

Increasing protein yields from transfected cells

by Dr S. Williams

Manufacturing recombinant proteins for therapeutic use requires the rapid generation of stable cell lines that express the protein of interest at high levels. Transfecting mammalian cell lines with plasmid DNA containing the relevant gene is the starting point for generating “cell factories”, but as the site of gene integration can determine the levels of protein produced, this step needs to be followed by methods to select clones of cells that are the most productive. This article discusses alternative approaches that have now been developed to overcome the integration-site-dependence of transgenic cell line productivity.

Some of the approaches to produce cells that express proteins at high levels 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. Such approaches are growing in importance as the numbers of protein drug candidates entering preclinical and clinical studies continues to rise. To generate a cell line that produces large amounts of a specific protein, the gene of interest must be inserted into the host cell genome, and then transcribed and translated efficiently. 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.

Picking productive clones

Given the variability of productivity following transfection, it is important to choose a high-producing cell from which to grow up a clonal cell line for protein manufacture. 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. Traditional approaches, for example using limited dilution to generate clonal cell lines and then employing ELISA (Enzyme-linked immunosorbent assay) to assess the levels of antibodies secreted, can take months to screen around 1,000 clones. As timing is critical in commercial scale-up, several companies are now offering high throughput screening methods that can increase the rate of screening.

Regeneron’s technology platform, FASTR (Flow cytometry-based Autologous Secretion Trap), is a flow cytometry-based method for the isolation of high quality cell lines. The company has engineered a parental Chinese Hamster Ovary (CHO) cell line to enable doxycycline-inducible expression of Fc receptor. When expression is induced in cells transfected to include the gene for an antibody therapeutic, the FcR binds to the Fc portion (the ‘stem’ of the Y-shaped antibody) inside the cell, and then displays it on the cell surface. Sorting by FACS using fluorescent anti-Fc enables the highest expressors to be selected from millions of cells. Following sorting, the expression of FcR can be turned off, to allow unhindered secretion of the antibody product for manufacturing purposes. ClonePix FL from Genetix is an alternative approach, which can screen around ten thousand secreting clones 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 2% from 10,000 clones, and out of nine of these, which were expanded to obtain productivity data, five produced more antibody than the best clone picked using the traditional method. The downside of these high throughput selection methods is that they are only really applicable to 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. This means that fewer cells need to be screened to identify one that can generate a highly productive line.

Chromatin structure influences productivity

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

One way of getting around the problem of integration site-dependent productivity is to target a specific site that is known to be within a region of active chromatin, and thus will produce the gene product at high levels. Both Regeneron and PDL Biopharma have generated technologies to target transcription 'hot spots'. PDL Biopharma's approach is to first transfect cells with a plasmid containing the gene for a monoclonal antibody that will be expressed on the surface of a cell. They then use FACS analysis 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, is then directed to take the place of the first plasmid within the open chromatin via site-specific recombination in positive clones. 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 site-specific recombination can be used for the expression of other transgenes.

Importing artificial chromosomes

Mammalian artificial chromosomes are an alternative method of introducing large amounts of DNA into a cell in an autonomously replicating, non-integrating format, and this approach avoids the need to consider where the transgene integrates. The ACE System is Chromos' answer to engineering cell-lines, based on the company's artificial chromosome technology [2]. The platform ACE 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'. Pfizer is currently evaluating the system for use in monoclonal antibody production. Although high levels of protein can be produced by this system, it still takes a long time to generate cell lines that can produce between 500 and 1000 mg of protein per

litre, typically 5-6 months. In addition expression levels tend to reduce over generations of the cell lines, though selection helps to offset the rate of this loss. This last point is important as where proteins are manufactured for use in humans the regulatory authorities require evidence that the process used for manufacture can provide stable production over 25 to 30 generations of culture.

Opening chromatin

Rather than trying to avoid the transcriptional repression that occurs when the transgene inserts into heterochromatin, several companies have been trying to overcome this by inserting factors into the vector 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. Many investigators have been working to identify elements on the chromosome that are involved in organising chromatin, specifically

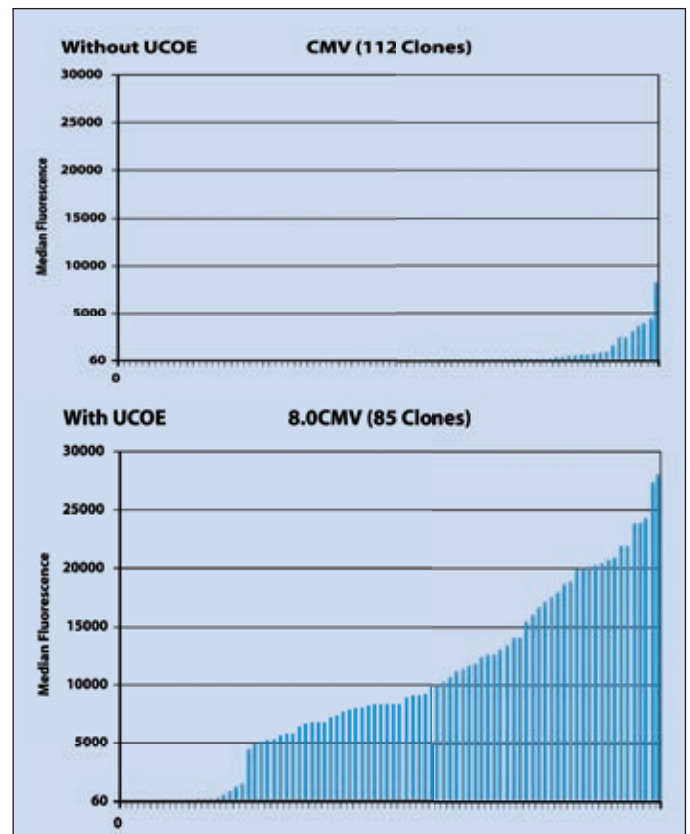


Figure 1. CHO cells were stably transfected with a human CMV-EGFP reporter gene combination with or without an upstream 8kb UCOE fragment. Median fluorescence indicates the level of reporter gene expression achieved in individual clonally-derived fragments.

those that have the power to hold chromatin open and maintain its transcriptional activity irrespective of the tissue type or integration site. Crucell have 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). Selexis has also identified regulatory regions of DNA. The ones on which they have focused are called Matrix-Attachment Regions (MARs), which 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 can boost the number of cells that actively produce protein after transfection by up to 20-fold, with production levels exceeding 80 p/c/d³. Cobra Biomanufacturing Plc, working in conjunction with researchers from King's College London, identified what they have called 'Ubiquitous Chromatin Opening Elements' (UCOEs) from genetic regions surrounding housekeeping genes that do just this [4,5]. UCOEs of 4-8kb in size, in combination with the promoter/enhancer sequence from the human cytomegalovirus (hCMV) that is commonly used to achieve strong, non-selective expression in mammalian cells, 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 that can routinely give antibody yields of 1.5-2g/L with specific productivities between 15-40 p/c/d and expression is stable over at least 50 generations [Figure 2]. Cobra has developed the technology to rapidly generate high stably expressing pools that can yield up to 0.8g/L EPO and 0.9g/L antibody at a 10L

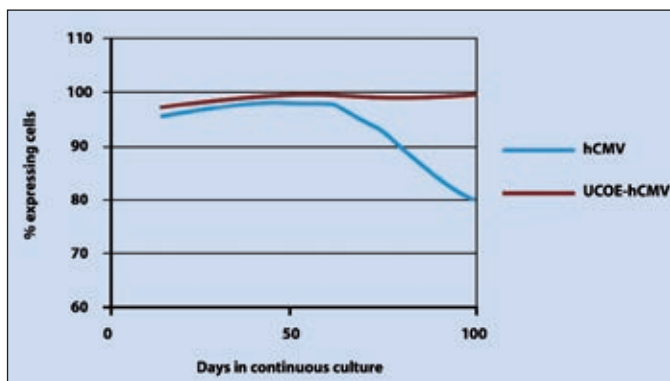


Figure 2. Pools of CHO cells selected for integration of an hCMV-GFP vector show increased stability of reporter gene expression in the presence of a UCOE.

scale within 28 days from transfection. This is clearly orders of magnitude higher than can be obtained in transient transfection with less resource.

Conclusion

Manufacturing recombinant proteins on an industrial scale requires technologies that can engineer stable, high-expressing cell lines rapidly, reproducibly and easily. Although high-throughput mechanisms now exist to screen vast numbers of clones to identify those where the transgene has integrated into active chromatin and are thus producing protein at high levels, a more promising approach is now upon us: to include in the vector elements that can influence the chromatin surrounding the inserted gene. The integration site no longer has to dictate the productivity of a transgenic cell line, and manufacturing large quantities of protein has never been so easy. Most of the current technologies are now able to produce high expressing clones in very much reduced time scales with very similar levels of productivity.

References

1. Barnes L M, and Dickson A J. Mammalian cell factories for efficient and stable protein expression. *Current Opinion in Biotechnology* 2006; 17: 381-386.
2. Lindenbaum M, Perkins E, Csonka E, Fleming E, Garcia L, Greene A, Gung L, Hadlaczy G, Lee E, Leung J, MacDonald N, Maxwell A, Mills K, Monteith D, Perez C F, Shellard J, Stewart S, Stodola T, Vandenborre D, Vanderbyl S, Ledebur H C Jr. A mammalian artificial chromosome engineering system (ACE System) applicable to biopharmaceutical protein production, transgenesis and gene-based cell therapy. *Nucleic Acids Research* 2004; 32: 21.
3. Fisch I. The Role of Matrix-Attachment Regions in Increasing Recombinant Protein Expression. *Bioprocess Int.* February 2007.
4. Antoniou M, Harland L, Mustoe T, Williams S, Holdstock J, Yague E, Mulcahy T, Griffiths M, Edwards S, Ioannou P A, Mountain A, Crombie R. Transgenes encompassing dual-promoter CpG islands from the human TBP and HNRPA2B1 loci are resistant to heterochromatin-mediated silencing. *Genomics* 2003; 82: 269-279.
5. Williams S, Mustoe T, Mulcahy T, Griffiths M, Simpson D, Antoniou M, Irvine A, Mountain A and Crombie R. CpG-island fragments from the HNRPA2B1/CBX3 genomic locus reduce silencing and enhance transgene expression from the hCMV promoter/enhancer in mammalian cells. *BMC Biotechnology* 2005; 5: 17.

The authors

Dr Steve Williams

Head of Mammalian Expression

and Dr Rocky Cranenburgh

Head of Research

Cobra Biomanufacturing Plc

www.cobrabio.com

Hotline: www.drugplusinternational.com & tick 30896