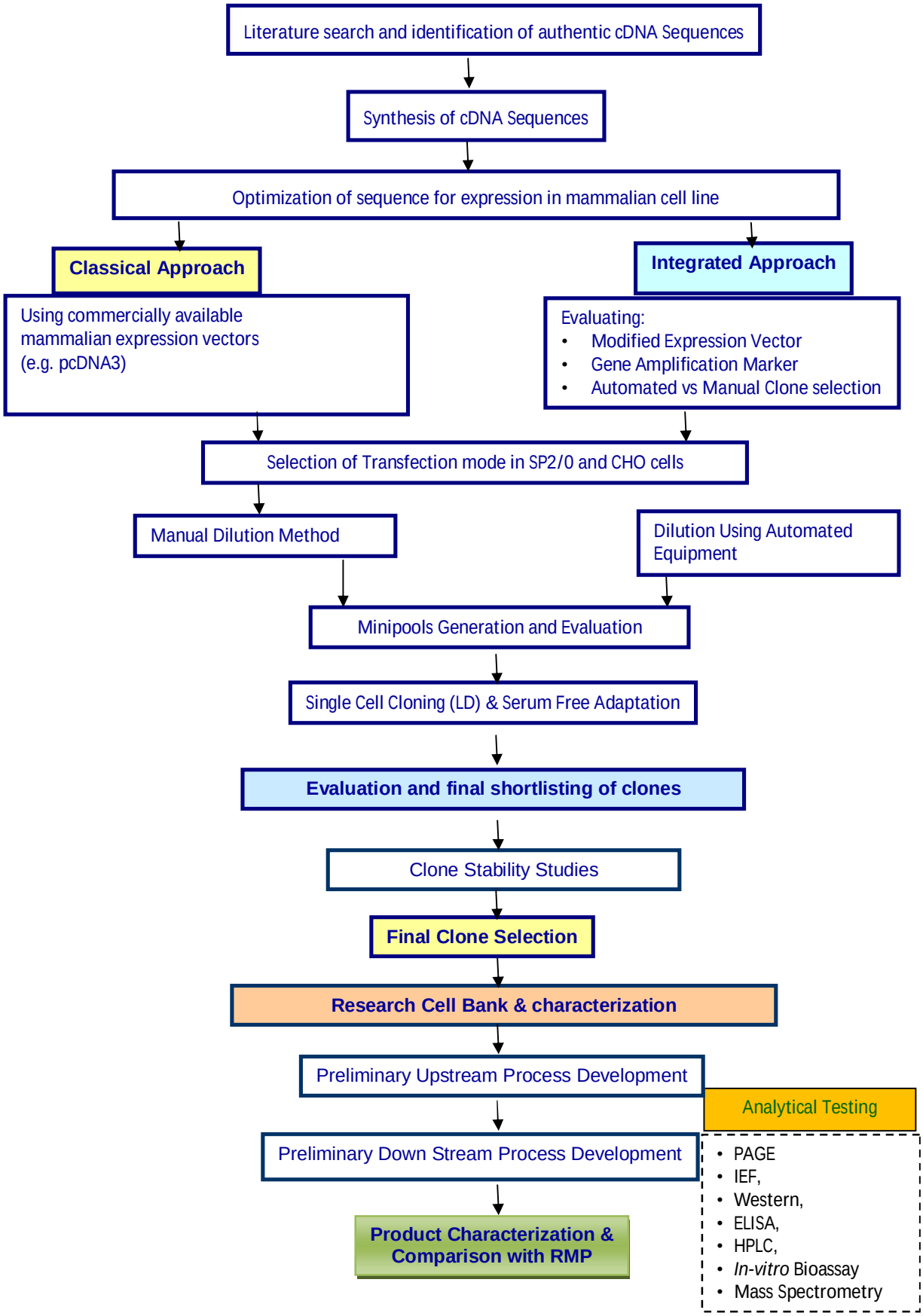
c. Pancreatic cancer (Abbruzzese, *et al* 2004).

1.9. Objectives

Cetuximab is produced by SP2/0 cells by the originator company. The mAb is unique amongst the mAbs listed in Table 1.6 because of the fact that it possesses two glycosylation site one in Fc domain and other Fab region. As described earlier, because of α1-2 linkage of galactose in the Fab motif, it induces anti-IgE response in humans (Platt-Mills, *et al* 2008). For avoiding this α1-2 linkage, the study of producing Cetuximab in CHO cells which will process α2-3 linkage was undertaken. Hence, the objective of study was laid down to clone and express Cetuximab in alternate host that is CHO. The study is covered under the following chapters:

1. Cloning and expression of biosimilar anti-EGFR antibody in an alternate host (SP2/0 → CHO)
   a. Recombinant vector construction for expression in SP2/0 and CHO cells
   b. Cell Line Development: Transfection, clone screening and clone stability studies.
2. Final Clone Selection
   a. Selection of the lead clone based on productivity and quality attributes
   b. Establishment of platform evaluation methods for screening of recombinant clones expressing anti-EGFR monoclonal antibody.
3. Upstream process development for improving expression of recombinant anti-EGFR monoclonal antibody in shake flasks.
4. Characterization of the expressed anti-EGFR mAb.

1.10. Work Plan



CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

Standard Chemicals and Reagents used frequently are summarized below or described as appropriate. Listing is based on the category of chemicals, reagents and materials:

2.1.1. Chemicals and Reagents

- 2-Mercaptoethanol, Sigma, Cat# M7154
- 2-propanol, Sigma, Cat# 19516
- Acetic Acid, MERCK, Cat # AC9A590101
- Acetone, Sisco, Cat # 129135
- Acrylamide-99%, Sigma Cat# A3553
- Acetone, SISCO, Cat# 129135
- Acetonitrile, MERCK, Cat# 61803025001730
- Agarose, Sigma, Cat# A9539
- Agar Powder, Sigma, Cat# A5306
- Ampicillin powder, Sigma, Cat# A0166
- Ammonium per sulfate, Sigma, Cat# A9164
- Bromophenol blue sodium,Sigma, Cat# B8026-25G
- Brilliant Blue R250, Sigma, Cat# B7920
- Bovine Serum Albumin, AMRESCO, Cat# 332
- Bisacrylamind, Sigma, Cat# M7279
- Butanol, MERCK, Cat# 61775805001730
- Citric Acid, Sigma, Cat# C0759
- Chloroform, SIGMA, Cat# C2432
- Calcium chloride, Merck Cat# 10238205001046
- Dextran Sulphate, AMRESCO, Cat# AM-0198
- Di-methyl Sulfoxide (DMSO) – Sterile, Sigma, Cat# 673439
- EDTA, Tetrasodium salt, Sigma, 03695-250G
- Ethyl alcohol 99% , Changshu yanguan chemical , GB678-2002
- Ethyl alcohol 99% , Jiangsu Huaxi international, 100202
- Ethidium bromide, Sigma, Cat# E7637
- Glutamine-(non-animal source), Sigma, Cat# G8540
- Glucose, Sigma, Cat# G7021
- Glycerol, Sigma , Cat# G5516-1L
- Glycine, Fluka, Cat# G8898-1KG
- Hydrochloric acid 35%, Merck, Cat# 61762525001730
- Isopropyl alcohol, SISCO, Cat# 92956

- Magnesium chloride, Sigma, Cat # M8266-100G
- MES , SIGMA, Cat # M3671
- Methanol, Merck, Cat # GR 60600925001730
- OPD: SIGMA, (Tablet), Cat no-P-5412, Lot no-077K-8225
- PhastGel Dry IEF, GE healthcare, Cat# 17-0677-01
- Ponceau stain, HIMEDIA, Cat# RM977
- Protein Assay Solution, BIO-RAD, Cat# 500-0006
- Skimmed Milk, DIFCO-BD, Cat# 232100
- Sodium Acetate Anhydrous, SISCO, Cat# 1944117
- Sodium chloride, MERCK, Cat# 60640450001730
- Sodium dodecyl sulphate, SIGMA, Cat# L4390
- Sodium hydroxide, SIGMA, Cat# S5881
- Sodium pyruvate, SIGMA, Cat# P5280
- TEMED, SIGMA, Cat# T 9281
- Transfection Reagents
  - Turbofect in vitro Transfection Reagent, MBI Fermentas, Cat# R0531
  - Lipofectamine-2000 Transfection Reagent, Invitrogen, Cat# 11660-019.
  - TransIT ® 2020 Transfection Reagent, Mirus Bio LLC, Cat # MR5404.
- Triton X 100, Sigma Cat # T8787-250ML
- Trizma base, Sigma, Cat # T6066-1KG
- Tryphan blue, Sigma, Cat# T6146
- TWEEN-20, Sigma, Cat# P1379
- TMB, Sigma, Cat# T0440 OR Vector Inc., Cat# SK-4400
- Urea, Sigma, Cat# U5378
- Xylene Cyanol, Sigma, Cat# X-4126.
- Pharmalyte (pH 3-10), GE, Cat # 170456.01
- IEF Dry Gel, GE, Cat # 57-6781-00.

2.1.2. Markers and Enzymes

❖ **DNA and Protein Ladder:**

- GeneRuler 1 Kb DNA Ladder, MBI-Fermentas, Cat# SM0313
- λ-DNA/EcoRI + HindIII, MBI-Fermentas, Cat# SM0193
- GenRuler 100 bp+ DNA Ladder, MBI-Fermentas, Cat# SM0321
- Prestained SDS-PAGE Standards, BioRad, Cat# 161-0318
- Precision Plus Protein Unstained Standards, BioRad, Cat# 161-0363

- Prestained SDS-PAGE Standards, low range, BioRad, Cat# 161-0305
- Precision Plus Protein All Blue Standards, BioRad, Cat# 161-0373
- Unstained Protein Molecular Weight Marker MBI-Fermentas, Cat# SM0431
- PageRuler Unstained Protein Ladder, MBI-Fermentas,Cat# SM0661
- PageRuler Prestained Protein Ladder, MBI-Fermentas,Cat#SM0671
- IEF Marker, GE, Cat # RPN800E

❖ **Restriction enzymes:**

Generally all the restriction enzymes used are from New England Biolabs, UK except where stated.

*Bgl*II (Cat# R0144S) , *Nhe*I (Cat#R131S), *Pvu*I (Cat # R0150L), *Xma*I (Cat# R0180L), *Bst*B1 (Cat# R0519S), *Bam*HI (Cat# R0136S), *Kpn*I (Cat# R0142S), *Nco*I (Cat# R0193S), *Pst*I (Cat# R0140S), *Pvu*II (Cat# R0151S), *Xba*I (Cat# R0145S), *Xho*I (Cat# R0146S), *Sal*I (Cat# R0138S), *Aat*II (Cat# R0117S), *Nde*I (Cat# R0111L), *Not*I (Cat# R0189S), *Afl*II (Cat# R0520S) *Hind*III (Cat# ER0505), *Eco*RI (Cat# ER0275), FastDigest *Hind*III (Cat# FD0508), FastDigest *Nde*I (Cat # FD0588), FastDigest *Bgl*III (Cat# FD0084)

❖ **Enzymes and Other Reagents:**

- Taq DNA Polymerase, Banglore Genei, Cat# MME5J
- Taq DNA Polymerase, recombinant, MBI-Fermentas, Cat# EP0404
- Phusion Pfu polymerase, Finnzymes, Cat# F-530S
- Pfu Turbo DNA Polymerase, Stratagene, Cat# 600250-52
- DNA Polymerase (Klenow), NEB, Cat #M0210S
- Alkaline Phosphatase (Calf Intestinal), Promega, Cat# M182A
- T4 DNA Ligase, NEB, Cat# M0202S
- T4 DNA Ligase, MBI-Fermentas, Cat# M0202S
- dATP, MBI-Fermentas, Cat# N0440S
- dTTP, MBI-Fermentas, Cat# N0443S
- dGTP, MBI-Fermentas, Cat# N0442S
- dCTP, MBI-Fermentas, Cat# N0441S
- Papain Carica Papaya, Merck, Cat# 5125
- Pepsin, Merck, Cat# 1071850100

- Endoproteinase Lys-C, Sigma, Cat# P3428
- Pfu Pyroglutamate Aminop, Takara, Cat# TAK 7334
- PNGase, Prozyme, Cat # GK80110

2.1.3. Antibodies

- Monoclonal Anti-human IgG (Fc Specific.)-M, Sigma, Cat# I6260
- Polyclonal Anti-Human (Fc Specific)-R, Sigma, Cat# I9135
- Antihuman Kappa Light Chain HRP Conjugate, Sigma, Cat # A7164

2.1.4. Reference Medicinal Product

- The anti-EGFR monoclonal antibody, Erbitux® available from Merck was used as Reference Medicinal Product (RMP).

2.1.5. Commercial Kits

- LookOut Mycoplasma PCR detection Kit, Sigma, Cat# MP0035
- EZ-PCR Mycoplasma Test Kit, Biological Industries, Cat# 20-700-20
- Qiagen Plasmid Midi Kit , Qiagen, Cat#12145
- Wizard® Plus SV Minipreps DNA Purification System, Promega, Cat # A1460
- GenElute Gel Extraction Kit, Sigma, Cat# NA1111
- QIAquick Gel Extraction Kit, Qiagen, Cat# 28704
- PureLink™ Genomic DNA Mini Kit, invitorgen, Cat # K1820-01
- GeneJET™ Plasmid Miniprep Kit, MBI-Fermentas, Cat# K0502
- QIAprep Spin Miniprep Kit, QIAGEN, Cat# 27106
- PureYield™ Plasmid Midiprep System, Promega Cat# A2492
- RNeasy Mini Kit, Qiagen, Cat# 74104
- HiTrap MabSelect, GE Healthcare, Cat# 28-4082-53
- MabSelect SuRe Resin, GE Healthcars, Cat# 17-5438-01
- OneStep RT-PCR Kit, Qiagen, Cat# 210212
- IEF calibration kit (pI 3-10) GE healthcare, Cat#17-0471-01

2.1.6. Primers

The list of primers is given in Table 2.1.1:

S.No.	Gene	Location	Purpose	Primer Sequence
SR-171	CMV Promoter		For Amplifying CMV Promoter	5'-GATGCGGCCGCCCCGATCCCCTAT-3'
SR-172	CMV Promoter		For Amplifying CMV Promoter	5'-AGTGGATCCCAGCTTGGGTCTCC-3'
SR-173	E18	8731	For Sequencing	5'- GGGTTATTGTCTCATGAGCGGA-3'
SR-174	E18	1074	For Sequencing	5'-CCAGTGGATGTTGGTGCCGAT-3'
SR-175	E18	1192	For Sequencing	5'-TCAACTCCGTGGAATCCGAG-3'
SR-176	E18	1494	For Sequencing	5'-CAGGGAGTAGGTGCTGTCCTT-3'
SR-177	E18	1582	For Sequencing	5' -TGACCAAGTCCTTCAACCGG-3'
SR-178	E18	2789	For Sequencing	5'-CGGACACGGTACAGGTGAT-3'
SR-179	E18	3012	For Sequencing	5-CCTACTATGACTACGAGTTCGC-3'
SR-180	E18	3200	For Sequencing	5-CAGAGTTCCAGGACACGGTCA -3'
SR-181	E18	3522	For Sequencing	5'-CGAGGACCCTGAAGTGAAGTT-3'
SR-182	E18	4244	For Sequencing	5'-CCCCCAGAATAGAATGACACCT-3'
SR601	CMV Promoter		For Sequencing	5'–CGCAAATGGGCGGTAGGCGTG-3'

Table 2.1.1 List of Primers used during the cell line development activity

Note: The primers were synthesized from Sigma-Aldrich. Generally 5µM of primers were used in PCR reactions unless stated otherwise

2.1.7. Vectors, Cells and Cell Lines

- pcDNA-3.1(-), Invitrogen, Cat# V795-20
- GeneArt Vectors: pMAT E18_L1 (bearing Light Chain) and pGA E18_H1 (bearing Heavy chain) were chemically synthesized from GeneArt (Invitrogen), Germany.
- *E.coli* DH5-α cells, MTCC, Cat# 1652
- Chinese Hamster (*Crisetulus griseus*) Ovary Cells (CHO), Invitrogen, Cat#1169-012
- SP2/0 -Ag14 (myeloma) cells, ATCC, CRL- CRL-1581
- A431NS, Human Epidermoid Carcinoma Cell Line, ATCC, CRL-2592

2.1.8. Media and Supplements

❖ **Bacteriological Media and Supplements**

- Agar (Bacteriological), Sigma, Cat# A5306
- Selected Soytone, DIFCO-BD, Cat# 212488.
- Yeast Extract, DIFCO-BD, Cat# 212750.
- Luria-Bertanii Broth, HIMEDIA, Cat# M1245

❖ **Cell Culture Media & Supplements**

The Basal Media and Feed Media used are listed later in Table 2.1.2 and 2.1.3 respectively.

- L-Glutamine, SIGMA, Cat# G8540
- D-Galactose, SIGMA, Cat# G5388
- Pluronic F68, SIGMA, Cat# P1300
- RPMI 1640, SIGMA, Cat# R8758 OR Hyclone, Cat# SH30027
- Dimethyl Sulphoxide (DMSO), Sigma, Cat# D2650
- HT supplement (100x), Gibco/Invitrogen , Cat# 11067-030
- MTX (Methatraxate), SIGMA, Cat# M8407
- MSX (Methionine Sulphoxamine), SIGMA, Cat# M5379
- Foetal Bovine Serum, SIGMA, Cat# F2442;
- Qualified FBS, Australia, GIBCO, Cat# 10099-133
- Foetal Bovine Serum, Euroclone, Cat# ECS150L.
- Penicillin- Streptomycin Solution, SIGMA, Cat #P0781
- Hygromycin, GIBCO/INVITROGEN, Cat# 10131-035
- Recombinant Trypsin, Cascade Biologics, Cat# R-009-50
- Cell Dissociation Soln (Non-enzymatic), BIOLOGICAL Ind., Cat# 03-071-1C
- MEM Amino acid Non-Essential, SIGMA, Cat# M7145
- Geneticin G418, SIGMA, Cat# A1720.
- Dulbecco's Modified minimal essential media, SIGMA, Cat # D5523
- RPMI-1640, SIGMA, Cat # R6504.

2.1.9. Consumables

- Labtip Blue (200-1000ul), THERMO SCIENTIFIC, Cat# 94300220
- Shake Flask 125mL, NALGENE, Cat# 4115-125
- Cryovials, NUNC, Cat# 368632
- Gene pulser cuvette 0.2cm, BIO-RAD, Cat# 165-2086
- Shake Flask 250mL, NALGENE, Cat# 4115-125

- Shake Flask 125mL, NALGENE, Cat# 4115-250
- Tissue Culture Flask 25 cm2 CORNING, Cat# CLS430639-200EA
- Tissue Culture Flask 75 cm2, CORNING, Cat# CLS430641-100EA
- 6-WELL Tissue Culture Flask, CORNING, Cat# CLS3506-100EA
- 12-WELL Tissue Culture Flask, CORNING, Cat# CLS3512-100EA
- 96-WELL Tissue Culture Flask, CORNING, Cat# CLS3599-100EA
- Serological pipette(Disposable) 5mL, BD, Cat# REF-357543
- Serological pipette(Disposable) 10mL, BD, Cat# REF-357541
- Serological pipette(Disposable) 25mL, BD Cat# REF-357525
- Erlenmeyer Flask (Disposable) 125 mL, Corning, Cat# 431143
- Erlenmeyer Flask (Disposable) 500 mL, Corning, Cat# 431145
- Sterilization grade 0.2 µ filters of different sizes, Sartorius / Millipore / MDI

2.1.10. Equipments

- pH meter (Lab-India)
- UV Visible single beam spectrophotometer (Eppendorf)
- Laminar flow (Esco)
- Bacteriological incubator (37 °C) (Memmet)
- Autoclave (Medical)
- Gel doc XR system (BioRad)
- Ultra freezer (-80 °C) (New-Brunswick scientific)
- Magnetic stirrer-cum hot plate (Schott)
- Vortex (Sphinix)
- Refrigerator (Samsung)
- Gel dryer (Amersham)
- Incubator shaker, refrigerated (Kuhner)
- CO_2 Incubator, NuAir / Thermo
- CO_2 incubator shaker (Kuhner) and NuAir
- Bioreactor, 2L, 20L, Model Biostat B Plus, B-Braun Sartorius
- Trans blot SD - Cell (Bio-Rad)
- Electrophoresis Apparatus (Bio-Rad)
- Electrophoresis Power Pack (Bio-Rad)
- Macro Centrifuges (Thermo Scientific)
- Ultra Rocking Platform Shaker (Bio-Rad)
- Weighing Balance (Sartorious)
- Peristaltic Pump (Pharmacia biotech)

- Vacuum Pressure Pump (Pall-Lifesciences)
- Microwave oven (LG)
- Master cycler gradient PCR machine (Eppendorf)
- Pipettes P2.5, P20, P200, P1000 (Eppendorf)
- Dispenser (Eppendorf)
- Milli-Q water purification system (MilliQ)
- Water bath (Julabo)
- Ice making machine (Icemate)
- Phast IEF System, GE
- Agilent HPLC Series 1100 / 1200
- Plate Reader, BioTek Instruments, Model - Synergy HT
- SYNAPT HDMD Mass Spectrometry, Waters.
- Procise 492, ABI Biosystems

2.1.11. Softwares:

- PlasMapper
- NEB RE Cutter
- PLA2.0®

2.2. Buffers and Media Preparation

2.2.1. Competent Cell Preparation

1. $CaCl_2$ (0.1 M)

   2.940 g of $CaCl_2$ (Mol. Wt. - 147.02) was dissolved in 150 mL of MilliQ / WFI. The final volume was made up to 200 mL.

   General usage: For competent cells preparation

   Sterilization: Autoclaved at 121 psi for 15'

   Storage: 4 °C

2. 0.1 M CaCl2 + 15% Glycerol

   15 mL of 100 % glycerol was added in 85 mL of 0.1 M $CaCl_2$ solution (prepared above).

   General usage: For washing cell pellet for competent cells preparation.

   Sterilization: Autoclaved at 121 psi for 15 min

   Storage: 4 °C

2.2.2. Glycerol Bank preparation

1. Glycerol (50%):

   50 mL of glycerol was added in 50 mL 0f autoclaved MilliQ / WFI and final volume was made up to 100 mL.

   General usage: As a cryoprotectant for competent cells

   Sterilization: Autoclaved at 121 psi for 15'

   Storage: 4 °C

2.2.3. Bacteriological Media

1. SY - Media

   This media contains 1.6% selected Soytone, 1.0% Yeast extract.

   4.0 g of Soytone and 2.5 g yeast extract was dissolved in ~200 mL of WFI / MilliQ. The final volume was made up to 250 mL with WFI / MilliQ.

   The media was sterilized by autoclaving at 15psi at 121 ^{0}C for 15 min.

   For preparing agar plates, 3.75 g of agar (1.5% agar) in 250 mL SY broth was added before sterilization.

   The media can be warmed if necessary to dissolve the completely.

2.2.4. Cell Culture Media

Note: All the cell culture media and supplements were filter sterilized using 0.2µ filter before using.

1. Growth Media for CHO-S cells:

   DMEM powder (13.4 g) was dissolved in 800 mL of WFI / MilliQ after which 3.7 g/L sodium bicarobonate was dissolved. The pH was adjusted to 7.2 by addition of 5N HCl. The total volume was made up to 1000 mL with WFI / MilliQ. Subsequently, 10% Foetal Bovine Serum, 1x HT supplements (from 100x), 1x MEM Non-essential amino acid mix (from 100x), 4 mM glutamine (from 200 mM stock) and 1x Penicillin -Streptomycin solution (from 100x) was added and filter sterilized.

   This media was used for growing cells before and during transfection. The cells were later adapted to chemically defined media as follows:

   In 1000 mL of CD-CHO (liquid), 4mM Glutamine, 1x HT supplements, 1x MEM Non-essential amino acid mix, 10 µg /mL Dextran Sulfate, 1x Penicillin - Streptomycin solution was added.

   Similarly, the media used during the upstream process development at shake flask were prepared with HT supplements, MEM Non-Essential amino acid, Dextran sulfate and Penicillin- Streptomycin solution.

2. Growth Media for SP2/0 cells:

   RPMI-1640 + 10% Foetal Bovine Serum + 1 mM Sodium Pyruvate + 0.4 g/L glucose.

3. Cryopreservation Media:

   The cell line specific cryopreservation media was prepared by addition of DMSO at rate of 10% in the respective growth media.

4. Feed Media :

   All the liquid form feed media listed in Chapter 5 were prepared as per the supplier's instruction and then addition of Penicillin-Streptomycin solution was done.

2.2.5. Electrophoresis Buffers & Reagents

1. SDS 20 %

   Procedure: In ~ 75 mL of warm WFI / MilliQ, 20 g of SDS (Mol wt:-288.38) was dissolved. The final volume was made upto 100 mL.

   General usage: 100 mL (for SDS-PAGE GEL Preparation)

   Sterilization: NA

   Storage: RT

2. Ammonium Persulphate (10 %)

   Procedure: 1 g of Ammonium Persulphate (Mol wt.:-228.2) was dissolved in 10 mL MilliQ.

   General usage: 10 mL (for SDS-PAGE Native and Reducing Gels).

   Sterilization: NA

   Storage: 4 °C for not more than 24 hrs.

3. Acrylamide: Bis acrylamide (29:1)

   Procedure: 29 g of Acrylamide (Mol wt.:-71.08) and 1 g of Bis-acrylamide (Mol wt.:- 154.17) was dissolved in 50 mL of WFI / MilliQ in a 250 mL beaker wrapped with aluminium foil. The final volume was made up to 100 mL and filtered through Whatman filter paper.

   General usage: SDS-PAGE and Native gels

   Sterilization: NA

   Storage: 4 °C

Precaution: Neurotoxic. Wear gloves and facemask while handling.

4. Tris –Cl pH 8.8 (1.5 M)

   Procedure: 182.1 g of Trizma base (Mol. Wt. - 121.14) was dissolved in 500 mL of WFI / MilliQ. The pH was adjusted to ~8.8 with conc. HCl. The final volume was made up to 1 L. Autoclaved and stored at RT.

   General usage: For Resolving PAGE

   Sterilization: Filtered through 0.45 µm filter.

   Storage: RT

5. Tris –Cl pH 6.8 (1 M)

   Procedure: 121.14 g of Trizma base (Mol. Wt. - 121.14) was dissolved in 500 mL of MilliQ. The pH was adjusted to ~6.8 usin conc. HCl. The final volume was made up to 1 L. Autoclaved and stored at RT.

   General usage: For stacking gel of PAGE.

   Sterilization: Filter through 0.45 µm filter.

   Storage: RT

6. Saturated Butanol (50%).

   Procedure: The butanol was diluted 1:1 (50 ml + 50 mL) in WFI/MilliQ and stirred for 30 min on magnetic stirrer. When the distinct layers appears, the upper layer was transferred to other bottle. This was used to overlay on polyacrylamide gels.

   General usage: Overlay on the resolving PAGE to make surface even

   Sterilization: NA

   Storage: RT

7. 10 X SDS-Tris Glycine Buffer:

   Procedure: 30.3 g of Trizma base (Mol. Wt.-121.14), 144 g of glycine (Mol .Wt - 75.07) and 10 g of SDS was dissolved in 850 mL of warm MilliQ. The final volume was made up to 1 L.

   General usage: Running of SDS-PAGE

   Sterilization: NA

   Storage: RT

8. Coomassie Blue Staining

Fixing Solution:

COMPONENT	VOLUME
Methanol	500 mL
Glacial acetic acid	100 mL
WFI	400 mL
Total	1000 mL

Storage: RT

General usage: Fixing protein on PAGE / IEF for staining.

Staining Solution:

COMPONENT	VOLUME
Methanol	50 mL
Glacial acetic acid	10 mL
WFI	40 mL
Coomasie Blue R 250	0.1 g
Total Volume	100 mL

*100 mL of fixing solution could be taken to prepare staining solution.-(100 mL fixing solution + 0.1 g Coomasie blue).

Storage: RT

General usage: Staining Proteins on PAGE / IEF gels.

Sterilization: NA

Destaining Solution:

COMPONENT	VOLUME
Methanol	400 mL
Glacial acetic acid	100 mL
WFI	500 mL
Total	1 L

Storage: 4 °C

General usage: Removal of excess stain from PAGE

Sterilization: NA

9. Protein Sample loading buffer: 4X Reducing.

	Final Conc. (1x)	**STOCK**	**5 ML**
Tris HCl (pH 6.8)	50 mM	1M	1.0
SDS	2 %	Solid	0.4 g
B-Mrcaptoethanol	1 %	14.7 M (100 %)	0.2 mL
Glycerol	10 %	100%	2 mL
EDTA, pH8.0	12.5 mM	0.5 M	0.5 mL
Bromophenol blue	0.2%	Solid	4 mg
H_2O	Make up	---	~1.5 mL

10. Silver staining solution

Preparation for 100 mL solution.

Solution A (Methanol –50%, Acetic acid-10%)

Methanol	50 mL
Acetic acid	10 mL
Water	40 mL

Storage: RT. Solution could be reused 5 times.

General usage: Fixing proteins on PAGE after gel run

Sterilization: NA

Solution B (Methanol – 5%)

Methanol	5 mL
Water	95 mL

Storage: RT.

The solution was prepared fresh every time.

Solution C (Sodium thiosulphate:-0.02 %)

0.02 g of Sodium thiosulfate (Mol wt: - 248.18) was dissolved in ~50 mL WFI / MilliQ in a clean plastic bottle. The final volume was made up to 100mL with WFI / MilliQ.

General usage:

Storage: The solution was prepared fresh every time.

Sterilization: NA

Solution D (Silver nitrate: - 0.2 %)

0.2 g of Silver Nitrate (Mol wt: - 169.88) was dissolved in ~50 mL of WFI in a clean plastic bottle The final volume was made up to 100mL with WFI.

General usage: Reagent for Staining Protein on PAGE or IEF gels

Storage: The solution was prepared fresh every time.

Sterilization: NA

Solution E (Developing Solution-Sodium carbonate:-3 %)

3 g of Sodium carbonate (Mol wt.:- 105.99) was dissolved in ~50 mL WFI. The final volume was made up to 100mL with WFI. The solution was prepared fresh every time.

To this solution 50 µL Formaldehyde and 2 mL (from 0.02%) Sodium Thiosulphate solution (Mol wt.:-248.18) was added just before its use during staining procedure for developing gel.

General usage: Reagent for developing silver stain PAGE / IEF Gels

Storage: the solution was prepared fresh solution every time.

Sterilization: NA

Solution F (EDTA Na2 Salt:- 1.4 %)

14 g of Sodium-EDTA (Mol wt.:-372.24) was added in ~70 mL WFI. It was dissolved by continuos mixing and dropwise addition of conc. HCl till EDTA dissolves completely. The final volume was made up to 1 L.

General usage: Stop Reagent for silver stain PAGE / IEF Gels

Storage: RT.

Sterilization: NA

Solution G

790 mg of Ammonium bicarbonate dissolved in WFI and the volume was made upto 100 mL with WFI.

General usage: Destaining of silver stain PAGE Gels

Storage: RT.

Sterilization: NA

Solution H

1.6 g of Sodium thiosulphate added in ~80 mL WFI and volume was made upt to 100 mL with WFI.

General usage: Destaining of silver stain PAGE Gels

Storage: RT.

Sterilization: NA

Solution I

1.0 g of Potassium ferricyanide in ~80 mL WFI and final volume was made upt to 100 mL with WFI.

General usage: Destaining of silver stain PAGE Gels

Storage: RT.

Sterilization: NA

11. TAE running buffer for agarose gel 50 X

121 g of Trizma base (Mol. Wt. - 121.14) was dissolved in 150 mL of MilliQ. Subsequently, 50 mL of 0.5 M EDTA (pH 8.0) was added, and then 28.55 mLof glacial acetic acid was added slowly. The pH was adjusted to ~8.3 and final volume made upto 500 mL.

General usage: 500 mL

Storeage : RT.

Sterilization: NA

12. Phosphate Buffered Saline (PBS):

8 g NaCl (Mol .Wt. -58.44), 0.2 g of KCl (Mol .Wt.) 1.44 g Na_2HPO_4 (Mol. Wt.:-141.96) and 0.24 g of KH_2PO_4 was dissolved in 400 mL of WFI / MilliQ. The pH was adjusted to 7.4 with conc. HCl. The final volume was made up to 1 L. [Final Conc. of components: 137 mM of NaCl ,2.7 mM of KCl ,10 mM of Na_2PO_4 and 2 mM of KH_2PO_4.]

General usage volume: 1 L.

Sterilization: Autoclave

Storage: RT

13. 1M Tris-Cl stock

121.14 g of Trizma base (Mol. Wt. - 121.14) dissolved in 500 mL of MilliQ. The pH was adjusted as per the requirement (eg 7.5, 6.8, 8.8, etc.) with conc- HCl. The final volume was made up to 1 L.

General usage volume: 1 L.

Sterilization: Autoclaved at 118 ^{0}C for 15 min.

Storage: RT

14. Tris Buffered Saline (TBS) 50 mM - for Western Blotting

Ingredients	Final Conc.	From Stock	Total Vol. (mL)
Tris HCl (pH 7.5)	**50 mM**	**1M**	**50**
NaCl	**150 mM**	**4 M**	**3.75**
Milli Q	**--**	**--**	**Make up to 1000 mL**

Storage: 4 °C

General usage volume: 1 L

Sterilization: NA

15. Wash Buffer (TBST)

In the above TBS buffer, Tween-20 at 0.1% was added. This solution was prepared fresh every time.

16. Transfer Buffer for Western Blotting

3.075 g of Trizma base (Mol wt:-121.14) and 14.4 g of glycine (Mol wt :-75.07) was dissolved in 500 mL of WFI / MilliQ. Subsequenly, 150 ml of methanol was added and final volume was made up to 1 L.

General usage volume: 1 L.

Sterilization: NA

Storage: 4 °C

17. Blocking Buffer For Western Blot:

5 g of Skimmed milk added in 50 mL of TBST. The skimmed milk was dissolved by vortexing and store at 4 °C.

General usage volume: 50 mL (for Blocking and Antibody preparation)

Sterilization: NA

Storage: 4 °C

18. TAE running buffer for agarose gel 50 X

121 g of Trizma base (Mol. Wt.- 121.14) dissolved in 150 mL of MilliQ. To this 50 mL of 0.5 M EDTA was added (pH 8.0) and then 28.55 mL of glacial acetic acid was added slowly. The pH was kept ~8.3 and finally volume was made upto 500 mL.

General usage volume: 500 mL

Sterilization: NA

Storage: RT

2.2.6. Protein A Purification

1. Preparation of Load Buffer (20 mM Phosphate Buffer pH 7.0):

0.3 g NaH_2PO_4 and 0.49 g Na_2HPO_4 was dissolved in 230 mL of Milli Q, and then 2.9 g NaCl was dissolved in it. The pH was adjusted to 7.00. The final volume was made up to 250 mL. Filtered with 0.45 µm filter before using for purification.

2. Preparation of Elution Buffer (75mM Glycine, 0.5 M NaCl buffer pH 3.0):

1.4 g Glycine and 7.3 g NaCl was dissolved in 230 mL of Milli Q. The pH was adjusted to pH 3 by addition of conc HCl. The final volume was made up to 250 mL. Filtered with 0.45 µ filter.

2.2.7. Protein A HPLC

1. Preparation of Mobile Phase A

8.2 g of Sodium Acetate anhydrous and 29.22 g of Sodium chloride was dissolved in 800 mL of WFI or MilliQ. The pH was adjusted to 7.0 with 1:5 times diluted Acetic Acid (5 -6 mL was generally required required). The final volume was made up to 1 L with WFI or MilliQ.

2. Preparation of Mobile Phase B

8.2 g of Sodium Acetate anhydrous and 29.22 g of Sodium chloride was dissolved in 500 mL of WFI or MilliQ and add approximately 350 mL of concentrated acetic acid was added. The pH was adjusted to 2.5 with

concentrated Acetic Acid. The final volume was made upto 1L with WFI or MilliQ.

2.2.8. Cation Exchange Chromatography

1. Preparation of Mobile Phase A (20mM MES (pH 6.75):

   3.9 g of MES was dissolved in ~800 mL of WFI / MilliQ. The pH was set at 6.5 with 1N NaOH. The final volume was made upto 1L with WFI or MilliQ or WFI. Filtered through 0.2µm filter. This buffer can be stored at 2 ^{0}C to 8 ^{0}C for 48 hrs.

2. Preparation of Mobile Phase B:

   3.9 g of MES and 29.22 g Sodium Chloride was dissolved in ~800 mL WFI or MilliQ. The pH was adjusted to 6.75 with 1N NaOH solution. The final volume was made upto 1L with WFI or MilliQ. Filtered through 0.2µm filter.

2.2.9. Reverse Phase Liquid Chromatography

1. Mobile phase A:

   70 % Acetonitrile (700 mL ACN + 300 mL WFI / MilliQ)

2. Mobile phase B:

   100 mM Na_2SO_4, pH 2.5 + 0.1.

   14.2 g Na_2SO_4 dissolved in 950 mL of WFI / MilliQ water, the pH was adjusted to 2.5 with concentrated H_2SO_4. The final volume was made upto 1L with WFI or MilliQ. Filtered through 0.2 µm filter.

2.2.10. Size Exclusion Chromatography

1. Preparation of Mobile Phase (Phosphate Buffer, pH 6.5):

   7.1 g of Di-sodium hydrogen phosphate anhydrous was dissolved in ~800 mL of WFI / MilliQ. The pH was adjusted to 6.5 with concentrated ortho-phosphoric acid. The final volume was made upto 1L with WFI or MilliQ. Filtered through 0.2µm filter.

2.2.11. *In-vitro* Bioassay

1. Growth Media for A431NS Cell line

Sr. #	Basal Media / Supplements	Quantity
1	Dulbecco's Modified Minimal Essential Media (DMEM)	870 mL
2	Glutamine (4mM)	20 mL of 200 mM
3	Fetal Bovine Serum	100 mL
4	Sodium Bicarbonate	3.7 g
5	Penicillin-streptomycin solution	10 L

All the above components were mixed aseptically in a sterile reagent bottle and vacuum filtered through 0.2 µm sterile filter. The media was stored at 2 °C to 8 °C. The sterility of media was checked by inoculation 1 mL of media in LB broth and Sabouraud's Dextrose broth for checking bacterial and/or fungal contamination. The sterility tubes were incubated for 14 days at 37 ^{0}C or RT respectively. The media was used within one month from the date of preparation.

2. Cell Fixing Solution

   The Formaldehyde solution (37%) available was diluted 1:10 in PBS to get 3.7% which is used for fixing cells.

3. Blocking solution

   3 g of BSA dissolved in TBS (pH 7.5). To be freshly prepared

4. Antibody solution

   The secondary antibody (Anti-IgG kappa specific HRP conjugate) was diluted 1:10000 in blocking solution.

5. Substrate solution

   TMB readymade solution used

2.3. Methods

2.3.1. RE Digestion

The reaction in general was set up as follows:

Component	Amount
10X RE specific Buffer	1X
100X BSA (If needed))	1x
Restriction Enzyme (RE)	1– 3 units per µg DNA
DNA	1 -2 µg per reaction
Milli Q Water	Make up volume to 15- 20 µL

The reaction mix was incubated at at 37 ^{0}C for 3-4 hrs. or overnight.

2.3.2. Ligation Reaction:

The reaction in general was set up as follows:

Component	Amount
Double digested, purified Vector DNA	1 mole
Double digested, purified Insert DNA	3 moles
10 x Ligation buffer	1.5-20 µl
T4 DNA ligase	1 µl
Milli Q Water	Make up volume to **15- 20 µl**

The ligaion mix was incubated for ≥ 16 hrs at 22 ^{0}C.

2.3.3. Gradient PCR:

The gradient PCR was set to find the optimal annealing temperature for further amplification of desired DNA fragment in bulk quantity. Typical reaction is as follows:

Component	Amount
10X Taq DNA Buffer	1x
Taq DNA Polymerase	2 -3 units
10mM dNTP mix	0.5 mM
5 µM Forward Primer	0.25 µM
5 µM Reverse Primer	0.25 µM
Template DNA	Min 100 ng
Milli Q Water	Make up volume to **20 µL**

Note: In case of PCR for the genes to be clones, Phusion *Pfu* polymerase or *Pfu* Turbo was used.

2.3.4. Plasmid and Genomic DNApreparation protocols were followed based onthe kit manual.

2.3.5. Competent cell Preparation *E.coli* DH5α

Inoculated *E. coli* cells from glycerol bank in 10 mL of SY media and incubated at 37 ^{0}C /200 rpm for 16-18 hrs in incubator shaker. The OD_{600} was measured of overnight grown culture following which the culture was splited at OD_{600} 0.2 in 100 mL of SY in a 500 mL conical flask. The flask was incubated futher under similar conditions until OD reaches to 0.8 to 1.0 (good log phase). The culture was dispensed in centrifuge tubes and cooled on ice for 30 minutes. The cells were pelleted by at 4000 rpm for 10 min at 4 ^{0}C. The cells were washed twice with 10 mL (1/10th volume of initial culture) of ice cold 0.1 M $CaCl_2$ and spin at 4000 rpm at 4 ^{0}C for 10 min. Following this the cells were resuspended in 1 mL (1/100th volume of initial culture) of 0.1M $CaCl_2$ and 15% glycerol solution. This resuspended cells were aliquoted in sterile micro-centrifuge tubes (50 µL each) and stored at 80 ^{0}C for future use.

2.3.6. Transformation of *E.coli* DH5α by Heat & shock Method

An aliquote of 50 µL aliquot of competent cells was thaw on ice for 1-2 minutes, to which 950 µL of cold 0.1M $CaCl_2$ was added and mix gently. This was resuspended in fresh microcentrifuge tubes, 200 µL each. To this competent cells 5-20 µL of the ligation mix (or 0.5 µL of supercoiled DNA for positive control) was added. After gentle mixing the cells and ligation mix was incubated on ice for 30 minutes. The cells were given a heat shock at 42 ^{0}C for 60 seconds and incubated on ice for 3 minutes. The SY media was added to make the final volume to 1 mL and then cells were incubated at 37 ^{0}C in a shaker at 200 rpm for 45-60 minutes. The transformed cells were plated at two different concentrations (10 % and 50%) on to the agar plates containing selection antibiotics. Incubated at 37 ^{0}C in biological incubator for 16–18 hrs (overnight) when the visible colonies appeared.

2.3.7. Cell Culture Methods

1. <u>Maintenance of Cell Lines</u>

The seeding density of CHO-suspension cells and SP2/0 cells were kept @ 0.5 x 10^6 to 0.75 x 10^6 cells / mL. When the cell density reaches 2-2.5 x 10^6 cells/ mL the cells were subcultured and reseeded at the same seeding density.

2. Transfection of CHO-S by Lipofection

The following steps for transfection were followed:

On Day 0: The cells were seeded in 6 well TC plates @ ~0.5 x 10^6 cells / well. The cells were observed for the confluency (generally ~60-70% confluency required for the Transfection). The media was changed completely before 3-4 hrs of transfection. For each well, DNA was diluted with medium without serum (e.g.OPTI-MEM medium) in sterile tubes / cryovials in the following way:

- Added transfection reagent in different ratios of DNA:Transfection Reagent (1:4 and 1:6); ie., 1 µg DNA + 4 µL Transfection reagent in OptiMEM media.
- Tube A: The Lipofectamine reagent with Opti-MEM media was added (4 µL Transfection Reagent to196 µL Opti-MEM media) and incubated at RT for 5 min.
- Tube B: Linearized vector (~1.0 µg DNA) in a total volume of 100 µL Opti-MEM.
- Tube 1: TEST=100 µL of Tube A + 100 µL of Tube B Linearized Expression vector
- Tube 2: Negative Control = 100 µL of Tube A + 100 µL of OptiMEM.

The tubes were incubated at RT for 20 min to allow DNA- transfection reagent complex formation. This mixture (200 µL) was added drop-wise in respective wells of cells by intermittent gentle shaking. One well of cells were kept as control well without DNA. Plates were incubated at 37^0C at 5 %CO_2 in a humidified incubator. After 48 hrs of transfection a complete medium change was done with complete 1.0 mL DMEM medium. The cells were passaged / subcultured when the confluency reaches to around 80-90% (generally after 24 hrs). The cells were subcultured at a ratio of 1:4 and maintained in 6 well plates. The supernatant was collected after 24 & 48 hrs of transfection were checked for transient expression analysis. The selection pressure was added after 48 hrs starting with lowest concentration 200 µg / mL Geneticin G418 and then increasing concentration to 750 µg / mL Geneticin G418. A cell bank of 1 vial each in complete medium + 5% DMSO was prepared as back-up and stored at LN_2 for future use. This is done at every critical stage of cell line development.

3. Transfection of CHO-S by Electroporation

The cells were trypsinized, centrifuged at 210 xg for 5 min at room temperature in swing-out bucket. The cells were resuspended in Hank's Balanced Salt Solution to get 0.75 x 10^6 cells/mL/cuvette/well. The cells were mixed with 1 µg linearized plasmid DNA. The cuvettes were cooled on ice for few minutes. The cells were the electroporated using following two programs:

- Program 1 – Exponential-130v, 600 µF, 4mm cuvette. Resulting in 15.7 mS, 129v.
- Program 2 – Time Constant, 200v, 20 mS, 4 mm cuvette. Resulting in 19.9 mS, 198v, 650 µF, ∞Ω

Folllowing electroporatiuon the cuvettes were kept at room temperature for 10 min.

The cells from the cuvettes were then added in respective wells in 6 well TC plate with 1 mL growth media. The plates were incubated at 37^0C at 5 % CO_2 in a humified incubator. The supernatant was collected after 24 & 48 hrs of transfection were checked for transient expression analysis. The selection pressure was added after 48 hrs starting with lowest concentration 200 µg / mL Geneticin G418 and then increasing concentration to 750 µg / mL Geneticin G418.

4. Generation of Minipools

The lead transfectant pool based on growth and productivity data were proceeded for minipools generation. Minipools are generated by plating 50-100 cells / well in 96 well TC plate. The minipools were labeled based on the position in plate that is the first letter is the alphabet of the row and second the sequencial number of the well of 96 well TC plate. The top minipools were selected based on cell growth and expression yield for single cell limiting dilution

5. Single Cell Limiting Dilution Cloning

The top two minipools were selected for limiting dilution to achieve single clonal population. The cells were seeded at 1-5 cells / well in 5 x 96 well plates. Following seeding, the wells were observed and marked for receiving single cell per clone using microscope. The growth and expression yield of the single clones were analyzed and final 6-8 clones were selected for further analysis.

2.3.8. Serum Free Adaptation of selected clones

The 8 clones shortlisted after single cell limiting dilution were gradually adapted to serum free culture. Individual clones were reseeded in T25 cm^2 TC flask at 0.5-0.6 x 10^6 cell/mL in reduced serum containing media 2%. The cells were subcultured for 2 passages in this media. Individual clones were reseeded in T25 cm^2 TC flask at 0.5-0.6 x 10^6 cell/mL in further reduced serum containing media 1% and 50:50 DMEM:CD-CHO media. The cells were subcultured for 3 passages where only the cells floating in suspension were collected and the adherent cells were discarded. Individual clones were then reseeded in T25 cm^2 TC flask at 0.5-0.6 x 10^6 cell/mL without serum containing CD-CHO media. The growth was monitored for 3 passages and following this the cells were adapted to suspension culture by seeding in 125 mL shake flaks containing 40 mL of CD-CHO media and seeding count of 0.75-0.9 x 10^6 cell /mL. The cells were grown for 3 passages and each passage the growth and productivity profile need to be measured.

2.3.9. Evaluation of clones

The clone were screened and evaluated at smaller scale, 6 well plate or T25 cm^2 TC flask. At this stage, the cells were seeded at 0.2-0.3 x 10^6 cell/mL and allowed to grow in incubator maintained at 37 ^{0}C and 5% CO_2. After three or four days the cell supernatant were collected and analyzed for product expression by ELISA or Protein-A HPLC as stated. The shortlisting of top 3 producer clones were done in 125 mL shake flask under suspension culture and agitation. Clone evaluation was carried out in 125 mL shake flask with 40 mL growth media (CD-CHO with 4 mM Glutamine). The cells were seeded at 0.5 - 0.75 x 10^6 cell/mL followed by incubation in humidified CO_2 incubator maintained at 37 ^{0}C temperature and 5% CO_2, the shaker speed maintained was 130-150 rpm. After achieving cell density of ~ 3-4 x 10^6 cells / mL and viability of >90% at Day 3/4, the temperature was reduced to 32 ^{0}C and cells were maintained till viability drops below 80% (generally 16 days). The cells were maintained by addition of Feed cocktail (Efficient Feed A + Efficient Feed B in ratio of 1:1) at 10% v/v on every alternate day starting from the day of temperature shift. Samples were collected on alternate days after temperature shift for cell count and productivity analysis (after Day 6). This is a preliminary process to start for the study of expression of protein and evaluation of clones. The Integral viable cell density (IVCD) was calculated using following formula:

$$IVCD(t_2) = IVCD(t_1) + \frac{VCD(t_1) + VCD(t_2)}{2} \times (t_2 - t_1)$$

Where VCD(t) is the viable cell density at time t.

The protein expression in supernatant was measured either by ELISA or Protein- A HPLC. The specific productivity (qP) of clone was calculated as:

qP = Expression Yield / IVCD

2.3.10. Upstream Process Development

The scope of upstream process development was to identify the most suitable basal media and feed supplement supporting growth and expression yield of the lead clone (D41E213).

1. Screening Basal Media

The clone D41E213 was seeded in the basal media listed in below table:

ID	Media Name	Mfg	Cat#	Form
A	Is-MAB-CD	Irvin Scientific, USA	91104	Liquid / powder
B	IS-CHO-CD-XP	Irvin Scientific, USA	91121	Liquid / powder
C	IS-CHO-CD-XP	Irvin Scientific, USA	91120	Liquid / powder
D	IS-CHO-CD-K-XP	Irvin Scientific, USA	91125	Liquid / powder
E	HyQCDM4CHO	Hyclone, USA	ARF26505	Liquid / powder
F	CDM4PerMab	Hyclone, USA	AWB94001	Liquid / powder
G	Serum FreeCHO	HiMedia, India	SFM002	Liquid
H	PowerCHO2CD	Lonza	BE12-771Q	Liquid
I	PF-CHO Media 1 OF 2	Hyclone, USA	SH303334.01	Powder
	PF CHO BASAL 2 OF 2		SH303335.01	
J	Excell 302	SAFC	24324C	Liquid / Powder
K	MAM-PF7	Animed, Israel	10-02575-I	Liquid
L	Mam-PF2	Animed, Israel	10-02F24-I	Liquid
M	CDM4Mab	Hyclone, USA	AVK80331	Liquid / Powder
N	CD-OptiCHO	Invitrogen, USA	12681	Liquid
O	Excell CD-CHO	SAFC	14360C	Liquid / Powder
P	Pro CHO4	Lonza	BE12-029Q	Liquid / Powder
Q	Pro CHO5	Lonza	BE12-766Q	Liquid / Powder
Z	CD-CHO	Invitrogen, USA	10743	Liquid

Table 2.1.2 List of Basal Media

The cells were first adapted in respective media by seeding at 0.2 - 0.3 x 10^6 cell/mL in 6-well TC plate for 2 passages. Following this the cells were reseeded at 0.2 - 0.3 x 10^6 cell/mL and allowed to grow at 37 ^{0}C and 5% CO_2. The cell supernatant was withdrawn on Day 3 and Day 5 and expression was measured by ELISA.

2. Screening Feed Media

The clone D41E213 was seeding in the shortlisted basal media from above list and expression of product from the clone was studies when the culture was run in a fed-batch mode using below listed feed media:

Sr. No.	FEED NAME	**MFG.**	**CAT #**	**FORM**
1	Efficient Feed A (10%)	Invitrogen	A10234	Liquid
2	Efficient Feed B (10%)	Invitrogen	A10240	Liquid
3	Gln (4mM) + Glu (2g/L)	NA	NA	Powder
4	Efficient Feed C (10%)	Invitrogen	A13275	Liquid
5	Extreme Feed (Excellgene)	Lonza	NA	Liquid
6	Cell Boost 2 (10%)	Hyclone	RR12927.01	Powder
7	Cell Boost 3 (10%)	Hyclone	RR12635.01	Powder
8	Cell Boost 4 (10%)	Hyclone	RR12800.01	Powder
9	Cell Boost 5 (10%)	Hyclone	RR12929.01	Powder
10	Cell Boost 6 (10%)	Hyclone	RRE114619	Powder
11	Lonza Feed (10%)	Lonza	NA	Liquid
12	Feed Power CHO2 (10%)	Lonza	BE12-771Q	Liquid
13	Is-CHO-Feed-CD-XP (10%)	Irvine	91122	Liquid / Powder
14	GS-Max (10%)	Hyclone	SH30586.01	Liquid
15	Efficient Feed A + B (5%)	Invitrogen	A10234 + A102340	Liquid
16	Efficient Feed A + B (7.5%)	Invitrogen		Liquid
17	Efficient Feed A + B (10%)	Invitrogen		Liquid

Table 2.1.2:

Table 2.1.3 List of Feed Media

The D41E213 cells growing in suspension culture were seeded at 0.2 - 0.3 x 10^6 cell/mL in 6-well TC plate in the shortlisted basal media from the media screening experiments. The plates were incubated at 37 ^{0}C and 5% CO_2 for four days on which

the first feed addition was done followed by second addition on day 6 of culture. The supernatant was collected on Day 6 (48 hrs. after first feed addition) and Day 8 (48 hrs. after second feed addition) for expression yield estimation by ELISA.

3. Fed Batch experiments in shake flask for media, feed screening and clone evaluation

For running experiments in shake flask the cells were seeded at 0.5 – 0.6 x 10^6 cell / mL in 40 mL volume in 125 mL Erlenmeyer flask. The flasks were incubated on shaker maintained at 120-150 rpm in CO_2 incubator (37 ^{0}C and 5% CO_2). On Day 4 feeding is started and continued on every alternate day. The incubation temperature was reduced from 37 ^{0}C to 32 ^{0}C in order to slow down cell growth and enhance production of the desired product. The viability was monitored every alternate days and when it reaches below 75% the cell supernatant was harvested (or on Day 18).

2.3.11. Analytical Methods

1. SDS-PAGE (Reducing and Non Reducing)

The 10% Resolving Gel was prepared as per the following recipe.

CONSTITUENTS	**5 ml (For 1 gel)**	**10 ml (For 2 gels)**	**20 ml (For 4 gels)**	**40 ml (For 8 gels)**
WATER	2.2 ml	4.45 ml	8.9 ml	17.8 ml
29% ACRYLAMIDE/1% BIS ACRYLAMIDE MIX	1.7 ml	3.35 ml	6.7 ml	13.4 ml
1.5 M TRIS-Cl **(pH8.8)**	1.05 ml	2.1 ml	4.2 ml	8.4 ml
20% SDS	25 µl	50 µl	100 µl	200 µl
APS (10%)	25µl	50 µl	100 µl	200 µl
TEMED	2.5 µl	5 µl	10 µl	20 µl

For casting 4-14% Resolving Gel, a 20% resolving gel was prepared and added in a chamber of gradient mixture while in other chamber MilliQ water was added. The gel was poured following guidance from the supplier's manual to cast the gradient gel.

The stacking gel (4%) was prepared as per the following recipe:

CONSTITUENTS	2.5 ml (For 1 gel)	5.0 ml (For 2 gels)	7.5 ml (For 3 gels)	10.0 ml (For 4 gels)
MILLIQ / WFI	1.5 ml	3ml	4.5 ml	6 ml
29% ACRYLAMIDE/1% BIS ACRYLAMIDE MIX	0.35 ml	0.7 ml	1 ml	1.35ml
0.5 M TRIS-Cl (pH 6.8)	0.625 ml	1.25 ml	1.88 ml	2.5 ml
20% SDS	12.5 µl	25µl	37.5 µl	50 µl
APS (10%)	12.5 µl	25.0 µl	37.5 µl	50 µl
TEMED	2.5 µl	5.0 µl	7.5 µl	10 µl

Running Gel

The gel apparatus was set as per the instructions in suppliers manual. 1x running buffer was poured in the upper as well as lower chambers of the running unit as recommended The samples and protein standard marker was loaded as per the lane pattern.

The gel running parameters for a minigel on the Power pack was set as:

- Constant Voltage: 100 V in stacking gel and 125V in resolving gel
- Max Current: 200 mA
- Run until dye front reaches the bottom of gel. (Usually about 1½ hrs).

Staining of the Gel:

Depending on the sensitivity required, either Silver or Coomassie Brilliant Blue staining was used.

Silver Staining of PAGE:

Step No.	Solution	Operation	Time
1	Sol A	Fixing	30 Min
2	Sol B	Incubation	15 Min
3		Wash 3 times with WFI	3 x 5 Min
4	Sol C	Incubation	2 min
5		Wash 3 times WFI	3 x 30 sec.
6	Sol D	Incubation	25 Min
7		Wash 2 times WFI	2x60 sec
8	Sol E	Developing	10 Min Max
9	Sol F	Stop developing	10 Min.
10	---	Wash with water	----

Coomassie Staining of PAGE:

The gel were removed from the gel plates carefully and transferred to box containing ~20-30 ml Coomassie Brilliant Blue stain. The staining was done at RT on rocking platform for at least 30 min at RT. The staining solution was removed and gel was rinsed with Milli Q. The gel was destained by destaining solution. The destaining was done till the clear bands without background observed. The image was captured using documentation system.

2. Western Blot:

This is an identity test to detect the expressed antibody using conjugated antibody. The SDS-PAGE was run under non-reducing conditions as per the procedure in described in above section. The bands were transferred from the gel onto the PVDF membrane using semi-dry western blot apparatus. The membrane was checked quickly by staining with Ponceau-S stain. The blot was then blocked with 5% skimmed milk for 30 min at RT under rocking conditions. The blot was washed twice with TBST buffer following which it was probed with secondary antibody (anti-IgG HRP conjugate). The blot was incubated at RT for 60 min under rocking conditions. The blot was washed thrice with TBST buffer and then detected developed by addition of TMB substrate.

3. Isoelectric Focussing

RMP and Sample Preparation

The RMP and Protein-A purified samples were concentrated and desalted using 35 kDa centricon. The concentrated sample was reconstituted in 25 µL of WFI / MilliQ water.

Focusing IEF Gel

The cassette was wiped with 70% IPA and then with MilliQ. The gel was placed on the cassette and clamped so that there should not be any air bubble trapped beneath the gel. The gel was soaked in Pharmalyte mixture (40% pH 5-8 + 60% pH 8 – 10.5 + 7 M Urea). The wells were checked for any leakage. 2 mL of pharmalyte was added. The gel was pre-focussed at 70 AVH. After pre-focussing samples and RMP was applied using sample applicator (to the assigned well) and then the gel was focused till the current reaches 512 AVH. Remove the gel and strip the pharmalytes by treating the gel with 10% TCA for overnight and then with 1% TCA for 1 hr. The gel was stained using silver staining protocol as described in the Phast System program.

4. Sandwich Indirect ELISA

- Coating Antibody:

  Mouse monoclonal anti human IgG (Fc specific) **Stock:** (1 mg / µL).

  Working Coating Concentration: 200 ng / 100 µL / well.

  The antibodies were added in each well, 100 µL. The plate was incubated for O/N at 2 – 8 °C or at 37 ^{0}C for 3 hrs. The plate was washed 3-times with TBST buffer.

- Blocking: 3% BSA in Wash Buffer.

  The blocking was done by addition of 300 µL blocking solution to each well. Plate was incubated for 60 min at 37 °C. The plate was washed 3-times with 300 µL of TBST buffer.

- Standard and Sample dilution:

  The RMP was available at a stock concentration of 5 mg/mL. It was diluted in PBS to get working concentration of 125 ng /mL.

  All the samples & standard dilutions were diluted in TBST buffer. Following this the working standard was serially diluted (250 µL + 250 µL) till 1.83 ng /mL. The standard dilutions were added in duplicates. The 100 µL of each dilution (standard and samples) were added in the pre-coated plate. The plate was incubated for 60 min at 37 °C and then washed 3-times with 300 µL of TBST buffer. **Note: The samples should be added as per the plate layout shown below.**

- Detection antibody & Plate Reading:

  Detection Antibody: Anti-mouse IgG kappa specific polyclonal antibody-HRP conjugated. **Stock:** (1mg/ml).

  Working Concentration: 1:5000 in 1% blocking solution

  This antibody was added at 100 µL / well and incubated for 60 min at 37°C. The plate was washed 3-times with 300 µL of TBST buffer.

  Substrate addition:

  100 µL substrate (TMB/OPD) was added into each well. The plate was incubated for 15-20 min under dark. When the sufficient color development was observed in standard wells, the reaction was stopped by addition of 50µl of the stop solution (1N HCl) to each well. Plate was read using plate reader after a brief shaking. (TMB: 450nm or OPD: 492nm).

Schematic Representation of Plate Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Std 125 ng / mL**	**Std 125 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	**Std 62.5 ng / mL**	**Std 62.5 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	**Std 31.25 ng / mL**	**Std 31.25 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	**Std 15.6 ng / mL**	**Std 15.6 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	**Std 7.8 ng / mL**	**Std 7.8 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	**Std 3.9 ng / mL**	**Std 3.9 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	**Std 1.83 ng / mL**	**Std 1.83 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Blank
H	**Std 0 ng / mL**	**Std 0 ng / mL**	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Blank

5. Protein A purification

Approximately 8 mL of harvest diluted in Load buffer (total 15 mL) was loaded onto the GE MabSelectSure resin at a pH of 7, conductivity of 24 ms/cm and 1ml/min flow rate. Washed with the load buffer at 1 ml/min for 10-20 CV.The protein was eluted using 75mM Glycine, 0.5 M Sodium chloride buffer, pH 3.0.

Load volume: 8ml Conc : 0.3mg/ml diluted to 15 ml with equilibration buffer pH7; conductivity: 24ms/cm.

6. Protein –A HPLC

Preparation of standards for calibration curve

Stock solution: - The RMP available is 5 mg/ mL. To create a standard curve the following dilutions were prepared and injected. The RMP was diluted to 1.25 mg/mL following which the further dilution was done as per the below mentioned dilution scheme:

Sr. No.	Conc. Ref. Standard (mg/ mL)	Volume of Stock 1 (µl)	Volume of Mobile Phase A (µl)	Total volume (µl)	Injection amt. (µg)	Injection volume (µl)	No. of injections
1	15	15	485	500	15	100	3
2	10	10	480	500	10		
3	5	5	495	500	5		
4	3	3	497	500	3		

Calibration Curve Preparation:

100 µl of each dilution was injected in triplicates. The run was programmed as:

Time (min)	Mobile phase(B)	Flow-rate
0.0	0%	2.0 mL /min
3.0	0%	
4.0	100%	
6.0	100%	
6.5	0%	
11.0	0%	
Detection wavelength	280 nm	
Sample manager temperature	2 °C to 8 °C	
Column temperature	25 °C	
Pressure	Min: 0 bar, Max: 250 bar	
Run time	11 min	
Injection Volume	100	

The Mean, Standard deviation (SD) and % RSD of area were calculated, (by applying the formula of % RSD as mentioned below). A graph of mean area vs known concentration was plotted and perform linear regression analysis. Calculate Slope of regression line and the squared correlation coefficient (R2 value). The back calculated concentration and back calculated % recovery was calculated by applying the formulas give below:

Note: Formulas used for calculation:

$$\%RSD = \left(\frac{\text{Standard Deviaition}}{\text{Average Area}} \right) \times 100$$

$$\text{Back calculated Con.} = \left(\frac{\text{Average area of the standard solution - intercept}}{\text{Slope of the calibration curve}} \right)$$

$$\text{Back calculated \% Recovery} = \left(\frac{\text{Back calculated Concentration mg/ml}}{\text{Actual Concentration (mg/ml)}} \right) \times 100$$

Acceptance criteria for Calibration Curve followed were:

1. The % RSD of the area between the triplicate injections in the calibration curve of reference standard should be ≤ 2 %.
2. The R2 value of regression should be ≥ 0.99 for the calibration curve.
3. Back calculated % recovery should be between 90 to110 % percent.

Preparation of Test Solution

This method was used for two types of samples: (a) Cell culture supernatant and Harvest samples (b) Partially purified samples after Protein-A purification.

The samples to be analyzed was injected as such (100 µL**)**.

Sample Analysis

The samples were prepared as discussed above. The column was equilibrated using obile phase B and with the above conditions for around 40 min (or till a stable base-line is obtained). The system suitability was analyzed following which the samples were analyzed. The samples were injected in singlet. The injection pattern is as follows:

Injection No.	Sample/ analyte	Injection volume (µL)	Number of injections
1	Blank	-	3
2	Standard solution (5 µg)	100	1
3	Test sample	100	1

Note: The blank which is just before the run of the samples should be taken for blank subtraction.

Equipment and Equipment Parameters

Instrument: - Agilent 1100/1200 series

Column	
Brand Name	Applied Biosystems
Catalog Number	1-5022-26
Type of Column	POROS-A/20
Specification of Column	Diameter: 4.6 mm, Length: 100 mm Particle size: 20 Micron
Guard Column	
Brand Name and specifications	Supelco, 0.5 µm
Catalog Number:	55214-U

7. RP-HPLC

Preparation of Standard (5µg / 100 µL)

The RMP stock available is 5 mg/mL, this was diluted 1:100 in Mobile Phase A. The volume injected was 100 µL, resulting in injection of 5 µg protein.

Preparation of Test Sample:

100 µL of cell culture supernatant was injected directly. The sample loading pattern is shown below:

Injection No.	Sample/ analyte	Injection volume (µl)	Number of injections
1	Blank	-	3
2	Standard solution (5 µg)	100	1
3	Test sample	100	1

Equipment and Equipment Parameters

Instrument: - Agilent 1100/1200 series

Column:	
Brand Name	Grace Vydac
Catalog Number	214TP54
Type of Column	Reverse phase C4 column
Specification of Column	Diameter: 4.6mm, Length: 250 mm Porosity: 300˚A, Particle size: 5 Micron
Guard column	
Brand Name	Zorbex 300 SB-C3 (Agilent)
Catalog Number	820950-921
Type of Column	Reverse phase Zorbax 300 SB-C3
Specification of Column	Diameter: 4.6mm, Length: 12.5 mm Porosity: 300˚A, Particle size: 5 µ

Chromatography Parameters

Method Parameter: <u>Buffer A</u>: 70% ACN **<u>Buffer B</u>**: 100 mM Na_2S0_4				
Time	%Buffer A	%Buffer B	Flow rate	Max. Press.
0	75	25	0.3 mL/min	250
2	75	25		
5	50	50		
10	20	80		
15	20	80		
17	75	25		
Absorbance		214 nm		
Column Temp.		75 ^{0}C		
Max. Press.		250 Bar		
Run time		25 min.		
Injection Vol.		100 µL		

8. <u>Cation-Exchange HPLC</u>

Preparation of Standard Solution

The RMP was diluted to a concentration of 1.0 mg/mL using mobile phase-A as mentioned in the point 2.2.8

Stock solution 5 mg / mL → 30 µL added in 120 µL of Mobile Phase A → 1 mg / mL

Preparation of test solution

The Protein-A purified test sample was diluted in Mobile phase A to get a concentration of 1.2 mg/ml (by OD280 nm)

The standard and samples were loaded in following pattern:

Injection No.	Sample	Injection volume (µl)	Number of injections
1	Blank	-	3
2	Standard solution (5 µg)	100	1
3	Test sample	100	1

Acceptance Criteria for System Suitability

- Retention time (Rt) of Major peak 1 of reference solution match with test solution (± 0.2 min)
- Resolution between Major peak 1 (which is K0: No lysine at C terminal) and Major peak 2 (which is K1:1 lysine at C-terminal) of the test solution should be more than 1.5 min.

Chromatography Parameter

Time (min)	% Mobile Phase A	% Mobile phase B	Flow-
0.0	91%	9 %	0.8 ml /min
2.0	91%	9 %	
23.0	70%	30 %	
24.0	70%	30 %	
26.5	0	100 %	
28.0	0	100 %	
28.5	91%	9 %	
50.0	91%	9 %	
Detection wavelength	220 nm		
Sample manager	2 °C to 8 °C		
Column temperature	30 °C		
Pressure	Max: 200 bar		
Run time	50 min		

Equipment and Equipment Parameters

Instrument: - Agilent 1100/1200 series

Column	
Brand Name	Tosho Hass
Catalogue Number	21966
Type of Column	TSK CM STAT

Specification of Column	Diameter: 4.6 mm, Length: 100 mm, Particle size: 7µ
Pre-column filter	
Brand Name and specifications	Supelco, 0.5 µm
Catalog Number:	55214-U

9. Size Exclusion - HPLC

Preparation of RMP

The RMP wa diluted to a concentration of 0.2 mg/mL using Mobile Phase (Point 2.2.10). For this the 30 µL of stock solution (5 mg / mL) was added in 120 µL of Mobile Phase A, resulting in 1 mg / mL. This was further diluted by adding 30 µL of 1 mg/mL in 120 µL of Mobile Phase A to achieve final concentration of 0.2 mg / mL.

Preparation of test solution

The protein concentration of the Protein-A purified samples were measured by OD280 nm. The test samples were diluted in mobile phase to get final concentration of 0.2 mg/ml.

The sample loading pattern is shown below:

Sr. #	Sample/Solution	No. of injections	Conc. (mg/ml)	Injection Vol. (µL)	Amt. injected (µg)
1	Blank (Mobile phase)	1	----	100	----
2	Reference solution	2	0.2	100	20
3	Test solution/s	1	0.5	30	15

The Retention time (Rt) and total area of the peaks obtained for reference solution was analyzed. The percentage recovery of reference solution using the calculation mentioned below was calculated

Average area of peak of Reference solution 2 = ‘X’

Total Area of peaks of the Reference solution 1= ‘Y’

The % recovery of Reference solution 2 = X*100/Y

Acceptance Criteria for System Suitability

Retention time (Rt) of Major peak 1 of reference solution match with test solution (± 0.2 min)

Chromatography Parameter

Time (min)	Mobile phase	Flow rate
0	100 %	0.5 ml/min
35	100 %	
Detection wavelength	214 nm	
Auto sampler temperature	2 °C to 8 °C	
Column temperature	25 °C	
Pressure	Min: 0 bar, Max: 100 bar	
Run time	35 min	

The column was equilibrated with the above conditions for about 60 min to 70 min. The RMP and sample was diluted as above.

Equipment and Equipment Parameters

Instrument: - Agilent 1100/1200 series

Column	
Brand Name	TOSHO HAAS
Catalog Number	08541
Type of Column	TSK3000SWXL, Size exclusion column
Specification of Column	Diameter: 7.8 mm, Length: 300 mm Porosity: 250 A°, Particle size: 5µ
Pre-column filter	
Brand Name and specifications	Supelco, 0.5 µm
Catalog Number:	55214-U

10. <u>*In-vitro* Bioassay</u>

Cell Line : A431NS

Growth Media – DMEM with 5% FBS and Pen-Strep solution

Antibody – Anti-Human IgG *kappa*-specific HRP conjugate

Blocking Buffer – 3% BSA

Substrate – TMB

The A431NS cell line expresses EGFR on the cell membrane and caveolar space. The binding on RMP and test sample to the receptor was measured by secondary detection with HRP labeled antibody

Day 0:

The cells growing in TC flask were trypsinized using Trypsin-EDTA solution and a single cell suspension was prepared. The cells were counted using automated cell counter. The cell suspension was diluted in growth media to achieve final cell concentration 2 x 10^5 cells /mL. 100 µL of diluted cell suspension was added in each well of 96-well TC plate leaving the side columns. In the outer wells 100 µL of PBS was added to avoid edge effect. The plates were incubated in incubator maintained at 37 ^{0}C and 5% CO_2 for 24 hrs.

Day 1:

Growthe media from the 96-well plate was discarded. The cells were gently washed by addition of 200 µL PBS. The cells were fixed to the plate surface by addition of 200 µL of 3.7% Formaldehyde and incubated for 15 min at RT. The monolayer was washed with 200 µL PBS twice. The surface of plate was blocked by addition of 200 µL / well 3% BSA solution incubated the plate at 37 ^{0}C for 1 hr. The plate was washed with 200 µL/ well PBS, twice. The diluted reference and test samples were added in following pattern:

	1	2	3	4	5	6	7	8	9	10	11	12
	PBS	RMP (ug/mL)			Sample 1		Sample 2		Sample 3			PBS
A	PBS	0.78125	0.78125	0.78125	1;2	1;2	1;2	1;2	1;2	1;2	Cell Ctrl	PBS
B	PBS	0.390625	0.390625	0.390625	1;4	1;4	1;4	1;4	1;4	1;4	Cell Ctrl	PBS
C	PBS	0.195313	0.195313	0.195313	1;8	1;8	1;8	1;8	1;8	1;8	Cell Ctrl	PBS
D	PBS	0.097656	0.097656	0.097656	1;16	1;16	1;16	1;16	1;16	1;16	Cell Ctrl	PBS
E	PBS	0.048828	0.048828	0.048828	1;32	1;32	1;32	1;32	1;32	1;32	Positive Ctrl	PBS
F	PBS	0.024414	0.024414	0.024414	1;64	1;64	1;64	1;64	1;64	1;64	Positive Ctrl	PBS
G	PBS	0.012207	0.012207	0.012207	1;128	1;128	1;128	1;128	1;128	1;128	Positive Ctrl	PBS
H	PBS	0.006104	0.006104	0.006104	1;256	1;256	1;256	1;256	1;256	1;256	Positive Ctrl	PBS

After addition the plate was incubated for 1 hrs at 37 ^{0}C. The cells were washed by addition of 200 µL/ well PBS, twice. The receptor-antibody complex was probed by addition of 100 µL/well 1: 5000 diluted anti-human IgG kappa specific HRP conjugate antibody (0.2 µg / mL). The plate of incubated for 1 hrs at 37 ^{0}C. The unbound secondary antibody was washed off by addition of 200 µL/well PBS, four times. 100 µL/well of substrate, TMB was added. The color development was done at RT for 15-20 min under dark conditions following which the over-development was stopped by addition of 50 µL/well of 5N HCl solution. The plate was read in plate reader at 450 nm. The relative activity of the samples in comparison with RMP was determined by calculating PLA software.

11. N-terminal Sequencing

The Protein-A purified samples were run on SDS-PAGE (10%) under reducing condition to separate heavy chain and light chain (Section 2.3.11 (2)). The bands were transferred from PAGE to nitrocellulose membrane using semi-blot apparatus (Section 2.3.11 (3)). The bands were visualized using by Ponceau-S stain and corresponding blots were cut and analyzed for N-terminal sequencing. The N-terminal sequencing was done by Edman degradation procedure using Procise 492 equipment from ABI Biosystems.

12. Glycan Analysis by MS

Equipment – SYNAPT, Waters

The Protein-A purified sample and RMP were limited Lys-C digested to release Fab and Fc fragment. The mAb concentration: Lys-C was standardized as 1:400, pH 8.0 at 37 ^{0}C for 10mins to achieve a single nick at the hinge region of heavy chain to produce two fragments of Fab and a single fragment of Fc to study the Fc and Fab glycans individually. The Lys-c digested sample was reduced by treating with 15 mM DTT at 37 ^{0}C for 25 min to get simpler profile and reduce complexity of the spectrum in LC-MS since both heavy chains are glycosylated. This digested and reduced sample was resolved on C8 Zorbax column with 70% Iso Propyl Alcohol (IPA) + 20% Acetonitrile (ACN) with a linear gradient of 25% to 40% in 30 min and the eluting species were detected with ESI-TOF. The raw spectrum was deconvoluted with standard parameters

Procedural flow of the steps followed to analyze sample by MS is as follows:

Step 1: Protein-A purification (Described in Sec 5)

Expressed mAb was captured from Cell culture supernatant by MabSelect Sure column.

Equilibration buffer: 100mM Phosphate buffer, 100mM NaCl pH 7.0

Elution buffer: 50mM Citrate pH 3.0

↓

Step 2: Limited Lys-C digestion

Digestion was carried out of the purified mAb and RMP with Lysine-C at a ratio of 1:400 at pH 8.0 at 37 ^{0}C for 10 min. to achieve a single nick at the hinge region of Heavy Chain to produce two fragments of Fab and a single fragment of Fc to study the Fc and Fab glycans individually

↓

Step 3: Reduction of digest

The Lys-c digest was reduced with 15mM DTT at 37 ^{0}C for 25 min for simplifying the complexity of the spectrum in LC-MS since both heavy chains are glycosylated. The digested was separated using RP-HPLC and then the peak were passed to LC-MS

↓

Step 4: RP-HPLC

The reduced Lys-C digest was resolved by RP-HPLC on C8 Zorbax with 70% IPA+ 20% ACN with a linear gradient of 25% to 40% in 30mins.

↓

Step 5: LC-MS

The eluting species were detected with ESI-TOF. The raw spectrum was deconvoluted with standard parameters.

CHAPTER 3

CLONING AND EXPRESSION OF BIOSIMILAR ANTI-EGFR ANTIBODY IN ALTERNATE HOST

3.1. Recombinant Vector Construction

3.1.1. Preamble:

A number of expression systems have been developed where *trans*gene expression can be regulated. They all have specific characteristics making them more suitable for certain applications than for others. Since some applications require the regulation of several genes, there is a need for a variety of independent yet compatible systems (Mullick, *et al* 2006). The designing of expression vector is the most critical aspect of cell line development. The selection of vector should be based on the available literature, past experience with other proteins and genetic stability.

In pursuit of establishing a stable high expressing cell line for anti-EGFR monoclonal antibody various vector constructs were designed with dual promoter system and evaluated. The dual promoter system facilitates expression of light chain and heavy chain of antibody (IgG) at similar molar concentration. Also, the integration of light chain and heavy chain sequence happen at the same region in the genome thereby making it convenient to establish and monitor the genetic stability and localization. The vector backbone used was pcDNA3.1(-) which is commercially available from Invitrogen, USA and is not covered under any patent terms. pcDNA3.1 (-) was selected based on features referred in Table 3.1.1

Feature	**Benefit**
Human cytomegalorvirus immediate-early (CMV) enhancer / promoter	Permits efficient, high-level expression of recombinant proteins (Gorman, *et al* 1989; and Graham, *et al* 1997.)
T7 promoter / priming site	Allows for *in-vitro* transcription in the sense orientation and sequencing through the insert
Multiple cloning site	Allows insertion of gene of interest and facilitates cloning.
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription / termination and polyadenylation of mRNA (Goodwin and Rottman 1992)
f1 origin	Allows rescue of single stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the selection marker like Neomycin or GS gene and episomal replication in cells expressing SV40 large T antigen
Neomycin Resistance gene	Selection of stable transfectant in mammalian cells (Southern and Berg 1982)
SV40 early polyadenylation signal	Efficient transcription / termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β- lactamase)	Allows selection of transformants in *E. coli*

Table 3.1.1 Features of pCDNA3.1 vectors (ref. Invitrogen pcDNA vector manual)

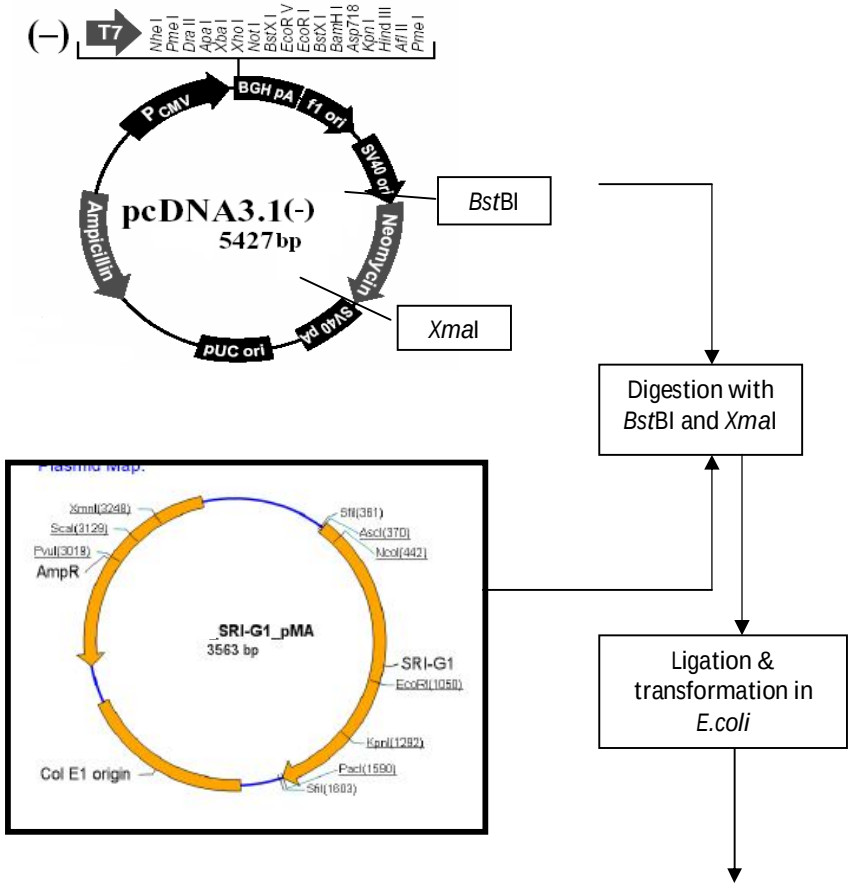
The following expression cassettes were designed for expression of recombinant IgG molecules. The recombinant vectors thus obtained would serve as a 'plug and play' system for any monoclonal antibody expression or hetero-multimeric proteins. The details of each constructs are described in the following text. The pSRM series of vectors denotes the specific modifications carried out in the pcDNA3.1(-) vector backbone.

- pSRM01 = pcDNA3.1(-) + Glutamine Synthetase gene
- pSRM02 = pSRM01 + CMV-2 promoter
- pSRM05 = pcDNA3.1(-) + CMV-2 promoter

3.1.2. Results and Discussion

3.1.2.1. Construction of pSRM01:

The schematic representation of construction is depicted in Figure 3.1.1:



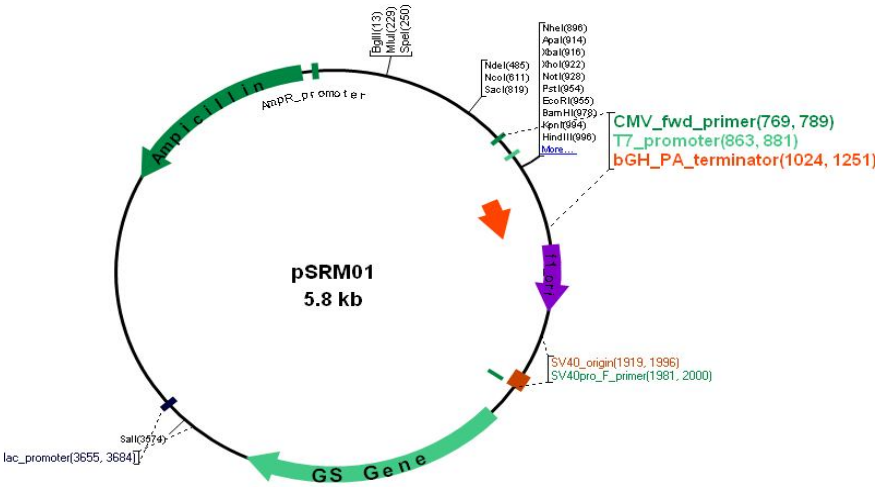


Figure 3.1.1 Flow diagram of construction of pSRM01 vector by modification of pcDNA3.1 vector so that neomycin trasferase gene was replaced with Glutamine synthetase gene (SRI-G1)

The Glutamine Synthetase (GS) gene sequence was taken from European Patent No. EP0333033which is expired in Sep 2009.

The gene was chemically synthesized from GeneArt, Germany and optimized for expression in CHO cells. The SRI-G1 pMA vector supplied by GeneArt possess GS gene. The restriction enzyme (RE) digestion of pcDNA3.1(-) and pMA vector with *Bst*B1 and *Xma*I resulted in a release of ~0.9 kb Neomycin gene and ~1.2 kb GS gene, respectively. The pcDNA3.1(-) backbone left was about ~4.5 kb as depicted in Figure 3.1.2.

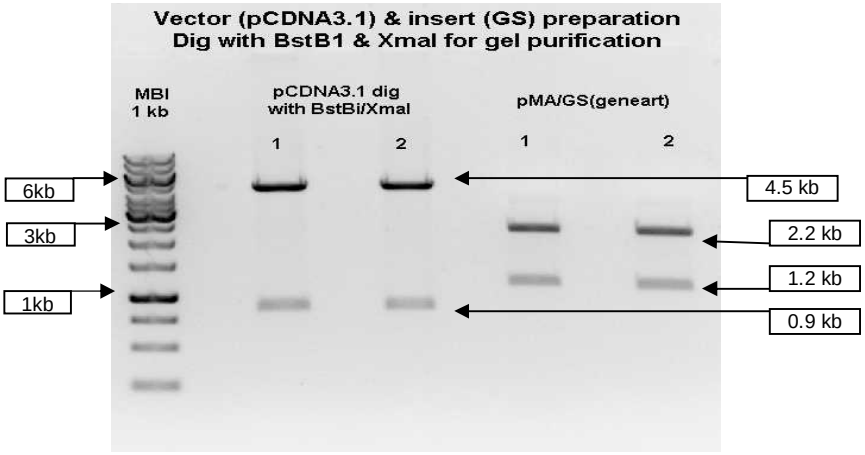


Figure 3.1.2 RE digestion of pcDNA3.1(-) and SRI-G1 pMA vector with *Bst*BI & *Xma*I.
Checked on 1% agarose gel

Further to this, the ~1.2 kb GS and ~4.5 kb pcDNA3.1 (-) fragments were gel purified using GelEluate kit (Sigma). These fragments were ligated using T4 DNA Ligase (NEB or Fermentas). *E. coli* DH5α cells were transformed by the ligation mix and about 500 colonies were obtained on ampicillin containing agar plates. Five colonies were selected for plasmid isolation and RE diagnosis. The RE diagnosis was carried out using *Bst*B1 and *Xma*I enzymes. A fragment release of ~1.2 kb was observed (Figure 3.1.3) in all 5 colonies which corresponds to the GS gene.

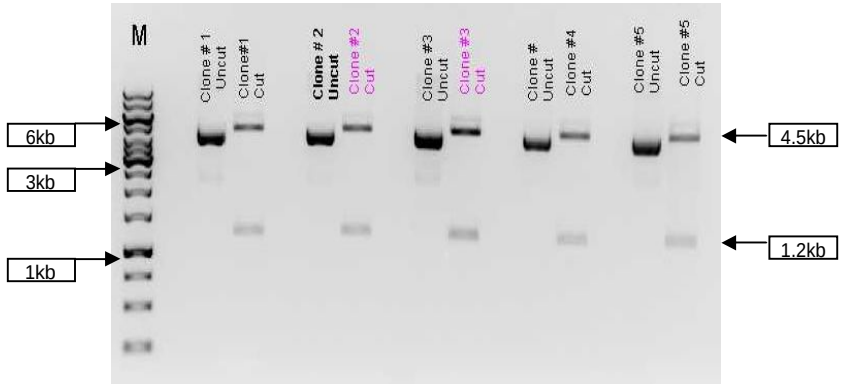


Figure 3.1.3 RE diagnosis of recombinant plasmids isolated from pSRM01 transformed *E.coli* DH5α cells.

M is the 1kb DNA ladder in first lane while the respective clones are labeled in the following lanes. Checked on 1% agarose gel

Clone # 2 & #3 were short-listed and sent for DNA sequencing. The DNA sequence of both these clones matched with the published GS sequence, hence were used for cloning. This vector was labeled as **pSRM01.**

In addition to the key feature of pcDNA3.1(-) mentioned in Table 3.1.1, the pSRM01 possesses GS gene in place of neomycin gene for selection and amplification of gene simultaneously.

3.1.2.2. Construction of pSRM02:

The pSRM01 vector constructed as above was modified to possess another CMV promoter for expression of two independent genes (eg. Light chain and heavy chain sequence). The schematic representation of construction of pSRM02 is depicted in Figure 3.1.4:

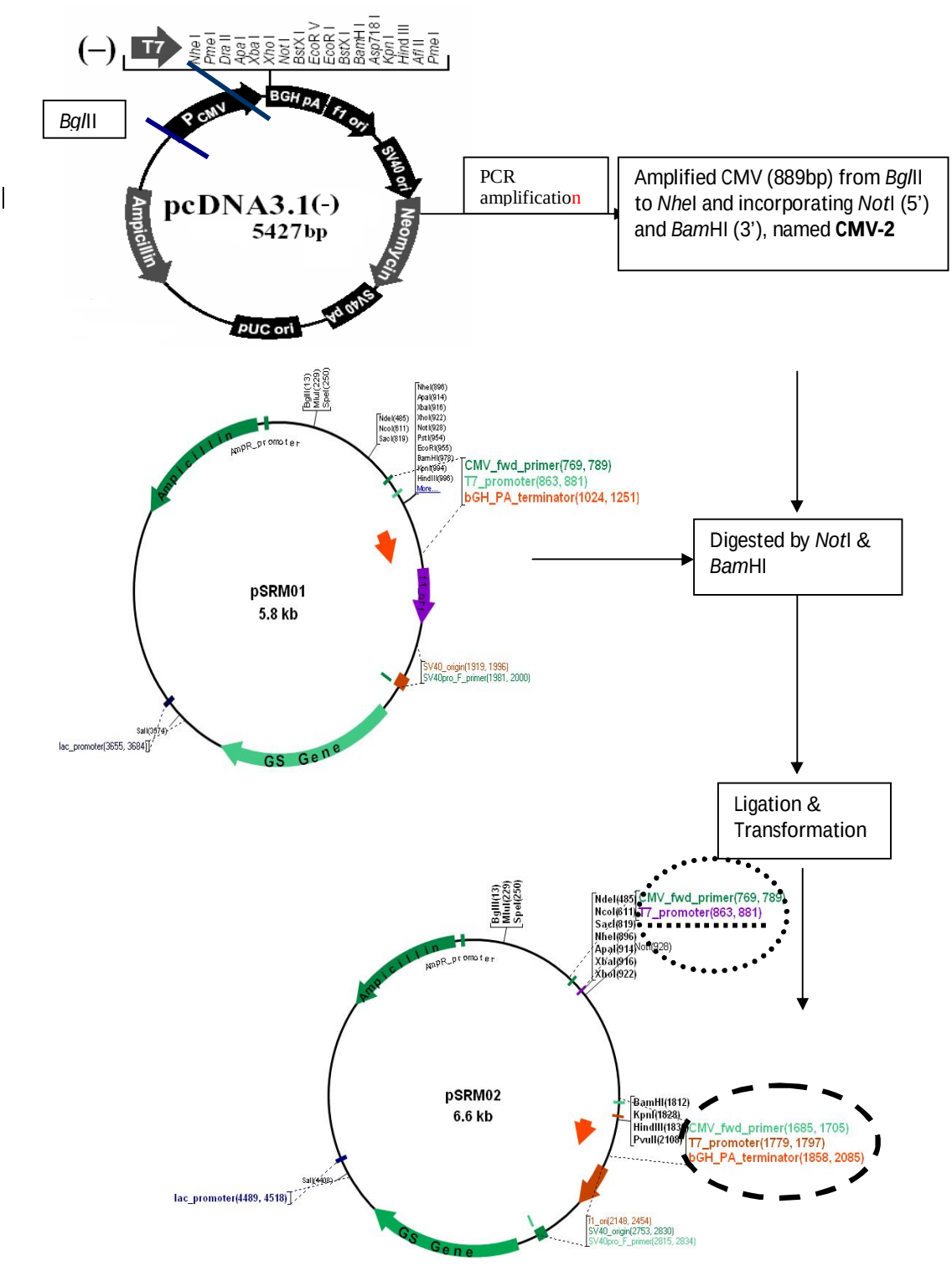


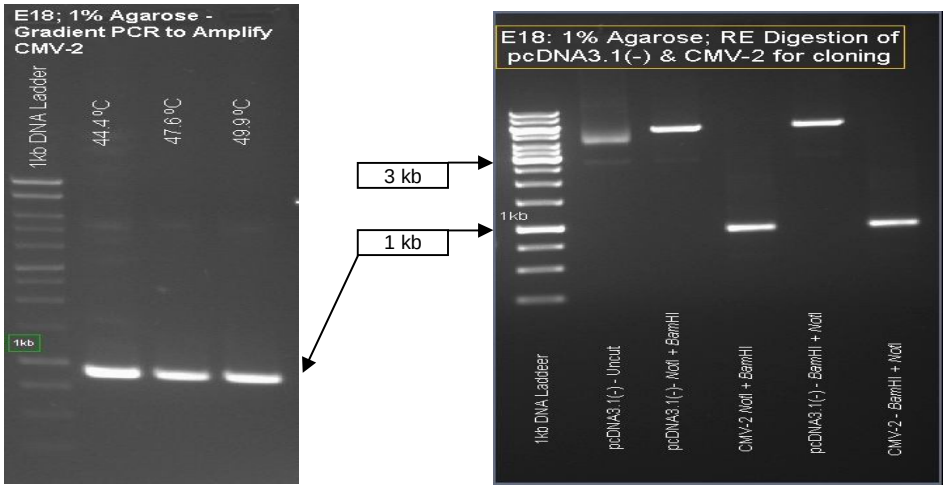
Figure 3.1.4 Flow Diagram of the Construction of pSRM02.

The dashed line represents the CMV promoters. The thin dotted line for CMV-1 while dark dotted line for CMV-2 promoter

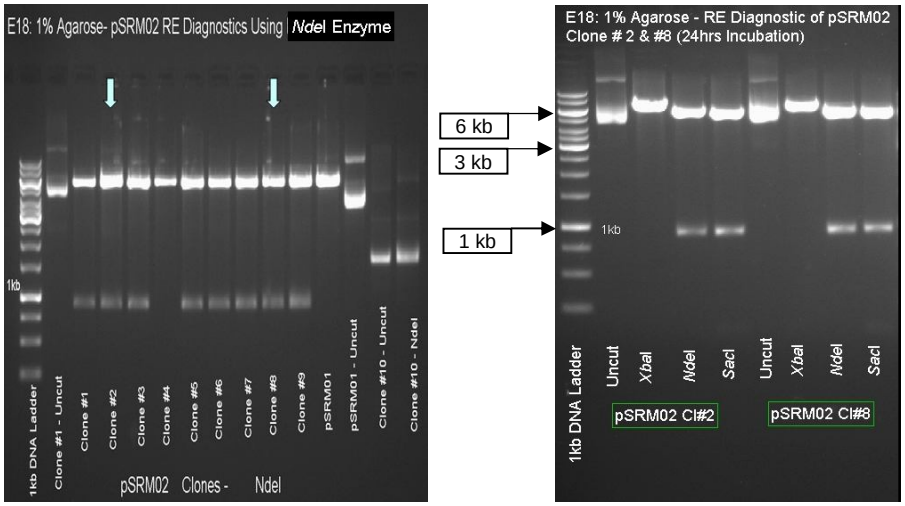
The CMV promoter from position 13 (*Bgl*II site) to 896 (*Nhe*I site) was PCR amplified using specific primers as follows:

Forward primer - SR171 ($^{5'}$-GATGCGGCCGCCCCGATCCCCTAT-$^{3'}$) and

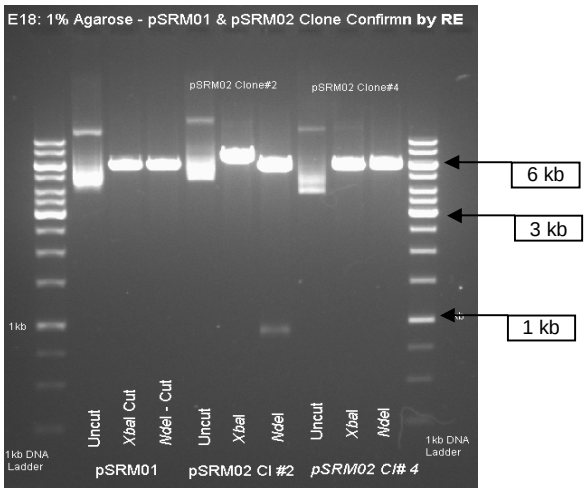
Reverse primer - SR172 ($^{5'}$-AGTGGATCCCAGCTTGGGTCTCC-$^{3'}$), and incorporating *Not*I and *Bam*HI site at 5' and 3' location, respectively. A gradient PCR was run to amplify this fragment which was named as CMV-2 promoter with size of ~908 bp (Ref. Figure 3.1.5A). The amplified CMV-2 and pSRM01 vector was digested sequentially using *Not*I and *Bam*HI enzymes (Ref. Figure 3.1.5:B). The ~0.9 kb fragment corresponding to CMV-2 and ~6.0 kb fragment of double digested pSRM01 was gel purified, ligated using T4 DNA ligase and transformed in *E.coli* DH5α cells. The transformation efficiency achieved was 2.73 x 10^3. Plasmid Miniprep from 10 colonies were used for RE diagnostics and screening for positive clones. When the plasmids from selected clones were digested with NdeI, two fragments of ~1 kb (corresponding to the size of CMV-2 insert) and ~6 kb (corresponding to pSRM01 vector) was observed. This was seen in all clones except clone #4 which was negative for ligation while clone # 10 showed unusual behavior (Ref. Figure 3.1.5:C). Clone #2 & #8 was selected for further experimentation while all other clones were discarded. pSRM02 Clone # 2 & #8 were further RE diagnosed using NdeI & SacI enzyme whereby two fragments as expected were observed (Figure 3.1.5:D).



(A) (B)



(C) (D)



(E)

Figure 3.1.5 Generation of pSRM02 vector.

(A) Gradient PCR to amplify CMV promoter sequence from pcDNA3.1(-), (B) Sequential digestion of pSRM01 and CMV-2 with *Not*I and *Bam*HI, (C) RE diagnostics for the positive clones of pSRM02. (D) RE diagnostics for pSRM02 clone # 2 & #8, (E) RE analysis to distinguish pSRM01 and pSRM02 (clone #2 & #4). All the DNA samples were checked on 1% agarose gel.

The distinguishable RE analysis of pSRM01 and pSRM02 (Figure 3.1.5:E) was done using *Nde*I enzyme. *Nde*I is present in CMV promoter site and hence when a second CMV-2 promoter was inserted there will be introduction of one more NdeI site; hence on digestion it releases two fragments of ~1 kb and ~5 kb size while pSRM01 and pSRM02 Clone #4 gave a linearized band. This indicates clone #2 & #8 possess CMV-2 introduced in pSRM01 resulting in pSRM02 vector while clone #4 is a negative clone lacking CMV-2.

3.1.2.3. Construction of pSRM05

This vector is a dual promoter vector with Neomycin transferase gene as a selection marker. The pSRM02 as explained above and pcDNA3.1(-) was digested using *Bam*HI (5') & *Not*I (3') to get release GS gene from pSRM02 and linearized pcDNA3.1(-). Figure 3.1.6 depicts the schematic representation of the construction of pSRM05.

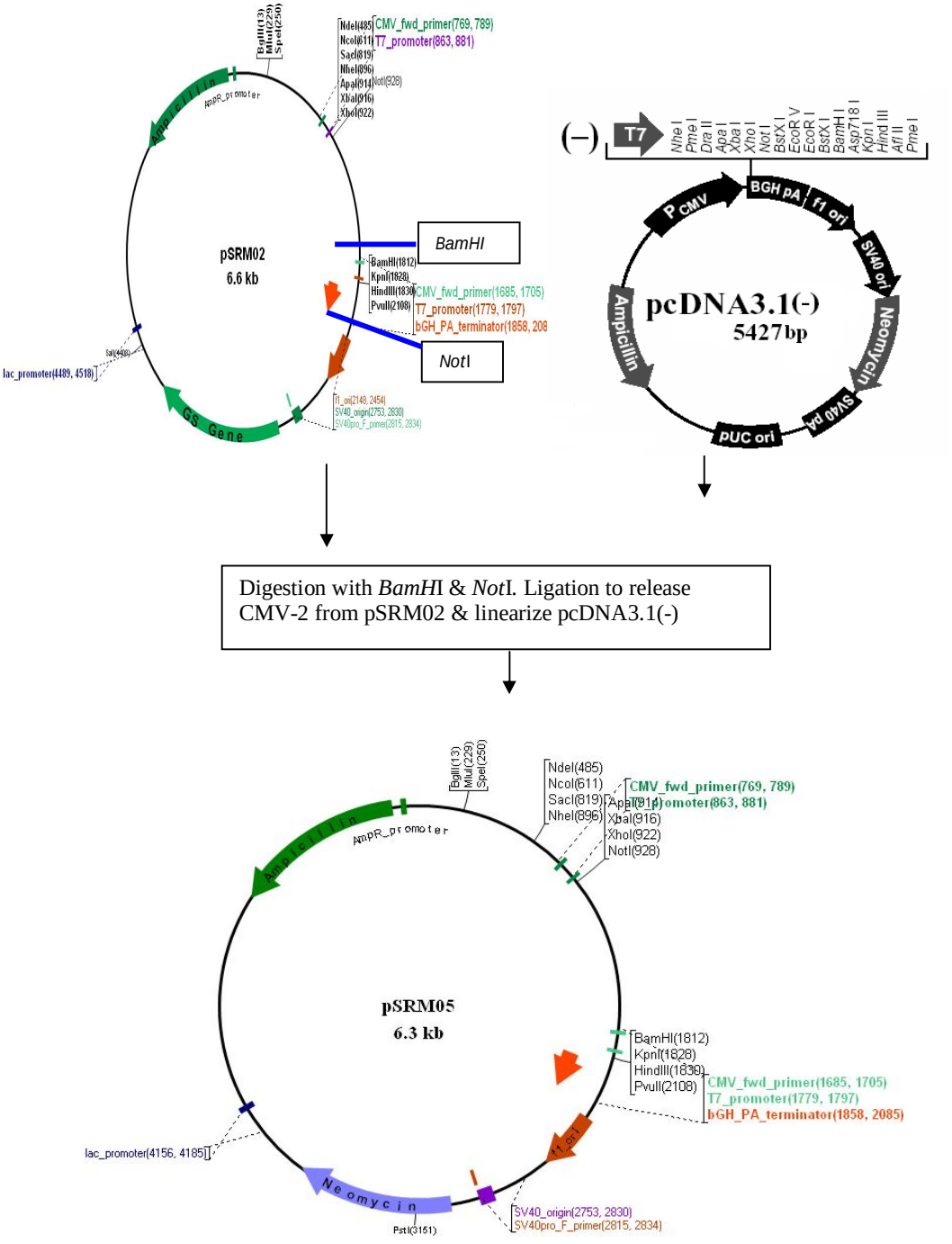
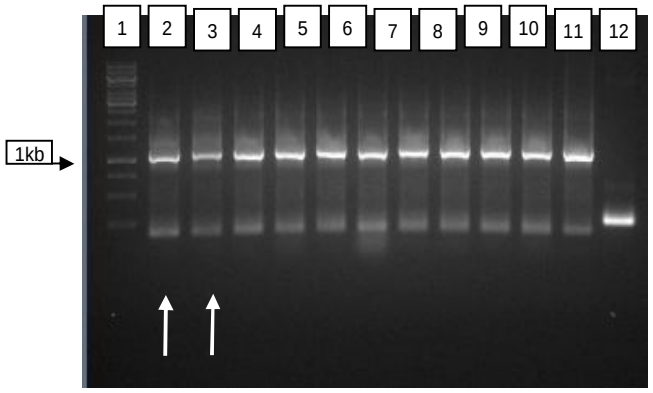


Figure 3.1.6 **Schematic representation of construction of pSRM05 vector**. This vector contains two CMV promoters and neomycine transferease as selection marker

The CMV-2 was ligated to pcDNA3.1 (-) using T4 DNA ligase to yield pSRM05 vector. *E. coli* DH5 α cells were transformed with the ligation mix. Ten colonies from the transformation plates were screened by colony PCR using primers :

Reverse Primer SR167 ($^{5`}$-TAGAAGGCACAGTCGAGG -$^{3`}$) and

Forward Primer SR601 ($^{5'}$–CGCAAATGGGCGGTAGGCGTG-$^{3'}$). The positive clones gave an amplicon of ~1.1 kb while the negative clones gave an amplicon of ~200 bp. As shown in Figure 3.1.7:, all the 10 selected transformants gave ~1.1 kb amplicon indicating ligation of CMV-2 in pcDNA3.1(-).



Lane 1 :1kbp DNA ladder
Lanes 2-11 :pSRM05 Clone #1-10
Lane 12 :pSRM02(+ve ctrl)

Figure 3.1.7 Screening of pSRM05 transformants of *Ecoli* HD5α by colony PCR.

Checked on 1% agarose gel.

Clone # 1 and #2 were selected for RE analysis. The plasmids isolated from these transformants were digested with BglII and EcoRI. The pSRM02 plasmid was expected to give two fragments of ~3.5 kb and ~3 kb size while the pSRM05 was expected to yield a single fragment of ~6.5 kb size. Based on RE analysis (Figure 3.1.8:) clone #1 and #2 were positive clones and used for construction of the final recombinant vector for antibody gene expression.

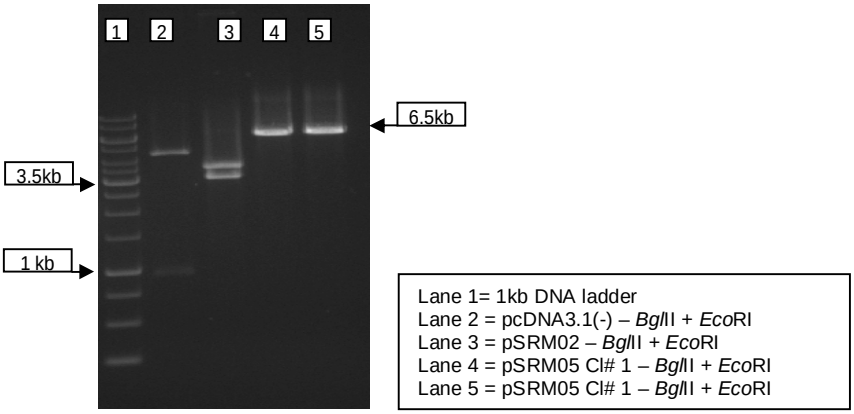
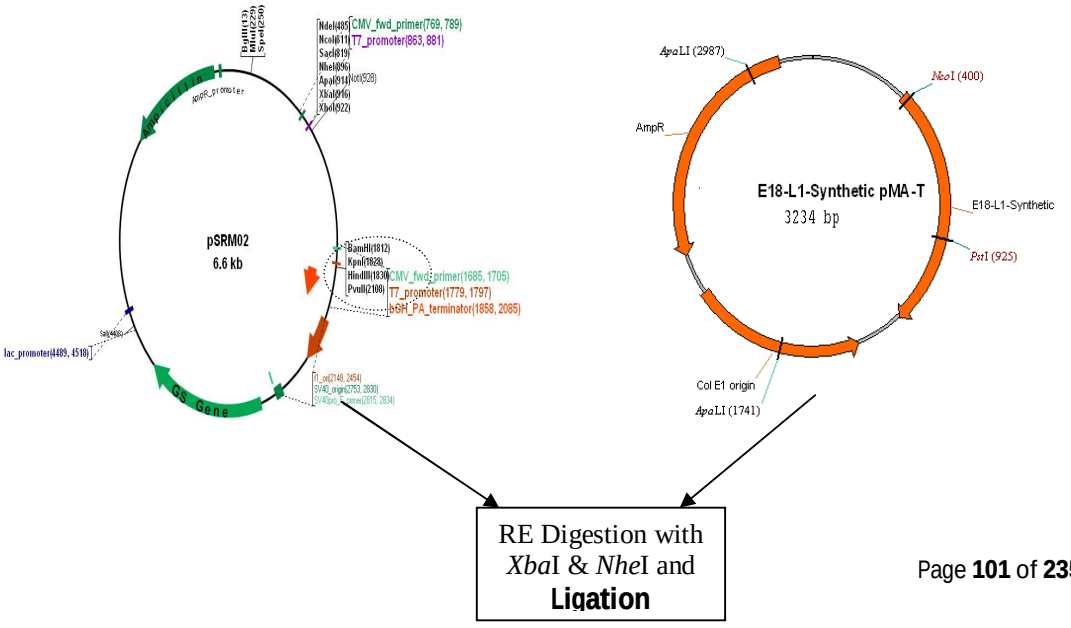


Figure 3.1.8 **RE Diagnosis of pSRM05 Clone #1 and # 2.** The plasmid from Clone #1 andd #2 was isolated and digested to check for the presence of two CMV promoters and neomycin transferease gene

3.1.2.4. Construction of Antibody Genes Expression vector (S8.1):

The Expression vector with GS as selection marker was named as S8.1 Expression Vector (pSRM02+L1+H1). As described in above section pSRM02 bears two CMV promoter and GS gene for selection and gene amplification. The genes of interest i.e., light chain (L1) and heavy chain (H1) genes of anti-EGFR monoclonal antibody (Cetuximab) were cloned sequentially. The light chain and heavy chain sequences were taken from the patent WO/2007/147001. Also the sequence was confirmed by 2D LC-MS/MS analysis of RMP (Data not shown). The light chain and heavy chain sequences were chemically synthesized from GeneArt, Germany.

For construction of the expression vector, the light chain and heavy chain sequences were cloned in the multiple cloning site (MCS) downstream of each promoter (CMV-1 and CMV-2). Below is the schematic diagram for construction of S8.1 expression vector:



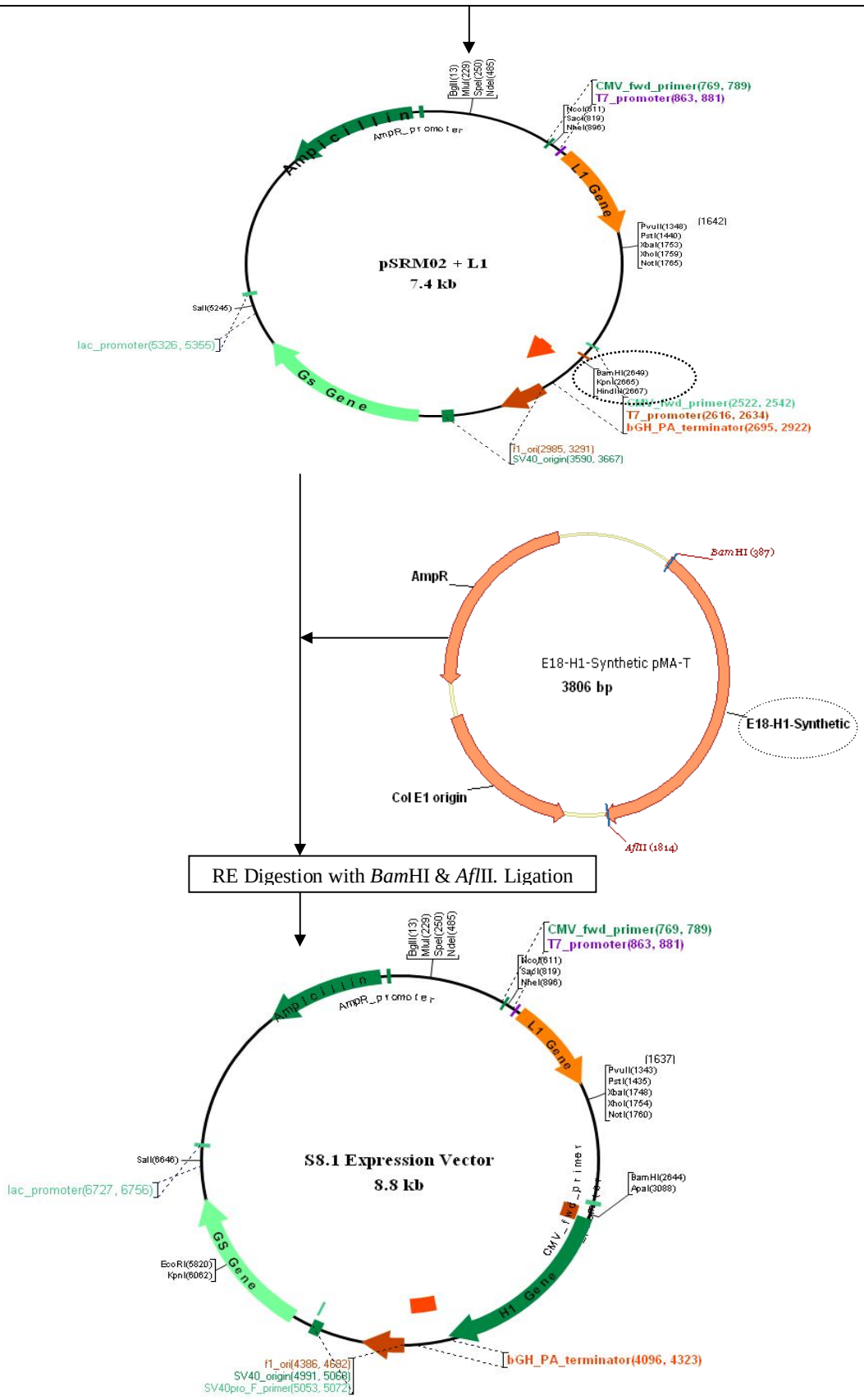


Figure 3.1.9 Schematic representation of construction of S8.1 expression vector.

The light chain & heavy chain sequence were chemically synthesized. E18-L1-Synthetic pMA-T vector bears light chain &E18_H1 synthetic pMA-T bears heavy chain

The E18-L1-Synthetic pMA-T vector recevived from GeneArt was digested with *Nhe*I and *Xba*I to release light chain sequence of 852 bp (this includes Kozak Sequence, CDS, and Bovine Growth Hormone PolyA). Similarly pSRM02 was also double digested with the same enzymes (Figure 3.1.10A). The fragment of interest and the digested pSRM02 were gel purified using GelElute kit and ligated using T4 DNA ligase. The ligation mix was used to transform *E.coli* DH5α and plated on SY media containing Ampicillin. Ten colonies were selected and tested for presence of recombinant plasmid by colony PCR. All clones gave an amplicon of ~300 bp as expected however, the signal in clone # B, G, H, I, and J was strong (Figure 3.1.10B). Clones # B & H were analyzed and used for further experimentation.

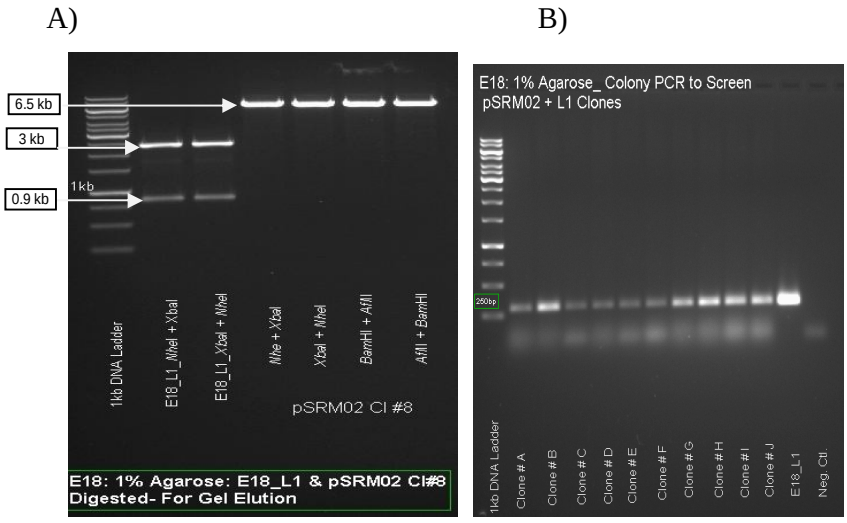


Figure 3.1.10 Cloning of Light Chain in pSRM02.

A) RE digestion of pSRM02 Clone # 8 and E18_L1-Synthetic pMA-T Vector (GeneArt),

B) Colony PCR for screening pSRM02 +L1 clones. Checked on 1% agarose.

pSRM02+ L1 clone # H was selected for cloning heavy chain sequence in it. As depicted in Figure 3.1.11A, E18-H1-Synthetic pMA-T Vector received from GeneArt and pSRM02+L1 Clone #H were RE digested with *Bam*HI and *Afl*II to get linearized pSRM02+L1 and fragment of ~1.4 kb (corresponding to heavy chain sequence) from GeneArt vector. These were then gel purified and ligated using T4 DNA ligase. The ligation mix was used to transform *E. coli* DH5α cells. A total of 13 colonies were screened by colony PCR using following primers:

Forward Primer - SR-177 ($^{5'}$-TGACCAAGTCCTTCAACCGG- $^{3'}$),

Reverse Primer - SR-178 ($^{5'}$-CGGACACGGTACAGGTGAT-$^{3'}$) primers. As shown in Figure 3.1.11B, an amplicon of ~1.2 kb size was observed in all the clones.

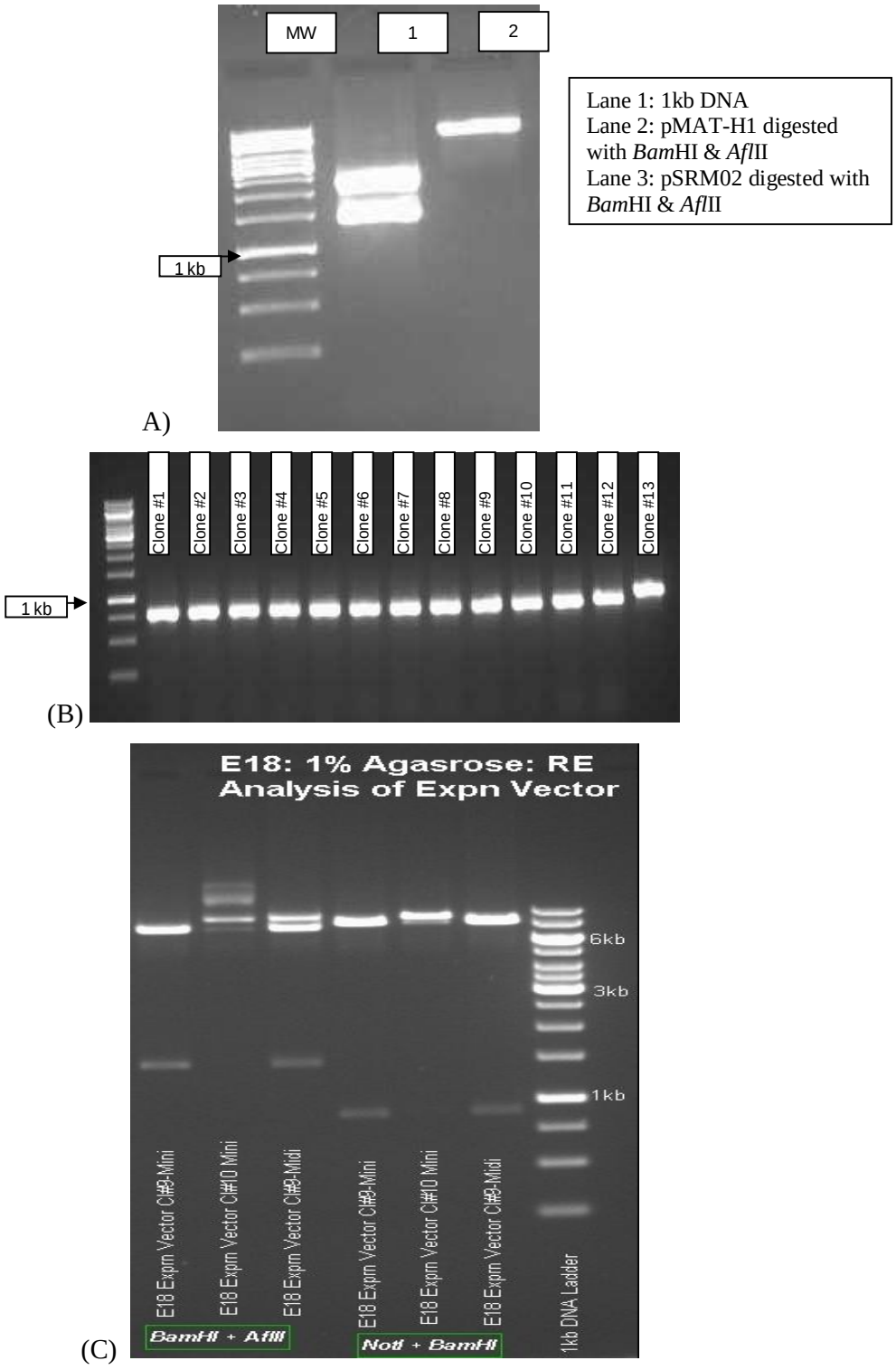


Figure 3.1.11 Cloning of Heavy Chain in pSRM02+L1.

A) RE digestion of pSRM02+L1 Clone # H and E18_H1 (GeneArt) Vector,

B) Colony PCR, (C) RE diagnosis of the final expression vector (pSRM02 + L1 + H1)

The SP2/0 cell line generated by transfection of this vector was designated as S8.1 and in CHO cell line as S8.2.

3.1.2.5. Construction of Expression vector (S8.3):

The expression vector S8.3 was designed to have Neomycin Transferase as selection marker and possessing two CMV promoters (pSRM05+L1+H1). It was generated from S8.1 expression vector by replacing GS gene with Neomycin transferase gene.

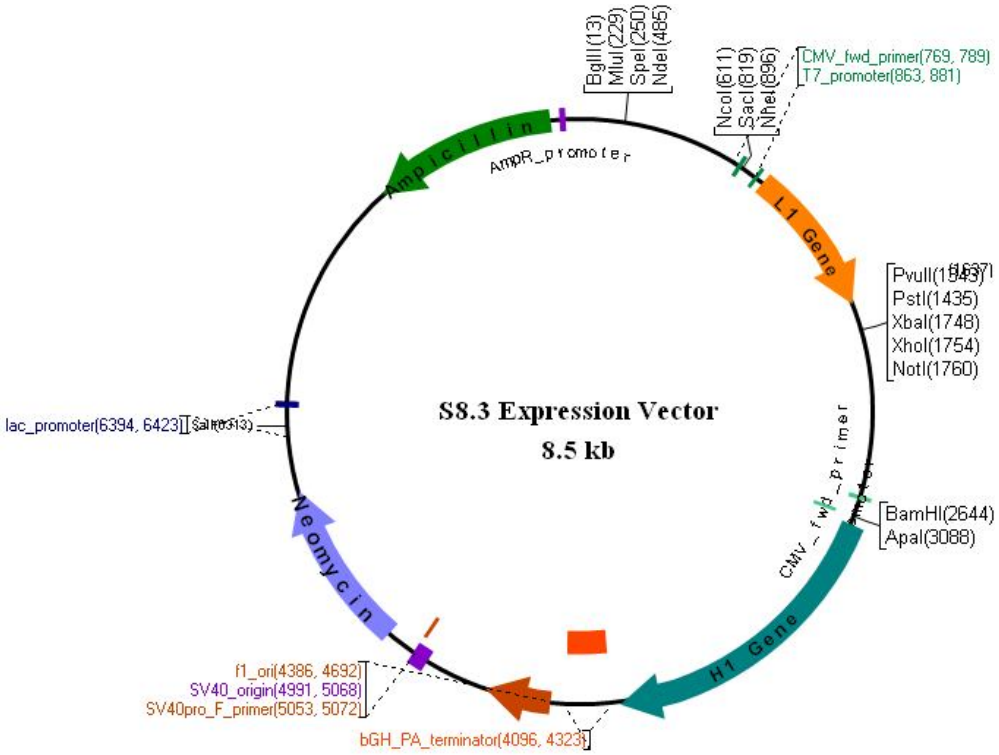


Figure 3.1.12 Schematic diagram of S8.3 expression vector

The S8.1 expression vector and pcDNA3.1 (-) were digested with *Bst*B1 and *Xma*I to release fragment of ~1.2 kb (corresponding to GS gene) from S8.1 expression vector and ~0.9 kb (corresponds to Neomycin Transferase gene) from pcDNA3.1 (-) (Figure 3.1.13A). The ~0.9 kb fragment corresponding to Neomycin transferase obtained from pcDNA3.1 (-) was ligated in S8.1 vector from where ~1.2 kb fragment corresponding to GS gene was removed.

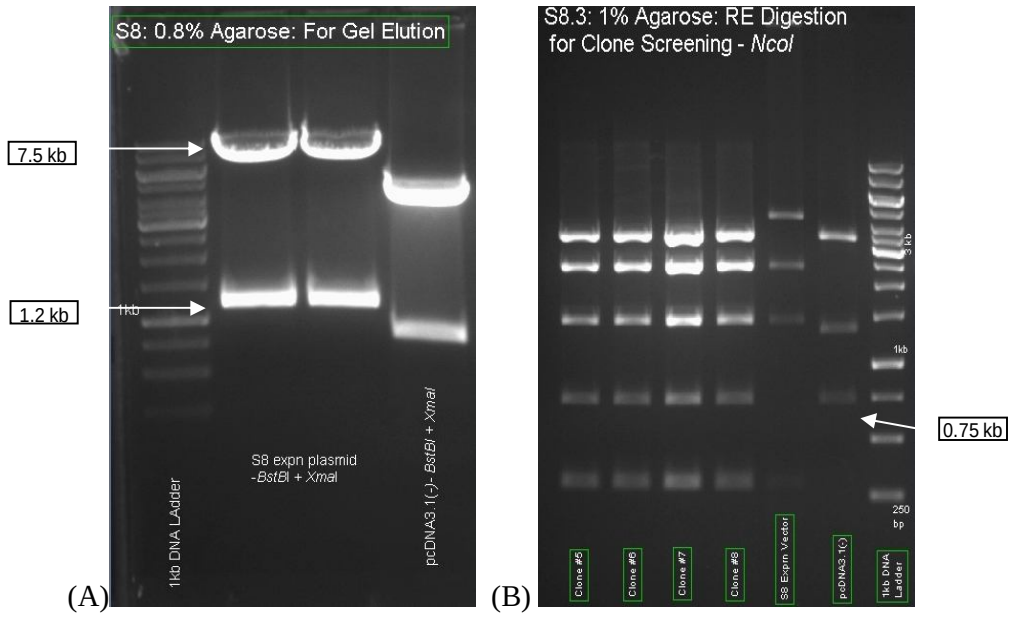


Figure 3.1.13 Cloning of Neomycin Gene in S8.1 Expression Vector.

(A) Gel elution (0.8% agarose) of neomycine transferase gene from S8.1 and RE digested pcDNA3.1(-)

(B) RE diagnosis of S8.3 recombinant plasmids isolated from transformants. Checked on 1% agarose.

The ligation mix was used to transform *E. coli* DH5α and 4 colonies were used for plasmid preparation and RE diagnosis. The plasmids thus prepared and named as **S8.3 expression vector.** S8.1 expression vector, pcDNA3.1(-) vector and isolated S8.3 plasmids were digested with *Nco*I. *Nco*I is an additional site present in Neomycin gene and not in GS gene. This resulted in an additional fragment of ~750 bp in S8.3 expression vector (Figure 3.1.13). After digestion clone number 5, 6, 7 and 8 gave 5 fragments as expected while S8.1 and pcDNA3.1(-) gave 4 bands. Clone #7 was used to transfect **SP2/0 cells and the recombinant cell line designated as S8.3 and CHO cells designated as S8.4.**

3.1.3. Conclusion:

The pcDNA3.1(-) was used as a back bone vector to generate various dual promoter vectors and different selection markers. In the present study, the expression vectors constructed possess CMV as the promoters to express anti-EGFR antibody. These vectors could be used for any future projects for cloning of two different genes under the control of same, CMV promoter.

The S8.1 expression vector constructed to bear GS as selection marker and S8.3 expression vector possess neomycin transferase gene as selection marker which will be used to generate stable cell line to be used at commercial scale.

3.2. Cell Line Development

3.2.1. Preamble

3.2.1.1 HOST SYSTEM:

- CHO (Chinese Hamster Ovary, *Cricetulus griseus*) Cells:

Until the later part of the 20th century, isolation and characterization of mammalian cell mutants for cytogenetic studies was a challenging exercise, fraught with failures because, unlike microbes, mammalian cells are generally diploid. The establishment of CHO cells by Dr. Theodore T. Puck of the Department of Medicine at the University of Colorado in 1957 enabled researchers to overcome this difficulty because these cells were functionally hemizygous for many genes, primarily due to gene inactivation. It was also noticed that CHO cells have low chromosome number of (2n=22) which made them particularly useful models in radiation cytogenetics and tissue culture studies (Jayapal, *et al*. 2007). CHO cells have, thereafter, been used in numerous biomedical studies ranging from analysis of intermediary metabolisms, cell cycle to toxicity studies, and commercially for manufacturing of therapeutic proteins, so much so, that they have been termed as the mammalian equivalent of the model bacterium, E. coli (Puck 1985).

CHO-K1 was derived from the original CHO cell line. It contains slightly lower DNA content than the original CHO. In this research work CHO-S cell line was used which is a stable aneuploid cell line distinguished as a separate sub-clone from the common CHO-K1 cell line and its history and stability is described in literature (Puck 1985; Deaven & Peterson 1973; and D'Anna 1996).

The CHO and CHO-K1 cell lines can be obtained from a number of biological resource centers such as the European Collection of Cell Cultures (ECACC) and American Type Culture collection (ATCC). The CHO-S used in this project was procured from Invitrogen (Cat # 11619-012).

CHO-S typically is a suspension adapted cell line but when grown in presence of serum and under static condition grows as adherent culture like CHO-K1 (Figure 3.2.1).

A) B)

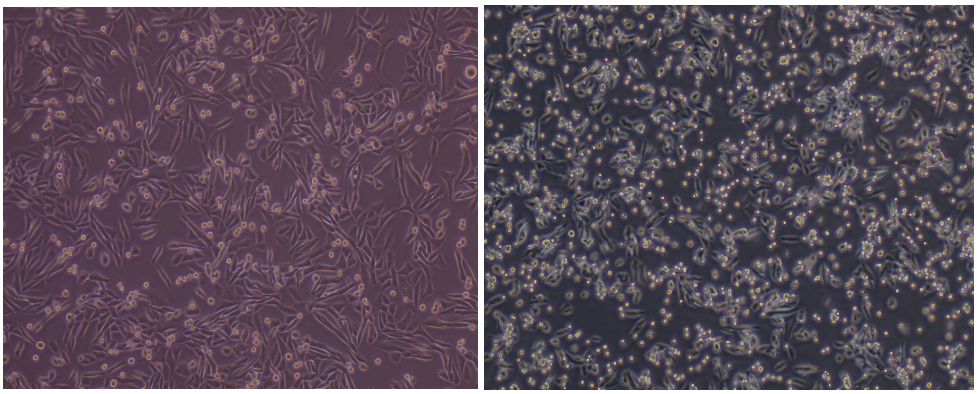


Figure 3.2.1 A typical Morphology of CHO-S adherent Cells.

A) Adherent cell morphology and B) suspension cell morphology. Observed under a magnification of 10x in inverted phase contrast microscope.

- SP2/0 AG14 (ATCC No. : CRL-1581) Cells:

The line was formed by fusing Balb/c spleen cells (from mouse immunized with sheep RBCs) with the P3X63Ag8 myeloma cell line. The cells do not secrete immunoglobulin, are resistant to 8-azaguanine at 20 µg/mL and are HAT sensitive (Shulman, *et al* 1978). SP2/0-Ag14 cells can be used as fusion partners for B cells in the production of hybridomas. It grows in suspension culture as shown in Figure 3.2.2.

A) B)

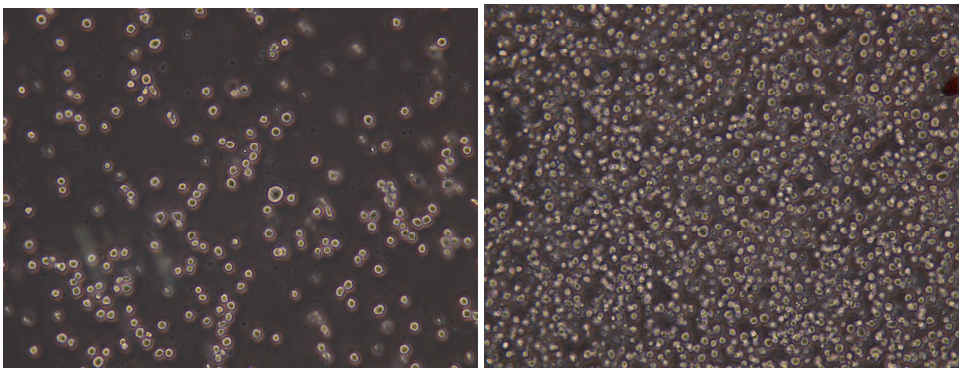


Figure 3.2.2 A typical morphology of SP2/0 AG14.

A) Cell grown at lower density (e.g. day 1 of seeding) and B) cell grown at higher density (e.g. day 3 of seeding). Observed under a magnification of 10x in inverted phase contrast microscope.

This cell line is also used for the expression and manufacturing of therapeutic monoclonal antibodies. However, it is not as widely used as CHO cells, and till date only four mAb molecules are commercially available including the one in discussion. In

the following studies the expression vectors (S8.1 and S8.3) constructed were transfected in both CHO-S and SP2/0 cells for evaluating expression of protein of interest.

3.2.1.2 TRANSFECTION:

Transfection of animal cells typically involves opening transient pores or "holes" in the cell membrane, to allow the uptake of DNA material. Transfection can be carried out using calcium phosphate, by electroporation, or by mixing a cationic lipid with the DNA material to produce liposomes, which fuse with the cell membrane and deposit their cargo inside. The principle of transfection techniques employed are discussed below:

- Lipofection

Lipofection or liposome mediated transfection is a technique used to inject genetic material into a cell by means of liposome, which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer. Lipofection generally uses a positively charged (cationic) lipid to form an aggregate with the negatively charged (anionic) genetic material. A net positive charge on this aggregate has been assumed to increase the effectiveness of transfection through the negatively charged phospholipid bilayer. In addition, this method is suitable for all transfection applications (transient, stable, co-transfection, reverse, sequential or multiple transfections). High throughput screening assay has also shown good efficiency in some *in-vivo* models. This method is the most preferred due to its low toxicity to the cell. Figure 3.2.3 represents the liposome mediated transfection.

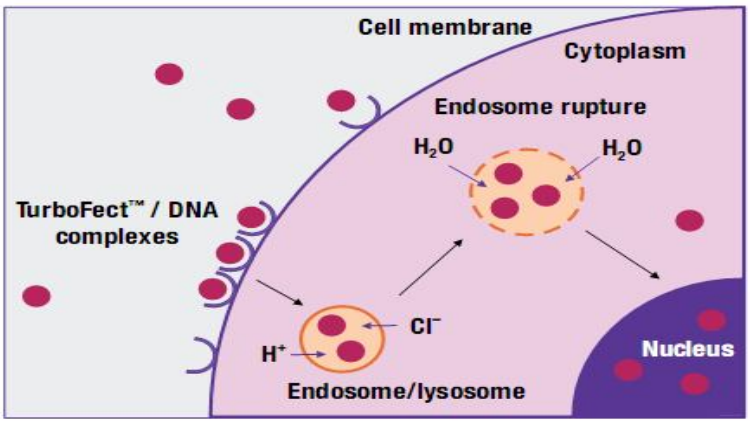


Figure 3.2.3 Liposome-mediated transfection.

Red rounds are the lipid DNA complexes. (Turbofect is a trade name for transfection reagent from MBI-Fermentas)

The expansion of biotherapeutics, most notably monoclonal antibodies and recombinant proteins, has created the need for rapid methods to produce protein, display correct co- and post-translational modifications, and create enough protein yields to facilitate further assay development validation, and high expressing cell line making commercial process economical. One of the most commonly used cell line is CHO cells because they are easily adapted to serum-free conditions in suspension cultures, generate high-level protein production and widely used in biopharmaceutical industry. Often researchers start with transient transfection methods during the optimization stage while stable clones are being screened for large-scale manufacturing. Optimization of transient transfection involves cell selection, media compatibility and reagent to DNA ratio, with the goal of having high yield at reasonable cost. With the time and development in the process many pharmaceutical companies are utilizing strains or variants that have enhanced secretion capabilities or suppressed apoptotic liability. Targeted proteins or variants may exhibit differential protein expression profiles requiring reagent to nucleic acid optimization and selection of ideal media growth conditions. The selection of reagent and cell culture media can greatly impact transfection performance and overall protein yield. For optimization, a range of reagent to plasmid ratios at selected culture densities is suggested, with choice of reagent impacted by media compatibility and protein yield. Another consideration is cost versus yield. Often though, higher yields are desired in which case lipid-based reagents may be preferred (Hayes 2010).

A wide range of transfection reagents are commercially available which are of various types and compatibility resulting in different transfection efficiency. The most widely use of following reagents are reported and hence these were tried to transfect SP2/0 and CHO cell lines. The reagents used are:

(a). Lipofectamine™2000

This is a proprietary formulation from Invitrogen, USA for transfection of nucleic acid (DNA and RNA) into Eukaryotic cells and provide following advantages:

- ✓ Highest transfection efficiency in many cell types and formats (e.g. 96well).
- ✓ DNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium, in the presence or absence of serum.

Lipofectamine™ 2000 may be used in the following applications:

- ✓ Transient and stable transfection of adherent and suspension cells
- ✓ High throughput transfections
- ✓ Delivery of Stealth RNAi and siRNA into cells.

The cell lines reported to be successfully transfected using Lipofectamine™ 2000 are (www.invitrogen.com):

HEK293F	HEK293H	HEK293
		BE(2)C (w/o serum)
CHO-K1	CHO-S (adherent)	CHO-S (suspension)
COS-1 (w/o serum)	COS7-L(w/o serum)	Primary Human Fibroblasts
HT-29 (w/o serum)	HT-1080	MDCK
MRC-5 (w/o serum)	PC12	SK-BR3
Vero	CHO	CHO-DG44
MCF7	MDA-MB-361	HCT 116
H1299	RKO	Hep3B, HepG2
HeLa	Rzneo	HOS
C3H/10T1/2	NIH3T3	Jurkat
K562	HUVECS	LoVo
A549		

(b). TransIT® 2020

This is a proprietory Broad Spectrum DNA Delivery reagent available from Mirus Bio, USA. It achieves high expression in many cell types, including hard to transfect cell lines and primary cells. TransIT®-2020 demonstrated higher protein yield when compared to other competitor reagents like FuGENE® HD, Lipofectamine™ 2000, and Lipofectamine™ 2000 CD. It is also compatible and superior for obtaining higher expression than other insect cell transfection reagents. It is an animal origin-free components providing high performance with maximum compatibility. In addition to the cell lines listed below, this reagent is known to successfully transfect following cell lines:

2B4.11	C6	CD-18Co
Colon 38	Daoy	DB-TRG-05MG
DI-TNC1	HL-1	INS-1
J774A.1	K-562	KYSE 410
LS180	MCF-7	MDA-MB-231
Monc-1	NCCIT	NCI-H522
NE-1	Neuro-2a	NRK-52E
PC-12	Phoenix™ Eco	RAW 264.7
RGC-5	Saos-2	SH-EP1
SH-SY5Y	SK-N-BE(2)	T-98G
TE-8	U2OS	

(c). TurboFect™:

TurboFect™ *in-vitro* Transfection Reagent available from MBI fermentas, USA is a sterile solution of a proprietary cationic polymer in water. The polymer forms compact, stable, positively charged complexes with DNA. These complexes protect DNA from degradation and facilitate gene delivery into eukaryotic cells. TurboFect™ is ideal for transfection of a variety of cells, including primary and secondary cell lines, adherent and suspension cell lines, differentiated and undifferentiated cell lines, and for stable and transient transfection. Transfection can be performed in presence or absence of serum. TurboFect™ demonstrates superior transfection efficiency and minimal toxicity when compared to lipid-based or other polymer-based transfection reagents.

This reagent is reported applications for:

- ✓ Plasmid DNA and oligonucleotide transfection.
- ✓ mRNA transfection.
- ✓ Co-transfection.
- ✓ Transfection of primary and secondary cell lines.
- ✓ Stable and transient transfection.

This reagent in addition to the entire above cell lines listed is used for following cell lines:

B50	BAEC	BHK-21
Calu1	COLO	HEL299
L929	MEF	MPC5
NTEPA2	RAW264	RBL-2H3
Sp2/Ag14	WEHI	

➢ Calcium-Phosphate:

As depicted in Figure 3.2.4, DNA-calcium phosphate precipitates are formed by mixing DNA with calcium chloride and gently bubbling in the saline/phosphate solution followed by incubation at RT. These precipitates were added onto the cells which adhere to surface of cells. The precipitates are taken up presumably by endocytosis. This co-precipitation method is widely used because the components are easily available at a reasonable price. The large precipitates thus formed upon incubation sometimes lead to cellular toxicity and reduced transfection efficiency.

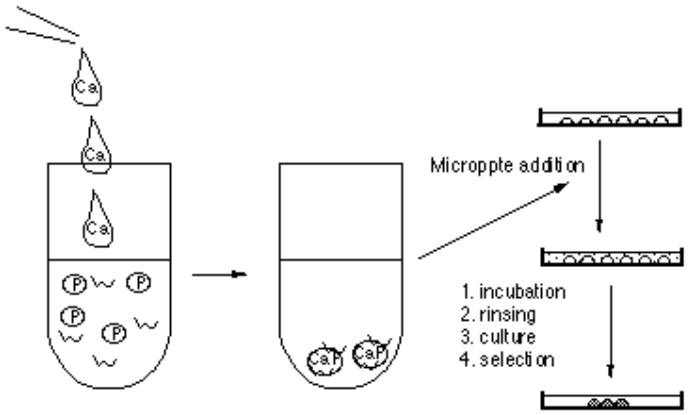


Figure 3.2.4 Calcium Phosphate Precipitation Method for transfection

➢ Electroporation

Electroporation or electro-permeabilization is a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field. It is usually used in molecular biology as a way of introducing some substance into a cell, such as loading it with a molecular probe, a drug that can change the cell's function, or a piece of coding DNA.

In molecular biology, the process of electroporation is often used for the transformation of bacteria, yeast, eukaryotic cells and plant protoplasts. The cells and plasmids are mixed together before applying several hundred volts across a distance of several millimeters are typically used in this process for transfer of DNA into the cells. This process is reported to be around ten times as effective as chemical transformation.

Electroporation allows cellular introduction of large highly charged molecules such as DNA which would never passively diffuse across the hydrophobic bilayer core. This phenomenon indicates that the mechanism is the creation of nm-scale water-filled holes in the membrane. During electroporation the lipid molecules are not chemically altered

but simply shift position, opening up a pore which acts as the conductive pathway through the bilayer as it is filled with water.

Electroporation is a multi-step process with several distinct phases as shown in Figure 3.2.5 (Weaver and Chizmadzhev 1996). First, a short electrical pulse must be applied, typical parameters would be 300-400 mV for < 1 ms across the membrane (note- the voltages used in cell experiments are typically much larger because they are being applied across large distances to the bulk solution so the resulting field across the actual membrane is only a small fraction of the applied bias). Upon application of this potential the membrane charges like a capacitor through the migration of ions from the surrounding solution. Once the critical field is achieved there is a rapid localized rearrangement in lipid morphology. The resulting structure is believed to be a "pre-pore" since it is not electrically conductive but leads rapidly to the creation of a conductive pore. Evidence for the existence of such pre-pores comes mostly from the "flickering" of pores, which suggests a transition between conductive and insulating states. It has been suggested that these pre-pores are small (~3 Å) hydrophobic defects. If this theory is correct, then the transition to a conductive state could be explained by a rearrangement at the pore edge, in which the lipid heads fold over to create a hydrophilic interface. Finally, these conductive pores can either heal, resealing the bilayer or expand, eventually rupturing it. The resultant fate depends on whether the critical defect size was exceeded which in turn depends on the applied field, local mechanical stress and bilayer edge energy.

This method needs a very fine standardization to suit the cells otherwise it can lead to high toxicity.

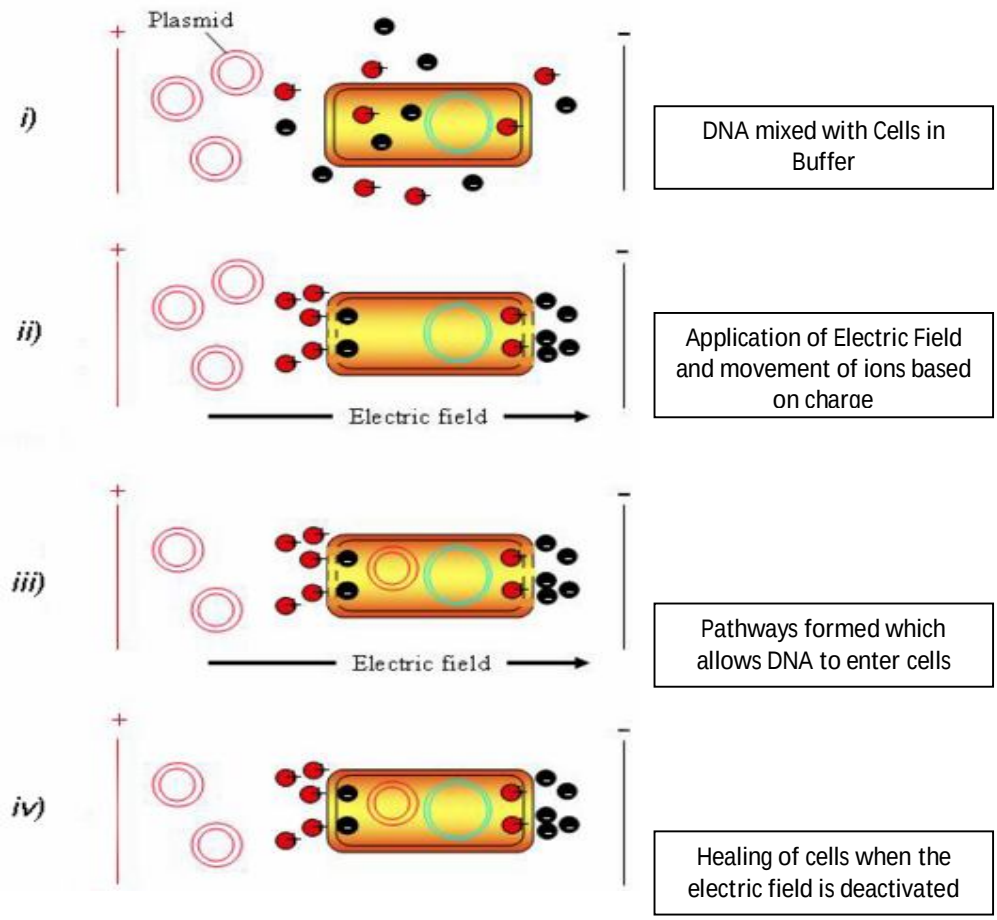


Figure 3.2.5 Electroporation as a mode of DNA transfer

- Nucleofection®

Nucleofection technology called Amaxa Nucleofector® Technology available from Lonza Bioscience is reported to yield higher transfection efficiency when compared with lipofection or any chemical based transfection. It is reported that Nucleofection® achieves 10-30 times higher expression rates in CHO cells compared to cells transfected with lipofection. A similar ratio has been determined for both the specific and the volumetric productivities (www.lonza.com). The picture of Amaxa Nucleofector® equipment is shown in Figure 3.2.6:

Figure 3.2.6 Amaxa Nucleofector® Equipment

The equipment comes with preset programs specific for most of the cell lines used for transfection in research and industry. The Nucleofection kit comes with the specialized cuvettes in which the cells were added after resuspension in the nucleofection reagent followed by exposure to the electric shock. In the present study lipofection, electroporation and nucleofection were used for transfection from which lipofetion was used for generating the recombinant CHO cell line mainly because of its easy availability and economic advantage. The same methods of transfection were also used to generate the SP2/0 cell line.

3.2.1.3 GENERATION OF POOLS AND MINIPOOLS

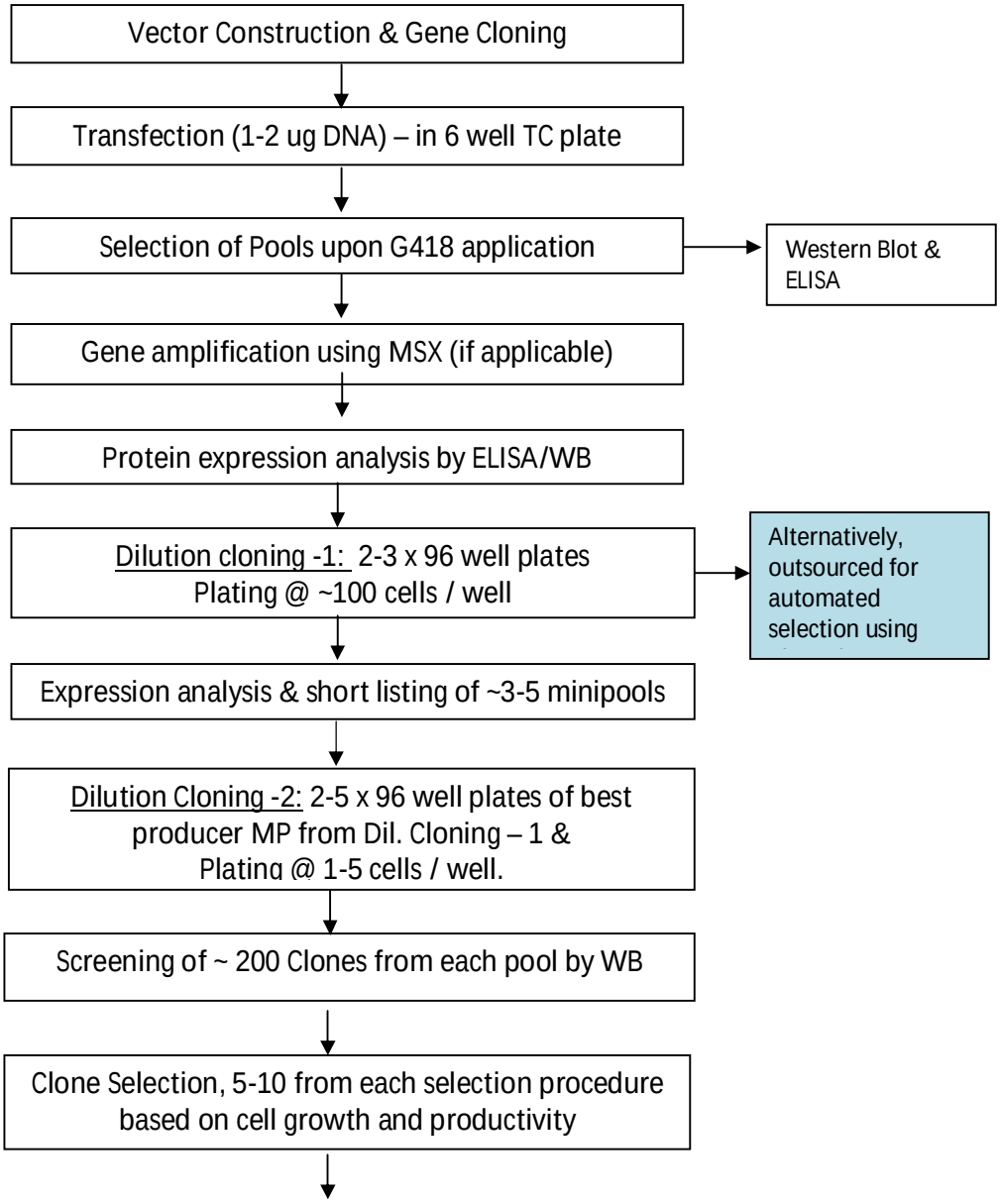
The cells after transfection with either method listed above were allowed to continue to grow for 48 hrs to recover from the shock given during the transfection. The cells were then exposed to the selection pressure (eg. Geneticin G418). The cells were subcultured if the cells overgrow, and the cells were named "Pool" labeled alphabetically. Following identification of top high expressing Pool, the cell population was diluted further to narrow down the heterogenity in the population by plating few cells per well called "Minipool". The minipools were evaluated based on cell growth and expression yield following which the top expressing minipool was diluted to clonality for achieving single cell population.

3.2.1.4 SELECTION OF HIGH-PRODUCING CLONES

The screening procedure for high-producing cell lines is extremely time- and labor intensive and costly, and is at present guided by an empirical approach based on individual experience. The main problem is that such high-producing subclones are rare and spend much of their energy on production, and thus have reduced growth rates. This

leads to overgrowth of non- or low-producing cells. The obvious solution would be to clone single cells by limited dilution methods. This is not always possible, however, due to the poor cloning efficiency of mammalian cells. As a compromise, the number of cells in each well can be increased to obtain a reasonable number of clones, which will be heterogeneous in nature (Minipool). Inevitably, cells with higher growth rates are more likely to comprise the main population in the microwell plate. The result is that many wells need to be screened and tested to finally find a clone with an increased production rate (Broth, *et al* 2001). The minipools were diluted either using manual limited dilution method or automated ClonePix FL® instrument to select the best clone.

3.2.1.5 TYPICAL FLOW OF CELL LINE DEVELOPMENT



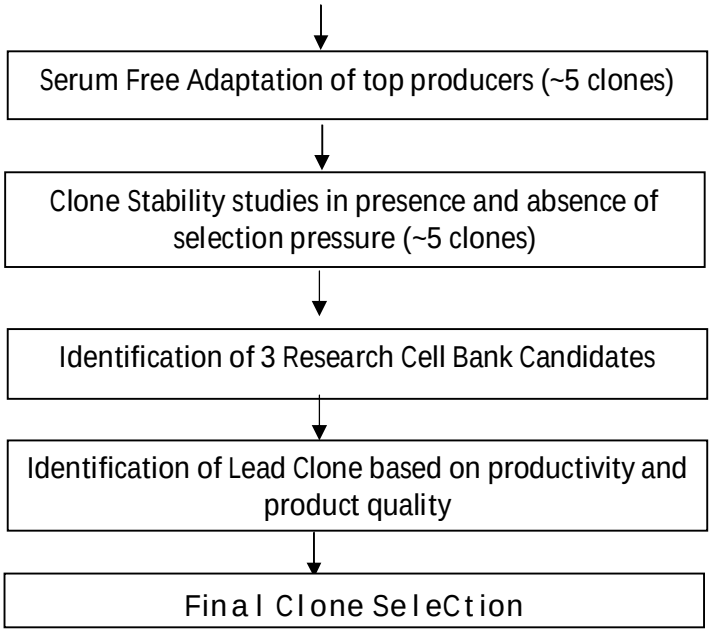


Figure 3.2.7 Flow Chart of Cell Line Development

Note: The above shown flowchart is a typical of the activity performed in-house except the automated selection of clone using ClonePix (light blue box). In case where automated system was used to select single clone Limiting Dilution 2 is not required

3.2.2. Results and Discussion

3.2.2.1. Evaluation of Different Transfection Reagents & Methods:

The transfection reagents listed above were used to transfect the expression vector in SP2/0 and / or CHO cells for generating SP2/0 AG-14 & CHO cell lines using S8.1 expression vector.

As per the conclusion drawn from section 3.1.2.6, S8.1 expression vector has GS as selection and amplification marker. After transfection, the cells were selected and gene amplification done with exposure of cells with increasing concentration of Methionine Sulphoximine (MSX).

- Transfection of SP2/0:

The SP2/0 cells were seeded at 0.4 x 10^6 cells/well (at 0.2 x 10^6 cells/mL) concentration a day prior to transfection. The transfection was carried out using different transfection reagents as listed above section 3.2.1 with different DNA : transfection reagent ratio. The S8.1 expression plasmid and pcDNA3.1(-) were co-transfected so that the initial

selection can be carried by antibiotic Geneticin G418 selection. The cell supernatant was analyzed for expression of antibody using western blot and ELISA after Protein-A affinity purification step. After 48 hrs of transfection ~1 mL of cell supernatant was collected, purified and concentrated using Protein-A column. The eluate was checked for the expression of antibody using anti-IgG kappa HRP conjugated antibody (Figure 3.2.8). **The cell line generated was labeled as S8.1**

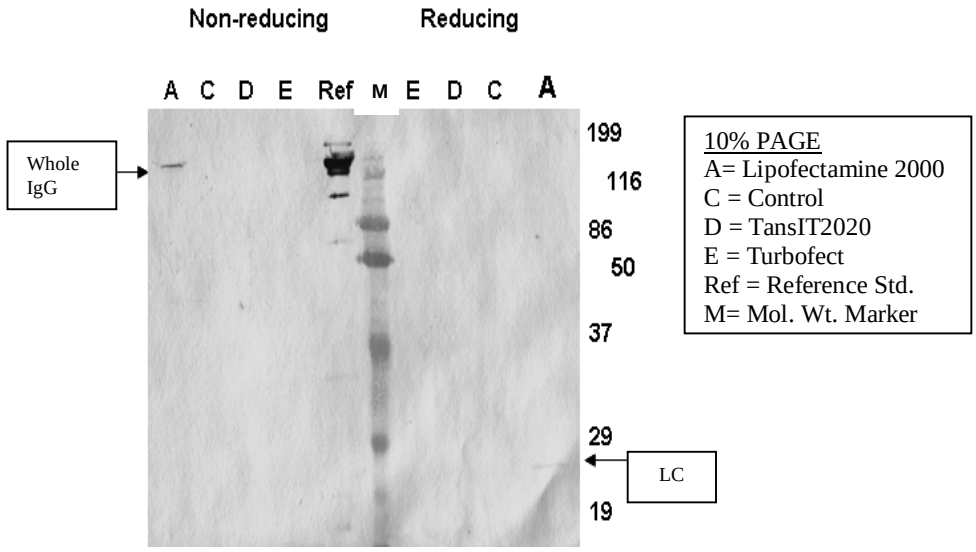


Figure 3.2.8 Western Blot Analysis of SP2/0 transfectants

The cell supernatant analyzed from S8.1 Cell Line generated by various transfection agents

A distinct band of ~200kDa in Non-reducing gel and ~25 kDa in reducing gel was observed in **Pool A** (ie. Transfection done using Lipofectamine 2000). A very faint band of similar size was observed in **Pool E** also (not visible in the above scan image but was seen at the time dot blot development), the case where transfection was done using TurboFect® reagent.

These two transfection pools were subjected for antibiotic selection and MSX amplification in 12-well TC plates. Each pool was split into two wells, in one well the cells were exposed to 500 µg/mL Geneticin G418 and in other well 500 µg/mL Geneticin + 50 µM MSX was added. The MSX concentration was increased gradually upto 200 µM. The cell supernatants from both Pool A and Pool E were collected and antibody concentration in them was estimated using ELISA. As shown in Figure 3.2.9, the expression of protein increased with increase in concentration of MSX reaching ~6 mg/mL at 100 µM MSX . In Pool E however, after 100 µM the expression was observed to be decreasing probably due to the inhibitory effect of high concentration of MSX.

There was no absorbance observed in Pool A indicating that there could be loss of gene or shut down of expression due to reasons unknown.

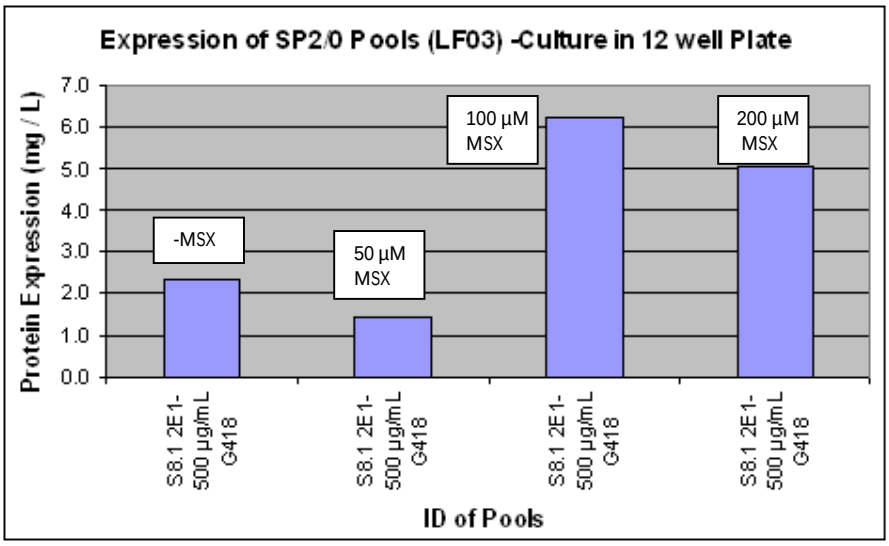


Figure 3.2.9 Expression Analysis of S8.1 Pool 'E' by ELISA

At early passages, the expression observed was low (~6 mg/L). Limiting dilution of Pool E was done to generate minipools. The cells were seeded at 100 cells/well of 96-well TC plate. The cells were incubated till sufficient cell density was achieved, generally 4-5 days after which the dot blot analysis of all these minipools was done using Bio-rad manifold system on a nitrocellulose membrane. Labeling was done using anti-IgG kappa specific HRP antibody and detection was done by adding TMB substrate. A typical Dot-blot scan is shown in Figure 3.2.10, and all the blots are not shown due to poor scanning quality. The top 5 minipools selected for further use were E1, E69, E70, E82 and E83.

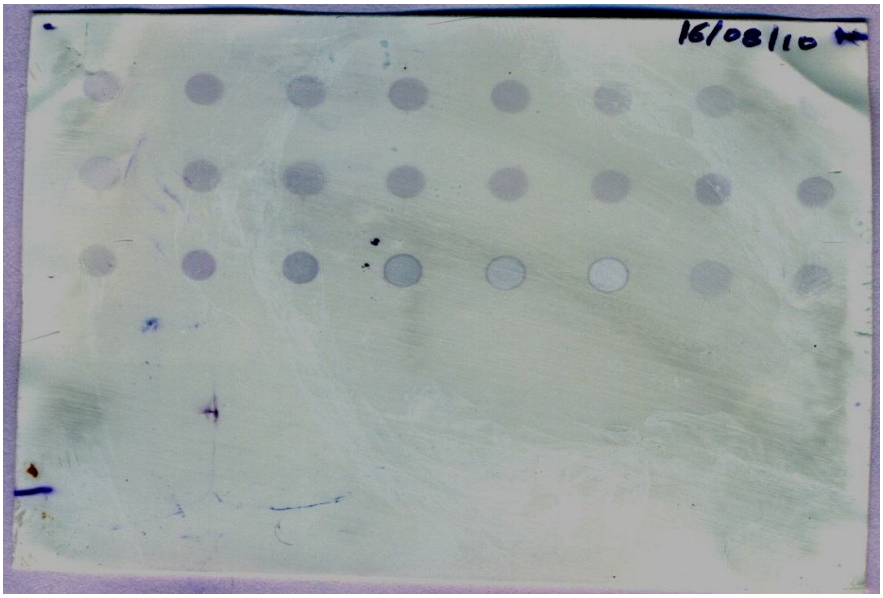


Figure 3.2.10 Representative Dot Blot for screening minipools

These five minipools were expanded in larger cell culture vessel from 96 well to 24 well to T25 flask (data not reported). It was observed that the expression was continuously decreasing and reduced to negligible. Hence, these S8.1 recombinant SP2/0 cell line and minipools generated using different transfection reagents were discontinued.

Stable recombinant SP2/0 cell line could not be generated when different transfection reagents were tried. The expression of protein of interest almost disappears after about 5 passages. This led to exploration of a nucleofector technology from Amaxa (Lonza) which facilitated transfer of nucleic acid into the cell. This technology is similar to electroporation whereby an electric pulse is generated using electrodes but the pulsing regime is different and specific reagents are used which could result in the enhanced transfection efficiency. The equipment comes with preset nucleofection procedures for different cell lines. For SP2/0 cell line the supplier has recommended programs X001, X005, B033, A033, and T020 which are already fed in the equipment. Out of these 5 programs one best program need to be identified to be used for generating recombinant cell line. The exponentially growing cells (~1.5 x 10^6 cells) were nucleofected with 2 µg reference plasmid (pMAX) expressing green fluorescent protein (GFP). The transfected pools (using different programs) were analyzed for the expression of GFP by measuring fluorescent by FACS after 24 hrs of transfection, refer Figure 3.2.11. The GFP expression of individual cell and as pools was analyzed. The cells localized in the left quadrant are GFP non-expressing cells, typically as in Mock cells. Based on the expression of GFP the cell localization shifts to the right hand side quadrant. The more cell localization in right hand side quadrant indicates more transfection efficiency. Also, based on the intensity of expression, the cells could be sorted at the first stage itself. The maximum transfection efficiency of 71% was observed in Program X001 hence this program was used for transfection of SP2/0 cells with S8.1 expression plasmid.

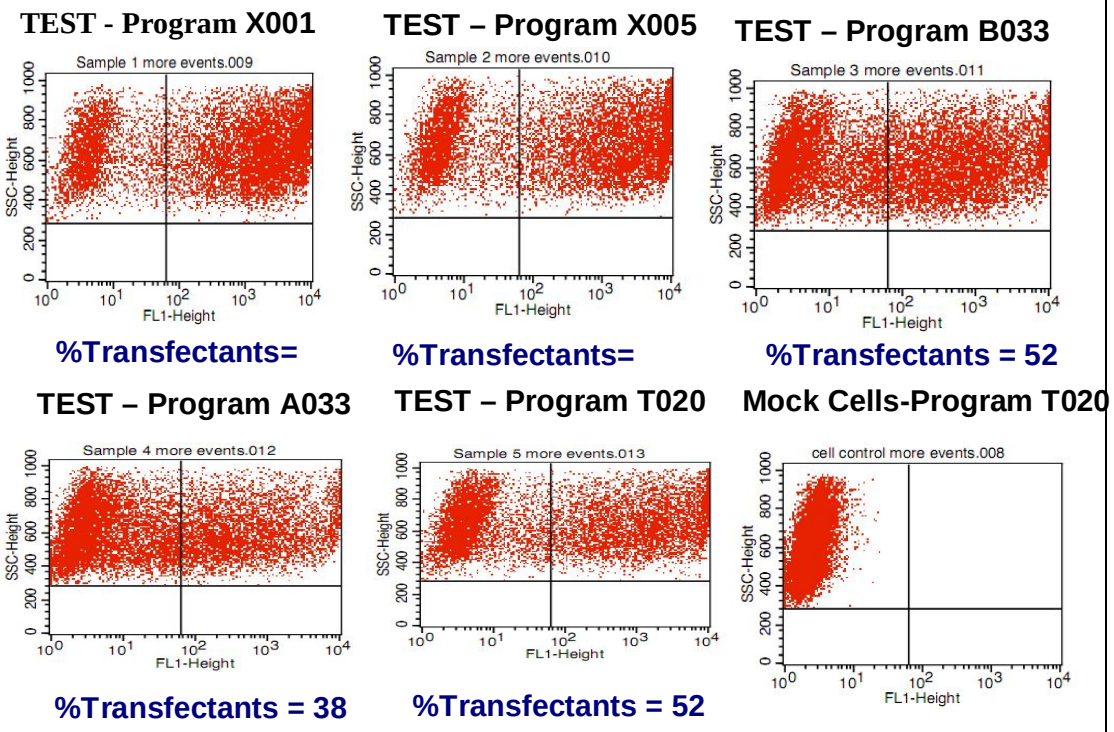


Figure 3.2.11 Optimization of Nucleofection Conditions

The SP2/0 cells grown for not more than 48 hrs were harvested, washed with PBS and finally resuspended in 0.1 mL of reagent supplied in the kit with effective cell concentration of ~1.5 x 10^6 cell / 0.1 mL. The cells were transferred to the cuvette and mixed with ~3 µg S8.1 expression vector. The cells were exposed to Nucleofection Program X001. After the exposure, the cells were seeded in 6 well plates for transient productivity analysis. A negative control that is without DNA was also kept which was labeled as Pool 'C'. The transfected pool was labeled Pool 'H'. After 48 hrs, the cells were put in selection pressure 50 µM MSX. Cell toxicity was observed after about 7 days and the cell number were also less. At this stage the cells were transferred to 12 well plates. Following further incubation, the cell density increased and hence were transferred to T25 flasks. The expression was checked from 12 well and T25 flask. Approximately 1 mL cell supernatant was purified and concentrated to 100 µL using Protein A resin, and was checked on 7% SDS-PAGE under non-reducing conditions and then probed with labeled antibody to be detected in western blot (Figure 3.2.12).

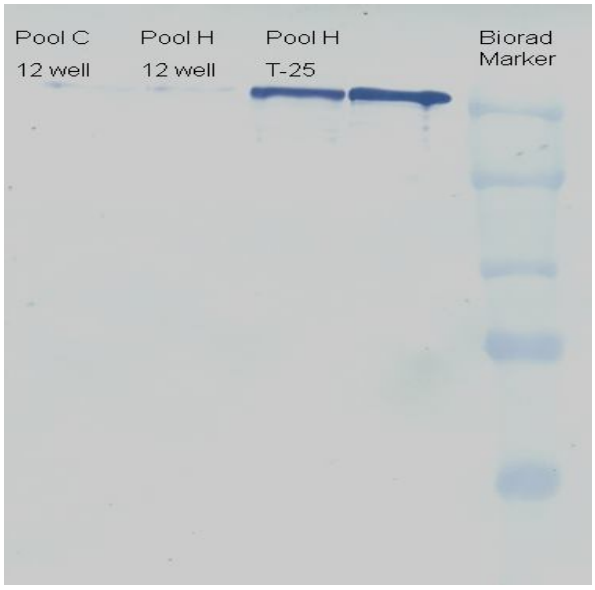


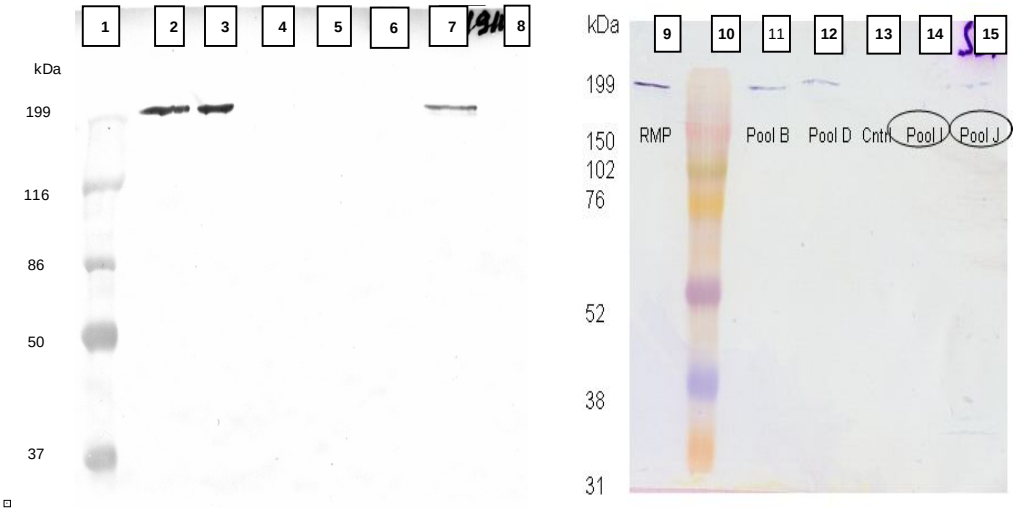
Figure 3.2.12 Western Blot analysis of SP2/0 transfectants

However, these cells also exhibited a loss of expression when grown further. It was also observed that the expression of protein of interest was low when different lipid based transfection reagents were used. Nucleofection was used in order to increase the transfection efficiency but to maintain stable expression was not possible and hence could not qualify to go ahead for further development at industrial scale. Also in comparison with CHO the expression is very less and not stable in SP2/0 (discussed later in following section).

3.2.2.2. Generation of SP2/0 & CHO cell lines using S8.3 expression vector:

An alternative strategy of generating stable transfectant was tried with S8.3 expression vector. As mentioned in section 3.1.2 point no. 7, this vector possess only Neomycin transferase gene as selection marker and the GS gene is removed.

The expression of protein of interest from different cell pools after transfection using different transfection reagents as shown in Figure 3.2.13. The SP2/0 transfected cells were labeled as S8.3/SP2 and CHO-S transfected cells as labeled S8.4/CHO. After 48 hours of transfection, cell supernatant was collected, purified using protein A and checked on western blot, Figure 3.2.13.



Lane 1 & 10=Rainbow Mol. Wt. Marker Lane 2 , 3 & 9 = RMP, Lane 4= SP2/0 Pool F (TurboFect), Lane 5 = SP2/0 Pool D (TransIT), Lane 6 = SP2/0 Pool E (Lipofectamine2000) Lane 7 = CHO Pool A (TurboFect), Lane 8 = SP2/0 control cells, Lane 11 = CHO Pool B (Lipofectamine 2000), Lane 12 = CHO Pool D (TransIT), Lane 13= CHO control cells, Lane 14 = SP2/0 Pool I (lipofectamine 2000), Lane 15 = SP2/0 Pool J (TransIT).

Figure 3.2.13 Western Blot for Expression Analysis of SP2/0 and CHO transfectants (S8.3 & S8.4 respy.)

As shown in above figure, a good detectable expression of mAb of interest was observed when SP2/0 were transfected using TransIT reagent (Pool J) compared to Lipofectamine2000 (Pool I). The TurboFect transfected cell pool of SP2/0 didn't show detectable protein expression (Pool F). Expression of protein was observed in all transfection sets of CHO-S but more in case where transfection was done using TurboFect (Pool D). In SP2/0 Pools 'I' & 'J', and CHO pools 'B' & 'D' antibiotic Geneticin G418 was added starting from 200 µg/mL and reaching 500 µg/mL. These pools were further expanded in different cell culture vessels (6 well plate, T25 and T75 flask) to study stable protein expression and selection of lead pool as assessed by Sandwich ELISA.

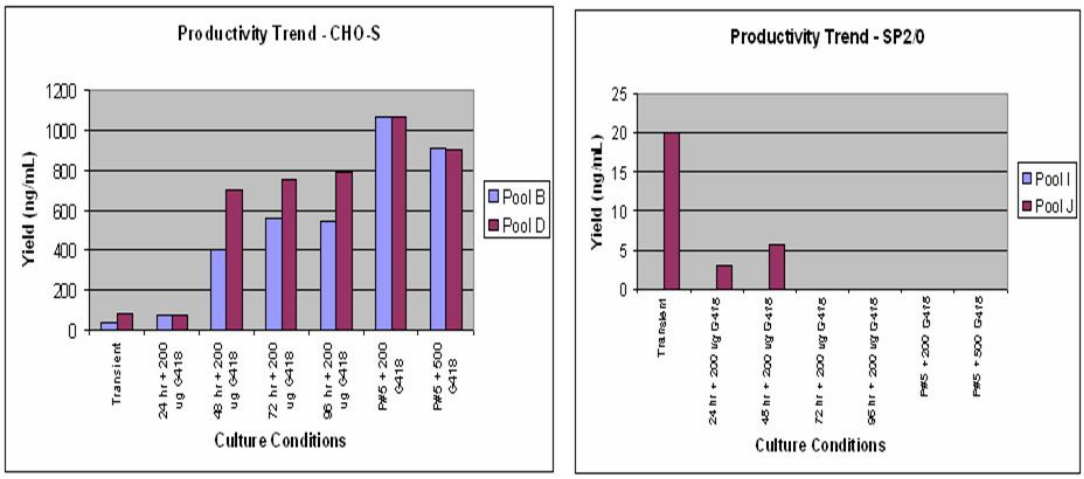


Figure 3.2.14 Expression Analysis of SP2/0 & CHO Pools by ELISA.

The samples were analyzed at different time intervals and different concentrations of G418.

A) Productivity trend in recombinant CHO-S cell pools,

B) Productivity trend in recombinant SP2/0 cell pools.

The expression in SP2/0 disappeared after about 72 hrs of addition of G418 which indicates that no integration of expression plasmid happened in the genome and the plasmid was lost during the subculturing procedure. On the other hand, the expression of protein in CHO pools B and D was increasing with increase in subculture time and concentration of G418. Both pools of CHO were giving similar expression of protein (Figure 3.2.14). It was also observed that the expression did not increase when more than 200 µg/mL G418 was added; in fact it appears to decrease at higher concentrations (Figure 3.2.15). When expression of Pool 'B' and 'D' was measured at passage #5, the Pool D excels in protein expression in case of recombinant CHO cells.

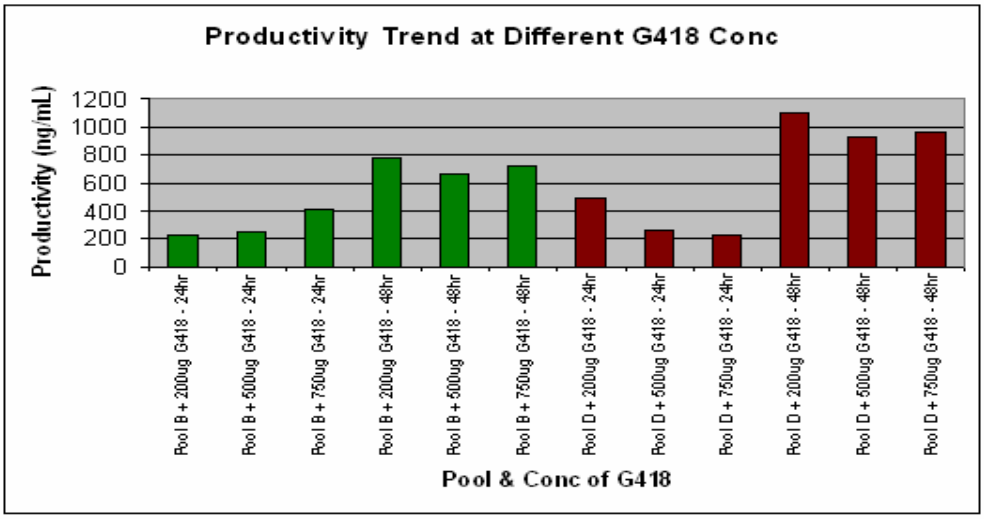


Figure 3.2.15 Expression comparison of in Pool B & D of CHO transfectants by ELISA

3.2.2.3. Generation and Evaluation of Lead Research Cell Bank Clones

The B & D pools of CHO-S transfectants were the high expressing pools at initial screening. These pools were taken further for generating minipools for which the cells were diluted in growth media (DMEM with 5% FBS) such that each well of 96 well TC plate received ~100 cell/well. A total of 2 x 96 well TC plates were seeded in a pattern as referred in Figure 3.2.16. A total of 120 Minipools were generated from each pool, i.e., 120 each from Pool B and Pool D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	PBS
C	PBS	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	PBS
D	PBS	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	PBS
E	PBS	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	PBS
F	PBS	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	PBS
G	PBS	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	Minipool or Clone	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 3.2.16 Plate Pattern followed for seeding cells for Minipool and Clonal population generation. Outer grey colored wells are not used and only PBS was added

The plates were incubated for a week's time or when the confluency reached ~70-80% before analyzing for expression using ELISA. About 100 µL cell supernatant was directly put in the ELISA plate pre-coated with Human IgG Fc-Specific monoclonal antibody. From the 120 minipools generated from Pool B, Minipool # B25, B80 & B96 gave highest expression and were shortlisted for further experimentation (Figure 3.2.17), B #1 and B #66 were not selected as it was observed that the growth of these cells are very sluggish. Similarly, from Pool D, Minipool #D7, 41 & 51 were selected (Figure 3.2.18) while D #1, D #37 and D #48 were not selected because of sluggish growth.

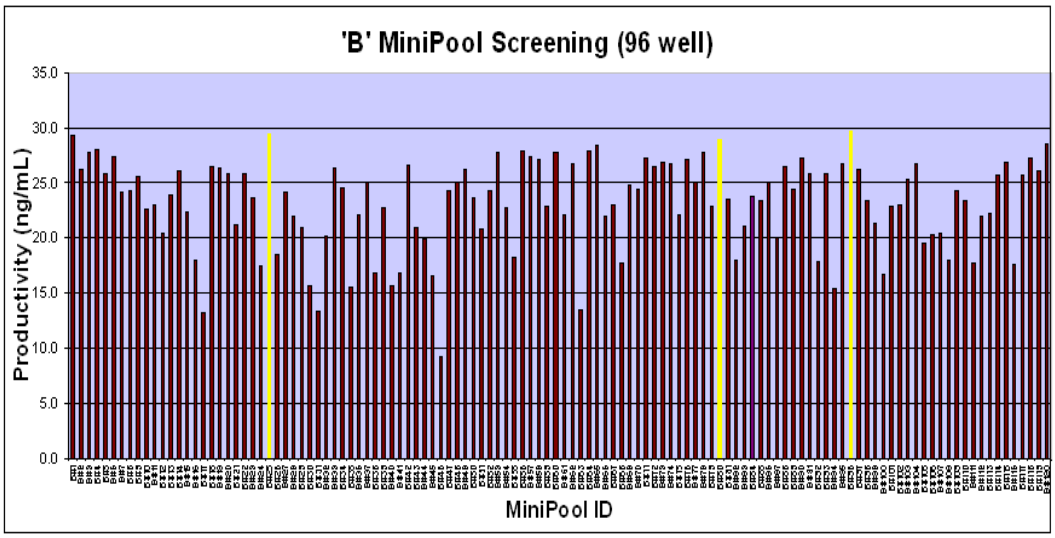


Figure 3.2.17 Comparison of Expression in Pool B Minipools by ELISA

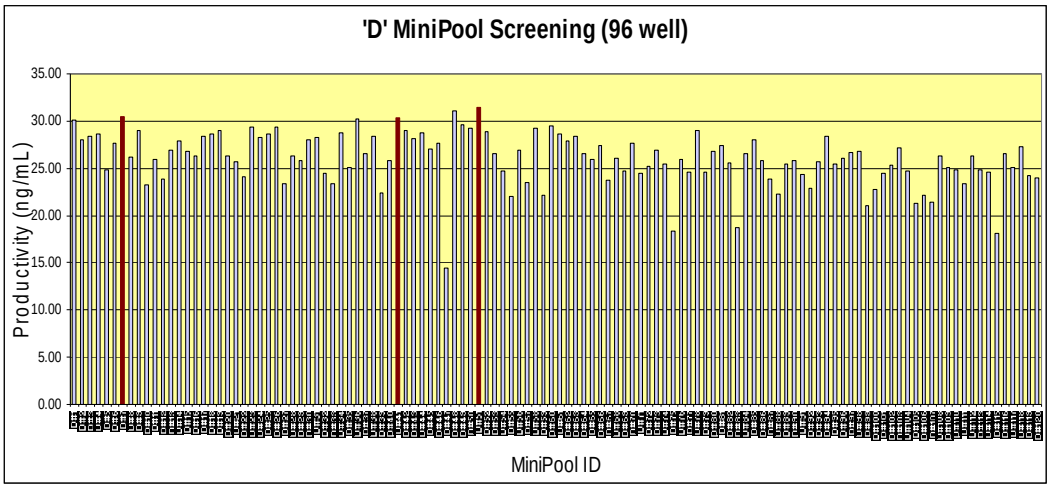


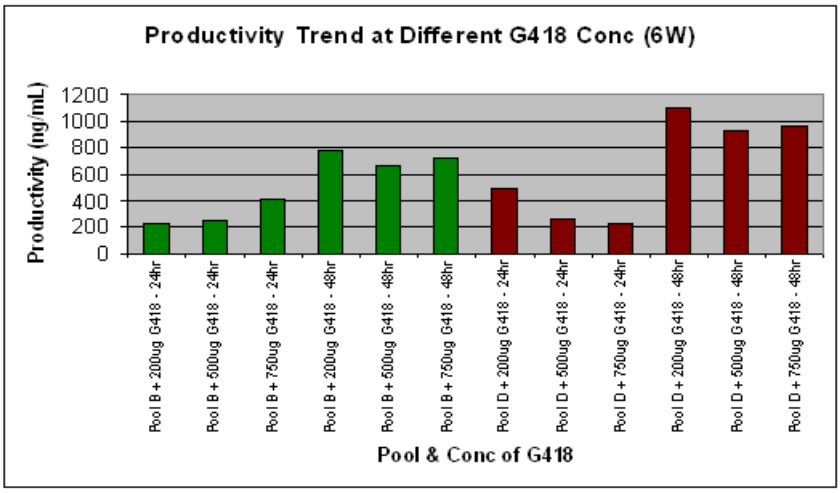
Figure 3.2.18 Comparison of Expression in Pool D Minipools by ELISA

The percentage confluency in Minipool D41 was less compared to other minipools, though it gave higher absorbance / higher expression yield, hence is considered as potential lead minipool.

The minipools thus generated were propagated in different culture vessels depending on the cell density achieved. The minipools generated from B & D pools were compared at 6 well plate and 12 well plates scale.

The minipools of D were leading when the expression was compared to B minipools (Figure 3.2.19); hence D minipools were considered for further clonal selection procedure.

A)



B)

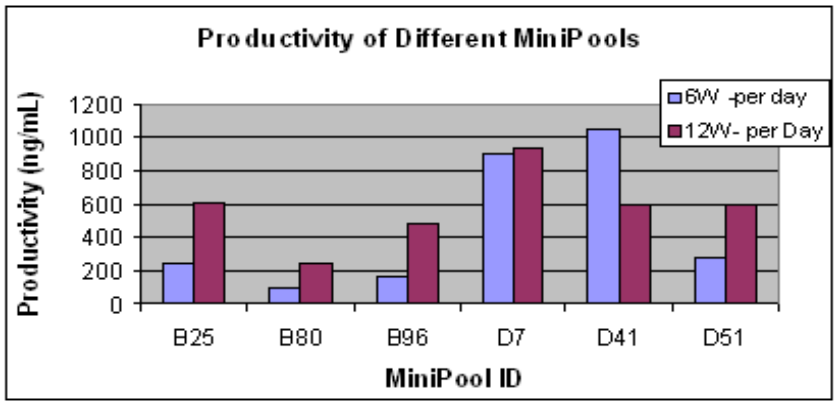


Figure 3.2.19 Comparison of expression amongst B & D Minipools by ELISA

A) Comparison of B & D Pools

B) Comparison of Minipools generated from B & D Minipools.

Amongst the shortlisted 'D' Minipools, D41 Minipool fairs ahead of other minipools as the expression was higher although the percentage confluency was lower than other minipools like D7 (observed microscopically). Hence D41 was selected for limiting dilution to generate single clones.

Minipool D41 was further taken for single cell limiting dilution in which the cells were seeded in multiple 96 well TC plate at a density of ~1-5 cells/well. A total of 300 wells were plated and incubated till sufficient cell density was achieved (Refer Plate layout in Figure 3.2.16). Out of these 300 wells, 21 wells were observed with single cell by microscopic analysis using inverted-phase contrast microscope. Protein expression was analyzed by ELISA from these single clones and represented graphically in Figure 3.2.20.

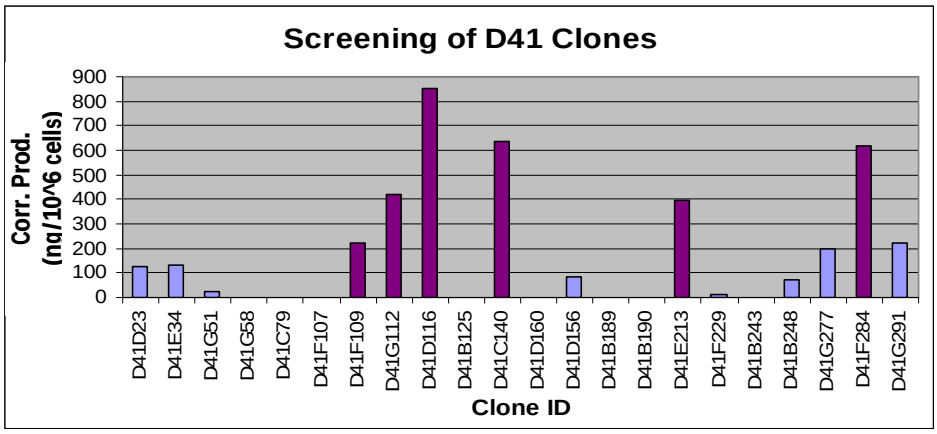


Figure 3.2.20 Comparison of expression of clones generated from Minipool D by ELISA
Corrected productivity was plotted which was calculated by dividing productiovity with viable cell count X10^6 cells.

The clone #D41F109, D41G112, D41D116, D41C140, D41E213, AND D41F284 (purple colored bars) are higher expressing and are potential top expressing clones which are selected for further analysis. The selected clones were propagated in the larger volume culture vessel (T25 cm^2 flask) and the expression was compared amongst the shortlisted clones (Figure 3.2.21). The cells were grown in static conditions for 72 hrs before the cell culture supernatant was analyzed for expression by ELISA.

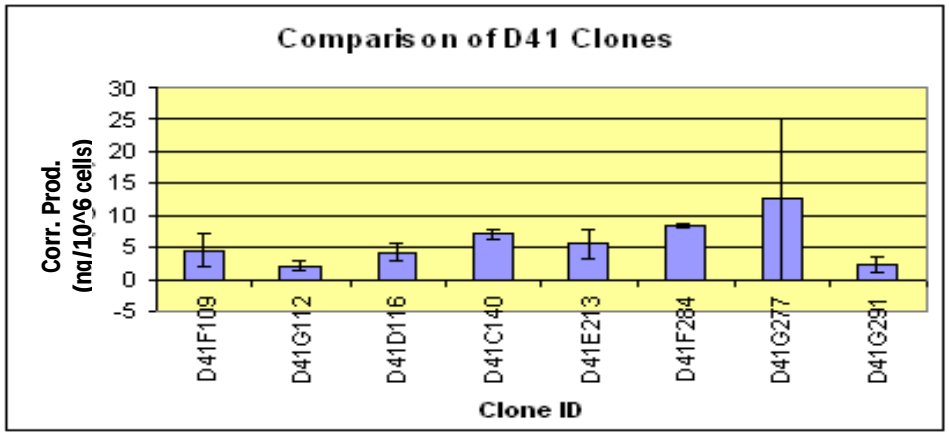


Figure 3.2.21 Comparison of D41 clones by ELISA
Corrected productivity was plotted which was calculated by dividing productiovity with viable cell count X10^6 cells. The data plotted are from two independent experiments.

It was observed that clone # D41D116, D41C140 and D41E213 gave higher expression than other clones. Clone # D41G277 gave higher expression in one experiment (this could be an outlier) and hence the error bar is too spread-out, while clone # D41G291 was observed to be very fast growing but have low expression level. Hence D41G277

and D41G291 were discontinued. These 6 clones (D41F109, D41G112, D41D116, D41C140, D41E213 and D41F284) were then gradually adapted in the serum free chemically defined (CD-CHO) media and then studied for the stable expression over an extended period of time in presence of selection pressure and absence of it.

The clonality was ensured microscopically when the cells were plated at 1-5 x 10^6 cells / well. The grown colonies of clone # D41D116, D41C140 and D41E213 are depicted in Figure 3.2.22.

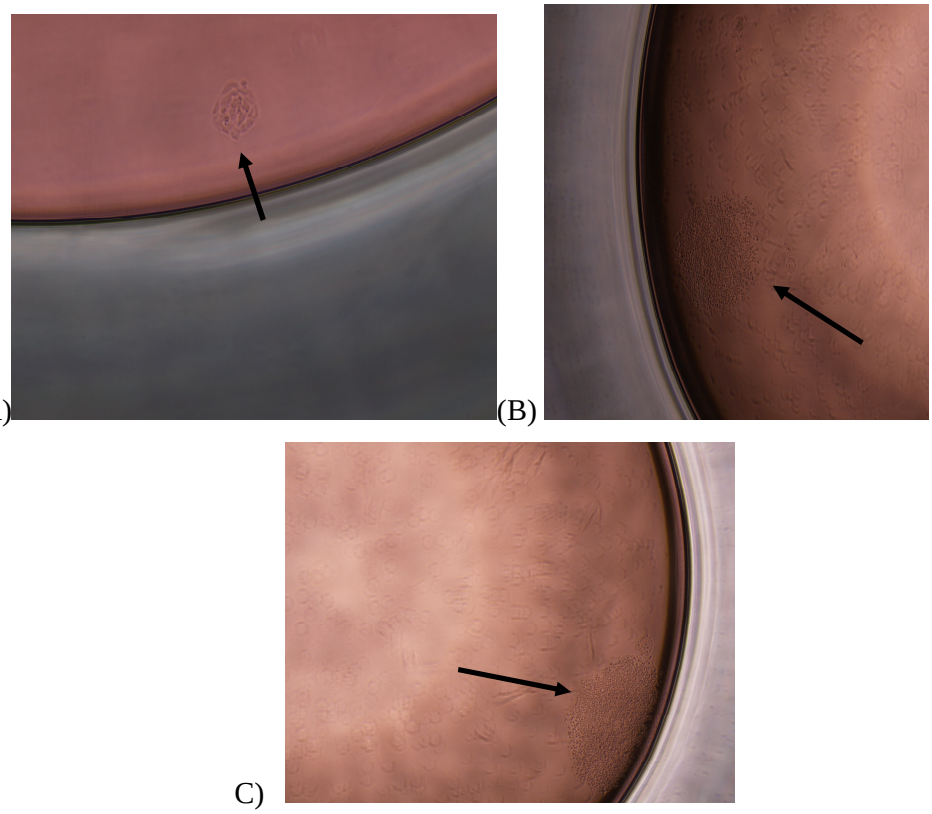


Figure 3.2.22 Clonality checking by microscopic observation

(A) Clone D41D116, B) Clone D41C140 and (C) Clone D41E213.

Image taken under inverted phase contrast microscope using 20x magnification.

3.2.2.4. Selection of clone using Automated Clone Selector

Conventionally manual limiting dilution method is employed since ages but, it is time consuming and laborious process where at least 2 round of dilution is required. In order to make process simpler and rapid, automated system could be engaged (eg, ClonePix FL, Make Genetix) to select the clone based on expression yield and growth of cells. ClonePix FL system available from Genetix, UK was used to analyze the higher expressing cells from the heterogeneous population and sort automatically into 96 well TC plate. As Pool D is high expressing pool it was selected for this activity. The cells were diluted and plated in 2 x 90 mm petri-dish in a semi-solid media. Once sufficient

sized colonies appeared, the cells were labeled with Recombinant anti-human IgG FITC (Cat # K8295 Lot # CDU1010A, Make Genetix). The fluorescence intensity depends on level of expression of antibody by the transfected cells (Figure 3.2.23)

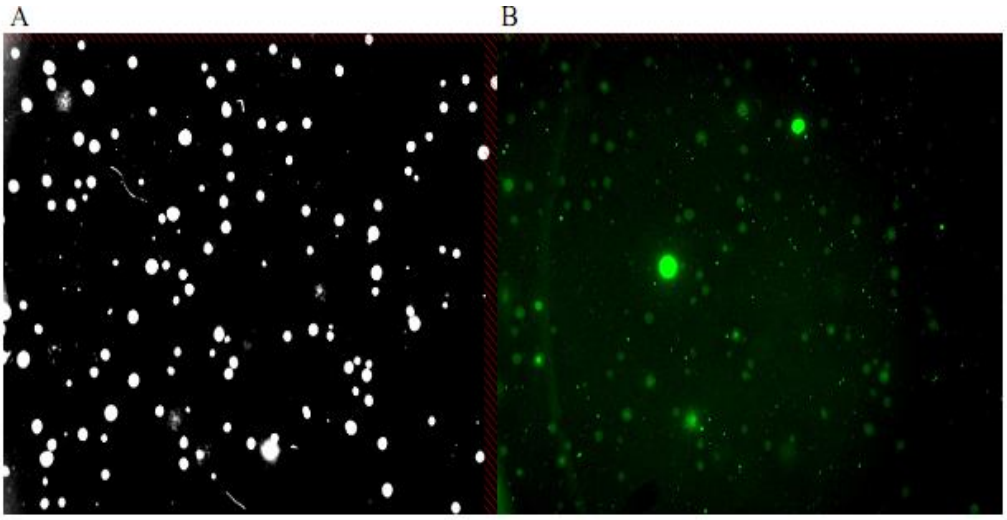


Figure 3.2.23 Image generated from ClonePix FL.

(A) Observation of colonies under white light

(B) Observation of colonies under Fluorescent mode

From both the plates ~1000 colonies were selected based on fluorescence and transferred with the help of automated arm in the 96 well plates for further growth and expression analysis. The outgrowing of cells and colony proximity was monitored by CSI (CloneSelect Manager Software) as shown in Figure 3.2.24.

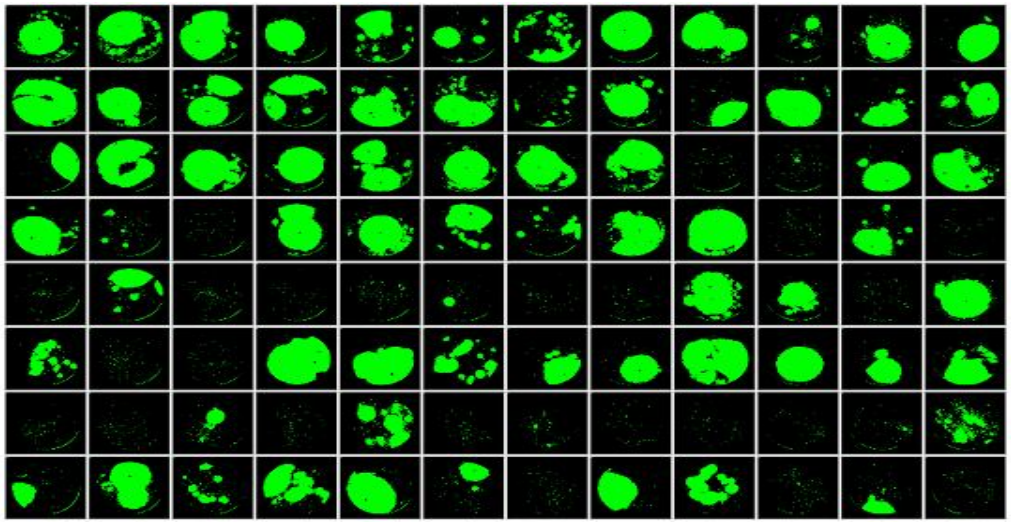


Figure 3.2.24 Image generated from the CloneSelect Imager to determine confluency (%)

The software gives information about the growth of the individual colony and the expression of the clone. The fluorescent, expression analysis and proximity number was

measured on Day 6 and analyzed by Clone Select Image software. Based on these analysis 30 clones were shortlisted for further analysis. The quantitative expression of recombinant mAb was done using Sandwich ELISA (Table 3.2.1)

	proximity	%confluence (day 6)	Conc (µg/ml)	Ratio
1B1	0,193	60	3,65	6,08
1A2	0,087	47	1,14	2,42
1B2	0,062	54	4,60	8,52
1D2	0,498	55	0,73	1,33
1F2	0,25	45	1,92	4,27
1G2	0,25	28	0,29	1,05
1C3	0,124	43	1,51	3,50
1H3	0,711	50	1,84	3,68
1B4	0,193	52	1,50	2,89
1C4	0,177	47	1,57	3,34
1D4	0,161	80	1,19	1,48
1G4	0,138	35	1,66	4,75
1A5	0,138	56	2,18	3,89
1B5	0,111	55	3,63	6,61
1C5	0,078	76	1,36	1,79
1B6	0,195	63	3,74	5,93
1F6	0,806	62	1,81	2,92
1A7	0,443	42	5,30	12,62
1C7	0,276	60	0,98	1,63
1D7	0,221	38	1,07	2,81
1E7	0,216	57	4,17	7,32
1F7	0,193	74	4,42	5,97
2F5	0,864	65	2,56	3,93
2G5	0,846	39	0,63	1,61
2H5	0,839	56	2,26	4,04
2B6	0,832	52	0,30	0,58
2E9	0,821	58	0,34	0,59
2G9	0,806	5	0,33	6,53
2A12	0,885	44	0,40	0,92
2H12	0,806	5	0,30	5,99

Table 3.2.1 Comparison of Clones Selected for analysis from ClonePix by ELISA

The ratio was calculated by using concentration (µg/mL; expression yield determined by ELISA) and %Confluency derived from CSI data of individual clones. The growth rates of the listed clones were monitored as shown in Figure 3.2.25.

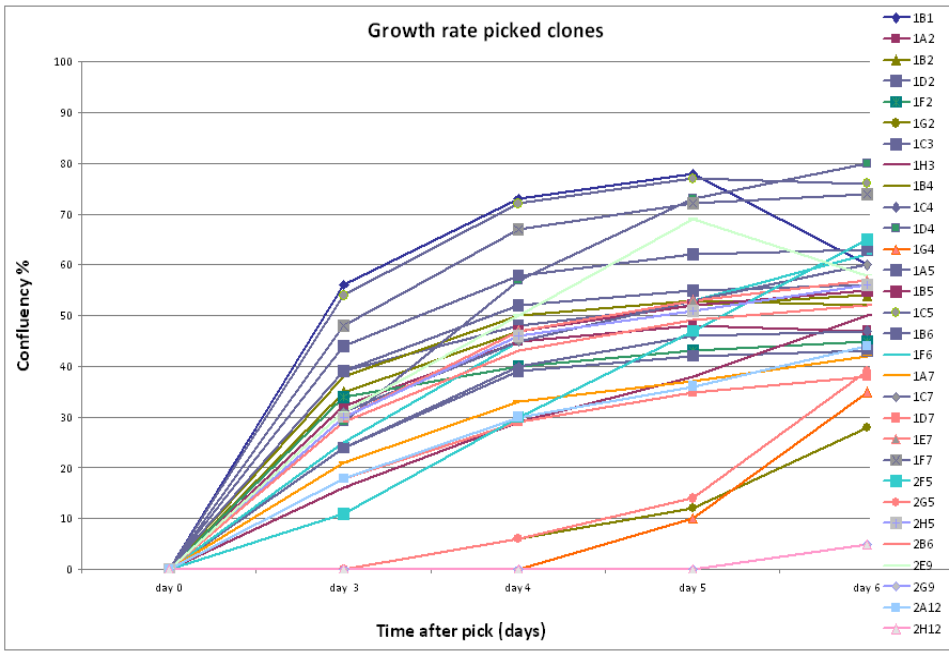


Figure 3.2.25 Growth Rate determination of top 30 shortlisted clones from ClonePix

Shortlisting of top 10 clones out of 30, the cell growth, expression and proximity values were considered and rated as tabulated in Table 3.2.2

Sr. #	Clone #	Corr. Productivity	Proximity	%Confluency	Growth
1	1A7	12.62	0.443	42	++
2	2G9	6.53	0.806	5	+
3	2H12	5.99	0.806	5	+
4	2H5	4.04	0.839	56	+++
5	1E7	7.32	0.216	57	+++
6	1H3	3.68	0.711	50	+++
7	2F5	3.93	0.864	65	++++
8	1F7	5.97	0.193	74	+++++
9	1B6	5.93	0.195	63	++++
10	1B1	6.08	0.193	60	+++

Table 3.2.2 Comparison of top 10 clones selected by ClonePix.

The selection was done based on expression yield, proximity and %confluency

Clones	pcd
1A7	1,59
1E7	0,73
1H3	0,07
2F5	3,07
2H5	2,14

Table 3.2.3 Specific productivity of the top 5 clones selected by ClonePix

The 5 clones highlighted in Table 3.2.2 were shortlisted for specific productivity determination and calculation (Table 3.2.3) based on which clone # 1A7, 2F5 and 2H5 was selected for further use.

3.2.2.5. Adaptation to Serum Free Media and Suspension Culture

All the cloning work and selection were done in DMEM media containing 5% Foetal Bovine Serum (FBS). FBS is a very complex animal derived product, having lot to lot variation and has regulatory concerns. Hence, it is not preferred at the industrial scale. Therefore, it was decided to remove FBS and grow the recombinant cell lines in chemically defined media (CD-CHO). CHO cells inheritably are adherent cells and this makes their large scale cultivation cumbersome apart from controlling its product quality. For industrial applications, the suspension culture-based manufacturing process has become the method of choice for therapeutically important proteins from rCHO cells

(Lee, *et al* 1999). The top 6 clones selected from manual limiting dilution and top 3 clones selected from ClonePix were gradually adapted to chemically defined media (CD-CHO, Invitrogen). The clones after adaptation in serum free media were adapted in suspension culture. All the 9 clones got adapted in serum free and suspension conditions except 2H5 (ClonePix) clone which didn't give good growth profile after adaptation. These clones were evaluated for productivity and quality assessment for expressed product.

3.2.2.6. Comparison of manually selected clones and ClonePix selected clones

The clones were grown in CD-CHO media in 6well and T25 cm^2 flask under static condition and in 125mL shake flask under shaking conditions. The expression yield was estimated on Day 4 by ELISA. Based on the cell growth and productivity data clone # D41D116, D41C140 and D41E213 (manual selection) and Clone # 1A7, 2F5 and 2H5 (ClonePix Selected) were shortlisted. These clones were compared across different scale of culturing for productivity analysis (Figure 3.2.26).

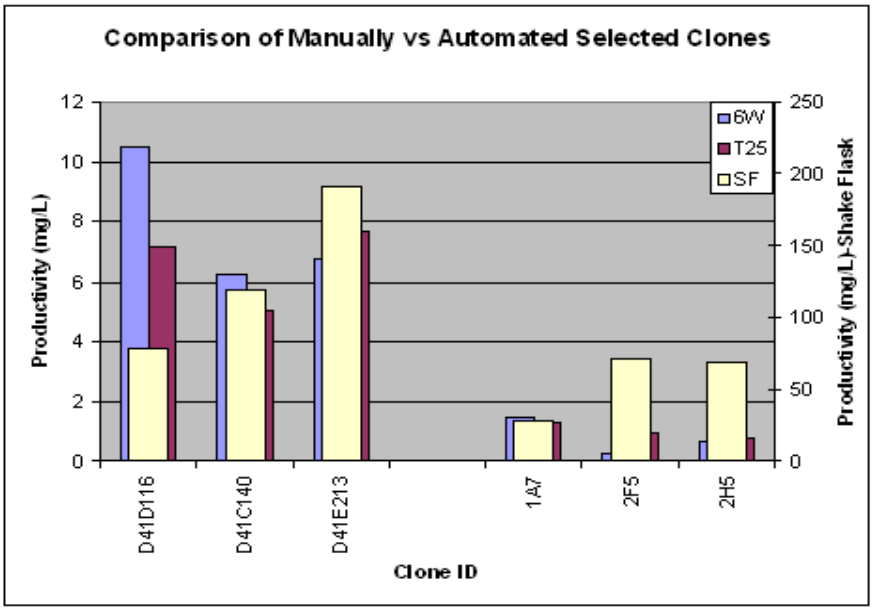


Figure 3.2.26 Productivity comparison of manually and automated selected clones.

The comparison was done across different scales starting from 6-well TC plate to T25 cm^2 TC flask to shake flask

The productivity in suspension culture (shake flask) is similar or more than the static cultures except for clone D41D116 where the productivity reduced in shake flask indicating some changes could have happened during adaptation phase.

An interesting observation was that the expression yield of clones selected using ClonePix were significantly less than the clones selected by manual limiting dilution. This observation was in contrast to the fact that selecting more number of clones and that too using automated system which possess all possible tools should give the best clones. Based on this observation clones D41D116, D41C140 and D41E213 were selected for further experimentation.

3.2.2.7. Preparation of Cell Banks

The cells generated at every stage starting from the transfection was preserved and stored in liquid nitrogen storage tank in vapor phase. When the growing cells reaches exponential growth phase, the cells were centrifuged and pellet resuspended in the cryoprotectant media containing DMSO as cryoprotectant. The number of cells preserved depends on the cell availability at different stages. At early stages the vials were prepared at cell density of ~2 x 10^6 cells/vial while for the primary cell bank the cell density preserved was ~10 x 10^7 cells/vial. The Primary Cell bank was prepared of clone #D41D116, D41C140 and D41E213 was revived from the earlier cell bank prepared and cells were propagated in shake flask till sufficient cell density was achieved to cryo-preserve ~10 vials at ~10 x 10^7 cells/vial.

3.2.2.8. Characterization of Primary Cell Bank

- Contamination Check

After preservation the cells were revived and tested for presence of any bacterial or fungal contamination by inoculating cell culture supernatant in LB-broth and Sabouraud's Dextrose broth, respectively. The LB-broth tubes were incubated at 37 ^{0}C while Sabouraud's Dextrose broth tubes after inoculation were incubated at RT for 14 days to monitor any microbial growth. No contaminant growth was observed in either tubes indicating the cell bank is free of any microbial or fungal contamination. Another important contaminant to be monitored is Mycoplasma which impact the cell line's growth and performance. As the growth and prevalence of mycoplasma could be at a low level hence a more sensitive and rapid method based on PCR principle was used (LookOut Mycoplasma detection Kit, Sigma Cat# MP0035). The LookOut® Mycoplasma PCR Detection Kit utilizes the polymerase chain reaction (PCR), which is established as the method of choice for highest sensitivity in the detection of Mycoplasma, Acholeplasma, and Ureaplasma contamination in cell cultures and other

cell culture derived biologicals. Detection requires less than 5 mycoplasma genomes per microliter of sample (~ 5 fg DNA).

The primer set is specific to the highly conserved 16s ribosomal RNA coding region in the mycoplasma genome. The PCR amplified product was run on agarose gel for detection of amplicon from mycoplasma, if any. This method can detect about 18 species of commonly found mycoplasma.

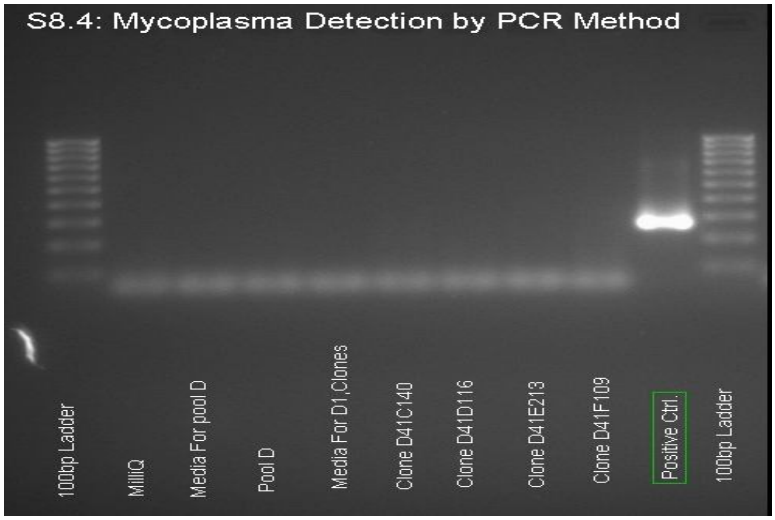


Figure 3.2.27 Detection of Mycoplasma by PCR based method.

The PCR amplified product was checked on 1% agarose gel.

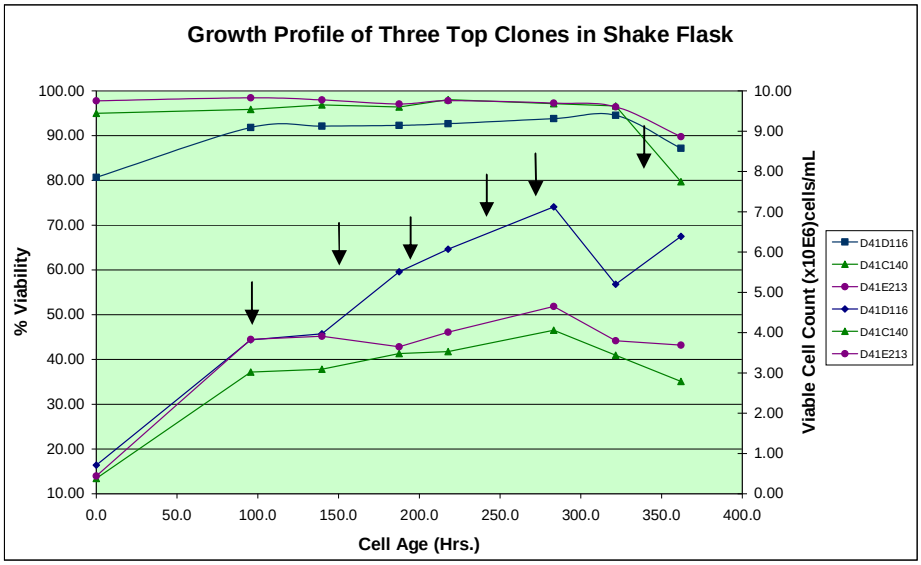
The case where the mycoplasma contamination is present a band of ~250 bp should be observed as shown in positive control. In all the clones tested no amplified product of ~250 bp observed indicating that all the clones tested were found to be mycoplasma-free (Figure 3.2.27).

- Cell growth profile and productivity

To monitor any changes happening during the freeze-thaw cycle of the cells, the cell growth and productivity profile was monitored for all the three clones. The frozen vial from the primary cell bank was revived in 125mL shake flask containing 40 mL of CD-CHO supplemented with 4mM Glutamine, 25 µg/mL Dextran Sulphate and Pencillin-Streptomycin solution. The seeding density maintained was ~0.25 x 10^6 cell/mL. The cells were grown for at least three passages before using it for the initiation of fed-batch culture for checking expression of target mAb. For initiating fed-batch the cells were seeded in shake flask at 0.5 x 10^6 cell/mL in a total volume of 40 mL. For the first three days cells were grown at 37 ^{0}C temperature after which the temperature was brought

down to 32 ^{0}C . The CO_2 and shaking speed was maintained at 5% and 120 rpm throughout the fed-batch. At every alternate day starting from Day 3, 10% v/v Feed cocktail {Efficient Feed A / Efficient Feed B (1:1, v/v)} was added. The fed-batch was terminated when the viability dropped below 80%.

A)



B)

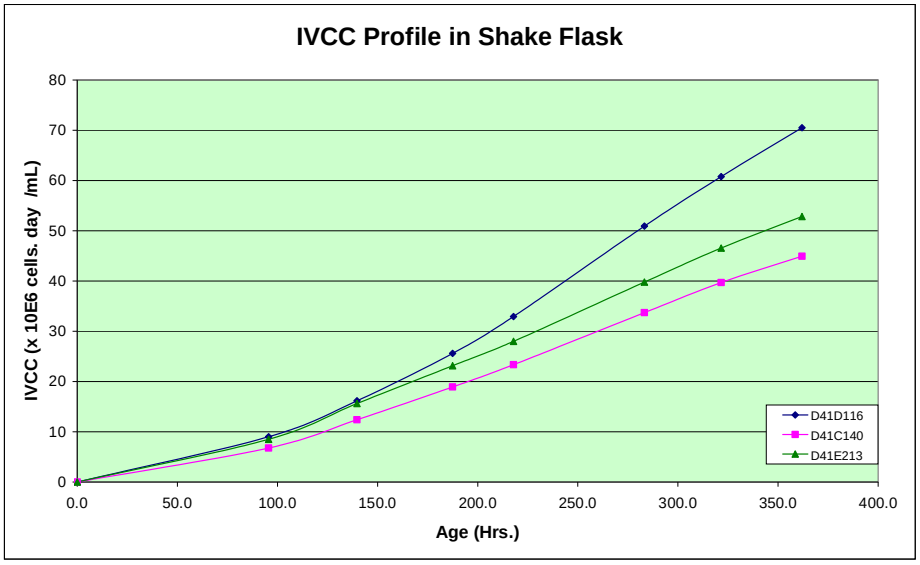
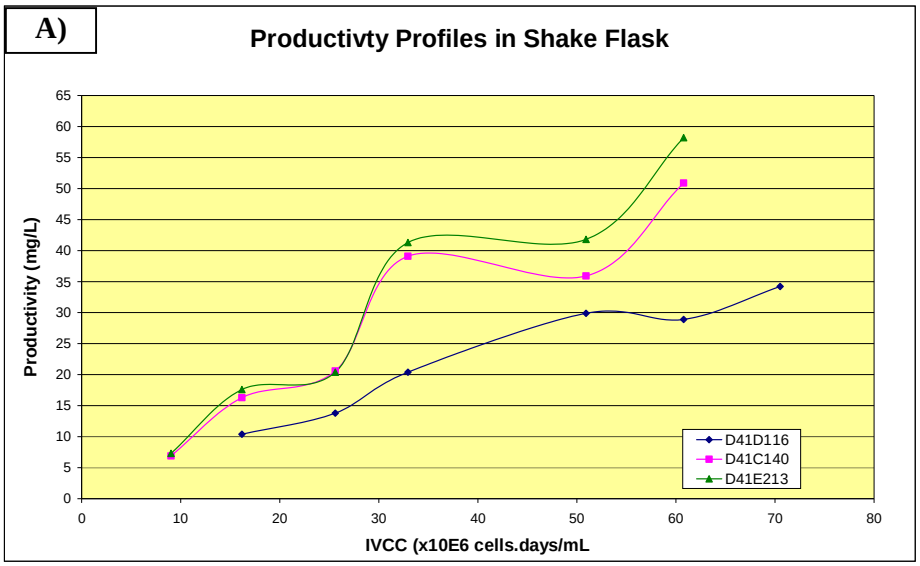


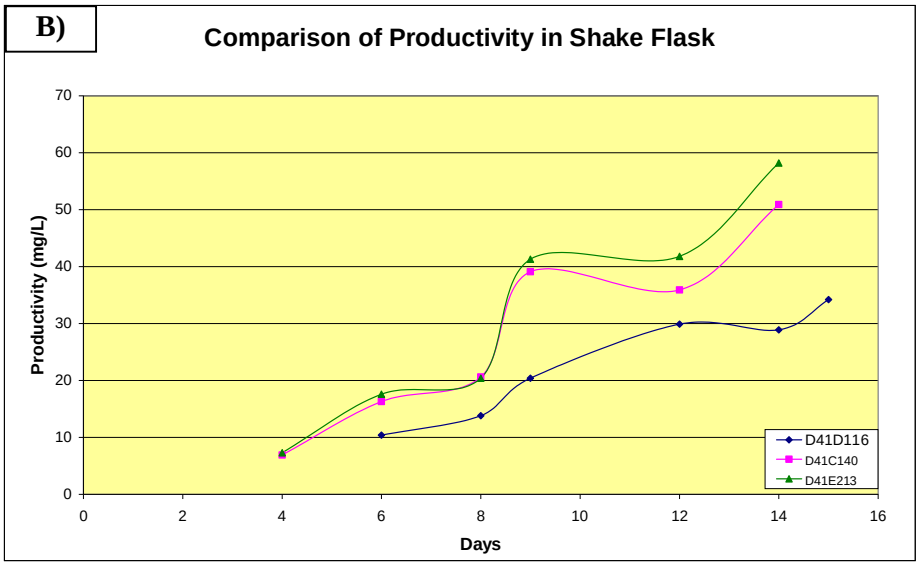
Figure 3.2.28 Growth Profile of three shortlisted clones of rCHO

(A) Viable cells and Viability Profile; arrow represents the time of addition of feed

(B) Integral Viable Cell Count (IVCC) profile. Arrows indicate the time when the feeding was done

As shown in Figure 3.2.28, all the cell growth profiles were comparable amongst the clones except clone D41D116 where the IVCC reached more than 6.0 x 10^7 cell/mL while in other two clones it was not more than 5.0 x 10^7 cell/mL.





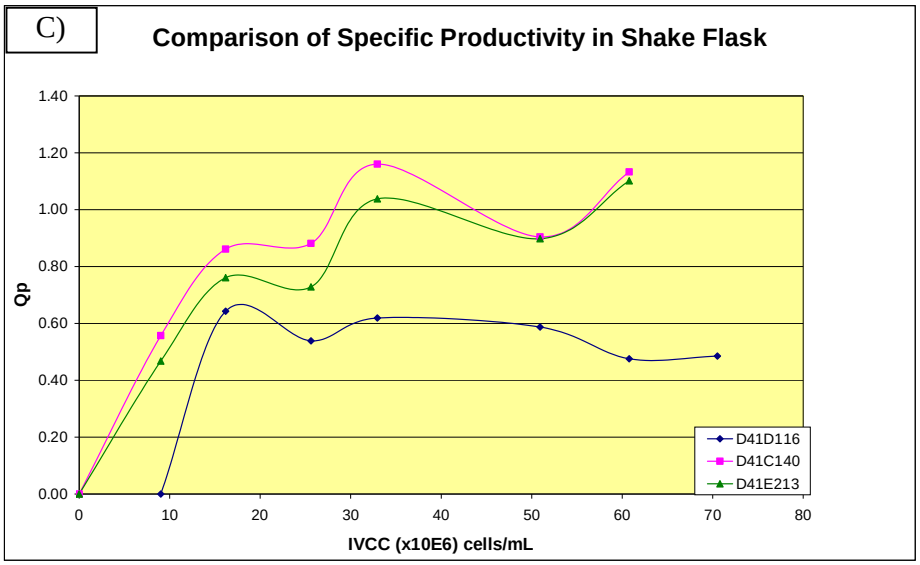


Figure 3.2.29 Productivity Comparison of top 3 clones of rCHO

(A) Productivity Profile – IVCC vs. Productivity; (B) Comparing productivity over a period of time in fed-batch culture; (C) Comparing specific productivity that is capacity of clone for production.

As shown in Figure 3.2.29: A & B, the expression yield increases with time and IVCC. The maximum expression yields observed are ~58, 52 and 34 mg/L in clone ##D41E213, D41C140 and #D41D116 respectively. Similarly, the specific productivity of clone #D41E213 was higher reaching to ~1.2 with increase in IVCC. The expression yield profile and cell growth profiles were similar to the earlier bank from which the primary cell bank was prepared.

3.2.2.9. Clone stability studies

The clone stability is an important parameter to be monitored for the recombinant cell lines. Generally, the industrial production of recombinant therapeutic monoclonal antibodies are operated at >500L in order to meet patient's dose and market demand. The recombinant cell line generated for expressing mAbs should be genetically stable for sustained expression for at least 50 generations. This is because of the fact that in each passage about 3-4 generations are passed (starting from master cell bank preparation to the end of cycle of final production stage). Hence, for master cell bank (MCB) preparation of ~200 vials, about 10 generations will be utilized. When one of these vials are scale up for production, starting from 40 mL → 200 mL → 1L → 5L → 20L → 150L →500 L, about 18-20 generations would be required. Finally, the cells would be grown in fed-batch mode for about 20 days (~8-10 generations). Thus, the cells should be stable for > 45 generations. Considering this, the clone stability of top 6 clones D41F109, D41G112, D41D116, D41C140, D41E213 and D41F284 was monitored by

in-vitro cell age for 76 days in a T25cm^2 TC flask in presence of selection pressure of antibiotic (Geneticin G418). The stability was determined by estimating recombinant protein production (Figure 3.2.30).

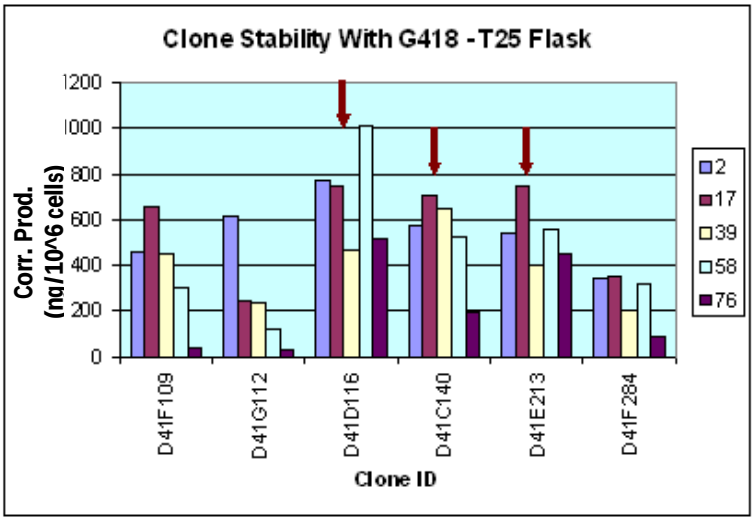


Figure 3.2.30 Clone stability in presence of selection pressure (G418) of rCHO

A drop in expression yield on Day 39 could be an outlier because following Day 39 expression profile came in trend**. Corrected productivity was plotted which was calculated by dividing productiovity with viable cell count X10^6 cells.**

At the end of 76 days culturing in presence of Geneticin-G418, a loss of expression to the extent of ~34%, 35% and 16% was observed in clones D41D116, D41C140 and D41E213, respectively. This loss is not significant compared to other three clones where the loss was ~ 91%, 96%, and 74% in D41F109, D41G112 and D41F284, respectively. The loss of expression in latter 3 clones is in presence of selection pressure hence in absence of selection pressure these would practically not produce anything when cultured for long time.

D41D116 and D41E213 clones showed a reduced expression on Day 39, however, in the next time point (Day 58) the productivity was more hence data of Day 39 is considered as an outlier.

The recombinant proteins are produced in bioreactor where agitation mode is used. Agitation is a physical parameter which could change the cell behavior hence there is need to prove the clone stability under shaking condition which mimics the bioreactor conditions. Therefore Clones D41D116, D41C140 and D41E213 were tested for clone stability in absence of selection pressure under shaking conditions. The cells at different

time points were subjected to fed-batch cultivation and the productivity at the end of batch was monitored. As shown in Figure 3.2.31, the productivity of clone D41E213 was maximum and the loss of productivity was only 5% when grown in absence of selection pressure for 48 days under shaking conditions. In clones D41D116 and D41C140 the loss of expression observed was ~11% & 9%, respectively which is also not significant.

Hence, clone D41E213 was found to be a high producer with minimal loss in productivity in absence of antibiotic.

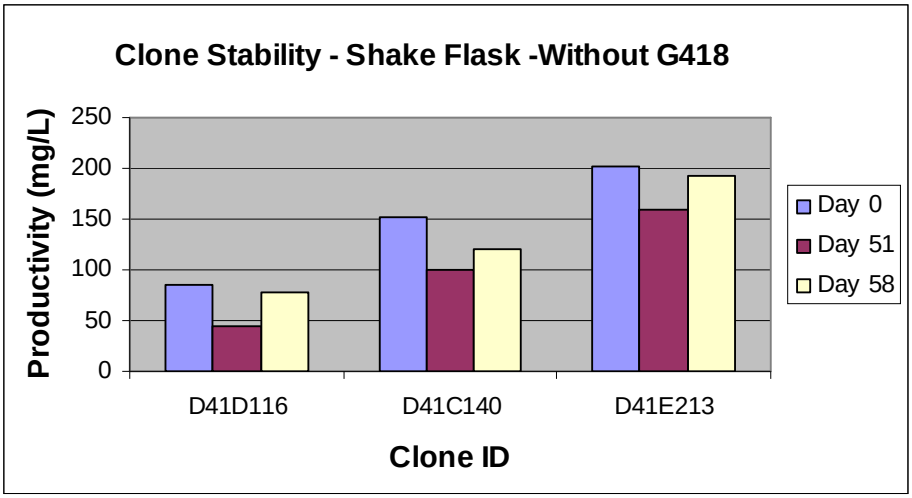


Figure 3.2.31 Clone stability in absence of selection pressure (G418) and under shaking Conditions of rCHO

3.3. Summary:

Chinese Hamster Ovary (CHO) cell line being most robust and widely used cell line for manufacturing recombinant therapeutic proteins was used as an alternate host to develop the cell line expressing anti-EGFR monoclonal antibody at higher levels which could not be achieved with the SP2/0 cell line (originally used for the expression of anti-EGFR mAb). Both these cell lines were evaluated for the expression of anti-EGFR antibody. Different transfection reagents/protocols were tried and SP2/0 was found to loose expression after a few passages as well the expression is at low level. Moreover, the expression of anti-EGFR antibody in SP2/0 declined with the passage and was short-lived. Hence we have evaluated alternate host system (CHO cell line) for expression of anti-EGFR antibody. Various transfection reagents were tried to get high efficiency and eventually high expression of protein of interest. Based on expression comparison TransIT reagent (supplied by Mirus Bio) and Turbofect (supplied by Fermentas) worked better and gave high level of anti-EGFR antibody expression when compared with

Lipofectamine2000 (supplied by Invitrogen) and electroporation technique. After transfection two methods were evaluated for obtaining clonal populations – the manual limit dilution method and the automated ClonePix method. The lead clones generated from both the methods were compared in terms of growth profile and productivity. It was observed that the clones generated through manual limit dilution gave higher expression levels than the clones selected using ClonePix.

Since cell line stability is also important along with growth profile and productivity, the lead clones were monitored over a period of time in presence and absence of selection pressure (Geneticin G418) for more than 50 generations under shaking conditions. Clone D41D116, D41C140 and D41E213 were stable in absence and presence of Geneticin G418 and also under shaking conditions. These clones were then evaluated as fed-batch cultures where D41E213 gave higher productivity than the other two clones.

The evaluation of product quality is critical for proving the biosimilarity of the expressed product, especially in the case where the expression host is changed. For this a number of analytical methods were developed and used for expression yield optimization. These methods give information above the structural as described in Chapter 3 at the early stage of clone screening. Following this high-end analytical technique of LC-MS/MS was used to assess the quality of product expressed by the lead clone in comparison with RMP. As shown in below Table 3.2.4, the quality of product expressed by the lead 3 clones were determined for checking identity, purity, quality and potency of the product. The detailed data are discussed in Chapter 6.

Parameters	Test	Clone D41D116	Clone D41C140	Clone D41E213	RMP (SP2/0)
Identity	Western Blot	Show positive band and size of band comparable with RMP			~150 kDa
	Protein A HPLC	Main peak in HPLC matches with main peak in RMP			Main peak at Rt
	N-terminal Sequencing	First five amino acid of Light chain and Heavy chain are identical to that of RMP			LC= DILLT HC = QVQLKQ
Purity	CEX-HPLC	The three major peaks overlaps with the RMP peaks. However, the basic peaks present in RMP are of lysine variants (as expressed in SP2/0) while it is not present in the protein expressed by CHO cells.			A total of 7 distinct peaks observed
	IEF	Shows anomaly in the band pattern	A total of 7 band matches with RMP		A total of 7 distinct bands observed
Quality	LC-MS/MS	Not Done	Not Done	The required forms of glycan are present except for the G2 in Fab which anyways is not desirable as it may casue immunogenic reaction (Chung C.H., et al 2008)	
Potency	*In-vitro* Bioassay	44%	$3%	55%	

Table 3.2.4 Summary of product characterization done using various analytical techniques.

3.4. Conclusion:

Based on the cell growth and productivity analysis, clone # D41E213 was observed and identified as lead clone for further experimentation.

CHAPTER 4:

A) SELECTION OF THE LEAD CLONE BASED ON PRODUCTIVITY AND QUALITY ATTRIBUTES

B) ESTABLISHMENT OF PLATFORM EVALUATION METHODS FOR SCREENING OF RECOMBINANT CLONES EXPRESSING ANTI-EGFR MONOCLONAL ANTIBODY

4.1. Preamble

Therapeutic monoclonal antibodies (mAbs), unlike other therapeutic proteins, are enormously complex drugs typically produced in mammalian tissue culture cells through recombinant DNA technology. As a result of naturally-occurring molecular heterogeneity, imperfect cellular processing, chemical and enzymatic changes during manufacturing and additional changes upon storage, antibody drugs display a wide variety of minor chemical changes, collectively termed heterogeneity or microheterogeneity. Common examples include glycan structural differences, deamidation, oxidation and glycation. This microheterogeneity is not due to variation in the primary protein sequence expressed by a single clone or pool of clones but arises due to cellular processes (Liu, *et al* 2007a).

Variants may result from either known or novel types of *in-vivo* (post-translational) modifications or from spontaneous (non-enzymatic) protein degradation, such as methionine oxidation piperazine formation, apsartate isomerization and deamidation of asparagines residues, or succinimide formation (Harris 1995).

Glycosylation is one of the most common modifications of proteins, and more than 50% of proteins are glycosylated. Carbohydrate moieties of such proteins are involved in expression of cellular functions including recognition, cell-to-cell signaling, protein folding, canceration, immune response, fertilization and differentiation (Kamoda, *et al* 2006). Control of microheterogeneity within predefined analytical specifications has been used in assessing the quality of the recombinant product expressed in biopharmaceuticals to guarantee consistent product quality during cGMP manufacturing (Flynn, *et al* 2010). Immunoglobulin G (IgG) is typically glycosylated in Fc regions and at times in Fab region, with a heterogeneous ensemble of structures (glycoforms) that are both highly reproducible (i.e. nonrandom) and site specific. In normal IgG, the two highly conserved oligosaccharides of the Fc region are found buried between the CH2 domains, forming specific protein-saccharide interactions with the Fc protein surface (Dwek, *et al* 1995). IgG antibodies possess a conserved N-glycosylation site, typically at Asn297, in the CH2 domain of the Fc portion. The variable (Fv) domain is glycosylated in approximately 20% of human IgGs. Glycosylation plays a critical role in the biology and physicochemical properties of an antibody. The biological functions influenced by glycosylation include resistance to proteases, binding to monocyte Fc receptors,

complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and circulatory half-life in vivo (Zhou, *et al* 2007). One of the functions attributed to the Fc oligosaccharides of normal IgG is to maintain the conformational arrangements of the Fc domains as well as the hinge regions. These structural features are necessary for Fc effector functions such as C1q and monocyte binding (Dwek, *et al* 1995).

Prior to designing a cell culture and purification process for mAb manufacturing, a quality target product profile provides a list of quality attributes (QA) and what levels are critical and hence need to be monitored and controlled. Since antibodies are a homologous class of molecules, knowledge gained through prior experience or from published studies may greatly aid in defining QAs. The definition for quality attribute is fairly broad and can potentially include raw materials in addition to features of the drug molecule itself (Flynn, *et al* 2010).

Critical Quality Attributes (CQA) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are generally associated with the drug substance, excipients, intermediates (in-process materials) and drug product [Ref: ICH Q8(R2)]. There are assigned Product Quality Attributes (PQA) which is product specific and most of the times dependent on the clone. The PQA like glycosylation and charge variants are cell line derived and changes from cell line to cell line due to variation in the metabolism from cell to cell. Hence, the PQA need to be determined at early stage of clone selection in order to identify the right clone so that there would not be surprises at the later stage of development thus, saving on the development time. Measuring and monitoring PQA will guide through determination of CQAs at the final process stage (Mire-Sluise, *et al* 2009).

The most recent regulatory document covering characterization of monoclonal antibodies was published by the European Medicines Agency (EMA) in July 2009 (Ref. EMEA/CHMP/BWP/1576532/2007). This EMA guideline entitled “Development, Production, Characterization and Specifications for Monoclonal Antibodies and Related Products” states that the monoclonal antibody should be characterized thoroughly before going for human use. This characterization should include the determination of physicochemical properties, purity, impurities and quantity of the monoclonal antibody,

in line with ICH-Q6B. As shown in Figure 4.1.1, the PQA assessment is done at different stages of the product life cycle employing similar or different analytical techniques at each stage.

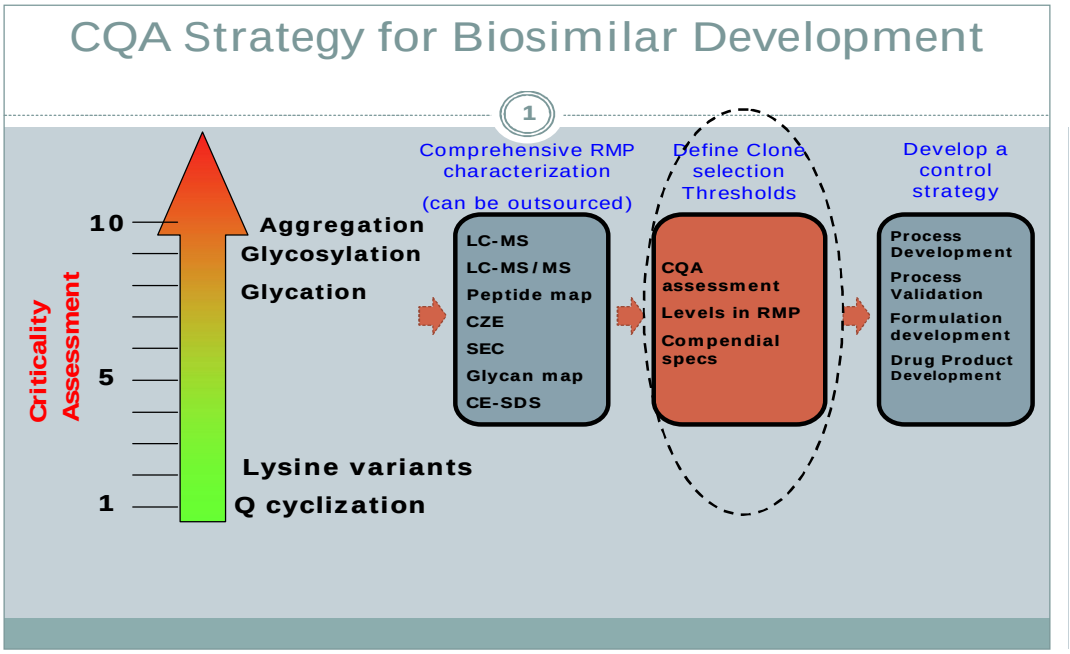


Figure 4.1.1 Importance and implementation of CQA in biosimilar development.

A typical strategy for the assessment and monitoring of CQA in a biosimilar drug development to assure the consistency in the product quality. As shown in the arrow diagram, the Lys variants are not as critical as the aggregation and glycosylation. Also, at the clone selection level, PQA assessment in comparison with the RMP is suggested in order to ensure biosimilarity at the later stages of development.

In this study the PQAs measured are highlighted in light yellow color in Table 4.1.1, to ensure the capacity of clone to process and produce the right molecule to go for the further development work. A typical PQA list is shown in Table 4.1.1 mentioning its impact on the biological function, however there could be little modifications to this depending on molecule and the function of it.

Product Quality Attributes -To be compared with RMP	
Attribute	**Impact on Function**
Afucosylation	Fucose content inversely proportional to ADCC
Galactose Content	Degalactosylation lead to three fold reduced CDC
High Mannose	High mannose forms lead to faster clearance from body due to their interaction with mannose receptors
Sialic Acid	Sialylated Glycans may lead to higher immunogeneity
Methionine Oxidation	Oxidation of Met252 reduces FcRn binding and also impact serum half life
Clips / Truncation	Hinge region fragmentation releasing one Fab+Fc and one Fab+CDR clips lead to conformational changes which will increase immunogenecity and reduces bioactivity
Aggregation	Covalent and non-covalent aggregation could lead to drop in potency
Biological Activity	Measure the potency of the product
Other PQA that have no significant impact on function but on purity of product	
C-terminal Lys Variant	Has no direct impact on biological activity
N-term pE	Spontaneous physicochemical conversion and has no functional significance
HCP	Indicator of consistency of process. Directly impacts product quality. HCP and resProteinA are immunogenic
resDNA	
resProtein-A	

Table 4.1.1 List of attributes having impact on the function of mAb.

[N-term pE (N-terminal pyroglutamate). HCP (Host Cell Protein) and DNA is the residual DNA to be measured in the final purified solution]

Conventionally, after transfection till MCB candidate identification, the clones are selected based on productivity and a few quality parameters. The selection of lead clone is critical as the process development and validation, which are time, labor and cost-intensive, would be carried out using the same clone as also the commercial production. Thus, the selected clone should satisfy all the quality parameters so that a need to change the clone in later stages does not arise.

In Chapter 3, the procedure of short listing the top three clones obtained either from manual selection (clones D41D116, D41C140 and D41E213) or from automated selection (clones 1A7, 2F5 and 2H5) has been described based on cell growth and expression yield. In this section, the lead clone (MCB candidate) was identified based on further analyses utilizing various analytical tools. Emphasis has been laid upon analytical tools used to assess the product quantity and quality as well as methods used to

establish biosimilarity with the commercially available RMP. The methods used to evaluate the lead MCB candidate are, SDS-PAGE, Western Blot, Iso-electric Focusing, Protein-A HPLC Weak-Cation Exchange Chromatography, RP-HPLC, and *in-vitro* bioassay.

4.2. Results and Discussion

4.2.1. Purification of target protein

The mAb molecule was effectively purified from the cell culture supernatant by affinity chromatography using Protein-A resin. This resin is also used for the analytical purpose for quantification of expressed mAb from the crude harvest as well as purified solution.

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (and / or group of proteins) and a specific ligand coupled to a chromatographic matrix (Figure 4.2.1). The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally very high.

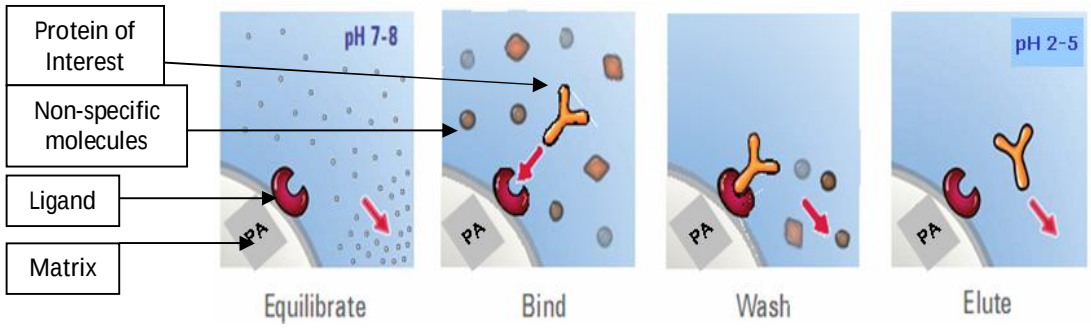


Figure 4.2.1 Principle of Affinity chromatography

Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure. Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography. The technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes of crude sample and also to remove specific contaminants. The basis for purification of IgG, IgG fragments and subclasses is

the high affinity of protein A and protein G for the Fc region of polyclonal and monoclonal IgG-type antibodies.

Protein A and protein G are natural bacterial proteins of *Staphylococcus aureus* and *Streptococcus* spp., respectively which, when coupled to Sepharose, create extremely useful, easy to use media for affinity capture of antibodies. Examples include the purification of monoclonal IgG-type antibodies, purification of polyclonal IgG subclasses, and the adsorption and purification of immune complexes involving IgG. IgG subclasses can be isolated from ascites fluid, cell culture supernatants and serum. (Ref. GE handbook). In the present study Protein A was used which is derived from *S. aureus* and contains five regions that binds to the Fc region of IgG.

The cell culture supernatants from the clones # D41D116, D41C140 and D41E213 were clarified by centrifuging the harvest at 1500 rpm for 10 min using swing out bucket rotor followed by filtration through 0.45 µ filter. On 5 mL Protein A (MabSelect SURE) resin column loaded with 15 mL (~3 mg target protein) of clarified harvest at 0.2 mL/min of flow rate followed by washing with phosphate buffer saline (pH 7.5) with 2 column volumes (15 mL) at 1.0 mL/min flow rate. The target protein was eluted with Glycine Buffer (pH 3.0) at a flow rate of 1.0 mL/min. The elution volume was ~2.0 mL. The chromatogram generated during the purification of harvest from clone #D41E213 is represented in Figure 4.2.2A.

The fractions generated during the purification process were checked on SDS-PAGE as depicted in Figure 4.2.2B.

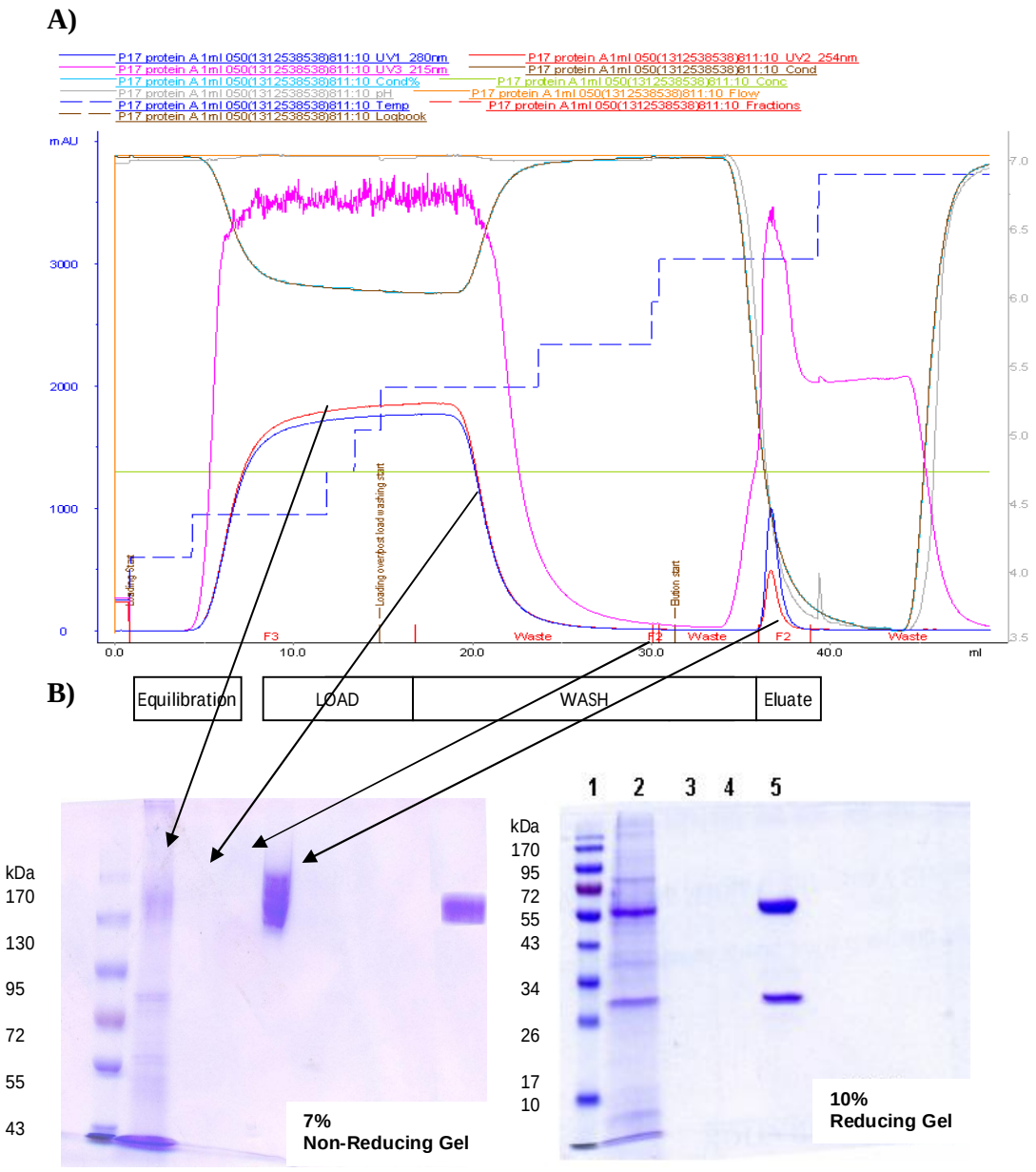


Figure 4.2.2 Affinity purification of Harvest.

A) Chromatograph with all process parameters. The detection is done at two absorbance frequency of 280 nm and 254 nm. For 280 nm: blue solid line is absorbance, aqua solid line-conductivity of solution and grey solid line for pH of solution. For 254 nm: red solid for absorbance, brown solid line for conductivity. Pink solid line is absorbance at 215 nm which give absorbance for peptide bonds, blue dotted line is for temperature and red dotted line is for fraction mark.

B) 7% Non-reducing PAGE profile of the purification run. Lane 1: 5 µL molecular weight marker, Lane 2: Clarified Harvest (10 µL), Lane 3: Flow Through (30 µL) Lane 4: Wash (30 µL), Lane 5: Eluate (5 µL); Lane 6 & & = Empty; Lane 8 = RMP (1µL = 5 µg)

C) 10% Reducing PAGE profile. The lane designation is same as Fig B except that the RMP was not added

The percentage recovery after purification was >90% as observed on the SDS-PAGE gel.

4.2.2. Identity of product

The demonstration of the identity is usually performed by comparing the expressed protein with RMP. For evaluating the identity of the target mAb expressed by the three lead clones the methods employed are Western Blot, Reverse Phase HPLC (RP-HPLC) and Iso-electric focusing (IEF).

4.2.3.1 Western Blot

Western blot is one of the simple techniques to establish the identity of expressed target protein by using detection of protein of interest with an anti-antibody. The Protein-A eluate supposedly containing target mAb from the three shortlisted clones were run on 10% SDS-PAGE under reducing conditions following which the separated proteins were transferred to nitrocellulose membrane. The nitrocellulose membrane was probed with Mouse Anti-human IgG (whole) HRP conjugated polyclonal antibodies following which bands were detected when the enzyme substrate TMB was added.

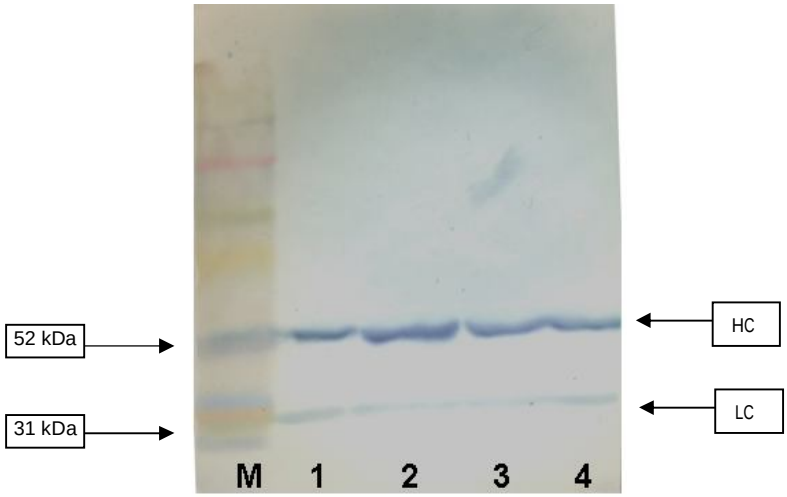


Figure 4.2.3 Western Blot Analysis for identity

M - Rainbow Molecular weight market (5µL); Lane 1 – RMP (2µg); Lane 2 – Clone # D41D16 (2µg) ; Lane 3 – D41C140 (2µg) ; Lane 4 – D41E213 (2µg)

As represented in Figure 4.2.3, immune-positive signal of size ~50 kDa and ~25 kDa corresponding to heavy chain and light chain, respectively was observed which is comparable with the RMP. The expressed protein in all the clones shows immuno-positive bands at similar sizes to that of RMP, thereby establishing the identity of protein to be an IgG molecule.

4.2.3.2 IEF:

IEF is an iso-protein analytical method and, therefore, very useful in demonstrating the antibody identity and charge heterogeneities. The charged isoform in the expressed product was analyzed in comparison with the RMP. IEF requires small sample amount either unpurified or purified; however to get better picture for comparison with RMP, partially purified sample was used. To obtain the isoform profile of the target protein, Protein-A purified samples were focused on the precast Dry-IEF gel (pH 3-10) available from GE Healthcare.

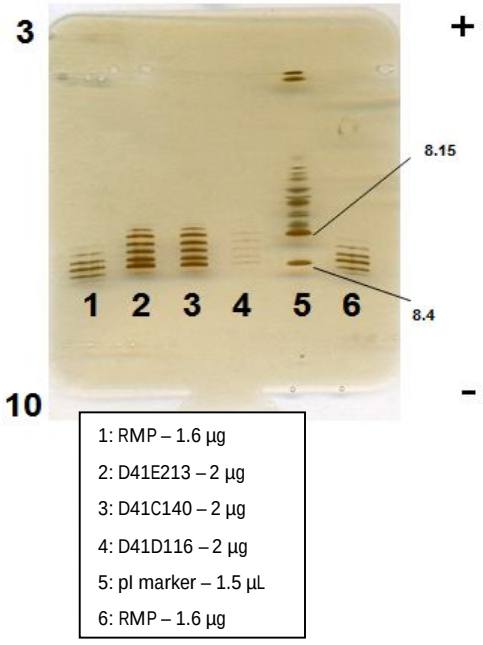


Figure 4.2.4 Isoelectric Focussing (IEF) for identity and purity

Precast Phast Gel with ampholyte range of pH3 to pH10 was used and focusing was done in the PHASTGel System from GE.

The theoretical pI of the target mAb without modifications calculated to be 8.6 (www.expasy.org). In the RMP mainly 5 bands are seen around pI 8.5 followed by 3 bands on the acidic side. A lower amount of sialic acid residues or modifications like deamidation or oxidation can lead to an increase in pI of the sample and thus a shift of the bands to a more basic region of the gel. The band pattern in clone # D41C140 and D41E213 are similar to the

banding pattern in RMP however; a couple of basic bands are not present in the expressed protein. This may be because of the absence of lysine variants in the recombinant expressed protein (as explained by CEX in the following section). The protein expressed by clone # D41D116 possess one or two bands less on both acidic and basic side therefore less comparable to the RMP banding pattern than the other two clones. Based on IEF results clone # D41C140 and D41E213 are expressing product similar to the RMP. These results were verified by CEX-HPLC in the section (Figure 4.2.7:).

4.2.3. Purity and Quality of Expressed mAb

There are various measures to ensure the purity of the product depending on the sensitive of the method used. Method starting from a simple SDS-PAGE to high-end HPLC methods was employed to ensure the purity and quality of the product.

4.2.3.1 SDS-PAGE (Reducing and Non-reducing)

SDS-PAGE is one of the most common methods to determine the purity (homogeneity of the protein sample) and stability of recombinant proteins in comparison with RMP. Commonly, the bands of IgG molecules appear at lower molecular weight on non-reducing SDS-PAGE due to the missing single light chain or single heavy chain (Liu, *et al* 2007a). The theoretical mass of anti-EGFR monoclonal IgG in discussion is 145.5kDa composed of two light chains and two heavy chains and possess glycan at Fab and Fc sites. The theoretical molecular weights of light chain and heavy chains of anti-EGFR mAb are 23.4 kDa and 49.37 kDa, respectively (www.web.expasy.org). Each light chain is connected to a heavy chain by one inter-chain disulfide bond, while each heavy chain is connected to the other heavy chain by two intra-chain disulfide bonds at hinge region of antibody molecule which is characteristic of IgG1 subtype. Each light chain possesses two intra-chain disulfide bonds while each heavy chain possesses four intra-chain disulfide bonds.

In theory, when analyzing an antibody on SDS-PAGE under non-reducing condition, there should be only one band: namely that of the intact antibody. Structurally, IgG is a homodimer of a heterodimer and due to improper assembly of the chains bands with both higher and lower molecular weights than intact IgG molecules are reported (Angal, *et al*

1993; Brody 1997; Hunt and Nashabeh 1999; Schauenstein, *et al* 1996; Schuuram, *et al* 2001; Taylor, *et al* 2006; Zhang and Czupryn 2002).

IgG1 and IgG2 hinge sequences are more optimal for inter heavy chain disulfide bonds (Angal, *et al* 1993; Schuurman, *et al* 2001). However, fragments of IgG1 and IgG2 have also been reported (Zhang and Czupryn 2002). The banding pattern of the fragments is very similar to the banding pattern of IgG after partial reduction (Virella and Parkhouse 1973), which suggests that similar to IgG4 half-antibody, fragments are likely formed related to the integrity of disulfide bonds. Low percentage of free sulfahydryl in IgG1 and IgG2 implies the existence of incomplete disulfide bonds (Zhang and Czupryn 2002), which can contribute to the presence of antibody fragments directly. Incomplete disulfide bonds can also contribute to the fragments directly by triggering disulfide bond scrambling.

SDS-PAGE was carried out on 4-14% gradient. The affinity purified product from the top 3 clones was checked and compared with the RMP under reducing and non-reducing conditions on SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250 followed by destaining and finally capturing the image using Gel Documentation system for visualization (Figure 4.2.5).

The band size of ~52 kDa and ~25 kDa under reducing conditions was observed corresponding to heavy chain and light chain of RMP. In case of non-reducing gel multiple bands are observed in all samples including RMP. As marked in the Figure 4.2.5, the intact IgG band appears around 200 kDa on non-reducing gel matching with the RMP. The band below the 200 kDa, at around 150 kDa could be the molecule with 1 heavy chain less, i.e., two light chains and one heavy chain linked to form theoretically ~100 kDa protein. Similarly other forms are available which are marked in Figure 4.2.5. The band pattern of expressed target protein may be because an alternate host (CHO) is used (Note: RMP is expressed in SP2/0 cells).

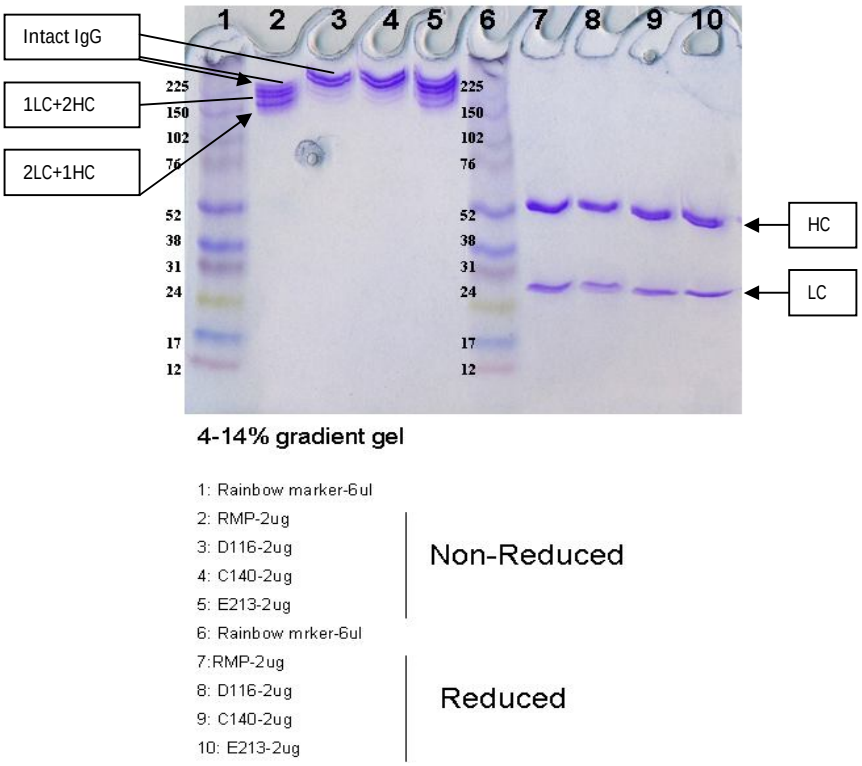


Figure 4.2.5 SDS-PAGE for molecular weight and purity determination of expressed mAb. Gel Electrophoresis with RMP. Lane 1 to 5 are Non-reducing condition where Lane 1= Rainbow Marker (6µL), Lane 2 = RMP (2µg), Lane 3 = Clone D41D116 (2µg), Lane 4 = Clone D41C140, Lane 5 = Clone D41E213 (2µg). The same loading pattern was followed for the reducing condition from Lanes 6-10.

4.2.3.2 HPLC Methods

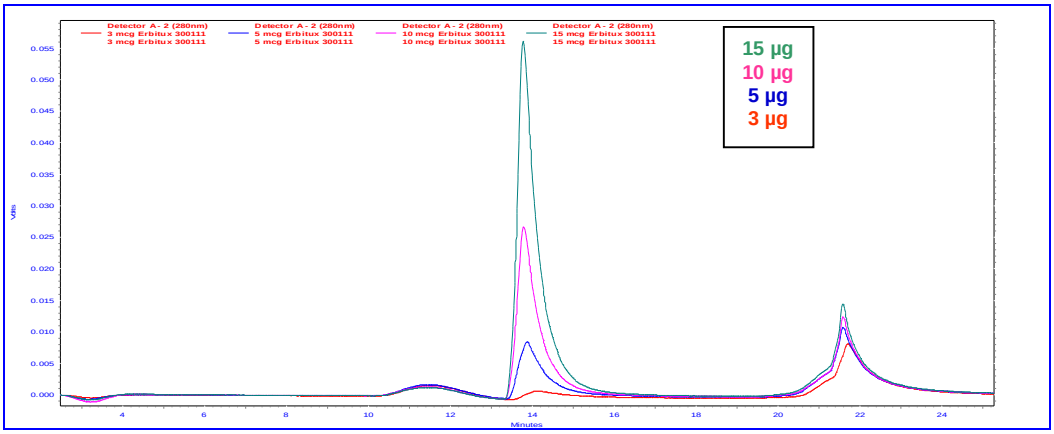
Separation by HPLC is a robust method used for getting information on antibody identity, concentration, purity, molecular weight and structure. Based on the PQAs of mAb, Cation Exchange (CEX) HPLC was used for determination of charge variants (Kaltenbrunner, *et al* 1993, Hamilton, *et al* 1987), whereas Size Exclusion (SEC) HPLC was used for determination of molecular weight, aggregation and degradants (discussed in Chapter 6). The HPLC profile or peak pattern obtained by analyzing Protein-A purified target mAb expressed from 3 lead clones was compared with the profiles generated from RMP.

- PROTEIN-A HPLC

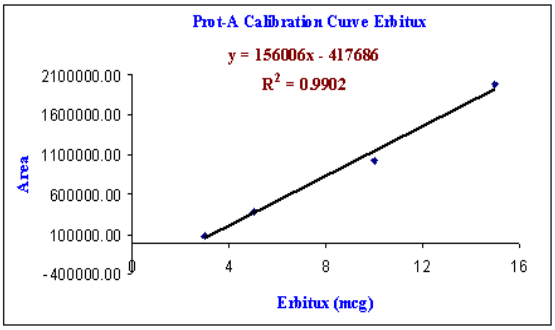
Protein-A affinity resin was used in analytical columns for quick estimation of target mAb in cell culture harvest as well as the purified sample. POROS column packed with Protein-A resin was used in Agilent 1100/1200 HPLC. A standard 4 point curve was

first established by injecting different concentrations of RMP and plotting peak area Vs concentration to establish the standard curve to be used for quantification. This method in addition to quantification was also used for determining the identity of mAb in comparison with RMP.

A)



B)



Injection amount µg	Peak Area	Back Calculated amount	% Recovery
3	85921.00	3.23	107.60
5	380144.00	5.11	102.28
10	1021373.00	9.22	92.24
15	1990031.00	15.43	102.89

C)

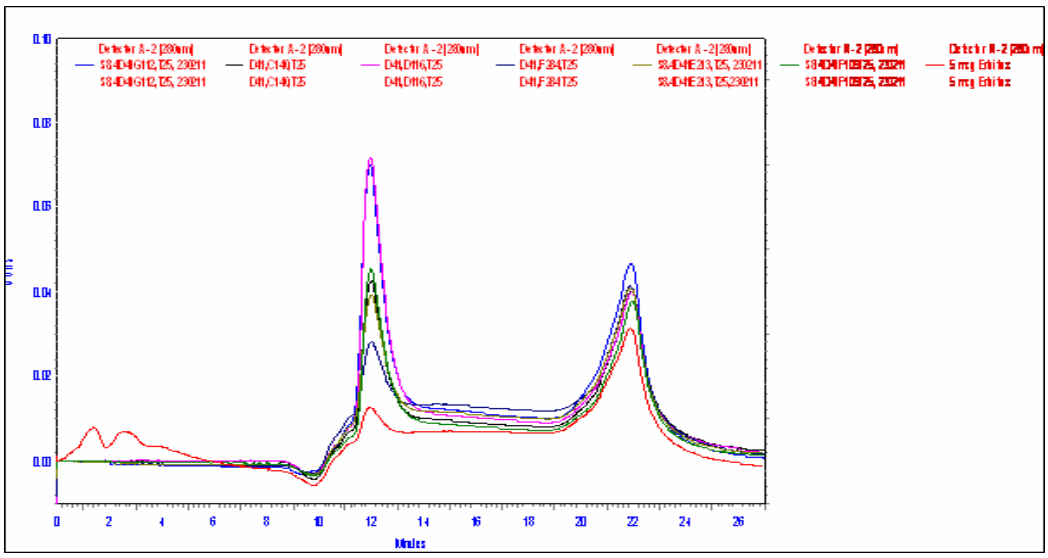


Figure 4.2.6 Protein-A HPLC for quantification of target mAb & establishing identity of target mAb.

A) Chromatogram of RMP obtained after injecting different concentration of RMP,

B) Calibration curve and table showing recovery of protein at different calibration point,

C) Overlay of chromatograph of three shortlisted clones with RMP.

The overlay of the product in cell culture supernatant obtained from two different fed-batch experiments were overlaid and represented in Figure 4.2.6. The mAb estimation in the cell culture supernatant is tabulated in Table 4.2.1

Date of receiving	Date of Analyis	Loading Vol. (ml)	Sample Name	Main peak area	Amount of mAb in the injected vol. (µg)	Calculated expression conc. (mg/L)	Comments
03.05.11	04.05.11	0.4	D116, Batch 3, Day 15	2377596	17.92	44.79	Prt A samples used for SDS-PAGE, RP-HPLC & IEX
		0.3	E213, Batch 3, Day 15	6978637	47.41	158.04	
		0.4	C140, Batch 3, Day 15	5837504	40.10	100.24	
		0.4	D116, Batch 4, Day 17	4459815	31.26	78.16	
		0.4	E213, Batch 4, Day 17	11548976	76.71	191.77	
		0.4	C140, Batch 4, Day 17	7036862	47.78	119.46	

Table 4.2.1 Protein-A HPLC quantification of cell culture supernatant from 3 lead clones.
Highlighted clone was observed to be highest expressing in shake flask condition at basal levels

The expression of target mAb is highest in clone #D41E213 (highlighted). The main peak of mAb at Rt 12 min was observed in all three clones (D41D116, D41C140 & D41E213) which matches with the peak of RMP (red color peak). The peak at Rt 22 could be some excipient peak of buffer.

➢ CEX PROFILE:

During the development and production of a therapeutic protein, characterization of structural variants is a critical challenge. In theory, characterization of recombinant proteins is a straightforward matter, as the requisite genetic engineering provides an expected amino acid sequences, with potential sites of post-translational modification identified on the basis of the known consensus sequences (Harris, 1995). In practice, however, a number of variations from the expected structure can be found. Variants may result from either known or novel types of *in-vivo* (post-translational) modifications (Wold, 1981) or from spontaneous (non-enzymatic) protein degradation, such as methionine oxidation, diketopiperazine formation (Battersby, *et al* 1994), aspartate

isomerization and deamidation of asparagines residues (Clarke and Greiger 1987), or succinimide formation (Kwong and Harris 1994).

The effect of various modifications on the elution of antibodies from ion exchange columns can be classified into three categories including direct contribution to charge difference, positional effect and conformational effect. Firstly, modifications can affect the elution of antibodies from ion exchange chromatography column by contribution to charge directly, e.g., a Lys residue introduces a positive charge. Therefore, on Cation Exchange columns, antibodies with no C-terminal Lys will elute first followed by antibodies with one C-terminal Lys followed by antibodies with two C-terminal Lys. Treatment with enzyme Carboxypeptidase B (CPB), which removes C-terminal Lys, changes the profile of the three peaks with different Lys residues into a single peak with no C-terminal Lys residues (Liu, *et al* 2007b). This indicates that the presence of C-terminal Lys affects the elution profile of antibodies from Ion Exchange columns by direct alteration of charge. The effect of sialic acid on elution is also based on net charge differences. Sialic acid introduces a negative charge and antibodies with oligosaccharides containing sialic acid groups will elute earlier on cation exchange column. Secondly, the same modification located at different positions of the antibody can have an effect on the retention time. For example, antibodies with the same number of pyroglutamate residues elute at different retention times depending on the position of the pyroglutamate (Moorhouse, *et al* 1997). These modifications in different permutation and combination could give multiple peaks at different retention time which at times would be difficult to characterize.

Determination of the charge variants present in the expressed target mAb from shortlisted clones after Protein-purification was carried out using Cation Exchange (CEX) HPLC as described by Gaza-Bulseco, *et al* 2008. In brief, TSK CM STAT (4.6 mm X 100 mm) column was used in an Agilent HPLC 1200 system for resolution of charge variant using MES buffer. This column has matrix of hydrophilic polymer with functional group of carboxymethyl cellulose. The column is packed with 7 or 10 µM mono-disperse non-porous resin particles of which the surface consists of an open access network of multi-layered cation exchange group.

The overlays of CEX-HPLC peak profiles generated from 3 lead clones were overlaid, compared with the profile of RMP (Figure 4.2.7) and the percentage distribution of each

peak was calculated and tabulated in Table 4.2.2. Peak #1 to 6 were observed in the expressed mAb from all the three clones which matched with the RMP however, the percentage was varying which could be due to the fact that the upstream process is not fully optimized and the RMP is produced using SP2/0 cells. Peak #8 to 11 are observed as distinct peaks in RMP while these are absent in the clones while peak #7 is very less in the clones. The peaks 8, 10 and 11 present in RMP are determined to be C-term Lys variants as confirmed by CpB digestion which clip-off the C-term Lys (This data is included in chapter 6 section 6.2.2 on Product Comparability). Peak #6 is the major peak corresponding to K0 form which accounts for ~31% in RMP and ~28% in clone D41E213 hence the best match. Peak number 1 to 3 are acidic variants mostly contributed by sialic acid present in all clones but are minimal in RMP. The comparison of clones with RMP peak by peak is tabulated in Table 4.2.2. Although, the C-term Lys variants are not significant in terms of immunogenecity, but contributes to the heterogeneity and purity of the product. In principle, the major charge variant form in RMP are K0 and peak 4 & 5 which is best compared with target mAb expressed by clone D41E213. The C-terminal Lys variant forms (Peak #1 to 3) need to be monitored and dealt with during the cell culture process fine-tuning at bioreactor scale to reduce these forms and come close to the pattern of RMP.

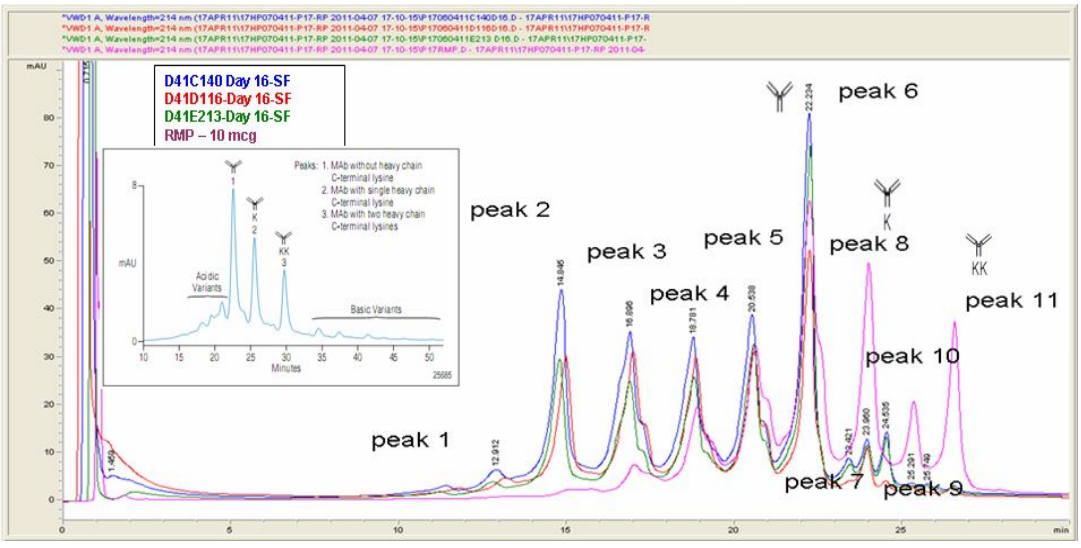


Figure 4.2.7 Cation Exchange HPLC for determination of charge variants.

The Protein-A purified cell culture supernatant from each clone was analyzed by CEX chromatography by injecting approximately 5 µg of partially purified mAb. The inset picture depicts a typical charge variant profile obtained for a mAb molecule.

S. No	Rt (mins)	% of individual isoforms				Remarks
		D116	C140	E213	RMP	
1	12.91	1.48	1.55	1.24	x	Low intense peak not present in RMP
2	14.84	16.06	17.33	14.30	x	High intense peak not present in RMP
3	16.89	20.15	17.32	14.75	3.28	Proportion not comparable with RMP
4	18.78	18.82	16.48	15.76	10.30	**E213 best match to RMP**
5	20.53	17.68	15.71	16.52	17.40	**All clones almost comparable to RMP**
6	22.23	21.66	24.63	28.25	30.87	**E213 best match to RMP**
7	23.42	x	1.83	2.47	x	Peaks contributed by lysine variants as confirmed by CpB digestion
8	23.96	3.32	2.58	3.27	19.30	
9	24.53	0.82	2.57	3.43	x	
10	25.02	x	x	x	6.87	
11	26.23	x	x	x	11.97	

Table 4.2.2 Percentges of peaks observed by CEX-HPLC in expressed mAb from 3 lead clones in comparison with RMP

- RP-HPLC

RP-HPLC analysis is a widely used analytical technique for monitoring the stability and production of biomolecules. The ability of this method to resolve nearly any form of chemical modification at a peptide and/or protein level and the direct adaptability to in-line mass spectrometry has proven to be a powerful tool. However, the use of RP-HPLC as an analytical tool for monitoring intact monoclonal antibodies has been limited because of the complex and hydrophobic nature of these large macromolecules causing poor recovery and limited resolution (Dillon. *et al* 2006). In this study RP-HPLC was used at the clone selection stage to monitor intact mAb and establishing identity, however because of the complex and hydrophobic nature of these large macromolecules causing poor recovery and limited resolution it is used with other orthogonal (complementary) test methods such as Protein-A HPLC or Western Blot to establish identity of the target mAb.

The cell culture supernatants from all the clones were tested on RP-HPLC. As shown in Figure 4.2.8, a major peak at Rt 10.8 min was observed in all clones corresponding to the one present in RMP which proves the identity. An associated peak at Rt 10.4 min was also observed in cell culture harvest which could be altered form of the target protein not present in RMP which is purified protein. The associated peak was not characterized as it was out of scope and since the testing was done on crude cell culture supernatant. The top 3 clones based on productivity data, i.e., D41D116, D41C140 and D41E213 plus additional one clone D41F109 was compared with RMP. Based on the main peak

comparison, the identity of mAb expressed was established by comparison to RMP in all the clones tested. There is no significant difference amongst the clones expressing target mAb observed. This method is also used for separation of the Fc and Fab fragments after digestion with enzyme Endopeptidase Lys-C (Lys-C) and to be further used for Mass spectrometry (Discussed in Chapter 6 section 6.2.4).

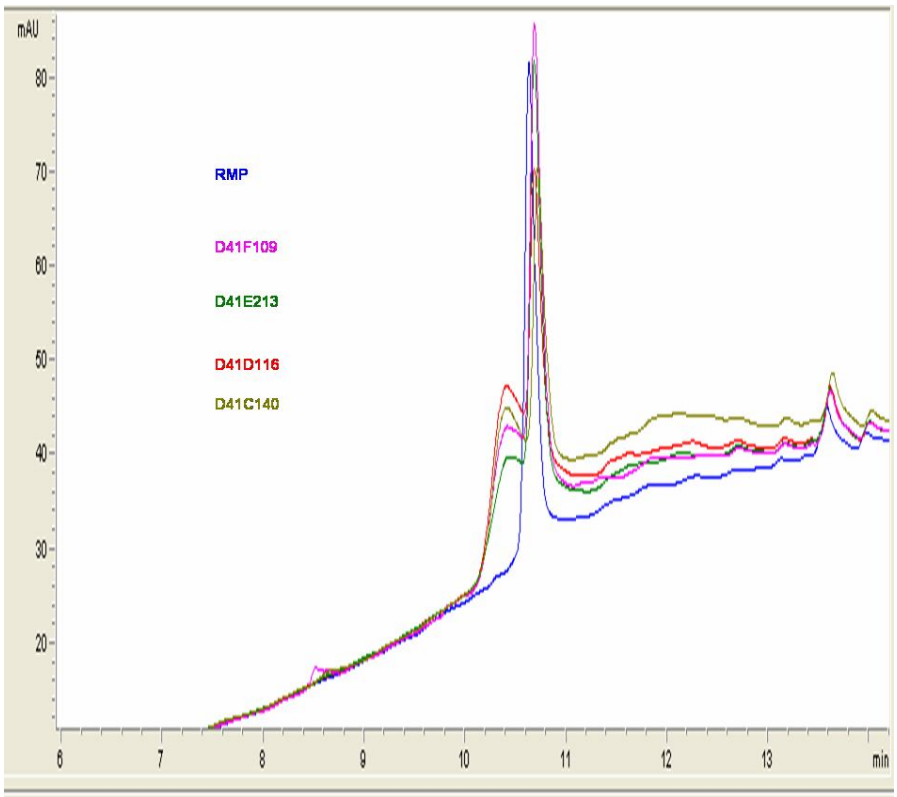


Figure 4.2.8 RP-HPLC for establishing identity of protein.

The protein expressed by the three lead clones and a next in line clone are represented.

4.2.3.3 *In-vitro* Bioassay

The epidermal growth factor receptor (EGFR) is a ubiquitously expressed *trans*-membrane tyrosine kinase receptor that binds six structurally related ligands and, in doing so, stimulates the proliferation of a wide variety of animal cell types. A tumor cell line A431 (ATCC, CRL# 2592) over-expresses EGFR and the majority of EGFR appears to be present in the caveolar fraction (Carpenter 2000). The cell binding of the target anti-EGFR mAb expressed by 3 lead clones was determined *in-vitro* by incubating A431 cells with target anti-EGFR mAb. The A431NS cells were grown in 96 well plates to ~70-80% confluency following which the cells were fixed using formalin solution to expose the EGFR receptor from the caveolar compartment. The RMP and expressed

anti-EGFR mAb was added to the fixed cells in different dilutions in replicates. The binding of anti-EGFR mAb with receptor was detected with the use of secondary antibody labeled with HRP. The enzymatic reaction after addition of substrate TMB results in yellow color which was measured using a spectrophotometer. The experiment was repeated on two different days and average with error bars are presented in Figure 4.2.9. The signal increased with the increase in the concentration of the protein till equilibrium between anti-EGFR concentration and number of EGFR was reached. The absorbance at 490 nm Vs. log concentration of anti-EGFR mAb was plotted for comparison with the RMP. The relative binding of the expressed mAb in comparison with RMP was calculated using PLA software. The percentage in-vitro binding potency of mAb expressed by clone #D41E213 was highest (~55% of RMP). For clone D41D116 and D41C140, the observed binding potency was ~44% and ~43%, respectively. The potency of expressed mAb from different clones was lower because of the fact that the upstream and purification process is not fully optimized. The mAb expressed is from the shake flask experiments which are not controlled and hence there could be variants/impurities, media or buffer components which could interfere with the binding of anti-EGFR mAb with the receptor. This trend was also observed in other mAb projects in which the *in-vitro* potency was less at early stage and later during the upstream process development improved considerably and came close to the RMP. Hence, there is a very good potential that the potency could improve once the upstream and purification process is fully optimized at the bioreactor scale because that would be the final scale for commercial manufacturing. The main highlight is the expressed anti-EGFR mAb follows similar binding kinetics as that of the RMP and clone D41E213 shows better binding activity.

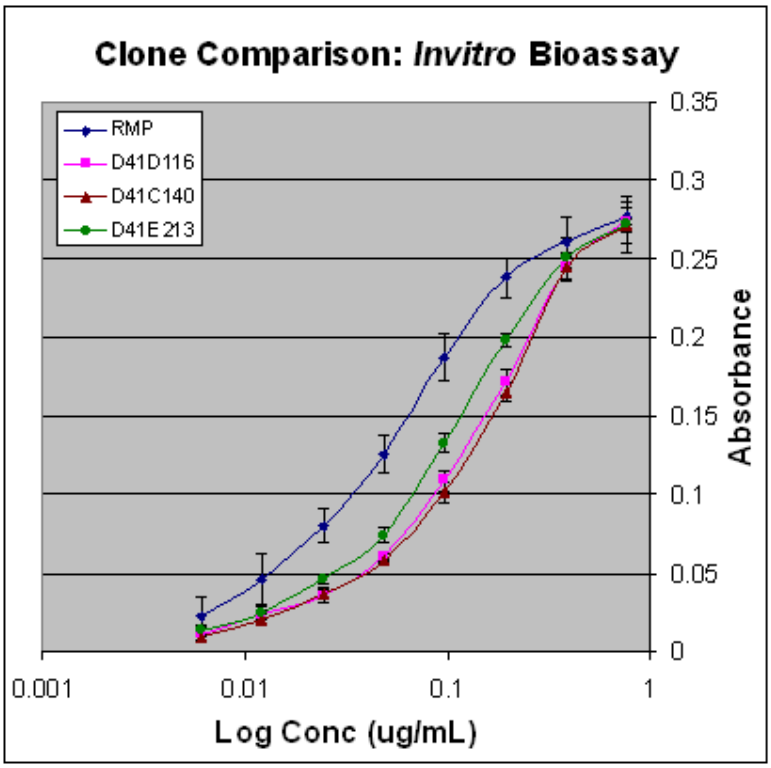


Figure 4.2.9 ***In-vitro*** **bioassay for determining the binding potency of the expressed mAb**

4.3. Conclusion

Commonly used techniques such as Western blot, Iso-electric focusing and HPLC are used to establish the identity and to some extent purity of mAb. There are, however, differences in the number of tests required for the various stages of drug development starting from R&D to commercial stage manufacturing. In the present study, an attempt was made to establish a battery of tests to be used during early stage development starting from the clone selection of the anti-EGFR mAb. Implementing tests like SDS-PAGE, Protein-A HPLC, RP-HPLC, CEX-HPLC, IEF and Western Blot will give information on the identity and purity of the molecule expressed by the clone. Determining the *in-vitro* biological activity of molecule at clone selection stage is important to ascertain complexity of the mAb molecule so that disappointments at later stages of product development could be avoided. Recombinant antibody production is generally shifting from murine hybridoma cells as production system to recombinant Chinese Hamster Ovary (CHO) cells to human cells (Per C.6). Irrespective of the host cell line employed, basically the regulations demand that the biopharmaceutical product manufactured are safe and are well characterized in accordance with the Product Quality

Attributes (PQA). These methods give considerable information at an early stage and are considered as platform methods to be used at clone selection stage for all mAb products in general.

Based on the analytical tests employed in final clone selection clone #D41E213 is the lead MCB candidate followed by D41C140 and D41D116. Therefore, clone D41E213 will be used for further experimentation such as upstream process development, purification process and product characterization.

CHAPTER 5

UPSTREAM PROCESS DEVELOPMENT FOR IMPROVING EXPRESSION OF RECOMBINANT ANTI-EGFR MONOCLONAL ANTIBODY IN SHAKE FLASK

5.1. Preamble

For a media to be qualified as 'ideal' for mammalian cell growth, a detailed investigation is needed to evaluate whether a particular media meets certain basic requirements. All microorganism or cell lines require water, sources of energy, carbon, nitrogen, mineral elements, trace elements, vitamins, lipids, etc. for survival. Optimal cell growth is routinely achieved using a defined basal medium supplemented with relatively high levels of serum or proteins. To manufacture highly purified biopharmaceuticals with consistent quality, a well-defined production process is required. Moreover, regulatory requirements dictate the elimination of serum or other animal-derived components from the culture media.

The development of serum-free media was initiated by Ham (1965), who reduced the amount of serum in media and optimized other medium components. Sato's group (Barnes and Sato 1980) investigated a range of components to promote cell growth and differentiation. Chemically defined, serum-free or protein free animal cell culture media offer lot-to-lot consistency and reduced production cost apart from eliminating the risk of potentially grave contaminants or microorganisms thus facilitating downstream purification processing, regulatory approval and biosafety.

Apart from the general media components which acts as source of carbon, nitrogen, phosphorous and sulfur, requirement of trace elements such as Fe, Zn, Cu, Se, Mn, Mo and V are significant for mammalian cells (Ham and McKeehan 1975). The chemically defined media contain contamination-free ultra-pure inorganic and organic constituents, and may contain pure protein additives, like insulin, epidermal growth factor, etc. that have been produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, fatty acids and specific amino acids. The CHO cell lines are widely used for being highly stable expression systems for heterologous genes, and for its relatively simple adaptation to adherence-independent growth in serum and protein free media. In contrast, protein free media do not contain any protein; they only contain non-protein constituents necessary for culture of the cells. The formulations such as Minimal Essential Media, Dulbecco's Minimal Essential, RPMI-1640, etc. are protein free where required protein supplementation is provided. Compared with serum supplemented media, use of protein-free media promotes superior cell growth and protein expression. The protein-free, chemically defined formulation allows the growth of many cell lines but at times makes the cell lipid-dependent, for example, NSO myeloma cells do not

grow in protein-free, chemically defined medium without further lipid supplementation. Through the use of cyclodextrin-based additives to medium, it is possible to solubilize significant quantities of sterols and other lipids and to maintain a protein-free, chemically defined cultivation environment for NS0 cells. The culture system can be kept entirely free of animal derived components if the supplement is made with plant-derived or synthetic lipids. A systematic approach was described to arrive at a chemically-defined protein-free medium called FMX-Turbodoma for the production of monoclonal IgA antibodies by hybridoma cells for IgA production (Stoll, *et al* 1996). The price of the protein-free medium is about 20% of the serum containing medium. This makes such a protein-free medium very convenient for laboratory and large scale production.

It is often observed that cells initially grown under serum-containing conditions and when adapted to serum- or protein-free conditions undergo a reduction in expression of the recombinant product. This is the consequence of reduced growth of the recombinant cells due to changed culture conditions. Cells which have been adapted to grow in serum- and protein-free medium for at least 40 generations and which expresses a recombinant product to desired levels can be considered stable. Thus, designing of fully defined media for mammalian cells is a daunting task and require in depth investigation. Therefore, media supplier companies market these media without disclosing the exact composition. This calls for evaluation of various media available from different sources based on availability, cost, convenience to use at large scale, regulatory approval, etc. These media should support the increasing demand for biopharmaceuticals produced using mammalian cell culture, coupled with capacity limitations and the requirement for improving productivity and robustness of the manufacturing processes. For larger scale of operations, the nutrient media should meet as many possible criteria as follows:

- Produce the maximum yield of product or biomass per gram of substrate used
- Produce the maximum concentration of product or biomass
- Permit the maximum rate of product formation
- Minimum yield of undesired products.
- Consistent quality and be readily available throughout the year
- Convenient during media preparation and sterilization.
- Cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment.
- Should be cost effective so that the product finally manufactured could be competitive.

Media optimization is an essential step when implementing a large scale process. Different combinations and sequence of process conditions need to be investigated to determine the optimal growth conditions that will produce biomass with the physiological state best constituted for product formation.

Media optimization by the classical method of changing one independent variable (nutrient, pH, temperature, etc.) while fixing all the others at a certain level can be extremely time consuming and expensive for a large number of variables. To make a full factorial search which would examine each possible combination of independent variable at appropriate level requires a large number of experiments, x^n, where x is the number of levels and n is the number of variables. This may be quite appropriate for three nutrients at two concentrations (2^3 trials) but not for the six nutrients at three concentrations. In this case 3^6 (729) trials would be needed. The commercial media formulations available are designed by the manufacturers keeping these trials in consideration and would have tried most of these combinations before they come up with the media to be used for a particular cell line and at times for a particular class of product. Screening these readymade available media will reduce the number of experiments at industrial level to optimize the conditions and achieve the expected cell growth and expression yield (Stanbury, *et al* 1997).

A fed-batch process requires a basal medium to support cell growth and productivity and a feed medium to prevent nutrient depletion, extend culture longevity, and maintain high volumetric productivity. A typical basal media constitutes about 50-70 components, while feed media contains fewer components but at a higher concentration. The basal media and feed media in synergy should be evaluated in the following three classes (Li, *et al* 2010):

Robustness	Preparation	Test Cell Lines
• Support consistent cell line stability at different cell age • Support consistent product quality • Suitable for early cell line development	• Filtration • Stability during storage • Scalability • Manufacturability	• Chinese Hamster Ovary cells as host • Selection system • Transfection method • Promoters • Development stage (Ph1, Ph3, commercial) • Growth properties • Specific productivity • Metabolism profile • Titer

Table 5.1.1 Properties of Basal Media and Feed Media to be look-into and evaluated for upstream process development

The basal media and feed media should qualify to fulfill the listed criteria in Table 5.1.1 and based on which the judicious selection of these should be done to take it further for optimization for the process conditions and parameters.

Continuous advances in media, feed supplements, culture vessels, and manipulation of temperature shifts, pH and numerous other methods make upstream process development to potentially be a never ending process for improving expression yield. The fact that optimal culture conditions are unique to each individual cell line, and that detailed upstream process development takes much time and resources, it becomes imperative that the MCB candidate cell line has maximum genetic potential for producing the desired quality of the target protein of interest before starting upstream process development (Bleck 2010). The media and feed combinations are important to achieve the higher expression levels. As mentioned earlier, in practice the number of experiments required to achieve the highest expression yield is numerous and never ending task. Hence, in the present study, the upstream process development was carried out in two rounds to achieve an enhanced expression level than the basal level. In the first round the basal media listed in Table 5.1.2 available from different manufacturers and based on some past experience were tried to screen the lead media which supports good cell growth as well as expression of mAb. The different media are denoted in alphabetic order and the control (CD-CHO Media) is labeled as “Z” in which the cells are already adapted and growing. The media used are available either in liquid or

powder forms or in both forms. The preference for powder form over liquid form is obvious because of the cheaper rates, shipping convenience, logistics and extended stability benefits.

ID	Media Name	Mfg	Cat#	Form
A	Is-MAB-CD	Irvin Scientific, USA	91104	Liquid / powder
B	IS-CHO-CD-XP	Irvin Scientific, USA	91121	Liquid / powder
C	IS-CHO-CD-XP	Irvin Scientific, USA	91120	Liquid / powder
D	IS-CHO-CD-K-XP	Irvin Scientific, USA	91125	Liquid / powder
E	HyQCDM4CHO	Hyclone, USA	ARF26505	Liquid / powder
F	CDM4PerMab	Hyclone, USA	AWB94001	Liquid / powder
G	Serum FreeCHO	HiMedia, India	SFM002	Liquid
H	PowerCHO2CD	Lonza	BE12-771Q	Liquid
I	PF-CHO Media 1 OF 2	Hyclone, USA	SH303334.01	Powder
	PF CHO BASAL 2 OF 2		SH303335.01	Powder
J	Excell 302	SAFC	24324C	Liquid / Powder
K	MAM-PF7	Animed, Israel	10-02575-I	Liquid
L	Mam-PF2	Animed, Israel	10-02F24-I	Liquid
M	CDM4Mab	Hyclone, USA	AVK80331	Liquid / Powder
N	CD-OptiCHO	Invitrogen, USA	12681	Liquid
O	Excell CD-CHO	SAFC	14360C	Liquid / Powder
P	Pro CHO4	Lonza	BE12-029Q	Liquid / Powder
Q	Pro CHO5	Lonza	BE12-766Q	Liquid / Powder
Z	CD-CHO	Invitrogen, USA	10743	Liquid

Table 5.1.2 List of Basal Media screened for optimizing expression

In the second round feed media listed in Table 5.1.3: was tried to screen the feed media which support sustained growth of the cells and increase in cumulative productivity.

ID	FEED NAME	MFG.	CAT #	Form
1	Efficient Feed A (10%)	Invitrogen	A10234	Liquid
2	Efficient Feed B (10%)	Invitrogen	A10240	Liquid
3	Gln (4mM) + Glu (2g/L)			Powder
4	Efficient Feed C (10%)	Invitrogen	A13275	Liquid
5	Extreme Feed (Excellgene)	Lonza	NA	Liquid
6	Cell Boost 2 (10%)	Hyclone	RR12927.01	Powder
7	Cell Boost 3 (10%)	Hyclone	RR12635.01	Powder

8	Cell Boost 4 (10%)	Hyclone	RR12800.01	Powder
9	Cell Boost 5 (10%)	Hyclone	RR12929.01	Powder
10	Cell Boost 6 (10%)	Hyclone	RRE114619	Powder
11	Lonza Feed (10%)	Lonza	NA	Liquid
12	Feed Power CHO2 (10%)	Lonza	BE12-771Q	Liquid
13	Is-CHO-Feed-CD-XP (10%)	Irvine	91122	Liquid / Powder
14	GS-Max (10%)	Hyclone	SH30586.01	Liquid
15	Efficient Feed A + B (5%)	Invitrogen	A10234 + A102340	Liquid
16	Efficient Feed A + B (7.5%)	Invitrogen		Liquid
17	Efficient Feed A + B (10%)	Invitrogen		Liquid

Table 5.1.3 List of Feed Media screened for optimizing expression

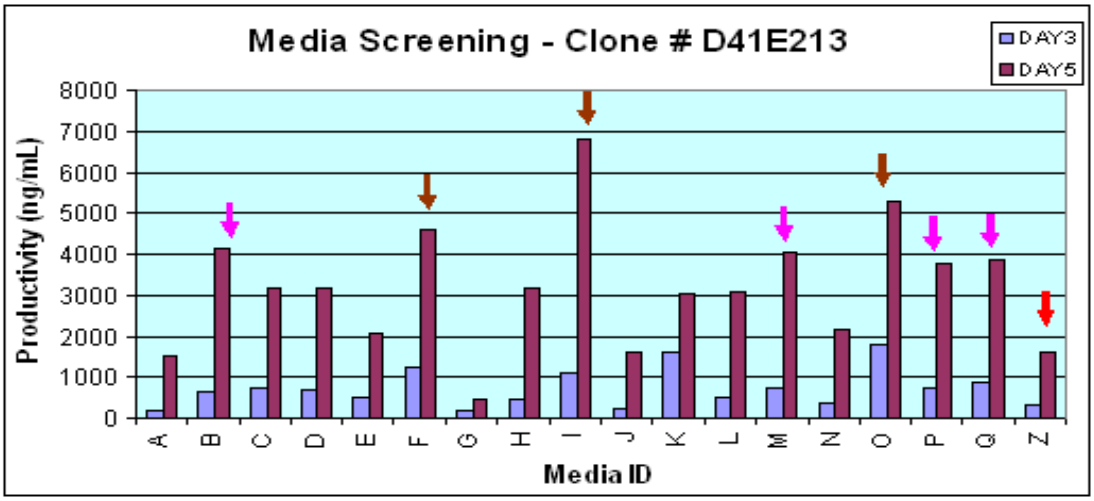
The scope of this study was to develop upstream process by screening basal media and feed supplements for the lead clone D41E213 at shake flask.

5.2. Results and Discussion

The clone #D41E213, already growing in CD-CHO media, was seeded at $0.2x10^6$ cells/mL in 6-well TC plate as control. The cells growing in CD-CHO media were adapted for growth in respective media for two passages before starting the experiments. After adaptation, the cells were reseeded in respective media and incubated at 37 ^{0}C and 5% CO_2. Samples (supernatant) were collected on Day 3 and 5 for expression analysis by ELISA.

As shown in Figure 5.2.1:A, the expression of target mAb increases with time resulting in a maximum yield in media PF-CHO (I) and Excell CD-CHO (O) on Day 5, followed by media CDM4PerMab (F), CDM4Mab (M), IS-CHO-CD-XP (B) and IS-CHO-CD-K-XP (D). The observed increase in expression yield was ~3 fold in PF-CHO and Excell CD-CHO media, ~2.5 fold in CDM4PerMab Media, ~2 fold in Media CDM4Mab and ~1.5 fold in Media IS-CHO-CD-XP and IS-CHO-CD-K-XP.

A)



B)

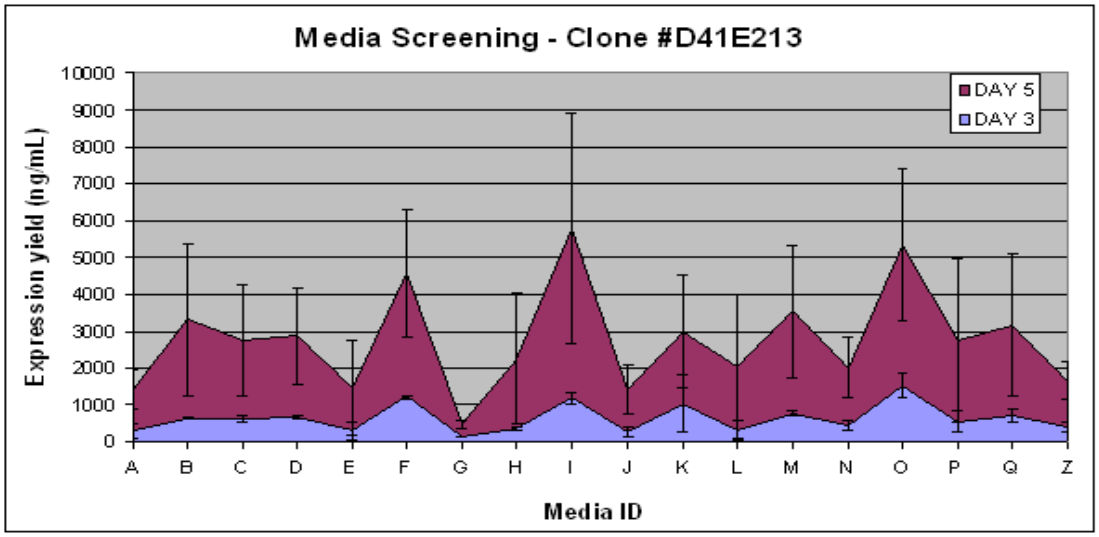


Figure 5.2.1 Media Screening to achieve higher expression yield for clone D41E213

(A) Showing the trend of productivity over time in an individual experiment. Arrows indicate media selected for further experimentation. The brown arrows represent top three media followed by next best (pink arrows) and the control flask (red arrow)

(B) Displays the trend of contribution of each value generated from two independent experiments over time. The experiments were done in duplicates in 6 well TC plates

From the above trend contribution graphs (Figure 5.2.1), Media PF-CHO, CDM4PerMab and Excell CD-CHO were the top media supporting higher expression of mAb. However, there is need to enhance the expression by implementing fed-batch process to achieve higher cumulative productivity. The error bars on Day 5 are extended probably due to the fact that the cells are undergoing apoptosis as they are growing in the same media without addition of any nutrients till five days. This nutrient limitation could also

affect the expression level heterogeneity within the same cell population. This was seen in feed optimization experiments as represented in Figure 5.2.3, where the error bars on later days were not spread out because of periodic addition of feed media. The variation is not significant in Day 3 samples as the cells are growing well and the nutrient requirements are sufficient to support the growth and expression of recombinant proteins. In addition to the above listed top 3 media (PF-CHO, CDM4PerMab and Excell CD-CHO, second best media IS-CHO-CD-XP and IS-CHO-CD-K-XP, IS-CHO-CD-K-XP and CDM4Mab were also selected as basal media to be tried with different feed media for further optimization experiments as listed in Table 5.2.1.

ID No.	Basal Media	Feed Media
F1	CDM4PerMab	Efficient Feed A (10%)
F2		Efficient Feed B (10%)
F3		Gln (4mM) + Glu (2g/L)
F4		Efficient Feed C (10%)
F5		Extreme Feed
F6		Cell Boost 2 (10%)
F7		Cell Boost 3 (10%)
F8		Cell Boost 4 (10%)
F9		Cell Boost 5 (10%)
F10		Cell Boost 6 (10%)
F11		Lonza Feed (16-07-11) (10%)
F12		Feed Power CHO2 (10%)
F13		Is-CHO-Feed-CD-XP (10%)
F14		GS-Max (10%)
F15		Efficient Feed A + B (5%)
F16		Efficient Feed A + B (7.5%)
F17		Efficient Feed A + B (10%)
B1	IS-CHO-CD-XP	Efficient Feed A + Efficient Feed B (50:50) added 10% v/v
D1	IS-CHO-CD-K-XP	
F1	CDM4PerMAB	
I1	PF-CHO	
M1	CDM4MAB	
O1	EXCELL CD-CHO	
Z1	CD-CHO	

B2	IS-CHO-CD-XP	Cell Boost 5 added 10% v/v
D2	IS-CHO-CD-K-XP	
F2	CDM4PerMAB	
I2	PF-CHO	
M2	CDM4MAB	
O2	EXCELL CD-CHO	
Z2	CD-CHO	

Table 5.2.1 Basal Media and Feed combinations used for improvement in expression yield.
Each combination is given an ID No. for ease of representation in graphical form and in text writing.

In the second round of optimization, basal media in combination with various feed media as listed in Table 5.2.1 were tried.

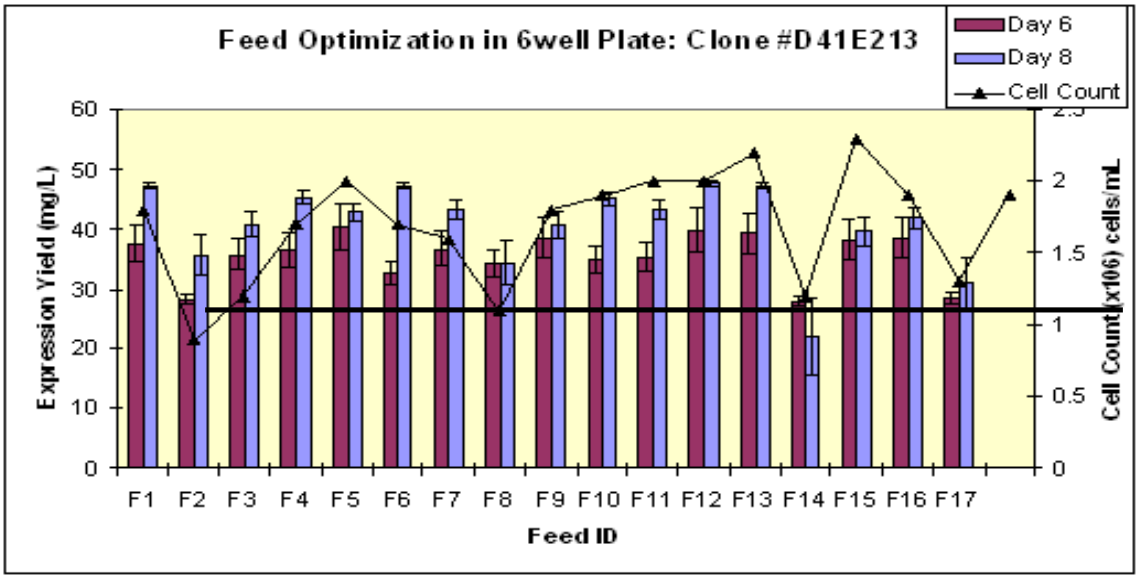


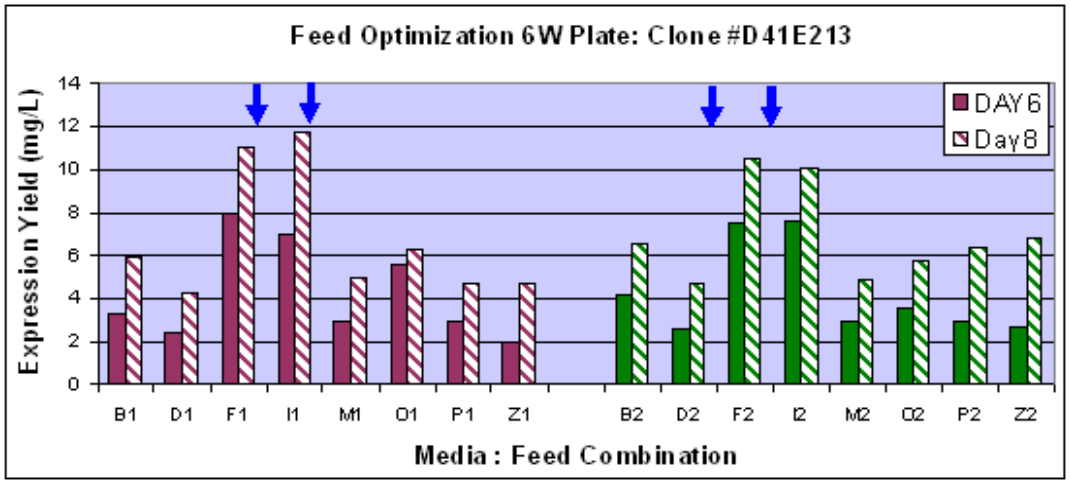
Figure 5.2.2 Feed Media Screening for fed-batch culture optimization
CDM4PerMab was used as the basal media to increase the expression yield. The expression of target antibody was measured using Sandwich ELISA. The experiments were done in duplicate in 6 well TC plates.

As mentioned earlier, the cells were seeded at 0.2 x 10^6 cells / mL in a total volume of 2 mL in 6-well TC plate in the CD-CHO media after adapting the cells for 2 passages. The media and feed media used were listed in Table 5.2.1. The respective feed media was added at 10% v/v on Day 4 and Day 6. The samples before addition of feed media were collected on day 6 and day 8 of total cell age and analyzed for expression yield by Sandwich ELISA. The cell count was also measured at the end of culture (Day 8) which is plotted as line curve in Figure 5.2.2.

An increase in expression yield was observed in feed combinations F1, F4, F6, F10, F12 and F13 used when CDM4PerMab was used as basal media but, was not significant in terms of fold increase.

The six basal media shortlisted above were tried in combination with two feed media (Efficient Feed A+ Efficient Feed B and Cell Boost 5). Although there are potential feeds identified but those didn't show significant improvement in terms of fold increase in expression yield, hence Efficient Feed A+ Efficient Feed B and Cell Boost 5 was shortlisted for further experimentation. Both these feeds were tried in combination with the shortlisted basal media. On Day 4 of cell growth, 10% v/v of each feed was added and cells were incubated further under similar conditions. The samples were collected on Day 6 (Day 2 after feeding) and Day 8 (Day 4 after feeding) for expression analysis by ELISA.

A)



B)

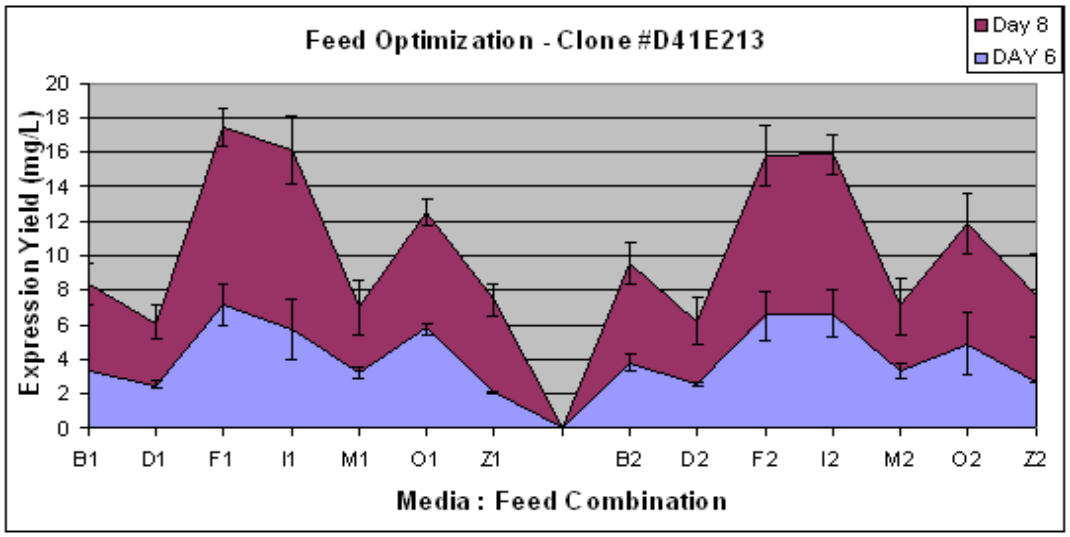


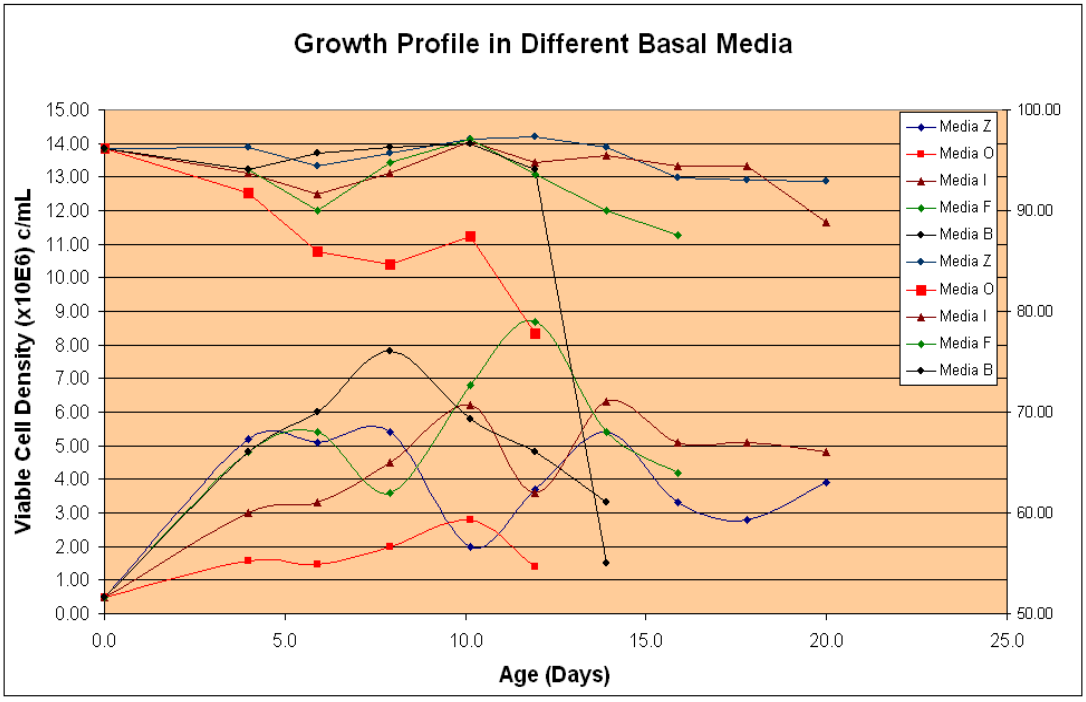
Figure 5.2.3 Combination of basal media and feed for fed-batch optimization

A) Showing the trend of expression yield over time in an individual experiment. Arrows indicate media selected for further experimentation. The green panel is where feed media Cell Boost 5 was added as feed and purple color is where Efficient Feed A +Efficient Feed B was added.

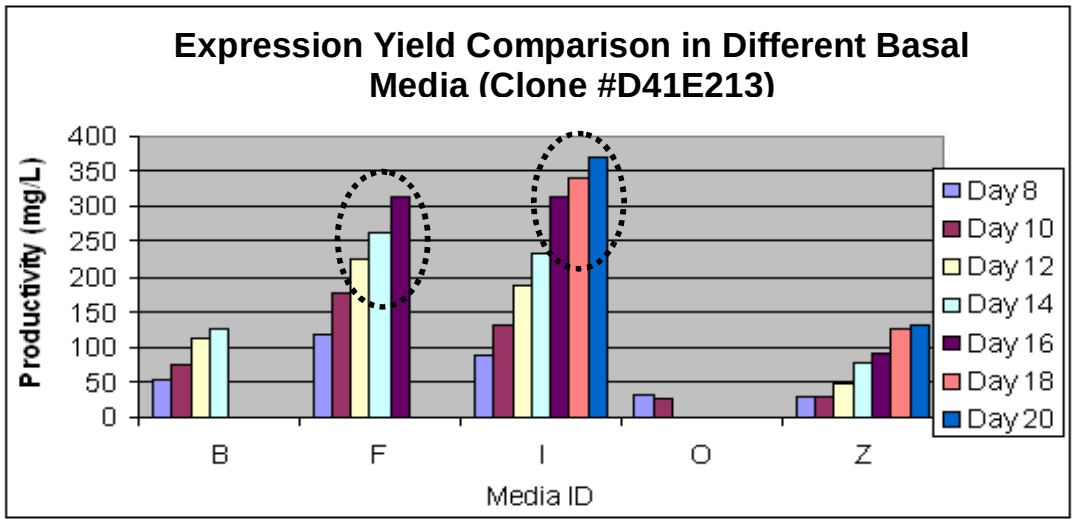
B) Displays the trend of contribution of each value generated from two independent experiments over time. The experiments were done in duplicate in 6 well TC plates.

From the trend contribution graphs depicted in Figure 5.2.3:B, both the feed media along with PF-CHO and CDM4PerMab basal media gave ~2 fold increase in expression yield while Excell CD-CHO basal media gave ~1.5 fold when compared to the control CD-CHO media. Hence, based on these observations, ease of use amongst the tried feed media and prior experience with these feeds, the CDM4PerMab as a basal media in combination with Efficient Feed A + Efficient Feed B was selected for further analysis at the shake flask level. In addition to these lead combinations the second in line combination using media IS-CHO-CD-XP, IS-CHO-CD-K-XP and CDM4Mab were also experimented. The cells were seeded in 125mL shake flask at 0.5 x 10^6 cells / mL and grown for 4 days at 37 ^{0}C and 5%CO_2 following which the shake flasks were shifted to a fed-batch mode where 10% v/v Efficient Feed A + Efficient Feed B (A+B) was added on every alternate day starting from Day 4. The cell growth was monitored by taking cell count at every alternate day and expression yield was estimated by Sandwich ELISA starting from Day 8 of culture. The fed-batch was terminated when the viability dropped below 80%. As shown in Figure 5.2.4:A, Excell CD-CHO and IS-CHO-CD-XP showed viability drop below 80% in early stages compared to CD-CHO (Z), PF-CHO (I) and CDM4PerMab (F) where the viability of more than 80% was maintained even after 20 days.

A)


Growth Profile in Different Basal Media
Viable Cell Density (x10E6) c/mL
Age (Days)
Media Z
Media O
Media I
Media F
Media B
Media Z
Media O
Media I
Media F
Media B

B)


Expression Yield Comparison in Different Basal Media (Clone #D41E213)
Productivity (mg/L)
Media ID
B
F
I
O
Z
Day 8
Day 10
Day 12
Day 14
Day 16
Day 18
Day 20

C)

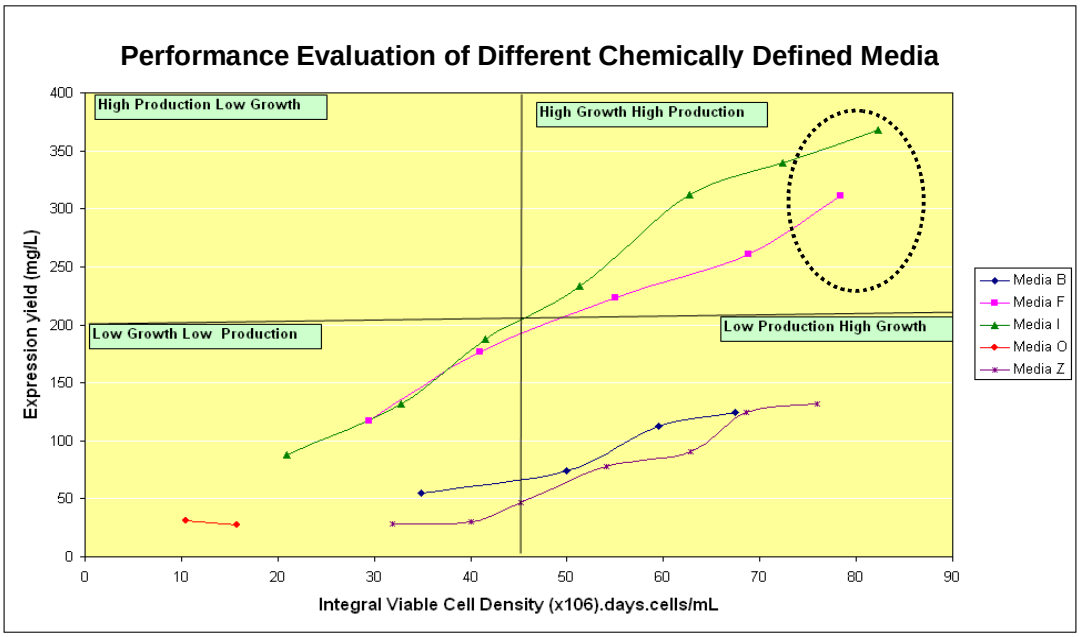


Figure 5.2.4 Comparison of Different Basal Media in Shake Flask Experiment

(A) Graph depicting the IVCC and %viability of cells to measure the cell growth. The cell count taken using automated cell counter Countess™,

(B) The expression yield was measured using Protein A HPLC every alternate day starting from Day 8 of culture

(C) Consolidated graph to evaluate performance of cells in terms of growth and productivity in various chemically defined media

The IVCC of clone D41E213 achieved at the end of fed-batch in different basal media are tabulated and rated in Table 5.2.2. The viability of cells in PF-CHO, CDM4PerMab and CD-CHO was around 90%, whereas it dropped steeply in IS-CD-CHO-Xp and Excell CD-CHO Media as shown in Figure 5.2.4:A and B.

Basal Media	Approximate IVCC and Days	Viability	Expression Yield (mg/L)	Rating
CDM4PerMab	80 x 10^6 cells (Day 16)	88%	311	*****
PF-CHO	80 x 10^6 cells (Day 20)	90%	370	****
Excell CD-CHO	20 x 10^6 cells (Day 10)	40%	28	*
IS-CHO-CD-XP	70 x 10^6 cells (Day 14)	55%	125	**
CD-CHO	80 x 10^6 cells (Day 20)	93%	132	***

Table 5.2.2 Comparing and rating the basal media by taking in account the cell growth and expression yield. *** represents the best and * poor combination**

The consolidated effect of IVCC and expression yield concluded that CDM4PerMab and PF-CHO Media supported higher growth and higher expression yield (Figure 5.2.4C). However, CDM4PerMab is the top media as the comparable IVCC was achieved on Day 16 which is 4 days less than that achieved in PF-CHO Media. This is a huge advantage in terms of operation and cost when the process is scale-up to larger volumes. Because of these obvious merits of CDM4PerMab Media in combination with Efficient Feed A+ Efficient Feed B these were selected as final basal media for further upstream process development at higher scale.

5.3. Conclusion

The most commonly used media for expression of recombinant proteins in CHO cells were screened of which two lead basal media (CDM4PerMab and PF-CHO) were identified. Out of these two media, CDM4PerMab was identified as the preferred media due to the fact that it is cheaper than PF-CHO media and is less complicated in preparation. With the CDM4perMab as a basal media various feed media based on the availability and experience were tried, out of which Efficient Feed A + Efficient Feed B was shortlisted as it gave good sustained cell growth and increase in expression yield. These media components (CDM4PerMab and Efficient Feed A + Efficient Feed B) are routinely used in other project and there is ease of using these due to availability in bulk resulting in establishment of platform media at competitive cost. The process was thus optimized for the expression of target protein at shake flask level using CDM4PerMab media with addition of Efficient Feed A + Efficient Feed B (50:50) at 10% v/v for further experiments.

CHAPTER 6

CHARACTERIZATION OF THE EXPRESSED RECOMBINANT ANTI-EGFR MONOCLONAL ANTIBODY

6.1. Preamble

Product characterization and comparability with the Reference Medicinal Product (RMP) are two important aspects for companies working on biosimilar molecules. Being biological in nature they have associated complexity. The expressed protein is host and process dependent. Therefore, the evaluations of critical quality attributes are of prime importance. The demonstration of comparability does not necessarily mean that the expressed product is identical to RMP however; it should be same in terms of primary sequence and, close in terms of quality, safety and potency. Characterization and determination of comparability at the early stages of development can be based on a combination of a few crucial analytical tests and *in-vitro* biological assay which gives preliminary confidence that the candidate clone selected will deliver a biosimilar product which shall meet all the PQAs.

Prior to designing a cell culture and purification process for mAb manufacturing, a quality target product profile provides a list of quality attributes (QA) and what levels are critical and hence need to be monitored and controlled. Since antibodies are a homologous class of molecules, knowledge gained through prior experience or from published studies may greatly aid in defining QAs. The definition for quality attribute is fairly broad and can potentially include raw materials in addition to features of the drug molecule itself (Flynn, *et al* 2010).

Critical Quality Attributes (CQA) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are generally associated with the drug substance, excipients, intermediates (in-process materials) and drug product [Ref: ICH Q8(R2)]. There are assigned Product Quality Attributes (PQA) which is product specific and most of the times dependent on the clone. The PQA like glycosylation and charge variants are cell line derived and changes from cell line to cell line due to variation in the metabolism from cell to cell. Hence, the PQA need to be determined at early stage of clone selection in order to identify the right clone so that there would not be surprises at the later stage of development thus, saving on the development time. Measuring and monitoring PQA will guide through determination of CQAs at the final process stage (Mire-Sluise, *et al* 2009).

The most recent regulatory document covering characterization of monoclonal antibodies was published by the European Medicines Agency (EMA) in July 2009 (Ref. EMEA/CHMP/BWP/1576532/2007). This EMA guideline entitled "Development, Production, Characterization and Specifications for Monoclonal Antibodies and Related Products" states that the monoclonal antibody should be characterized thoroughly before going for human use. This characterization should include the determination of physicochemical properties, purity, impurities and quantity of the monoclonal antibody, in line with ICH-Q6B. As shown in Figure 4.1.1 of Chapter 4, the PQA assessment is done at different stages of the product life cycle employing similar or different analytical techniques at each stage. The list of PQAs which with the impact on product is referred in Table 4.1.1:, and the PQAs studied for the mAb of interest are highlighted in yellow color which ensures the capacity of clone to process and produce the right molecule to go for the further development work. However, there could be some minor modifications to this depending on molecule and the function of it.

To identify the impact of a product characteristic change or manufacturing process change, careful evaluations of all foreseeable consequences for the product is performed by Innovator / Originator Company and are usually available in the form of published reports or literature.

For example, as depicted in Figure 6.1.1, there are a number of modifications possible in a mAb molecule. It shows the modifications of mAb molecule with one glycosylation site, and if we consider the mAb in discussion (anti-EGFR mAb, Cetuximab) where there is an additional glycosylation site then the possible number of variants could be much more than 10^8 (theoretically $10^8 + 10^8 = 2 \times 10^8$). Hence, this is a very complex molecule to deal with and product characterization is critical at an early stage so that the right clone is selected.

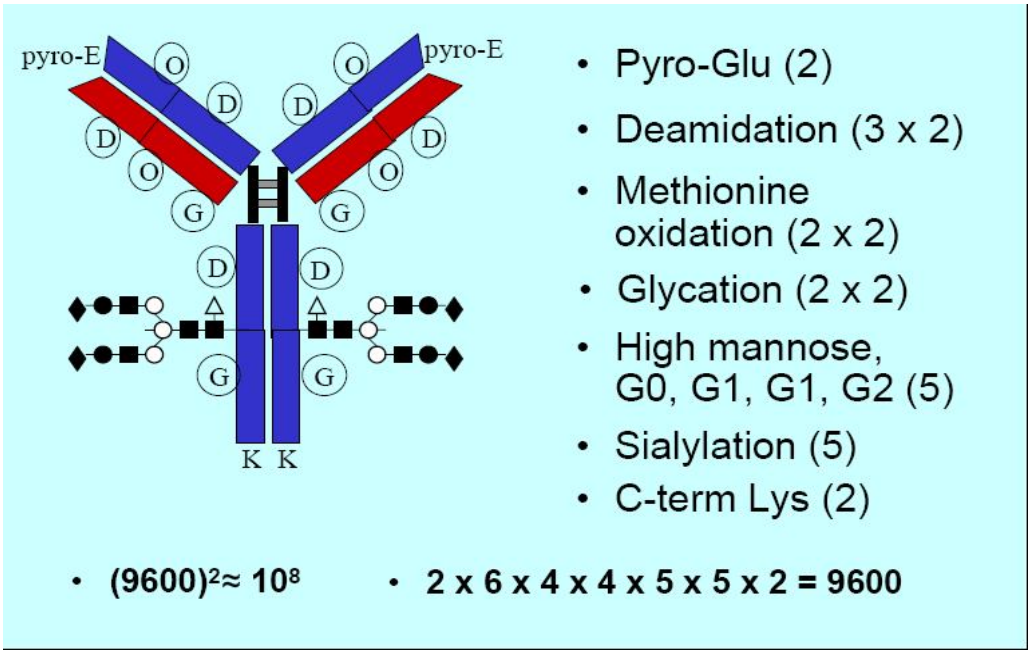


Figure 6.1.1 General product quality variables for mAbs.

(Figure taken from presentation by Steven Kozlowski, M.D., Director Office of Biotechnology Products OPS/CDER (6/21/06). There are possibilities of 2 pyroglutamate formation (Pyro-E), 6 deamidation (D) prone sites, 4 oxidation (O) prone sites, 4 glycation sites (G), 5 sialylation (◆)and 2 C-terminal lysine (K) sites. These all results in ~9600 variants of IgG molecule.

These modifications could be cell line or process driven and assigned as PQAs based on their impact on overall product quality and bioactivity. The list of PQAs and their importance is described in Chapter 4 section 4.1. As shown in Table 4.1.1, the most important PQA for mAbs is aggregate content while Q-cyclization is the least. The other kinds of modifications have criticality falling between these two extremes. Hence, these attributes should be carefully studied before finalizing on the lead clone for the manufacture of biosimilar therapeutic monoclonal antibody. In the mAb of interest, the preliminary analytical testing is already covered in Chapter 4 (Final Clone selection), however, high end analytical testing like Mass Spectrometry, Size Exclusion Chromatography and characterization of peaks observed in CEX-HPLC was carried out to further characterize the product expressed in an alternate host, CHO cells. The following quality attributes were studied:

6.1.1 Aggregation and Degradation of Protein

Protein aggregation is a common issue encountered during manufacturing of biotherapeutics. It is possible to influence the amount of aggregate produced during the cell culture and the purification process by carefully controlling the environment (e.g. media components) and implementing appropriate strategies to minimize the extent of aggregation. Steps to remove aggregates have been successfully used at a manufacturing scale. Care should be taken when developing a process to monitor the compatibility of the equipment and process with the protein to ensure that potential aggregation is minimized (Cromwell, *et al* 2006).

Aggregation is a general term that encompasses several types of interactions or characteristics. Protein aggregates are defined very broadly as high molecular weight proteins composed of multimers of natively conformed or denatured monomers. Aggregates of proteins may arise from several mechanisms and may be classified in numerous ways, including soluble/insoluble, covalent/noncovalent, reversible /irreversible, and native/ denatured. For protein therapeutics, the presence of aggregates of any type is typically considered to be undesirable because of the concern that the aggregates may lead to an immunogenic reaction (small aggregates) or may cause adverse immunological events on administration (particulates) (Rosenberg 2008). There is no consistent definition of what is meant by a “soluble” aggregate, so working definitions are often employed. For the purpose of this discussion, soluble aggregates refer to those that are not visible as discrete particles and that may not be removed by a filter with a pore size of 0.22 µm. Conversely, insoluble aggregates may be removed by filtration and are often visible to the unaided eye. Both types of aggregates are undesirable for the development of a therapeutic protein. Covalent aggregates arise from the formation of a chemical bond between 2 or more monomers. Disulfide bond formation resulting from previously unpaired free thiols is a common mechanism for covalent aggregation (Andya, *et al* 2003). Oxidation of tyrosines may also result in covalent aggregation through the formation of bityrosine (Giulivi and Davies 1994). For some proteins, a covalent interaction between monomers is required to form a stable protein structure that is to form a homo- or hetero-dimeric protein (eg. Follicle Stimulating Hormone, Antibodies).

Just as there are many types of interactions (hydrogen bond, disulfide linkage, covalent linkage) that can lead to protein aggregation, there are many environmental factors that

can lead to aggregation (Chi, *et al* 2003). Solution conditions such as temperature, protein concentration, pH, and the buffer ionic strength may affect the amount of aggregate observed. The presence of certain ligands, including specific ions, may enhance aggregation. Stresses to the protein such as freezing, exposure to air, or interactions with metal surfaces may result in surface denaturation, which then leads to the formation of aggregates. Finally, mechanical stress may also lead to aggregation. Each of these environmental factors is typically encountered during bioprocessing starting from the cell line to cell culture, purification, formulation, filling and storage processes (Cromwell, *et al* 2006).

There are several opportunities for protein aggregation to occur during cell culture process. During expression, accumulation of high amounts of protein may lead to intracellular aggregation owing to either the interactions of unfolded protein molecules or to inefficient recognition of the nascent peptide chain by molecular chaperons responsible for proper folding (Zhang, *et al* 2004). During a typical production of a monoclonal antibody from a mammalian cell culture, the protein is secreted from the cell into the cell culture medium. The medium usually contains cells, ions, nutrients for the cells, host cell proteins (including proteases), dissolved oxygen, and other species. This cellular suspension at near neutral pH is held at temperature above 30 ^{0}C for several days for getting the accumulated expressed protein. These conditions which could be unfavorable to proteins may lead to aggregation. Judicious selection of expression system and culture condition is important to minimize the aggregation (Cromwell, *et al* 2006). Hence the measurement of aggregation using rapid and simple method is essential at an early stage to monitor the cell line behavior.

It has been reported that mAbs expressed in a CHO cell line sometimes contain unpaired thiols (Zhang and Czupryn 2002) which leads to aggregate formation during cell culture process. The presence of a free thiol may affect long-term stability of the protein. Noncovalent aggregates have also been observed to be formed during cell culture (Cromwell, *et al* 2006). Protein aggregation poses risk in terms of generation of immune responses to the therapeutic protein product. Of principal concern are those immune responses associated with adverse clinical effects: neutralizing antibody that inhibits the efficacy of the product or, worse, cross reactively neutralize an endogenous protein counterpart; and severe immediate hypersensitivity responses such as anaphylaxis. Among the qualitative factors critical in inducing antibody responses are molecular

weight and solubility. While low MW aggregates such as dimers and trimers appear inefficient in inducing immune responses, large multimers whose MW exceeds 100 kDa are efficient inducers of immune responses. Moreover, it has been long known that particulate (insoluble) antigens are very rapidly endocytosed by antigen-presenting cells, which initiate immune response (Rosenberg 2006).

Proteolytic degradation of recombinant proteins represents a major problem related to production of gene products in heterologous hosts (Murby, *et al* 1996). Protein can undergo several degradation mechanisms. These can be both chemical (i.e. reactions that involve specific residues which results in a change in the primary structure of the proteins) and physical (i.e. partial unfolding, conformational changes, adsorption, dissociation and aggregation). Oxidation and deamidation are the most common chemical degradation reactions. Cells have both intracellular and extracellular pathways for degrading proteins. The major extracellular pathway is the system of digestive proteases, which break down proteins to polypeptides. These include endoproteases such as trypsin and chymotrypsin; exopeptidases such as aminopeptidase, carboxypeptidase and peptidase, contributed by lysed producer cell or a contaminant.

Protein turnover within the cell is a normal process that is highly regulated. Degradation- destined proteins are degraded via distinct pathways. For example, a newly synthesized protein, yet misfolded protein is degraded via the Ubiquitin Proteosome System (UPS) or Endoplasmic Reticulum Associated Degradation (ERAD) pathways in an ubiquitin dependent manner. In contrast, protein aggregates or oxidative modified proteins are predominantly degraded via autophagy. Degraded proteins could generate immune response hence the levels need to be estimated (Hermiling, *et al* 2004).

6.1.2 Heterogeneity of Monoclonal Antibodies

Monoclonal antibodies, like other proteins, are subject to many posttranslational modifications, including modifications of the termini, glycosylation, deamidation, isomerization, oxidation, fragmentation, aggregation, etc. Almost all of these changes can alter surface charge properties of the antibody either directly by changing the number of charged groups or indirectly by introducing structural alterations. Even a small perturbation of the structure may change the local distribution of charged residues or

modify their pKa, thus changing the overall surface-charge distribution of the antibody (Vlasak and Ionescu 2008).

Molecular alterations can take place at every stage of manufacturing: cell culture, purification, formulation, and storage. Since chemical modifications can affect the biological activity of a protein, detection and identification of molecular changes are important analytical issues in biopharmaceutical development. Antibody modifications can occur enzymatically or non-enzymatically, e.g. through unfavorable storage conditions which may cause charge or size heterogeneity. Common modifications of the primary sequence include N-glycosylation, methionine oxidation, proteolytic fragmentation, and deamidation (Jefferies, *et al* 1990, Jefferis and Lund 1997, Manning, *et al* 1989, Volkin, *et al* 1981).

In general the heterogeneity in mAbs is caused by chemical modifications which in turn lead to non-covalent interactions, conformational diversity and aggregation.
The chemical modifications cover the following but not limited to post-translational modifications happening either *in-vivo* or *ex-vivo:*

- Disulfide bonds includes free sulfhydryl and reactive cysteine residues, incomplete disulfide bond formation between inter-chain and intra-chains, disulfide bond breakage due to β-elimination and disulfide bond scrambling.
- Variation in the oligosaccharide structure due to culture conditions and media composition.
- Formation of N-terminal pyroglutamate from glutamate and glutamine.
- C-terminal Lysine processing of the heavy chain.
- Deamidation of asparagines through formation of succinimide intermediate.
- Isomerization of asparatate residue in the sequence Asp-Gly through formation of succinimide intermediate,
- Oxidation of methionine
- Glycation resulting from the non-enzymatic reaction between reducing sugars and the N-terminal primary amine or the amine group of lysine side chains,
- Peptide bond cleavage especially at the hinge region which is most susceptible in mAb structure,
- Cross-linking of light chain and heavy chain by non-reducible covalent bonds,
- Mutation, Insertion and truncation,

- Other chemical modifications like modification of the N-termini of either light chain or heavy chain by maleuric acid, C-terminal lysine residue or amidation of a newly exposed C-terminal proline after removal of both C-terminal lysine and glycine residues. The non-covalent interaction with riboflavin and metal ions like copper could change the charge property of mAb.

Conformational heterogeneity of protein exists as an ensemble of different conformations in equilibrium instead of a single rigid structure (Liu, *et al* 2007b).

Since most human IgGs have a basic isoelectric point, cation-exchange HPLC (CEX) is typically used. Variants that elute earlier than the main peak are referred to as acidic because they are less positively charged and therefore bind less tightly to the column. Variants that elute later than the main peak are referred to as basic. In CEX, the net surface-charge, its distribution, and the overall geometry of the protein play a role in the separation. For instance, deamidation events in different parts of the molecule can be resolved by CEX. Table 6.1.1 summarizes different modifications that were detected in antibodies by CEX. As described in Table 6.1.1, ProPac WCX-10 column (Dionex Corp.), based on the polymeric tentacle resin has become the gold standard for antibody analysis (Weitzhandler, *et al* 1998). However, newer version of gel based resins such as TSKgel CM-STAT (Tosoh Bioscience) gives better resolution of peaks in shorter run time.

Modifications	**Column Used**	**Elution**	**Explanation**
Lack of abnormal glycosylation	**ProPac WCX-10**	**Basic**	**Conformational**
Sialylation of glycans	**ProPac WCX-10**	**Acidic**	**New COOH**
C-terminal lysine cleavage	**ProPac WCX-10** **PL-SCX (Polymer Labs)** **Mono S HR5/5(Pharmacia)**	**Acidic**	**Loss of NH_2**
C-terminal amidation	ProPac WCX-10	Basic	Loss of COOH
N-terminal glutamine cyclization	PL-SCX (Polymer Lab) ProPac WCX-10	Acidic	Loss of NH2
Maleuric acid adduct	ProPac WCX-10	Acidic	Loss of NH2
Thiosulfate adduct	BakerBond WP Carboxy sulfone (J.T. Baker)	Acidic	New acidic group
Citrate or succinate adduct	Not specified	Acidic	New COOH
Oxidation, HC M252 & M428	ProPac WCX-10	Basic	Conformational

Cysteinylation, HC, C104	ProPac WCX-10	Acidic	Conformational
Disulfide related	ProPac WCX-10	Basic	Conformational
Deamidation, HC N55	ProPac WCX-10	Acidic	New COOH
Deamidation, HC N55, N384, N389	ProPac WCX-10	Acidic	New COOH
Deamidation, LC N30, HC N55	BakerBond CSX	Acidic	New COOH
Deamidation, LC N161, HC N141	Mono S HR5/5 (Pharmacia)	Acidic	New COOH
Succinimide LC D30	ProPac WCX-10	Basic	Loss of COOH
Succinimide, HC D102	BakerBond CSX	Basic	Loss of COOH
Isomerization, HC D102	BakerBond CSX (J.T. Baker)	Basic	Conformational

Table 6.1.1 Modifications Detected by CEX in the Full-Length Ab.

LC – light chain, HC – heavy chain. The ProPac WCX-10 is manufactured by Dionex Corp. – The elution is indicated relative to the unmodified antibody, for some variants, like those related to C-terminal lysine cleavage or to pyroglutamate formation the main population may carry the modification. - Formation of an amide bond between the carboxyl of maleuric acid and the N-terminal primary amine (Vlasak and Ionescu 2008). **Bold represent the modifications studied during this work**

Monoclonal antibodies intended for therapeutic use require a comprehensive characterization of their structural integrity, purity, and stability. Charge heterogeneity is a common phenomenon in Abs and mAbs. Manufacturers of pharmaceutical proteins are expected to define and characterize the pattern of heterogeneity and assure batch-to-batch consistency for approval by regulatory authorities (Points to Consider, FDA, USA, 1997).

In this study we have monitored two major modifications, C-terminal lysine and glycosylation

6.2.4.1. Heterogeneity due to C-terminal Lysine cleavage.

All human IgGs contain a lysine residue at the C- terminus of the heavy chain. Lysine and arginine residues at the C-terminus of proteins are usually removed by cellular enzymes called carboxypeptidases. Typically, the removal is incomplete, resulting in two, so called lysine variants, with lysine on either one or both heavy chains. In some antibodies the main population could still contain lysine on both heavy chains, because of a lower level of carboxypeptidases in the producing cell line (Vlasak and Ionescu 2008). Lysine variants can not be identified by method like RP-HPLC but other methods, including CEX (Weitzhandler, *et al* 1998 and Gadgil, *et al* 2006) and IEF where

antibodies with the C-terminal lysines elute as basic peaks due to the presence of the lysine. Removal of C- terminal lysines with carboxypeptidase B (CPB) can be used as a quick method to determine the amount of lysine variants and to identify their peaks in the chromatogram (Vlasak and Ionescu 2008). The presence of lysine variants complicates analytical characterization because every species is accompanied by its own lysine variants. Consequently, the number of peaks increases three fold and can lead to peak overlaps. Treatment with CPB prior to analysis can significantly simplify the chromatograms (Perkins, *et al* 2000).

Lysine variants are not expected to affect efficacy or safety. In fact, similar processing occurs in natural human antibodies (Vlasak and Ionescu 2008). In addition, lysine removal was found to have no effect on CDC (Antes, *et al* 2007). Lysine variants, however, need to be monitored to ensure consistency of the manufacturing process and can serve as a surrogate marker of product quality (Vlasak and Ionescu 2008).

6.2.4.2. Heterogeneity due to Glycosylation

Glycosylation is an important aspect to monitor to ensure the bioactivity of molecule and heterogeneity in the product, although there is no unifying function for oligosaccharides (Dwek, *et al* 1995). Recombinant IgG antibodies are typically glycosylated at a consensus sequence Asn-Xxx-Ser/Thr which is conserved site at Asn_{297} in the CH2 domain of the Fc portion. The antibody in discussion, anti-EGFR monoclonal antibody (Cetuximab), is a unique monoclonal antibody possessing three N-linked glycosylation sites; one in the CH2 domain which is conserved amongst all antibodies, second and third site within the framework 3 of the variable portion of the heavy chain.

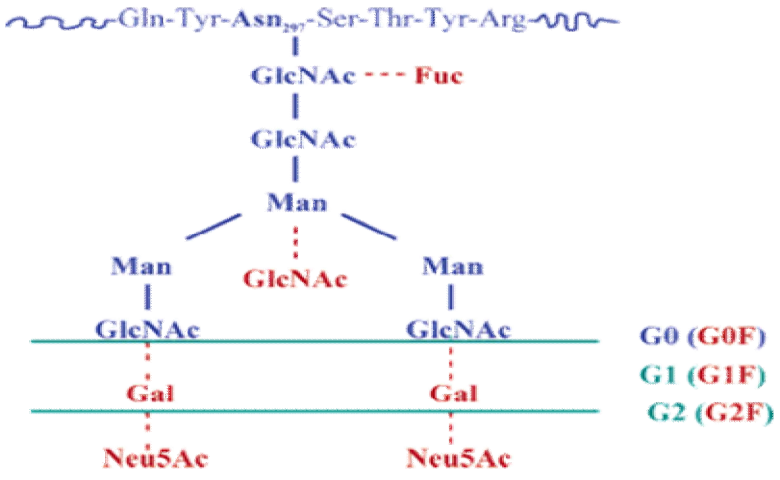
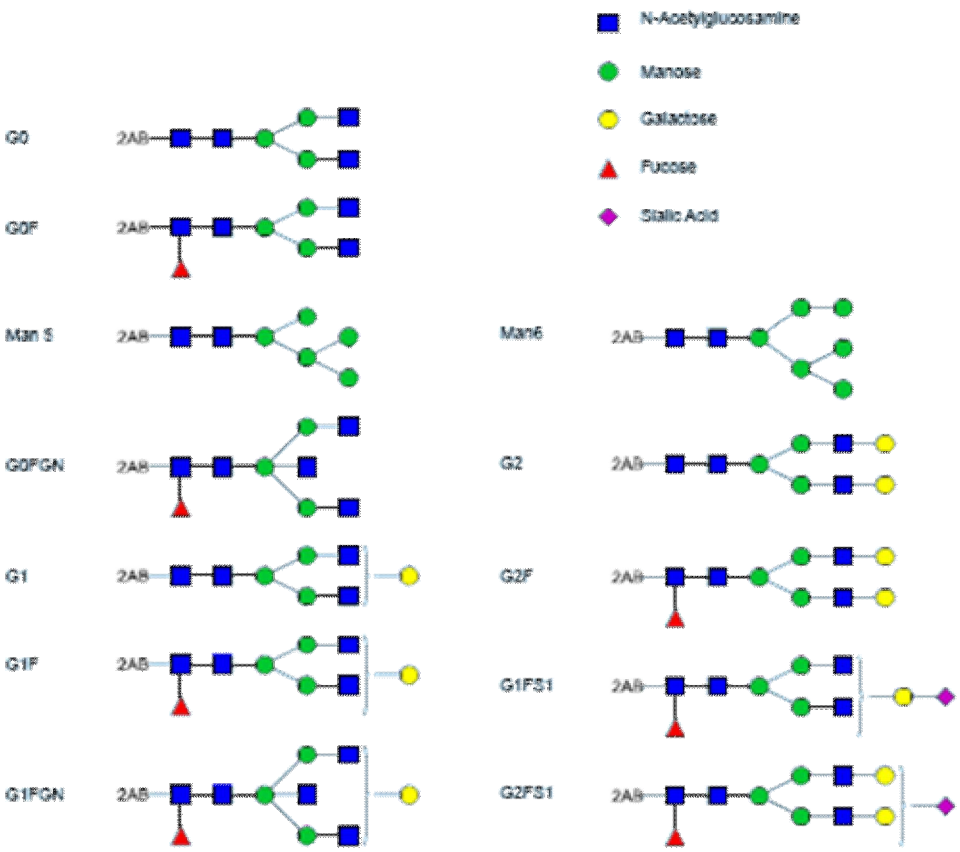


Figure 6.1.2 Depiction of conserved N-linked glycosylation site at Asn_{297} of Fc domain

In the case of Cetuximab, the typical characteristic is that the N-linked glycosylation site in Fc portion is at Asn_{299} and not Asn_{297}. The second site of glycosylation in Cetuximab is in variable domain at Asn_{88} while the third side is not glycosylated. The oligosaccharides located at the two sites are a complex mixture of different glycans, making them challenging for structural characterization (Qian, *et al* 2007). In general, glycosylation at $Asn_{297/299}$ is known to influence the biological, pharmacological and physico-chemical properties of IgGs (Kamoda, *et al* 2006). The biological functions influenced by glycosylation include resistance to proteases, binding to monocyte Fc receptor, complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and circulatory half-life in-vivo (Qian, *et al* 2007). Glycans represent only a small fraction of an antibody structure (2-3%) but add complexity and heterogeneity which plays a unique role in effector functions (Beck, *et al* 2008). N-linked oligosaccharides produced in different cell lines (CHO, SP2/0 or NS0) are usually fucosylated, complex bi-antennary structure with different degrees of galactosylation: no terminal galactose (G0F), one terminal galactose (G1F) and two terminal galactose (G2F).

A standard format for the depiction of glycosylation is represented in Figure 6.1.3 adapted from Harvey, *et al* 2009.

A)



B)

Symbol	Saccharide	Abbreviation
■ ■	*N*-acetylglucosamine	GlcNAc
□ □	Glucose	Glc
◇ ◇	Galactose	Gal
◆ ◆	*N*-acetylgalactosamine	GalNAc
◈ ◈	Fucose	Fuc
○ ○	Mannose	Man
★ ★	*N*-acetylneuraminic acid	NeuNAc (NANA)
☆ ☆	*N*-glycolylneuraminic acid	NeuNGc (NGNA)
△ △	Xylose	Xyl

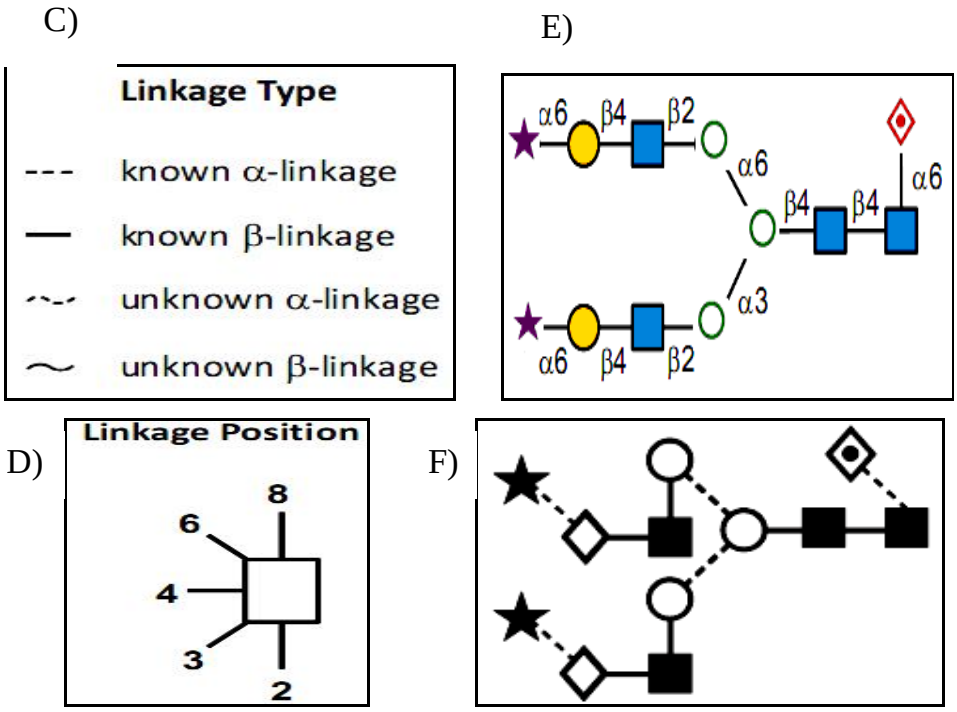
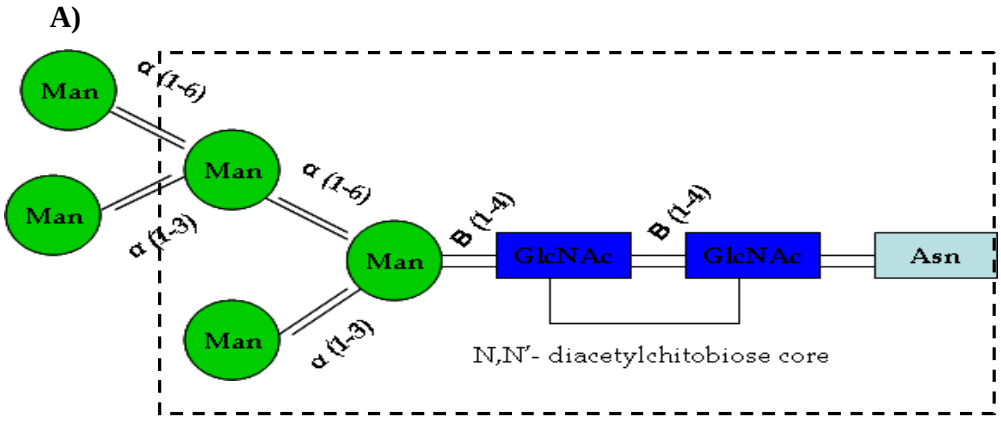


Figure 6.1.3 Format for depicting of glycosylation.

A) Different forms typically present in the glycosylated proteins. B) Abbreviations and symbols for different forms. C) Type of Linkages observed. D) Carbon atoms where the bond formation is possible, E) Representation of glycosylation structure.



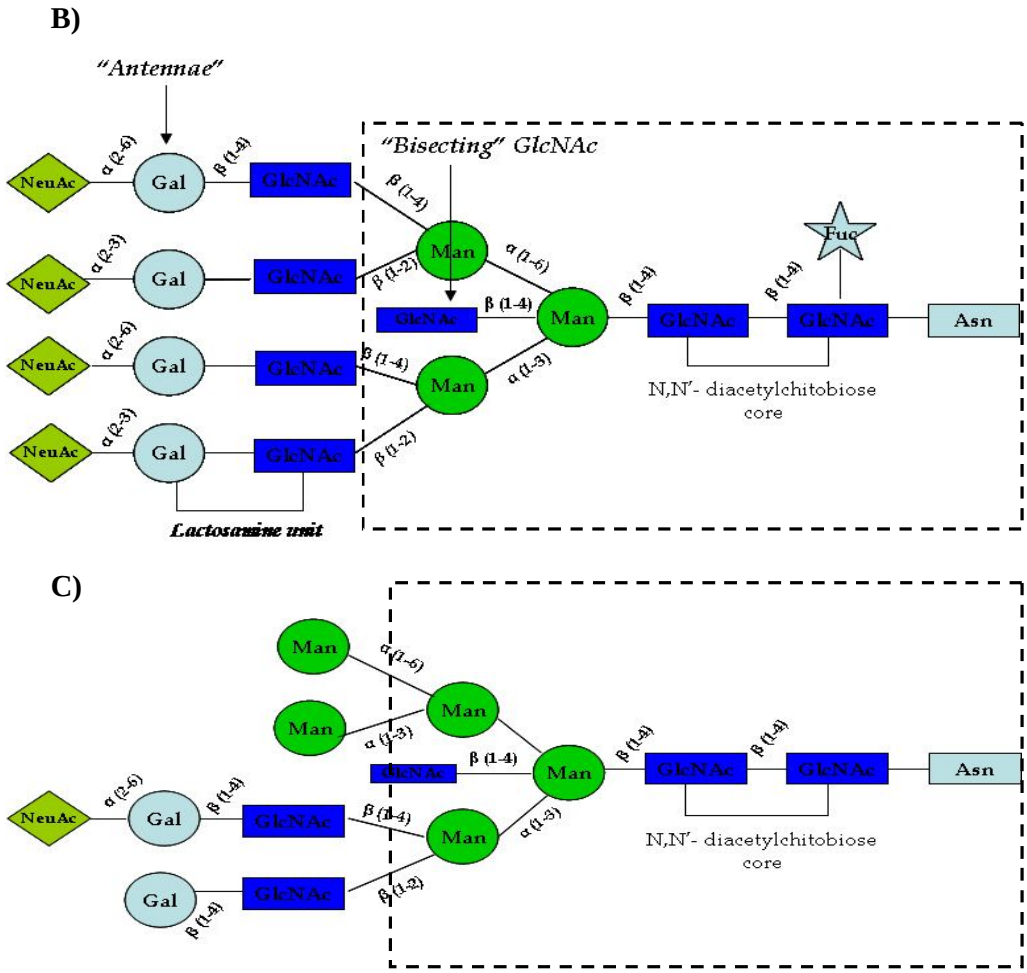


Figure 6.1.4 Three subclasses of N-glycan structure observed in mammalian cells.

A) High mannose structure in which there are mannose structures at the terminal positions, no fucosylation in core GlcNAc structure, represents early stage in the biosynthesis of glycans and typically found in yeast, B) Complex structure which exists in bi-, tri-, and tetra antennary forms, lactosamine units can be found in repeats, sialic acid present at the terminal galactose molecule, represents a late stage in the biosynthesis of O-linked oligosaccharides, C) Hybrid structure: The N-linked glycan of complex type is of pharmaceutical interest. Dashed box represents the core

Oligosaccharides in recombinant monoclonal antibody are generally composed of a heterogenous mixture of the G0F, G1F, and G2F structures (Figure 6.1.4). The relative ratios of the G0F, G1F, and G2F structures are dependent on cell line type, clone and cell culture process. The change in carbohydrates is observed in the terminal residues after the addition of mannose (green block). The glycan structure and carbohydrate linkages are similar in human and CHO cells except for the terminal N-AcetylNeuraminic acid (NANA). There are two possibility of bond formation in human α2,3 or α2,6 while in CHO only α2,3 is observed. In case of murine cell line like NS0 or SP2/0 terminal

galactose is linked with N-GlycolylNeuraminic acid (NGNA) with the help of α1,3 bond (Figure 6.1.5).

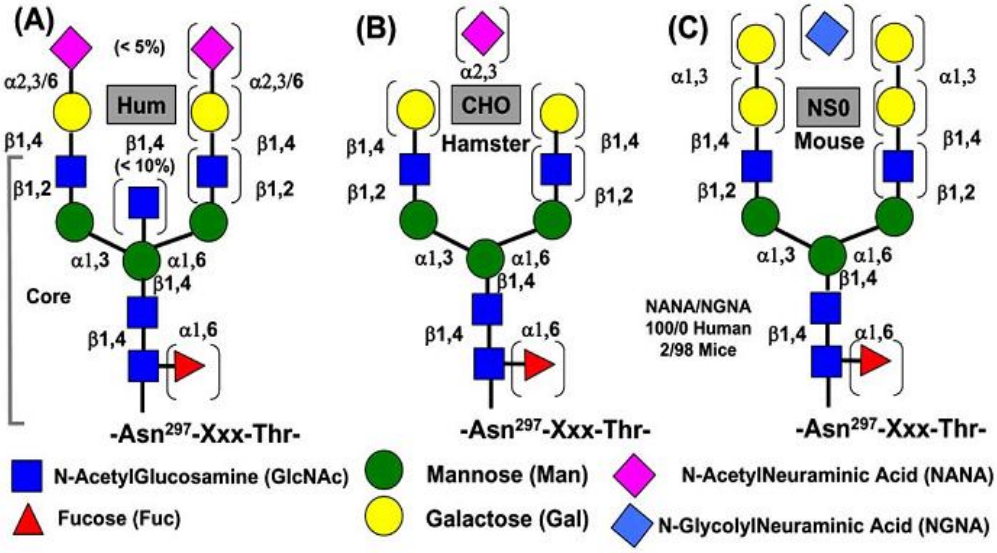


Figure 6.1.5 Various carbohydrate moieties observed in different cell lines.

The carbohydrate moieties shown are typically observed in recombinant monoclonal IgG molecules expressed in different cell lines. Typical glycosylation observed (A) Human, (B) CHO and (C) NS0 cells or SP2/0.

Glycosylation possess critical function for the proteins especially for therapeutic use. There are many cell functions of proteins which are affected by the characteristics of glycosylation such as protein folding, intracellular trafficking, biological activity, protein solubility, modulation of antigenic determinants, circulatory life-time, protein heterogeneity and protease protection and cell-to-cell recognition (Varki 1993). In addition to these functions, changes in the antibody glycosylation profiles can have a significant impact on antibody effector function. Therefore, glycosylation is considered as 'Product Quality Attribute (PQA)' which is important to be controlled for biosimilar mAbs and the glycosylation profiles should be similar to the RMP and consistent from lot-to-lot indicating that the cell culture process is well controlled and reproducible. In addition, mAb expression in CHO and murine myeloma cell lines may give rise to atypical glycosylated structures under some conditions which could have adverse effects on *in-vivo* efficacy, immunogenicity, or clearance (Jefferis 2005).

Furthermore, specific changes in oligosaccharide structure affect biological function. Various enzymatic and non-enzymatic modifications of oligosaccharide structure are

introduced during protein synthesis and assembly, cell culture process, purification, formulation, storage and incubation under various accelerated conditions often convert homogeneous population into a heterogeneous one. Thus, the oligosaccharides of mAb have micro-heterogeneity and their profile is often altered even under a defined set of culture and purification protocol. Apart from this the inherent property of the clone to make a particular glycan with a specific ratio of G0, G1, G2 forms are critical and required to establish biosimilarity of mAbs. The careful selection of single cell clones and optimization of cell culture conditions have been shown to impact the relative abundance of various antibody glycan structures. The enzymatic modification varies from cell to cell, hence determinination of the oligosaccharide / glycan structure is crucial and necessary for the identification of a Master Cell Bank (MCB) candidate clone.

6.2. Results and Disussions

From the results described in Chapter 5, Clone # D41E213 is the lead clone hence the mAb produced by this clone is characterized further using high-end analytical tool.

6.2.1. Determination of aggregates and degradants (clips) by Size Exclusion-HPLC

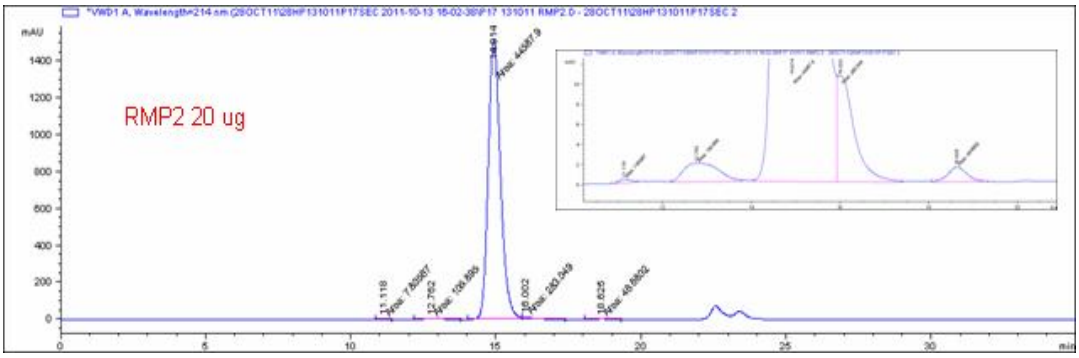
SE-HPLC is a method which separates molecules based on their size and hydrodynamic volume. Molecules pass through the column and depending on their sizes, elute through a stationary phase at different rate. Smaller molecules travel through the pores of the stationary phase while larger molecules are unable to enter all the pores and hence a separation is obtained where larger molecules elute first and the smaller molecules later. As the method described can resolve related forms based on their molecular size of mAb, it is used for quantitation of high molecular weight and low molecular weight related forms for purity analysis. There are numerous studies dealing with aggregation and dissociation processes, unfolding and refolding experiments available. The estimation of molecular mass in combination with size exclusions, crystallization studies and the biophysical characterization of the influence of salt concentration, pH and concentration of protein behavior in solution is important (Jangbauer, *et al* 2003).

High-performance size exclusion chromatography (SEC) is a simple method for molecular mass estimation, detection and quantification of aggregates formed and degradants generated during the process of culturing. This method suffers from a few restrictions that prevent correct molecular mass determination. Retention difference in SEC is caused by different diffusivities of the proteins into pores. In addition

electrostatic interaction and hydrophobic interaction with the matrix may give rise to aberrant retention. Aggregation of protein is a major problem in biopharmaceutical industry because of a possible loss of potency and visual appearance of the product. Although large aggregates are present in extremely low concentration, they may have a big impact on the quality of the product. SEC has been widely used to characterize protein aggregates present in human IgG (Jangbauer, *et al* 2003). Monitoring of aggregates at very early stage is important as clones differ in the levels of aggregates and therefore it is important to pick a clone which has low levels of aggregates. SEC also helps in monitoring the low molecular weight degradants or clips which could again be clone specific and/or process specific.

The Protein-A purified product from clone #D41E213 was tested for SEC-HPLC. TSK3000SWXL (Cat# 08541, Tosho) was used for the estimation of aggregates and degradants / clips. As represented in Figure 6.2.1, a main peak of mAb heterodimer contributing approximately 99% of total peak area was observed at Rt of ~15 min in RMP as well as test sample. A minor pre-peak observed before the main peak, contributing to 0.25% and 0.48% in RMP and test samples, respectively is indicative of aggregates present. The percentage degradants / clips are similar in RMP as well as test sample which appears as a hump associated with the main peak plus a small peak at Rt of ~18 min (Refer inset picture in Figure 6.2.1). The percentage of aggregates and clips are represented in Table 6.2.1.

A)



B)

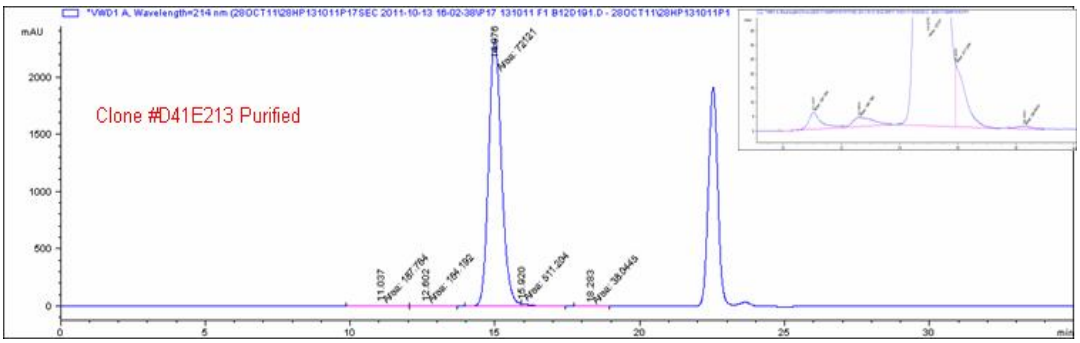


Figure 6.2.1 Comparison of expressed mAb from clone #D41E213 with RMP by SEC-HPLC.

A) Profile of RMP with inset picture zoomed to visualize pre and post peaks of main peak.

B) Profile of partially purified sample from clone #D41E213 with inset picture zoomed to visualize pre and post peaks of main peak.

Sample Name	Main Peak Area %	Total Aggregates Area %	Total Clips Area %
P17 RMP	99.033	0.245	0.721
P17-D41E213 Purified	98.766	0.482	0.752

Table 6.2.1 Comparison of RMP with expressed mAb from clone #D41E213 with RMP by SEC-HPLC

Although the percentage of aggregates is more in test samples than in RMP, it is not significantly higher and could be removed by incorporating another purification step during the final downstream process development while the total percentage of clips / degradants was almost similar (Table 6.2.1). The overall purity of Protein-A eluate of clone #D41E213 was ~98%. Hence the most important PQA of the mAb molecule is met.

6.2.2. Characterizing the charged forms observed on CEX-HPLC

The CEX-HPLC profile obtained for the Protein-A purified mAb from different clones are already explained in Chapter 4 (Final Clone Selection). The profile is depicted below for ease of understanding the further steps.

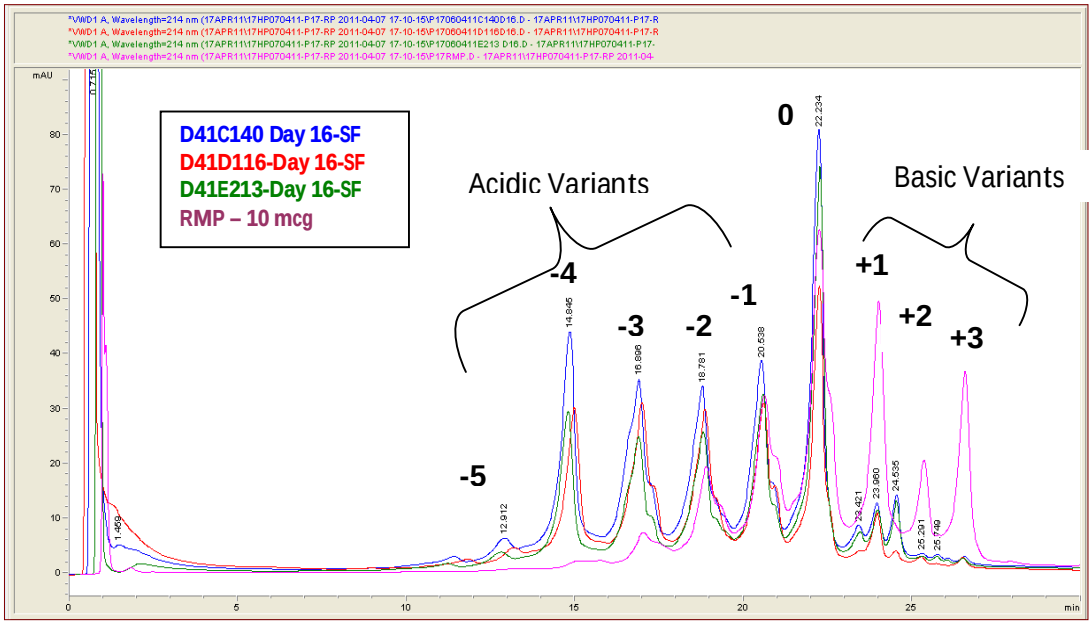


Figure 6.2.2 Cation Exchange HPLC for determination of charge variants (Same as Figure 4.2.7: The Protein-A purified harvest from each clone was analyzed for CEX profile by injecting approximately 5µg of protein. The peak labeling is done by assigning the K0 peak as 0.

About 15 µg RMP was digested with 0.8 µg carboxypeptidase to remove the C-terminal Lysine. The digested sample was then checked on CEX-HPLC. The basic peaks (+1, +2 and +3) shown in Figure 6.2.2 disappeared indicating that these peaks are indeed contributed by the C-terminal Lysine. These peaks are present in very small quantities in the expressed mAb as compared to that in the RMP hence there is less heterogeneity in the expressed mAb which in turn is advantageous in achieving batch to batch consistency during the final process and scale-up studies.

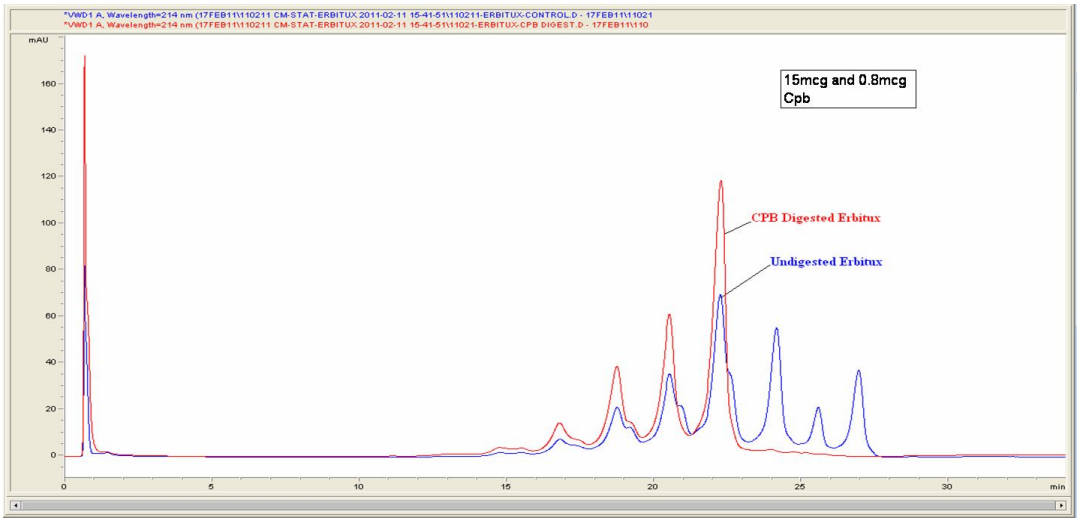


Figure 6.2.3 CEX-HPLC profile of RMP before and after CpB digestion.

The blue profile is charge heterogeneity observed in the product available in the market while the pink profile is after CpB treatment whereby the C-terminal Lysine is digested thereby reducing the heterogenity in the product.

6.2.3. Determination of N-terminal amino acid sequence by Edman Degradation Method

N-Terminal Sequencing uses a chemical process based on the technique developed by Pehr Edman (Edman 1950) and then automatized by Hugh Niall (Niall 1975) where by:

- The N-terminal amino acid reacts with phenylisothiocyanate (PITC).
- The derivatizing process results in a phenylthiohydantoin (PTH) – amino acid.
- This amino acid is then sequentially removed while the rest of the peptide chain remains intact.
- Each derivatization process is a cycle.
- Each cycle removes a new amino acid.
- The amino acids are sequentially analyzed to give the sequence of the protein or peptide.
- Routinely used for the analysis of membrane bound gel electrophoresis separated proteins, HPLC separated tryptic digest fragments, etc.
- Compatible with proteins and peptides prepared from a variety of sources, such as HPLC separation, PVDF blotting following electrophoresis, and peptide synthesis.

The RMP and protein A purified sample from clone #D41E213 was run on 10% SDS-PAGE reducing conditions and then transferred to PVDF membrane from where it was taken for N-terminal sequencing using Procise 492 (ABI) instrument.

In the heavy chain, the first amino acid is Glutamine which is present in the form of pyroglutamate. The sequencing reaction is blocked by this residue hence the first amino acid was chopped using Glutamyl aminopeptidase enzyme isolated from *Pyrococcus furiousus* (Werner, *et al* 2005).

The first five amino acid of light chain and heavy chain matched with that of the RMP as represented in Table 6.2.2

N-terminal Amino Acid	**1**	**2**	**3**	**4**	**5**	**6**
Light Chain – RMP	D	I	L	L	T	-
Light Chain – Test	D	I	L	L	T	-
Heavy Chain – RMP	Q	V	Q	L	K	Q
Heavy Chain – Test	Q	V	Q	L	K	Q

Table 6.2.2 N-terminal amino acid sequence comparison using Edman Degradation Method

The start of light chain and heavy chain protein sequence is same as that of the RMP thereby establishing the authenticity of the primary sequence.

6.2.4. Analysis of expressed product by LC-MS/MS

Mass spectrometry strategies for the identification of recombinant protein modifications and degradation products vary somewhat depending on the modification of interest and the type of MS instrumentation available. Despite these variations, most approaches involve the analysis of either intact proteins ("top down" approach) or peptides generated from enzymatic digestion ("bottom up" approach or "peptide mapping" approach). In either scenario, LC-MS is generally used for the analysis of process intermediates or final protein products when the presence of salts or other matrix components are not MS

compatible. The analysis of cell culture supernatant may require an additional "capture" step (eg. Affinity chromatography) to isolate the protein of interest from the spent cell culture media which contains a variety of feed components, host cell proteins, and cell debris (Battersby, *et al* 2001).

A general overview of an MS method for the analysis of intact and enzymatically digested recombinant proteins is depicted in Figure 6.2.4. This basic approach is widely used in proteomics application and biotechnology development and can provide verification of amino acid sequence, identification of post-translational modifications (PTMs), and degradation products, elucidation of glycoprotein oligosaccharide profiles and mapping of disulphide linkages for recombinant proteins of varying size (Nemeth-Cawley, *et al* 2003).

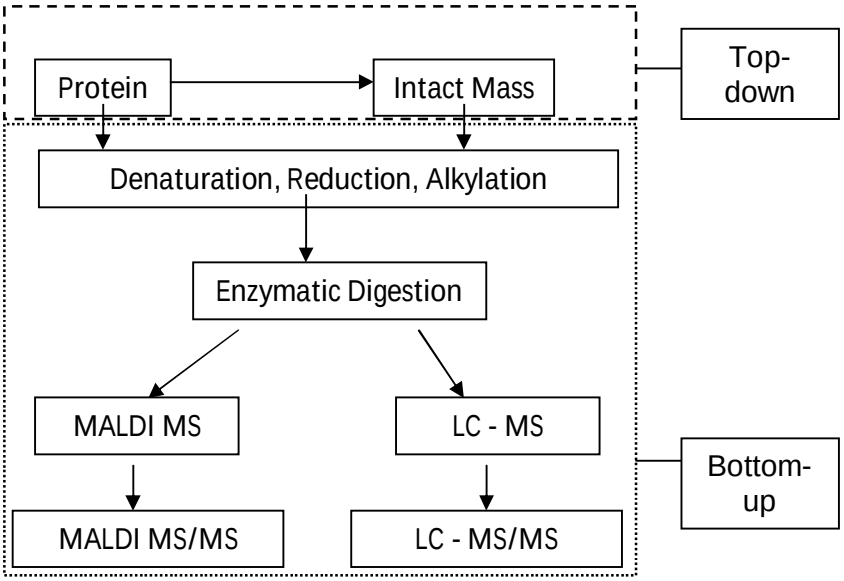


Figure 6.2.4 General approach for MS characterization of recombinant therapeutic proteins.

6.2.4.1. Analysis of Intact Proteins ("Top-Down" Approach)

The advantage afforded by MS analysis of intact proteins include: complete sequence coverage, assessment of overall protein heterogeneity, identification of labile post translational modifications (PTMs), and confirmation of N- and C-terminal sequences (e.g., N-terminal signal peptides). The approach also involves limited sample handling which may result in protein modifications that may not be related to the manufacturing process (e.g., Asn deamidation, carbomylation, Met/Try oxidation) [Barnes and Lim 2007].

Anti-EGFR mAb, Cetuximab has two consensus glycosylation sites at Asn_{299} and Asn_{88} on the Heavy chain. To interpret the complicated glycosylation pattern at the intact mass level would be an arduous task since two completely different glycans may have overlapping masses. We therefore attempted to reduce the mass of the antibody (IgG1) by cleaving the molecule with enzymes that can cleave the solvent exposed hinge region resulting in Fab and Fc fragments. This reduction in complexity would aid the individual analysis of the glycosylation sites. These fragments can be generated by papain, pepsin or a limited digestion with LysC. All of them have characteristic cleavage sites in the hinge region whose activity yields Fab (fragment antigen binding) and Fc (Fragment crystallizable) fragments. Papain makes a nick above the hinge region disulphides which is the case with LysC too. This reaction produces two individual Fab fragments composed of the light and the VH and CH1 domains of the heavy that are linked by a single disulphide. The other complementary fragment that is produced is the single Fc fragment which is composed of the heavy chains below the hinge region linked by 2 disulphides (CH2 and CH3 domains) with their respective glycans. The hinge region contains either two (IgG1 and IgG4) or four (IgG2) disulfide linkages between closely spaced Cys residues on the antibody heavy chains which are essential for formation of the antibody heterodimer (Burton 1985). This fragmentation enables the study of the modifications that pertain to the Fc and Fab areas individually without actually opting for the more endearing peptide mapping.

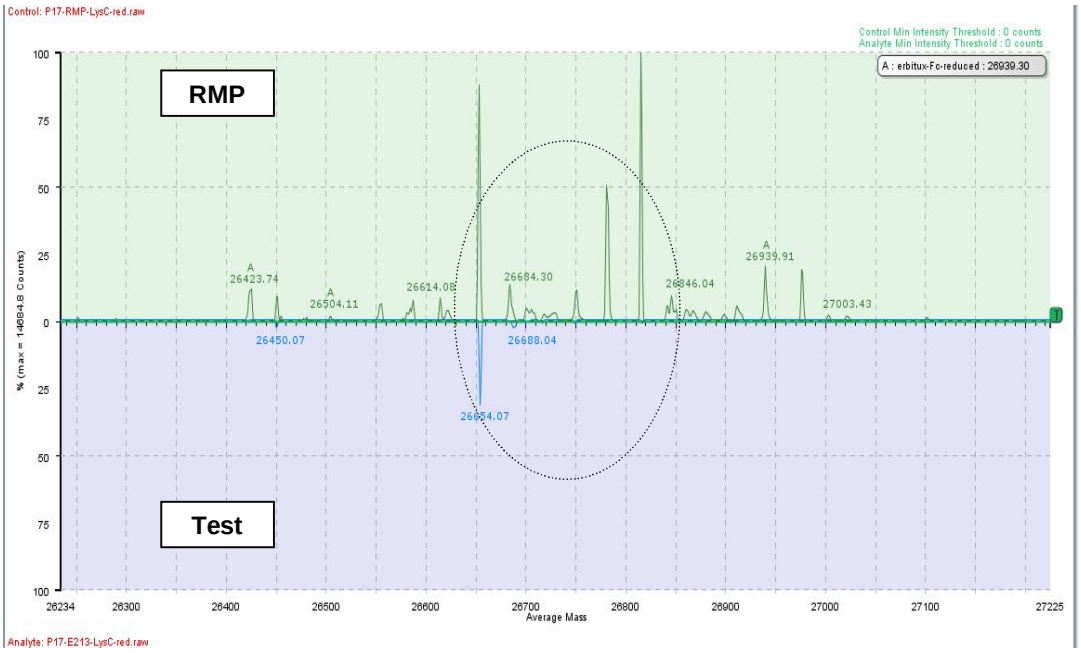


Figure 6.2.5 Deconvoluted spectra of Fc fragment after LysC digestion and reduction for molecular mass estimation.

The comparative profile is depicted for RMPand expressed mAb. The upper panel is for the RMP while the lower panel is for Test sample.

As shown in Figure 6.2.5, the main peak/species present is of MW 26654.07 daltons present in both RMP and test sample which corresponds to Fc fragment. The difference of 1.19 Da is due to the system variation. The other minor peaks may be due to various possible modifications as listed previously (e.g. deamidation, oxidation, etc.).

Similarly, the main peaks present in the Fab fragments obtained after Lys-C digestion and reduction are shown in the deconvulated spectra in Figure 6.2.6:.

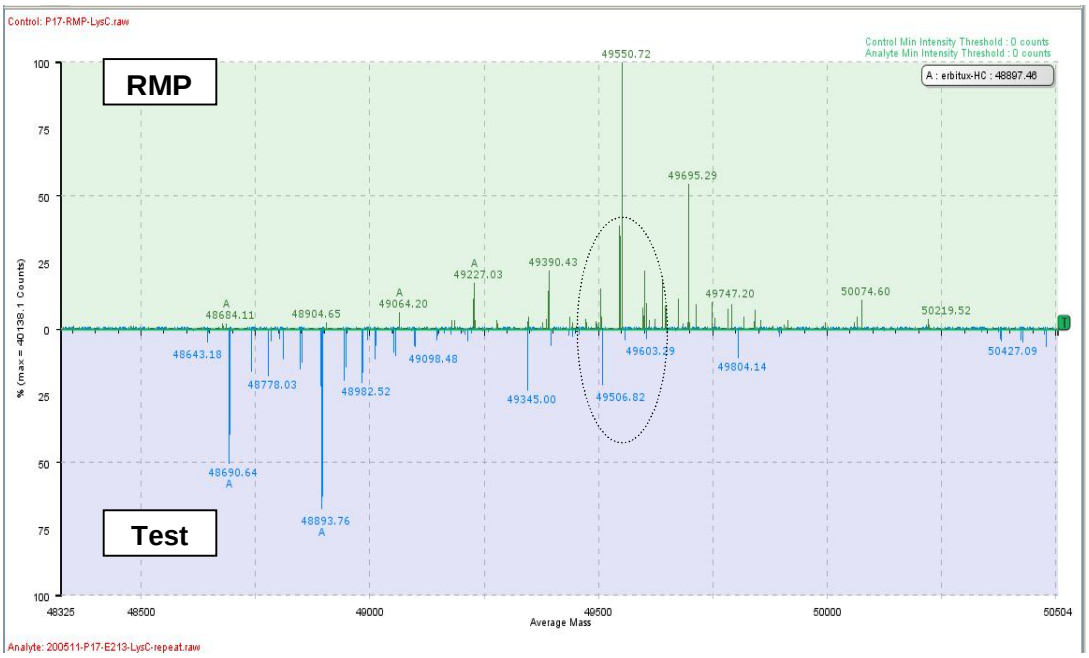


Figure 6.2.6 Deconvoluted spectra of Fab fragment after LysC digestion for molecular mass estimation.

The comparative profile of RMP and expressed mAb is shown. The upper panel is for the RMP while the lower panel is for the Test sample.

The expressed mAb (Cetuximab) was characterized by using the top down approach by LC-MS wherein sample integrity is preserved, avoiding chemical modifications intrinsic to proteolytic digestion. This high throughput method would indicate the intact mass of the RMP and expressed mAb sample for comparability and would indicate the impurities present. Intact mass analysis by LC-MS was performed on Waters SYNAPT mass spectrometer configured with the standard ESI source and in the positive ion mode.

Intact mass analysis of the recombinant and RMP LC and HC after reduction revealed the following masses (in Daltons):

	Observed Molecular Mass (Da)		
Fragment	**RMPs**	**Expressed mAb**	**Difference (Da)**
Fc	26652.14	26653.33	1.19
Fab	49503.43	49506.82	3.39
	49551.84	49556.86	5.02

Table 6.2.3 Determined mass of Fc and Fab fragment.

By employing the top-down approach the mass of Fc and Fab was confirmed and matched with the RMP to establish the identity of the expressed mAb.

6.2.4.3.Analysis of Glycan ("Bottom-up" Approach)

Analysis of the component peptides from enzymatic digestion of recombinant proteins can be used to locate PTMs, amino acid degradation product, glycosylation sites, and disulfide linkages within specific regions of the protein sequence. This approach is used commonly in biotechnology development as a fingerprint for identity testing, for process monitoring, and to demonstrate product comparability following manufacturing changes (Barnes and Lim, 2007).

The 'bottom-up" or peptide mapping approach is outlined in Figure 6.2.4 and generally involves denaturation, reduction, and alkylation of Cys residues prior to enzymatic digestion of the protein. The most common enzymes used for digestion are trypsin and endoproteinases Lys-C, Asp-N, and Glu-C. The resulting peptide mixture is then analyzed using MALDI MS or ESI MS with online liquid chromatographic separation of the digested peptides (LC-MS). To study the Fc and Fab glycosylation, the RMP and the expressed mAb, after Protein-purification, were partially digested with endoproteinase Lys-C to achieve a nick at the hinge region to generate two fragments Fab and Fc. The Lys-C digested fragments were reduced with DTT to simplify the complexity of the spectrum in LC-MS since both the heavy chains are glycosylated and there are two sites of glycosylation per heavy chain. The reduced Lys-C product was resolved to get Fab and Fc fragment on the CEX C8 Zorbax column (Figure 6.2.7) and

the eluting species were detected with ESI-tof. The raw spectrum was deconvoluted by standard parameters.

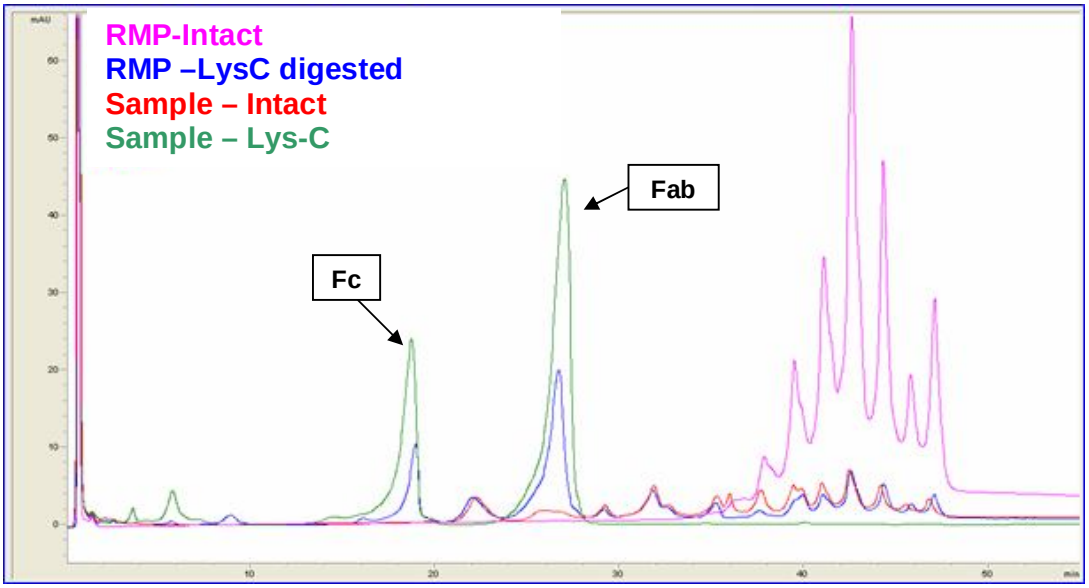


Figure 6.2.7 CEX-HPLC profile of RMP and expressed mAb digested with Lys-C and reduced
The pink profile is of the intact RMP while red is intact expressed mAb. The Lys-C reduced profile for RMP and expressed mAb is represented as green and blue, respectively.

The Fab and Fc reduced was then analyzed by ESI-tof LC-MS/MS.

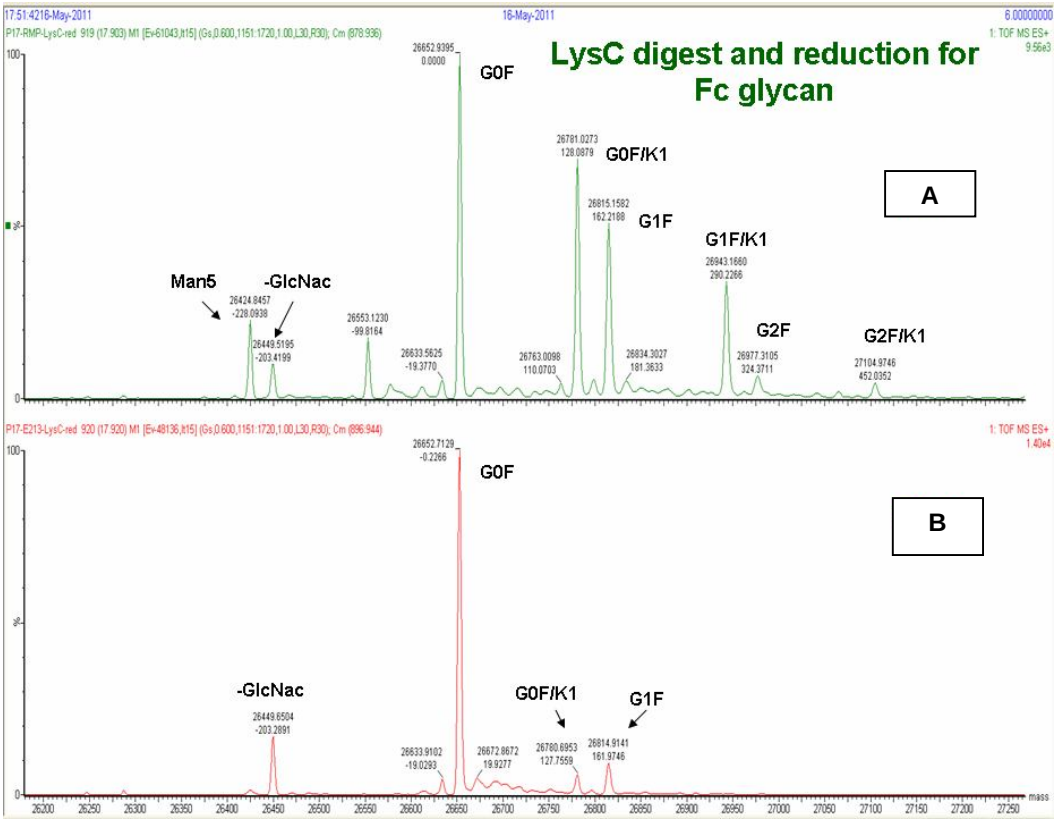


Figure 6.2.8 Deconvoluted spectra of Fc fragment to determine the Glycan forms.

A) Profile of RMP and B) Expressed mAb (clone #D41E213). The peak labels refer to the various glycoforms identified based on the deconvoluted mass. For deconvolution, m/z range from 26000 to 28000 Da was used.

As shown in Figure 6.2.8:, the G0F, G0F/K1 and G1F forms are present in both RMP and expressed mAb. The percentage of each forms are depicted in Table 6.2.4:

Glycan %	RMP	Clone # D41E213
Man5	12.70	0.00
G0F-GlcNac	3.18	12.81
G0F	**53.77**	**80.07**
G1F	**26.83**	**7.12**
G2F	3.52	0.00

Table 6.2.4 Percentage composition of glycan present on the heavy chain region of Fc domain.

The percentage of G0 and G0F are important and indicative of the glycosylation property of the host system. The other forms are not matching with the RMP because of the fact that the expression is in different host systems (CHO) and not in SP2/0. The G1 and G2 forms which are less than RMP can be improved during the fine tuning upstream process development at the bioreactor level. In this study the objective was to get the expressed mAb with as many forms as possible especially G0 and G1. .

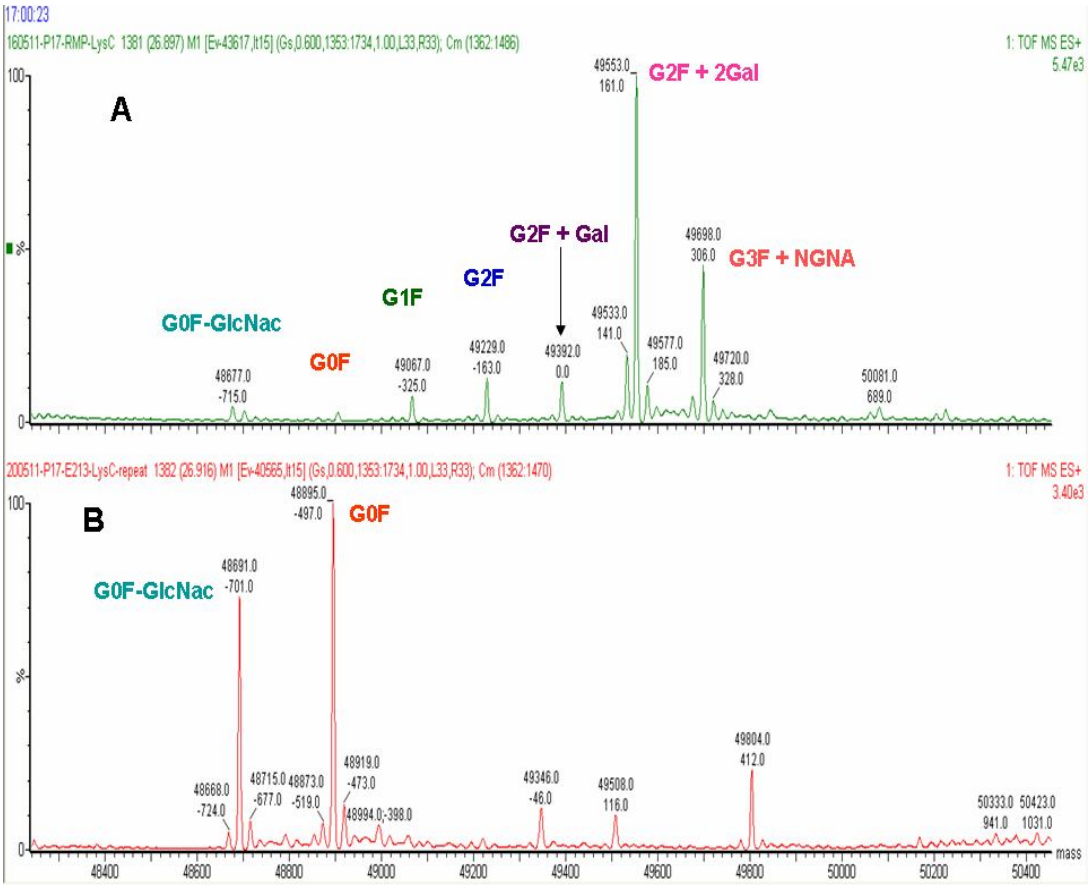


Figure 6.2.9 Deconvoluted spectra of Fab fragment to determine the Glycan forms.

A) Profile of RMP and B) Expressed mAb (clone #D41E213). The peak labels refer to the various glycoforms identified based on the deconvoluted mass. For deconvolution, m/z range from 47000 to 51000 Da was used.

As shown in 0, the G0F, G0F-GlcNac forms are present in both RMP and expressed mAb. The forms such as G1F, G2F, G2F+Gal, G2F+2Gal, and G3F+NGNA are not present in the expressed mAb. These forms are not preferred forms as they induces anaphylactic reactions and IgE- specific for Galactose α-1,3-galactose response in humans (Platts-Mills, *et al* 2008).

The percentage content of each forms are depicted in Table

Glycan %	RMP	Clone #D41E213
G0F-GlcNac	**0.00**	**42.17**
G0F	**0.00**	**57.83**
G1F	4.36	0.00
G2F	7.23	0.00
G2F+Gal	6.54	0.00
G2F+2Gal	56.34	0.00
G3F+NGNA	25.53	0.00

Table 6.2.5 Percentage composition of glycan present on the heavy chain region of Fab domain.

6.3. Conclusion

The recombinant mAb expressed (Cetuximab) by the lead clone #D41E213 was characterized to a greater extent using SEC-HPLC, N-terminal sequencing, CEX for charge variants and Mass Spectrometry for the final mass determination and glycan composition. The selected clone does not generate high levels of aggregates and clips/degradants which are not desirable to develop a simplified protein purification process. The heterogeneity in the expressed product is less compared to the RMP which will ensure better batch-to-batch consistency when the process is scaled-up to larger volumes. The detailed analysis of protein mass and glycans was done using ESI LC-MS/MS which revealed that the non-preferred forms of glycans (e.g. G2F, G3F, G3+NGNA, etc.) in Fab region are not present in the recombinant biosimilar mAb expressed in an alternate host (CHO), thereby indicating that the resulting biosimilar product from Clone #D41E213 would be less immunogenic. Also, the required forms in the Fc region are present (e.g. G0 and G1) which dictates the effector function of the mAb in-vivo, the levels of which can be tweaked around to make it comparable to RMP through upstream process development at bioreactor level.

Hence the recombinant CHO cell line expressing recombinant biosimilar anti-EGFR mAb (Cetuximab) do not produce any unwanted isoforms or glycan structures. Detailed comparison need to be established once the process is scaled-up in large bioreactors for the manufacture of the commercial product.

The quality of the product matches with the RMP to a considerable extent, not similar. This is understandable given that the biosimilar is expressed in an alternate host (SP2/0 → CHO) using different upstream process. However, the desirable forms (which determine the Product Quality Attributes) are all present while the undesirable forms (such as K1, K2 [CEX profile], G2, G2F-Gal, G2-Gal and G2-NGNA) are not found (or in very little proportion) in the CHO expressed product than the RMP. This makes CHO a better platform for production of recombinant biosimilar anti-EGFR mAb (Cetuximab). The functional significance of Fab glycosylation has not been fully evaluated but, it could have a neutral, positive or negative influence on antigen binding (Jefferis 2009). This molecule could be approved as biosimilar as the Fab glycosylation seen in molecule expressed in CHO do not have NGNA forms thereby have possibility of reduced anti-IgE response.

CHAPTER 7

SUMMARY

For the marketed version of anti-EGFR antibody, Cetuximab (Erbitux®), the production platform used by the originator company is myeloma cells, SP2/0. Typically a monoclonal antibody (mAb) molecule possess N-linked glycosylation site at Asn_{287} (Fc region) but Cetuximab possess a site Asn_{299} (Fc site) and also at Asn_{88} (Fab site). The complexity of this mAb is evident by the presence of 21 different glycoforms with around 30% capped by at least one α-1, 3-galactose residues, 12% capped by a NGNA residues and traces of oligomannose. It was reported by Platt-Mills *et al* in 2008, that the NGNA form in the Fc glycosylation site induces anaphylaxis and elicit IgE response in the humans. Hence the scope of this work was to clone, express and produce recombinant biosimilar Cetuximab in alternate host system, CHO cells which are known as not to produce NGNA forms. The regulatory perspective on manufacturing of biosimilar molecules is that changing a host system is allowed provided that the Product Quality Attributes especially the post-translation modifications, which are crucial for optimal biological activity, remains comparable with the Reference Medicinal Product (RMP) and should be safe for human use.

In pursuit of establishing a stable high expressing cell line for anti-EGFR antibody various vector constructs were designed with dual promoter system and selection marker were evaluated. The dual promoter system facilitated expression of light chain and heavy chain of antibody (IgG) at similar molar concentration. The vector backbone used was of pcDNA3.1 which is commercially available from Invitrogen, USA.

Various modes of transfections like lipofection, electroporation and nucleofection were evaluated to generate high expressing cell line. The expression of target mAb in SP2/0 cells was observed to be unstable and it weathered after few days post transfection. On the other hand, when the transfection was done in CHO cells the expression post transfection observed was high and stable during the *in-vitro* culture. Due to instability of SP2/0 cells and the advantages of using CHO cells for expression of recombinant mAb, the rCHO was selected for the further experimentation.

After transfection the clonal population was generated using manual limiting dilution and automated clone selection method (ClonePix FL®). Three top clones based on the productivity and cell growth analysis scored highest amongst hundreds of clones and two methods of selection were selected further as D41E213, D41C140 and D41D116. The clone stability, expression analysis and determination of Product Quality Attributes

conferred that clone D41E213 was the lead clone. The analytical techniques such as Western Blot, Iso Electric Focusing, SDS-PAGE, Protein-A HPLC, Cation Exchange Chromatography, RP-HPLC and *in-vitro* bioassay were used for determining the PQAs and identifying the lead clone. These methods gave considerable information at an early stage and are considered as platform methods to be used at clone selection stage for all mAb products in general.

The selection of cell cultivation media was important for the sustained and higher growth of the cells and to support the expression of recombinant protein. Upstream process development for screening basal media and feed supplements were carried out, whereby 18 different basal media and 18 different feed supplements (commercially available) were tried to increase the titer. A significant increase of 2.5 fold in product titer (~390 mg/L) was observed when basal media CDM4PerMab (Hyclone) and CHO Efficient Feed A: CHO Efficient Feed B = 50:50 (Invitrogen) mixture was used.

The recombinant mAb expressed (Cetuximab) by the lead clone # D41E213 was further characterized to a greater extent using SEC-HPLC, N-terminal sequencing, CEX for charge variants and Mass Spectrometry for the final mass determination and glycan composition. The selected clone did not generate high levels of aggregates and clips/degradants which were not desirable to develop a simplified protein purification process. The heterogeneity in the expressed product was less compared to the RMP which ensured better batch-to-batch consistency when the process was scaled-up to larger volumes. The detailed analysis of protein mass and glycans was done using ESI LC-MS/MS which revealed that the non-preferred forms of glycans (e.g. G2F, G3F, G3+NGNA, etc.) in Fab region were not present in the recombinant biosimilar mAb expressed in an alternate host (CHO), thereby indicating that the resulting biosimilar product from Clone #D41E213 was less immunogenic. Also, the required forms in the Fc region are present (e.g. G0 and G1) which dictates the effector function of the mAb *in-vivo*, the levels of which can be tweaked around to make it comparable to RMP through upstream process development at bioreactor level.

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CHAPTER 8

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PUBLICATIONS

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1. **Hatim Motiwala**, Archana Gattupalli, Brajesh Varshney, Rustom Mody (2012), Developing an alternate host for production of biosimilar anti-EGFR monoclonal antibody. Intl Jl of Pharmacy Biol Sci, 2(4): 90-100.

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2. **Hatim Motiwala**, Sona Jain, Archana Gattupalli, Brajesh Varshney, Rustom Mody (2013), A comparative study of manual and automated clone selectilon methods for recombinant CHO cells.. Bioprocessing Journal, USA (Accepted)

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3. April 2012: Bioprocess International Conference, Prague, Czech Republic. (www.ibclifesciences.com/BPI)
4. September 2012: International conference and exhibition on biowavers and biosimilars, San Antonio, USA. Abstract published in Journal of Bioequivalence Available, Volume 4(7):103. ISSN: 0975-0851
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