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, aspartate 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.
Afucosylation & Fucose content inversely proportional to ADCC

Galactose Content & D’egalactosylation 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 immunogeneity and reduces bioactivity

Aggregation & Covalent and non-covalent aggregation could lead to drop in potency

Biological Activity & Measure the potency of the product

<table>
<thead>
<tr>
<th>Other PQA that have no significant impact on function but on purity of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-terminal Lys Variant &amp; Has no direct impact on biological activity</td>
</tr>
<tr>
<td>N-term pE &amp; Spontaneous physicochemical conversion and has no functional significance</td>
</tr>
<tr>
<td>HCP &amp; Indicative of consistency of process. Directly impacts product quality and immunogenecity</td>
</tr>
<tr>
<td>resDNA &amp; Indicator of consistency of process. Directly impacts product quality and immunogenecity</td>
</tr>
<tr>
<td>resProtein-A &amp; Indicative of consistency of process. Directly impacts product quality and immunogenecity</td>
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</tbody>
</table>

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 \textit{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 \textbf{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 (1 μL = 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.

![Western Blot Analysis for identity](image)

Figure 4.2.3 Western Blot Analysis for identity

M - Rainbow Molecular weight marker (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 immunopositive 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.

![Image of IEF gel](image.png)

**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 pl of the target mAb without modifications calculated to be 8.6 (www.expasy.org). In the RMP mainly 5 bands are seen around pl 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 pl 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.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.
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 sulfhydryl 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) 

C) 

<table>
<thead>
<tr>
<th>Injections amount µg</th>
<th>Peak Area</th>
<th>Back Calculated amount</th>
<th>% Recovery</th>
</tr>
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</tr>
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<td>15</td>
<td>1990031.00</td>
<td>15.43</td>
<td>102.89</td>
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</table>
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

<table>
<thead>
<tr>
<th>Date of receiving</th>
<th>Date of Analysis</th>
<th>Loading Vol. (ml)</th>
<th>Sample Name</th>
<th>Main peak area</th>
<th>Amount of mAb in the injected vol. (ug)</th>
<th>Calculated expression conc. (mg/L)</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
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</tbody>
</table>

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.

![Cation Exchange HPLC for determination of charge variants.](image-url)

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

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<th>% of individual isoforms</th>
<th>Remarks</th>
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</table>

† 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.
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.