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 transgene 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

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 promoter / priming site</td>
<td>Allows for in-vitro transcription in the sense orientation and sequencing through the insert</td>
</tr>
<tr>
<td>Multiple cloning site</td>
<td>Allows insertion of gene of interest and facilitates cloning.</td>
</tr>
<tr>
<td>Bovine growth hormone (BGH) polyadenylation signal</td>
<td>Efficient transcription / termination and polyadenylation of mRNA (Goodwin and Rottman 1992)</td>
</tr>
<tr>
<td>f1 origin</td>
<td>Allows rescue of single stranded DNA</td>
</tr>
<tr>
<td>SV40 early promoter and origin</td>
<td>Allows efficient, high-level expression of the selection marker like Neomycin or GS gene and episomal replication in cells expressing SV40 large T antigen</td>
</tr>
<tr>
<td>Neomycin Resistance gene</td>
<td>Selection of stable transfectant in mammalian cells (Southern and Berg 1982)</td>
</tr>
<tr>
<td>SV40 early polyadenylation signal</td>
<td>Efficient transcription / termination and polyadenylation of mRNA</td>
</tr>
<tr>
<td>pUC origin</td>
<td>High-copy number replication and growth in E. coli</td>
</tr>
<tr>
<td>Ampicillin resistance gene (β- lactamase)</td>
<td>Allows selection of transformants in E. coli</td>
</tr>
</tbody>
</table>
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. The details of each construct are described in the following text. The pSRM series denote the specific modifications carried out in the parent pcDNA3.1(-) vector.

- 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:

![Construction Diagram]

Digestion with BstBI and XmaI

Ligation & transformation in E.coli
The Glutamine Synthetase gene sequence was taken from European Patent No. EP0333033 which is expired in Sep 2009.
The gene was chemically synthesized from GeneArt, Germany and optimized for expression in CHO cells. The pMA vector supplied by GeneArt possess GS gene. The restriction enzyme (RE) digestion of pcDNA3.1(-) and pMA vector with BstBI and Xmal 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.

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 DH5a 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 plasmid isolated from pSRMO1 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 (BglII site) to 896 (NheI site) was PCR amplified using specific primers as follows:

Forward primer - SR171 (5'-CGCTCGAGATCGGGCTTGGGATTCC-3') and
Reverse primer - SR172 (5'-AGTGGATCCCAGCTTGGGTCTCC-3'), and incorporating NotI and BamHI 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 NotI and BamHI 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 10³. Plasmid Miniprep from 10 colonies were used for RE diagnostics and screening for positive clones. When the plasmids from selected clones were digested with Ndel, 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 Ndel & SacI enzyme whereby two fragments as expected were observed (Figure 3.1.5:D).
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 NolI and BamHI.
(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 Ndel enzyme. Ndel is present in CMV promoter site and hence when a second CMV-2 promoter was inserted there will be introduction of one more Ndel 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 BamHI (5') & NorI (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.

![Schematic representation of construction of pSRM05 vector.](image)
The CMV-2 was ligated to pcDNA3.1 (-) using T4 DNA ligase to yield pSRM05 vector. 
*E. coli* DH5α 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(-).

![Figure 3.1.7 Screening of pSRM05 transformants by colony PCR.](image)

Checked on 1% agarose gel.

Clone #1 and #2 were selected for RE analysis. The plasmids isolated from these transformants were digested with BgIII 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.
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
Construction of S8.1 Expression Vector.
The E18_L1 (GeneArt) Vector was digested with *NheI* and *XbaI* 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.](image)

A) RE digestion of pSRM02 Clone # 8 and E18_L1 (GeneArt) Vector,
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, GeneArt Vector pMAT E18_H1 and pSRM02+L1 Clone #H were RE digested with *BamHI* and *AflII* 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'-CGGACACGCTACAGTGAAT-3')

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)
This vector was transfected in SP2/0 cells which is designated as S8.1 and in CHO cells designated 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. The S8.1 expression vector and pcDNA3.1 (-) were digested with BstB1 and XmaI to release fragments 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 3.1.12A). 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.

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 and pcDNA3.1(-) were digested with Ncol. Ncol 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 3.1.12). 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 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 with GS as selection marker and S8.3 Expression Vector with Neomycin Transferase as selection marker was constructed and 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).
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.

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.
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 is 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.](image)

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:
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:

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Plasmid DNA</th>
<th>mRNA Transfection</th>
<th>Co-transfection</th>
<th>Transfection of Primary and Secondary Cell Lines</th>
<th>Stable and Transient Transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>B50</td>
<td>BAEC</td>
<td>BHK-21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calu1</td>
<td>COLO</td>
<td>HEL299</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L929</td>
<td>MEF</td>
<td>MPC5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTEPA2</td>
<td>RAW264</td>
<td>RBL-2H3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp2/Agl4</td>
<td>WEHI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(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:

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Plasmid DNA</th>
<th>mRNA Transfection</th>
<th>Co-transfection</th>
<th>Transfection of Primary and Secondary Cell Lines</th>
<th>Stable and Transient Transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>B50</td>
<td>BAEC</td>
<td>BHK-21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calu1</td>
<td>COLO</td>
<td>HEL299</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L929</td>
<td>MEF</td>
<td>MPC5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTEPA2</td>
<td>RAW264</td>
<td>RBL-2H3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp2/Agl4</td>
<td>WEHI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
> 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](image)

> 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:
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 heterogeneity in the population by plating few cells per well called “Minipool”. The minipoools 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

Vector Construction & Gene Cloning

Transfection (1-2 ug DNA) – in 6 well TC plate

Selection of Pools upon G418 application

Gene amplification using MSX (if applicable)

Protein expression analysis by ELISA/WB

Dilution cloning -1: 2-3 x 96 well plates
   Plating @ ~100 cells / well

Expression analysis & short listing of ~3-5 minipools

Dilution Cloning -2: 2-5 x 96 well plates of best producer MP from Dil. Cloning – 1 & Plating @ 1-5 cells / well.

Screening of ~ 200 Clones from each pool by WB

Clone Selection, 5-10 from each selection procedure based on cell growth and productivity

Western Blot & ELISA

Alternatively, outsourced for automated selection using...
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 $0.4 \times 10^6$ cells/well (at $0.2 \times 10^6$ 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

\[
\begin{array}{cccccc}
\text{A} & \text{C} & \text{D} & \text{E} & \text{Ref} & \text{M} \\
199 & 116 & 86 & 50 & 37 & 29 \\
19 & 29 & 37 & 50 & 86 & 116 \\
\end{array}
\]

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.
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.
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.
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 10⁶ 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).
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.
As shown in the above figure, a good detectable expression of mAb of interest was observed when SP2/0 were transfected using TransIT reagent (Pool J) compared to Lipofectamine 2000 (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.
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.

![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.](image)

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

The percentage confluence 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.

Figure 3.2.18  Comparison of Expression in Pool D Minipools by ELISA
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.
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 cm² 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.

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](image)

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
The fluorescence intensity depends on level of expression of antibody by the transfected cells (Figure 3.2.23).

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.

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
30 clones were shortlisted for further analysis. The quantitative expression of recombinant mAb was done using Sandwich ELISA (Table 3.2.1).

<table>
<thead>
<tr>
<th>Proximity</th>
<th>%Confluency (day 6)</th>
<th>Conc (ug/ml)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB1</td>
<td>0.193</td>
<td>60</td>
<td>3.65</td>
</tr>
<tr>
<td>IA3</td>
<td>0.287</td>
<td>67</td>
<td>1.16</td>
</tr>
<tr>
<td>IB3</td>
<td>0.292</td>
<td>56</td>
<td>0.80</td>
</tr>
<tr>
<td>ID2</td>
<td>0.498</td>
<td>55</td>
<td>0.72</td>
</tr>
<tr>
<td>IE2</td>
<td>0.25</td>
<td>45</td>
<td>0.93</td>
</tr>
<tr>
<td>ID3</td>
<td>0.35</td>
<td>28</td>
<td>0.29</td>
</tr>
<tr>
<td>IA4</td>
<td>0.334</td>
<td>42</td>
<td>0.71</td>
</tr>
<tr>
<td>IB8</td>
<td>0.711</td>
<td>50</td>
<td>1.86</td>
</tr>
<tr>
<td>IB4</td>
<td>0.193</td>
<td>52</td>
<td>1.50</td>
</tr>
<tr>
<td>IC4</td>
<td>0.27</td>
<td>47</td>
<td>1.87</td>
</tr>
<tr>
<td>ID6</td>
<td>0.161</td>
<td>80</td>
<td>0.118</td>
</tr>
<tr>
<td>IS4</td>
<td>0.248</td>
<td>82</td>
<td>0.56</td>
</tr>
<tr>
<td>IB2</td>
<td>0.38</td>
<td>55</td>
<td>2.18</td>
</tr>
<tr>
<td>IS5</td>
<td>0.111</td>
<td>55</td>
<td>3.63</td>
</tr>
<tr>
<td>IA5</td>
<td>0.278</td>
<td>70</td>
<td>1.54</td>
</tr>
<tr>
<td>IB8</td>
<td>0.295</td>
<td>68</td>
<td>3.74</td>
</tr>
<tr>
<td>IB5</td>
<td>0.805</td>
<td>62</td>
<td>1.81</td>
</tr>
<tr>
<td>IS7</td>
<td>0.493</td>
<td>43</td>
<td>5.33</td>
</tr>
<tr>
<td>IA7</td>
<td>0.276</td>
<td>50</td>
<td>1.98</td>
</tr>
<tr>
<td>IB6</td>
<td>0.321</td>
<td>48</td>
<td>1.79</td>
</tr>
<tr>
<td>IB7</td>
<td>0.216</td>
<td>57</td>
<td>6.17</td>
</tr>
<tr>
<td>IB7</td>
<td>0.193</td>
<td>74</td>
<td>6.12</td>
</tr>
<tr>
<td>IB9</td>
<td>0.864</td>
<td>65</td>
<td>3.56</td>
</tr>
<tr>
<td>IB5</td>
<td>0.845</td>
<td>39</td>
<td>0.68</td>
</tr>
<tr>
<td>IB8</td>
<td>0.893</td>
<td>50</td>
<td>3.26</td>
</tr>
<tr>
<td>IB6</td>
<td>0.893</td>
<td>52</td>
<td>0.83</td>
</tr>
<tr>
<td>IB6</td>
<td>0.421</td>
<td>54</td>
<td>0.54</td>
</tr>
<tr>
<td>IB9</td>
<td>0.805</td>
<td>53</td>
<td>0.39</td>
</tr>
<tr>
<td>ID12</td>
<td>0.885</td>
<td>46</td>
<td>0.46</td>
</tr>
<tr>
<td>IA12</td>
<td>0.806</td>
<td>5</td>
<td>0.82</td>
</tr>
</tbody>
</table>

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

![Table 3.2.2](image)

Table 3.2.2   Comparison of top 10 clones selected by ClonePix.

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

![Table 3.2.3](image)

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 CloncPix 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 (CloncPix) 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 CloncPix selected clones
The clones were grown in CD-CHO media in 6well and T25 cm² 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 (CloncPix Selected) were shortlisted. These clones were compared across different scale of culturing for productivity analysis (Figure 3.2.26).

![Comparison of Manually vs Automated Selected Clones](image)

**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 cm² 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 $\sim 2 \times 10^6$ cells/vial while for the primary cell bank the cell density preserved was $\sim 10 \times 10^7$ 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 $\sim 10$ vials at $\sim 10 \times 10^7$ 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 °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.](image)

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 10⁶ 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 10⁶ cell/mL in a total volume of 40 mL. For the first three days cells were grown at 37 °C temperature after which the temperature was brought...
down to 32 °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)

**Growth Profile of Three Top Clones in Shake Flask**

(B)

**IVCC Profile in Shake Flask**

**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 $6.0 \times 10^7$ cell/mL while in other two clones it was not more than $5.0 \times 10^7$ 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 → 500L, 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 T25cm² TC flask in presence of selection pressure of antibiotic (Geneticin G418). The stability was determined by estimating recombinant protein production (Figure 3.2.30).

![Clone Stability With G418 - T25 Flask](image)

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.

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.

![Clone Stability - Shake Flask -Without G418](image)

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Test</th>
<th>Clone D41D116</th>
<th>Clone D41C140</th>
<th>Clone D41E213</th>
<th>RMP (SP2/0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>Western Blot</td>
<td>Show positive band and size of band comparable with RMP</td>
<td></td>
<td></td>
<td>~150 kDa</td>
</tr>
<tr>
<td></td>
<td>Protein A HPLC</td>
<td>Main peak in HPLC matches with main peak in RMP</td>
<td></td>
<td></td>
<td>Main peak at Rt</td>
</tr>
<tr>
<td></td>
<td>N-terminal Sequencing</td>
<td>First five amino acid of Light chain and Heavy chain are identical to that of RMP</td>
<td></td>
<td></td>
<td>LC = DILLT HC = QVQLKQ</td>
</tr>
<tr>
<td>Purity</td>
<td>CEX-HPLC</td>
<td>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.</td>
<td></td>
<td></td>
<td>A total of 7 distinct peaks observed</td>
</tr>
<tr>
<td></td>
<td>IEF</td>
<td>Shows anomaly in the band pattern</td>
<td>A total of 7 band matches with RMP</td>
<td></td>
<td>A total of 7 distinct bands observed</td>
</tr>
<tr>
<td>Quality</td>
<td>LC-MS/MS</td>
<td>Not Done</td>
<td>Not Done</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>In-vitro Bioassay</td>
<td>44%</td>
<td>33%</td>
<td>55%</td>
<td></td>
</tr>
</tbody>
</table>

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