

Improving Clone Production for Increased Protein Yield from Mammalian Cell Lines

A new generation of technologies is enabling the development of highly productive, stable mammalian cell lines more quickly than traditional methods.



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Optimising the cell culture process for production of recombinant therapeutics represents one of the most critical factors in determining the commercial feasibility of a biological product. The success of this stage requires the identification of transfected cells that produce the protein of interest at high levels, and the rapid generation of a stable cell line. This can be a lengthy and difficult process, and a number of factors can affect the levels of protein produced, including the site of gene integration. A range of novel strategies has been developed to overcome these limitations.

GENE INTEGRATION

While media optimisation, feed strategies and bioreactor parameters all play an important part in optimising protein production, the starting point is transfection of the mammalian cell with plasmid DNA. The site of gene integration can determine the levels of protein produced and so this step needs to be followed by methods to select the most productive clones. Approaches have been developed to overcome the integration site-dependence of transfected cell line productivity, some of which involve including elements in the transforming vector that can modify the chromatin structure surrounding the integrated gene of interest (1). These approaches can both boost the protein yield by an order of magnitude, and reduce the time taken to reach the industrial scale-up stage by several months.

The usual method followed to produce highly productive cell lines is to insert the gene coding for the protein of interest into the host cell genome. Although gene integration can be readily achieved via transfection with a plasmid, several factors can influence the productivity of a cell line produced from an individual transfected cell; these include the sites of integration, the number of integration events and the stability of the transgene.

SELECTION OF PRODUCTIVE CLONES

It is important to choose a high-producing cell from which to develop a cell line for protein manufacture. Using traditional approaches – such as dihydrofolate reductase (DHFR)/methotrexate (MTX) selection and amplification in Chinese Hamster Ovary (CHO) cells – multiple rounds of screening and selection are often required to create cell lines that can produce acceptable yields of around 1g of protein per litre of culture, and thousands of clones are frequently screened in order to generate one high-producing cell line. This is both labour-intensive and time-consuming. As timing is now more critical in the commercial scale up from clone to clinic, several companies are offering high-throughput screening methods that can increase the rate of screening.

Regeneron's technology platform, Flow cytometry-based Autologous Secretion Trap (FASTR), is a flow



cytometry-based method for the isolation of high quality cell lines. The company has engineered a parental (CHO) cell line to enable doxycycline-inducible expression of Fc receptor (FcR). In cells expressing an antibody therapeutic, the FcR binds to the Fc portion of the molecule (the 'stem' of the Y-shaped antibody) inside the cell, and then displays it on the cell surface. Fluorescent-Activated Cell Sorting (FACS) using fluorescent anti-Fc enables the highest expressers to be selected from millions of cells. Following sorting, the expression of FcR can be turned off to allow unhindered expression of the recombinant protein for manufacturing purposes.

An alternative approach for the screening of secreting clones is the use of cell imaging systems such as CellXpress marketed by Sigma-Aldrich Corporation, and ClonePix FL from Genetix Ltd. ClonePix FL can screen around ten thousand in three weeks. In a test to identify the clones of CHO-S cells secreting the highest levels of a humanised IgG, ClonePix FL picked the top two per cent from 10,000 clones; of these, nine were expanded to obtain productivity data, five of which produced more antibody than the best clone picked using the traditional method.

A significant disadvantage of these high-throughput selection methods is that they are specifically designed for secreted proteins, and the equipment needed is often expensive. In addition, FACS analysis relies on good detection antibodies being available. An alternative approach is to use technologies that increase the productivity of all transfected cells without the need for gene amplification. This means that fewer cells need to be screened to identify one that can generate a highly productive line.

THE EFFECT OF CHROMATIN STRUCTURE

The level of transcription of a transgene depends on whether it integrates into a region of the chromosome that is being actively transcribed (euchromatin) or a condensed area of the chromosome that is transcriptionally silenced (heterochromatin). These different areas are characterised by epigenetic modifications of the DNA and histones. Housekeeping genes, which are found within euchromatin, are usually transcriptionally active owing to a high level of histone acetylation; heterochromatin is characterised by extensive histone deacetylation. In addition, the DNA and the histones within heterochromatin tend to be more extensively methylated.

TARGETED INTEGRATION

Targeting a specific site that is known to be within a region of active chromatin – and thus will produce the gene product at high levels – can circumvent the problem of integration site-dependent productivity. PDL Biopharma has generated a technology to target transcription 'hot spots', based on clone selection by FACS analysis but taking the process a step further. The first step of this process is to transfect cells with a plasmid containing the gene for a monoclonal antibody that will be expressed on the surface of a cell. FACS analysis is then used to identify those clones where the transgene has integrated into euchromatin, and thus produce the antibody at high levels. A second plasmid, which contains the gene for a secreted therapeutic candidate, then replaces the first plasmid within the open chromatin via site-specific recombination. This process ensures that the gene of interest is actively transcribed.

Again, a problem with this method is the high cost associated with the equipment required for the first round of analysis. In addition, the need for two rounds of transfection does increase the time required to generate a cell line that can be used for scaled-up protein manufacture. However, once a cell line has been isolated with a favourable integration hot spot, it can be used for the expression of other transgenes through the same process of site-specific recombination.

MAMMALIAN ARTIFICIAL CHROMOSOMES

Mammalian artificial chromosomes allow researchers to introduce large amounts of DNA into a cell in an autonomously replicating, non-integrating format. This approach avoids the need to consider where the transgene integrates and so gets around the problem of integration site-dependent productivity. Chromos Molecular Systems, Inc, has developed the Artificial Chromosome Expression (ACE) System for the engineering of cell lines (2). The ACE platform is a murine artificial chromosome that contains multiple recombination acceptor (*attP*) sites. Cell lines containing this platform ACE can be transfected with the ACE-targeting plasmid and a helper plasmid containing the ACE integrase. After DNA recombination, a selectable marker that was initially present in the targeting vector is activated and this enables efficient identification of cells carrying 'loaded ACEs'.

This system can successfully produce cell lines that can deliver 0.5-1g of protein per litre, but this still involves a

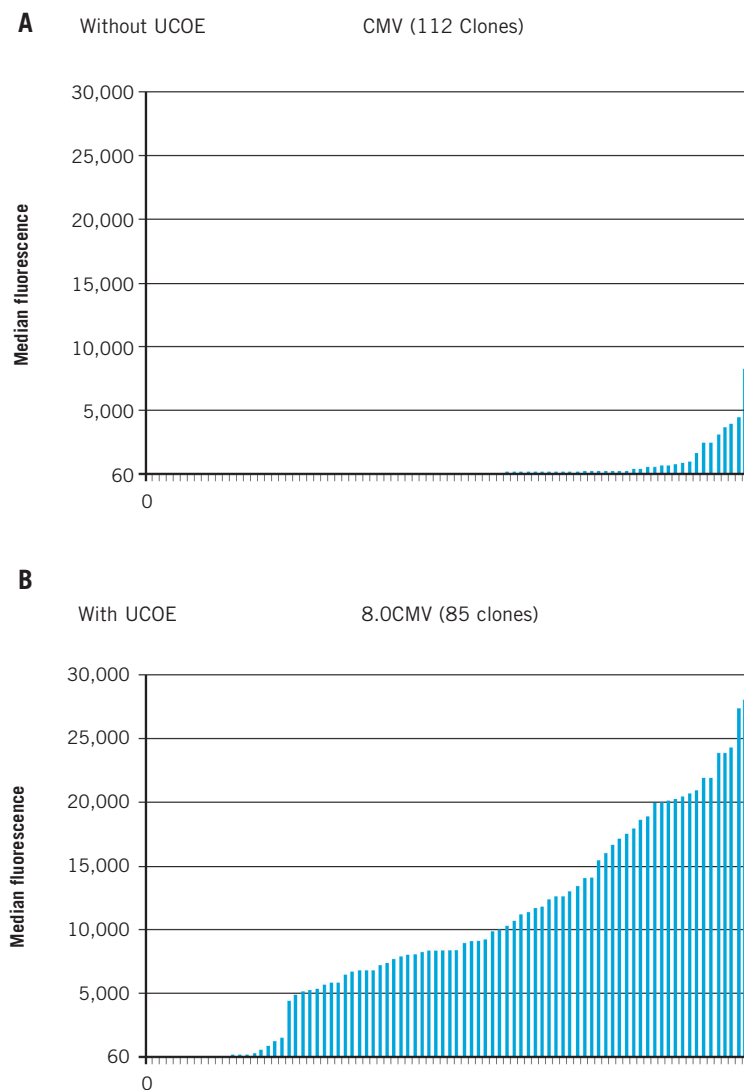


Figure 1: CHO cells were stably transfected with a human CMV-EGFP reporter gene combination with or without an upstream 8kb UCOE fragment. Median fluorescence indicates the level of reporter gene expression achieved in individual clones.

significant time commitment of 5-6 months. In addition, expression levels tend to fall over generations of the cell lines, although selection helps to reduce the rate of this loss. This is of significance when proteins are manufactured for use in humans, as the regulatory authorities require evidence that the process used for manufacture can provide stable production over 25 to 30 generations of culture.

CHANGING CHROMATIN STRUCTURE

Rather than trying to avoid inserting the transgene into heterochromatin and risking the transcriptional repression that this incurs, several companies have been developing techniques that can either remodel chromatin by altering the epigenetic environment of the DNA surrounding the integrated transgene, or physically prevent the surrounding chromosomal environment

from influencing transcription. The benefits of this approach include the fact that only one round of transfection is required to generate highly productive cell lines, and so fewer cells need to be analysed before a cell line suitable for scale-up for manufacturing can be picked.

A considerable research effort has focused on identifying chromosomal elements that are involved in organising chromatin, specifically those that have the power to hold chromatin open and maintain its transcriptional activity irrespective of the tissue type or integration site. Crucell has identified highly conserved DNA elements – called STAR® elements – that are able to counteract epigenetic gene repression. If these are used to flank a gene of interest, the cell lines generated demonstrate much higher expression of this gene than if the STAR elements are not used. The company claims that only 30-50 clones will need to be analysed to obtain a cell line producing between 25 and 50 picograms per cell per day (p/c/d). The STAR technology has further been improved by the development of a system, STAR-select, which increases the stringency of selection resulting in fewer but higher expressing clones.

An alternative set of DNA elements, identified by Selexis and called Scaffold/Matrix-Attachment Regions (S/MARs), contain AT-rich nucleotide motifs that are believed to become unpaired and function to unwind DNA. These regions influence gene expression by anchoring active chromatin domains to the nuclear matrix. Selexis has generated a synthetic S/MAR which, when integrated into the host genome, can boost the number of cells that actively produce protein after transfection by up to 20-fold, with production levels exceeding 80p/c/d (3). More recently, Selexis has reported the development of smaller, more powerful elements, which are claimed to increase antibody expression even further.

At Cobra Biomanufacturing, we have developed a system – maxXpress – that utilises the properties of DNA elements called ‘Ubiquitous Chromatin Opening Elements’ (UCOE). These were identified in collaboration with researchers from King’s College London, from genetic regions surrounding housekeeping genes that maintain an open chromatin structure (4,5). UCOEs of 1.5-8kb in size, in combination with the human cytomegalovirus promoter (hCMV), have been incorporated into a eukaryotic expression vector and result in a 10-25-fold increase in productivity. Figure 1 illustrates that high expressing clones (in this case GFP) can be more easily isolated when using a UCOE-containing plasmid.

The technology has enabled the selection of high-expressing clones in six to eight weeks, which can routinely generate antibody yields of 1.5-2g/L, with specific productivities between 15-40 p/c/d and stable expression over at least 50 generations (see Figure 2). Cobra has further developed the technology to rapidly generate stable, high-expressing pools that can yield up to 0.8g/L EPO and 0.9g/L antibody at a 10L scale within 28 days from transfection. This is clearly significantly higher than from transient transfection, whilst requiring fewer resources. maxXpress is therefore well suited to both supplying therapeutic proteins for early-stage development (such as optimisation of downstream processing and purification), and for high-level production and commercial manufacture from stably expressing clones.

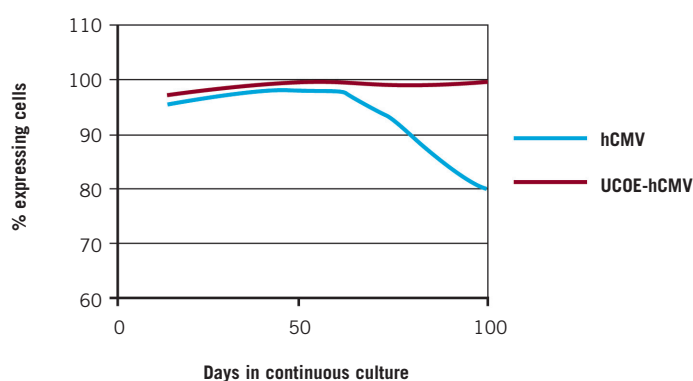
CONCLUSION

The selection of stable, high-expression cell lines is a critical first step in the successful manufacture of recombinant proteins on an industrial scale. Development of high-throughput mechanisms for the screening of vast numbers of clones has somewhat relieved the laborious process of identifying those clones where the transgene has integrated into active chromatin, and are thus producing protein at high levels. A more promising approach is the recent innovation of including elements in the vector that can influence the chromatin structure surrounding the inserted gene, preventing the site of integration from being a limiting factor in the production of high-expressing cell lines. This new generation of technologies enables the development of highly productive cell lines in very much reduced time scales compared with traditional methods, with very similar levels of productivity.

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Figure 2: Pools of CHO cells selected for integration of an hCMV-GFP vector show increased stability of reporter gene expression in the presence of a UCOE

