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Three steps to start you on the path to obtaining the 'best' recombinant CHO cell line

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Introduction

The keys to developing biomanufacturing processes which are efficient, robust and of high quality start during cell line and process development. It is therefore imperative that the expression system ('host cell line' and 'vector') and the process to isolate and identify the best clonal cell line ('clonal cell line development process') are of the highest quality. Getting it 'right first time' at the start of development can allow one to avoid later challenges. At FUJIFILM Diosynth Biotechnologies (Fujifilm), a team of scientists invested more than 20 FTE years undertaking innovation work which has culminated in the development of a new host cell line and vector, along with an optimised clonal cell line development process (the Apollo™ mammalian expression platform).

Host Cell Line

A host cell line that is traceable and both well-characterised and compatible with production requirements can save a substantial amount of development time in the future. Key elements when selecting a new host cell line include the ability to grow well in chemically-defined, suspension conditions and the capability for high expression of recombinant proteins. If a host cell line displays these superior characteristics, there is a good chance that this attribute will be heritable in recombinant cell lines. The benefits, for example, of improved growth include the potential for decreased cell line development (CLD) timelines and the potential to decrease the manufacturing seed train duration. The benefit of having the capability for high expression speaks for itself. It is also advantageous to have created a fully-characterised and documented cGMP cell bank for the host cell line. This ensures a high quality start point for any cell line development work, that is proven to be free from adventitious agents. Furthermore, if the host cell bank has tested negative for bovine and porcine viruses, this then removes the need to repeat such tests if no animal derived products are used during the CLD process, thereby reducing future costs.

So how can you develop a new and improved host cell line? In recent years, much interest has been placed into targeted genetic engineering of specific cellular processes to create new CHO cell populations with improved functionality[1]. However, the identification of suitable engineering targets has proven difficult, success had been varied and it is highly likely that intervention at multiple targets would be required. Furthermore, uncertainties about intellectual property and "freedom to operate" surrounding targets and the methods by which they are engineered can make this approach an undesirable proposition. An alternative approach is one which falls under the heading 'directed evolution'[2].

CHO cell populations are functionally heterogeneous i.e. a mixed population of individual cells each with its own particular set of functional attributes which render it either a suitable or unsuitable production vehicle. Indeed, this clonal variation is already exploited during cell line development when large numbers of potential cell lines are screened to isolate those with the most desirable attributes for biomanufacturing. 'Directed evolution' based approaches therefore aim to control/manage this clonal variation by either improving the functional capability of, or removing unsuitable variants from, the parental host population. The selection of CHO cell variants with improved characteristics is typically an iterative process comprising several rounds of induced selective pressure. Examples include relatively simple approaches such as extended cultivation of cell lines in altered subculture regimes or limiting dilution cloning. Indeed, efforts to adapt cell lines to serum-free, chemically-defined media will be a familiar 'directed evolution' approach to many. Other, more complex examples include using fluorescence-activated cell sorting (FACS) to enrich for cells with extended viability and growing cells in chemostat culture.

During the development of a new host cell line, a very large panel of potential new host cell lines can be obtained. It is therefore important to have a well-designed hierarchical screening strategy to gradually decrease these numbers and identify those cell lines with superior characteristics. Therefore, any screens used in this process should also assess the multiple desirable attributes you wish to identify in recombinant cell lines. For example, growth characteristics, expression capability and ability to 'fit' with available screening technologies. At Fujifilm, we have demonstrated that it is possible to identify host cell lines with improved characteristics using such techniques (see Figure 1). Indeed, the host cell line which forms part of the Apollo™ mammalian expression platform originates from this directed evolution work.

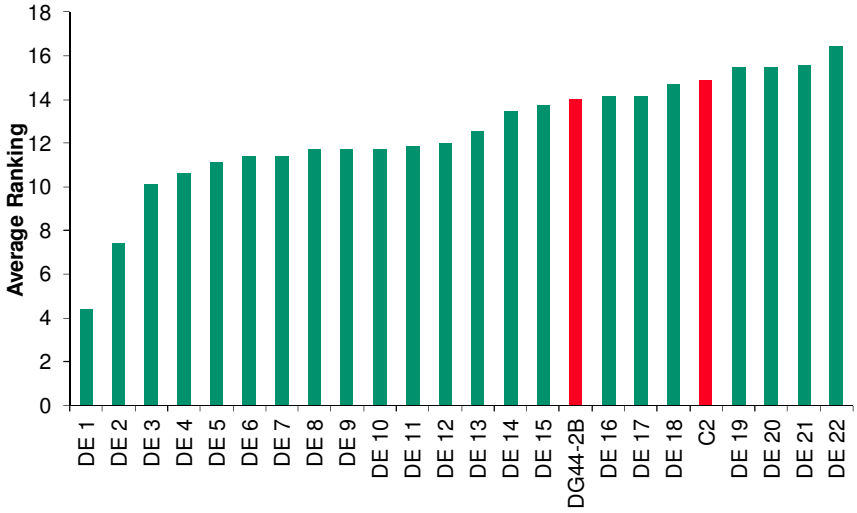

Figure 1: Average ranking for 15 out of 22 potential new host cell lines improved compared to that of an original host cell line (DG44-2B). *Following assessment in multiple screens, average ranking calculated for each host cell line, with 1 being the highest and 18 the lowest rank. Key: DG44-2B = original host cell line; C2 = new host cell line following adaptation to chemically-defined medium and suspension culture; DE = new host cell line following directed evolution approach applied to host cell line C2.*

Vector

A mammalian expression vector comprises of multiple components which contribute to the successful expression of a recombinant protein. Components include selection markers, transcriptional regulatory elements (e.g. promoters, enhancers), Kozak translation initiation sequences, leader sequences and polyadenylation signals. Here we will explore some of these components in a little more detail.

A sequence for a selectable marker protein is normally included in the vector, as well as the gene(s) of interest (GOI), as it enables the selection of a cell line producing the recombinant protein. Selectable marker genes used typically confer antibiotic resistance or some other easily detectable phenotype[3]. The two selection systems most commonly used in industry are (i) Dihydrofolate Reductase (DHFR) or another selectable marker in conjunction with DHFR and (ii) Glutamine Synthetase (GS).

Taking the DHFR system as an example to explore use of selection markers further: When using the DHFR expression system, host cell lines that are deficient in endogenous DHFR are typically used (e.g. DG44 and DUXB11), but it is not an absolute requirement. DNA replication requires DHFR as it catalyses the synthesis of a purine (hypoxanthine), thymidine and glycine[4]. Cells that are deficient in DHFR therefore require hypoxanthine, thymidine and glycine in order to grow. Consequently, if cells lacking this enzyme have the gene for DHFR introduced into them, they will be capable of growth in medium lacking these components[5]. And if the vector contains the GOI along with the DHFR gene, those cells which are capable of growth in such media are also likely to produce the recombinant protein.

The drug methotrexate (MTX) can irreversibly block the action of DHFR, and by increasing the concentration of MTX in the medium, drug-resistant cell lines can be isolated. These cell lines are able to overcome the effect of increased MTX by producing more DHFR, which is possible with increased copies of the DHFR transgene. This process of increasing gene copy number is known as amplification. As DNA adjacent to the DHFR gene also tends to be amplified, the GOI is frequently amplified[6]. It is this phenomenon that is often responsible for the isolation of higher producing cell lines[5]. Due to this action of MTX, DHFR expression vectors may employ a different selection marker, such as a gene conferring antibiotic resistance, and the DHFR itself may just be present as an amplifiable marker.

Fujifilm's new expression vector, part of the Apollo™ mammalian expression platform, employs the use of DHFR as a selectable marker in conjunction with a DG44 derived host cell line. However, it is not used as an amplifiable marker. Fujifilm is able to generate high producing cell

lines without the need for amplification, thereby avoiding some of the downsides of amplification such as longer timelines to complete amplification steps and the hypothetical risk of increased cell line instability.

A promoter is required for the transcription of the gene under its control. For high level expression of the GOI a strong promoter is required which will allow high levels of transcription. However, the promoter driving expression of the selectable marker is typically weak, allowing a lower level of expression. The consequence of this is that it increases stringency of selection: To be able to survive, the vector must have integrated into a highly transcriptionally active site for the cell to produce adequate amounts of the selectable marker protein. The cell then finds itself in a favourable position whereby the GOI is also in that highly transcriptionally active loci and high levels of transcription of the gene can be achieved.

The leader sequence encodes a leader peptide that is required for the translocation of the newly synthesised protein product into the lumen of the endoplasmic reticulum. This process can be one of the rate limiting steps in the secretion of proteins from the cell. An important parameter in determining the efficiency of this step is the sequence of the leader peptide. The native sequence is not necessarily the most efficient and alternative sequences can alter the rate of secretion. At Fujifilm, efficient expression of recombinant proteins is achieved through the use of a proprietary leader sequence in the new expression vector, which was identified from internal proteomic work.

With multiple components that can affect expression to take into account, work to develop a new expression vector can quickly become impractical with such large numbers of combinations possible. When developing a new expression vector at Fujifilm, an approach was taken whereby vector components were first assessed individually before moving to assess the most favourable components in combination (for example data see Figure 2). Additionally, early work was performed in a transient system, moving to assess more favourable components / combinations in more resource intensive and time-consuming stable cell line generation.

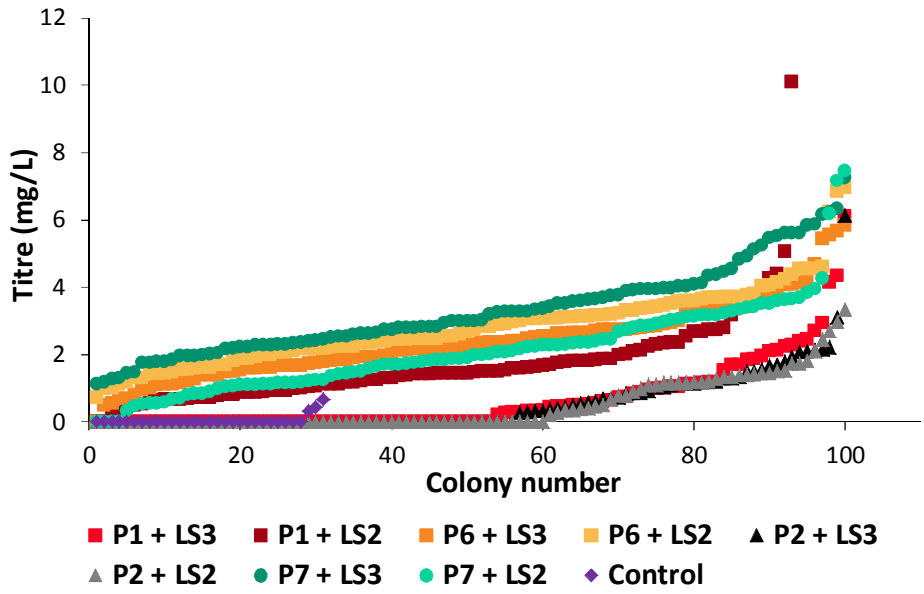

Figure 2 : Expression levels for a 'difficult-to-express' mAb in 96-well plate mini-pools. *First round assessment of vectors with more favourable components in combination. Key: P = promoter; LS = leader sequence*

Clonal Cell Line Development Process

When developing recombinant cell lines, the aim is to identify cell lines with higher titres within shortened development times and in a manner that meets regulatory acceptance. An important prerequisite to an optimised cell line development process is, of course, the use of a superior expression system comprising a host cell line and expression vector. Other key components include: (i) the introduction of platform screening procedures which are relevant to platform production processes; (ii) having the ability to demonstrate an acceptable level of probability of monoclonality (P(monoclonality)) with a minimal number of formal cloning rounds; (iii) creating high expressing cell lines without the need for multiple rounds of gene amplification and; (iv) using a process free of animal-derived components.

The disadvantages associated with amplification and the possibility of achieving high product concentrations without doing this were highlighted in the previous section. Ensuring the entire CLD process is animal component-free has become a standard expectation, which Fujifilm does comply with. The advantages of not using animal-derived components are that the risk of contamination with known (e.g. viruses, TSE) and unknown agents is reduced. Also, it reduces process variability that can be introduced by undefined materials. Finally, as discussed earlier, there is the potential to remove the need for bovine and porcine testing on the recombinant cell line bank if the host cell line has been pre-tested and no animal-derived components have been used in the CLD process.

It has previously been shown that it is of great importance to design and use assessment stages during cell line development which are demonstrated to be highly predictive of behaviour in the final production process[7]. If such screens are not included, there is a risk that the 'best' cell line from which to manufacture will not be identified. Efforts to improve the selection of the 'best' recombinant cell lines have therefore focused on the introduction of screens early in cell line development that are more comparable to and therefore more predictive of performance during manufacturing. Examples include the use of microbioreactors and shaken multi-well plate platforms which are operated in suspension mode and have feeds applied. In fact, Fujifilm has developed a fed-batch screen which is performed in suspension culture in 24 deep-well plates and employed during the early stages of cell line development (Figure 3). In addition, Fujifilm has introduced a screen in the ambr15™ microbioreactor system as the final step in the cell line development process. This screen replaces the classical batch and fed-batch shake-flask screens typically used at the end of cell line development with one single, higher quality screen.

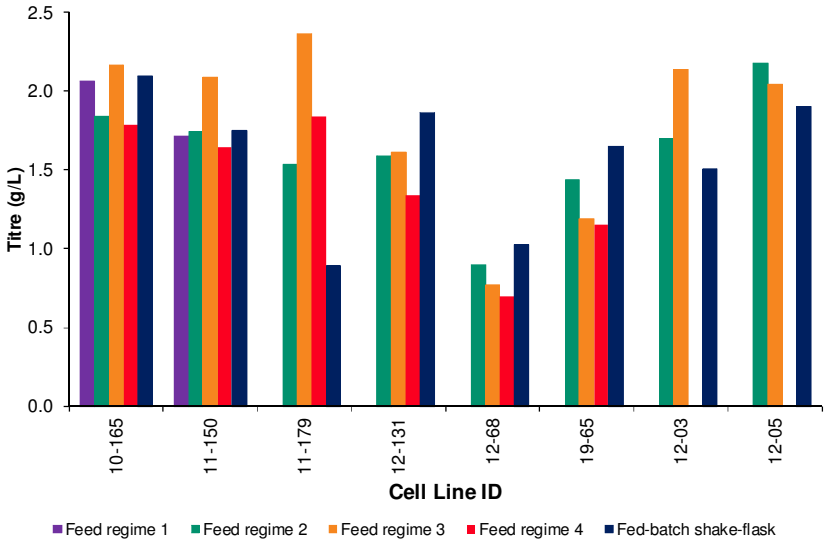

Figure 3 : Optimising a fed-batch, shaken multi-well plate screen. *Comparing titre data from different feed regimes in the fed-batch, shaken multi-well plates with that in fed-batch shake-flasks. Feed regime 1 was the initial adaptation of the shake-flask feed regime to the multi-well plates; feed regime 2 was selected as the lead process.*

The term 'clone' is applied to a population of cells that originate from a single parental cell. Regulatory guideline documents, relevant to the production of biotherapeutics from mammalian cells, convey that the production cell line will be derived from a single parental cell[8,9]. It is therefore standard procedure to include at least one cloning step during the development of a recombinant cell line for therapeutic protein production.

Many different techniques are used for cloning cell lines, including limiting dilution, “spotting”, micromanipulation, FACS, and cloning rings[10]. Automated systems such as the Quixell™ from Stoelting, the ClonePix™ system from Molecular Devices (Genetix), the LEAP™ platform from Intrexon (formally Cyntellect) and the CellCelector™ from Aviso are also available for cloning, selecting and expanding clonal cell lines. Some of these automated systems utilise semi-solid media to immobilise single cells which then expand into colonies; there are also groups that use semi-solid media and manually pick colonies they believe are clonal. Statistical approaches are commonly used to calculate P(monoclonality) following use of many of these methods. Many groups also now use plate imaging systems to try to visually confirm the existence of a single cell in a vessel (e.g. well of a 96-well plate). Some groups combine both approaches. However, none of the methods or approaches highlighted can completely guarantee that the arising population of cells is derived from a single cell.

Fujifilm is aware of increased interest from regulatory bodies regarding the method of cloning used and P(monoclonality) achieved during CLD. In a recent presentation at the 18th Symposium on the Interface of Regulatory and Analytical Sciences for Biotechnology Health Products (2014), the FDA’s Sarah Kennett outlined a number of examples where insufficient information on the cloning approach or P(monoclonality) values have been included in submissions.

Overall the quality of the final cell line is paramount. At Fujifilm careful consideration and extensive work has been undertaken to try to ensure such quality, with respect to monoclonality, is reached. Fujifilm’s two-step cloning strategy has been designed using cloning methods which have robust experimental and statistical evidence to support their use for generating cell lines with a ≥99.78% P(monoclonality).

Summary

The importance of an expression system (host cell line and vector) with superior growth characteristics and expression capability has been discussed, along with routes to develop them. Additionally, the importance of optimising the cell line development process itself, to increase the chance of isolating the ‘best’ recombinant cell line, from a functional and quality perspective, has been reviewed. Fujifilm has recently developed and launched a new mammalian expression platform, Apollo™, which was developed with all these considerations in mind.

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