Rapid Isolation of Monoclonal Antibody Producing Cell Lines

Selection of Stable, High-Secreting Clones

by Julian F. Burke and Chris J. Mann

With reports suggesting a compound annual growth rate for monoclonal antibodies (MAbs) of 43%, a predicted rise of biopharmaceutical products is driving the development of systems for isolating the best producers among genetically engineered cell lines (**1**). Isolation of a high-producing cell line can decrease the time it takes to get to a phase 1 clinical trial and ultimately lower the cost of large-scale production. Assuming that 3 kg of a product is required for phase 1 and 2 trials and that the yield after purification is 60%, it has been suggested that the number of runs for a 1000-L batch-fed reactor can vary from 10 for a cell line producing 0.5 g/L to a single run for a cell line producing what is probably the current limit of 5 g/L (**2**).

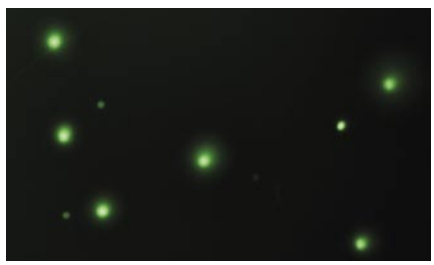
Not only does yield affect the speed at which a product can be made (the number of runs), it can greatly influence overall cost in terms of capital plant depreciation. For example, Wyeth's recently completed biopharmaceutical campus at Grange Castle in Ireland cost over US$1.8 billion and has 75,000 L of fermentation capacity (**2**).

When the first recombinant proteins were produced for biotherapeutic use, the emphasis was on molecular cloning of a gene, then transfering it into a cell line and growing that cell line. Early MAb products were bulk-produced in the same cell lines that produced them in research laboratories. Over the past five years, production groups have increasingly influenced research molecular biologists: Not only must a protein product have a specified activity, it also must be produced in a cell line that has been defined for productivity and purification criteria.

Making Therapeutic Antibodies

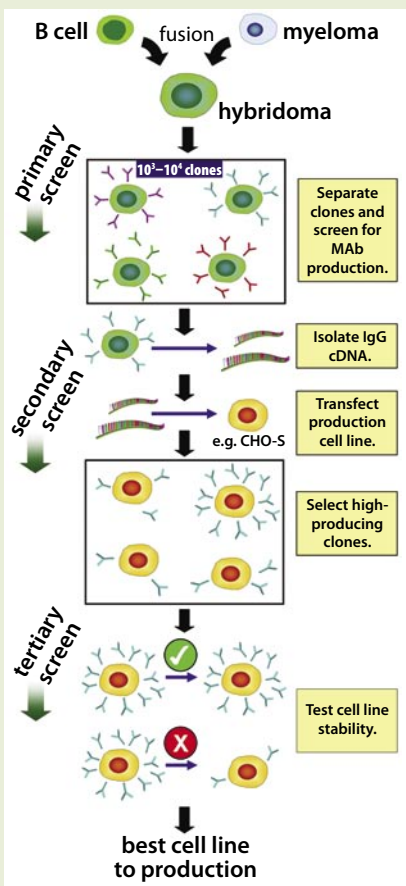
The standard method of isolating a therapeutic MAb (Figure 1) is to take a population of B cells believed to have at least a few expressing the antibody of interest, then fuse them to a tumor cell line (a myeloma) to establish immortal MAb-producing cells. Classic techniques could generate 1000–10,000 clones in each experiment. A substantial amount of work would then be required to identify those cells that produce the appropriate antibody and then isolate them for clonal propagation.

Identification of an appropriate clone was followed by a molecular cloning step that copied the predominant messenger RNA (mRNA) species into complementary DNA (cDNA). That was then placed in a construct so that expression could be driven by a powerful promoter in a heterologous cell line. The choice of cell line is now generally governed by development groups within each organization. The most commonly



GENETIX, LTD. (WWW.GENETIX.COM)

Figure 1: Overview of therapeutic antibody production (antibodies represented by Y shapes), the aim of which is to select the most productive cell line producing a specified antibody



used lines are CHO cells adapted for growth in suspension culture.

Subsequent steps determine whether cell lines produced are stable and which can be incorporated into a master cell bank (MCB), from which working cell banks (WCBs) are derived. Traditionally, all those steps involve the use of limiting dilutions by which individual cells are plated in 96-well plates at a concentration such that any well is likely to contain only one cell. In statistical terms, each plate must be seeded with 13 cells on average. But to screen a population of 10,000 fusions at that density would require 769 plates!

Biological methodologies have improved over time so that, for example, larger populations can be screened. Those can include "humanized" B-cell populations in which an animal heavy chain is replaced in vivo by a human IgG heavy chain integrated into the genome. Thus are the molecular cloning steps simplified. Alternatively, it is possible to screen millions of immunoglobulin gene constructs by using "libraries" of immunoglobulin genes encoded in phages (phage display) or bacteria. Some constructs will have a human constant region, again eliminating the need for some molecular cloning steps.

By whatever process the appropriate antibody-producing cells are obtained or phages identified, a common step is transfection of the production cell lines with an expression vector. Traditional processes again use the limiting-dilution process for screening and single-cell isolation.

Figure 2: Comparing three methods for hybridoma screening; the approximate time taken for this step (postfusion) depends on the method used (for limiting dilution, a total time of ~42 days; for ClonePixFL, a total time of ~14 days; for FACS ~42 days.

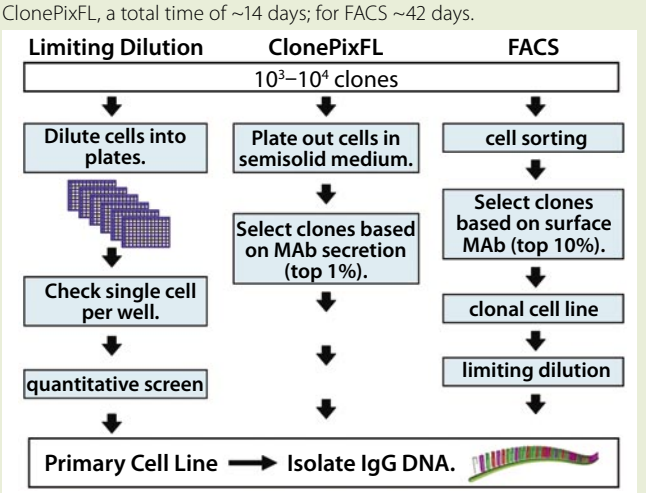
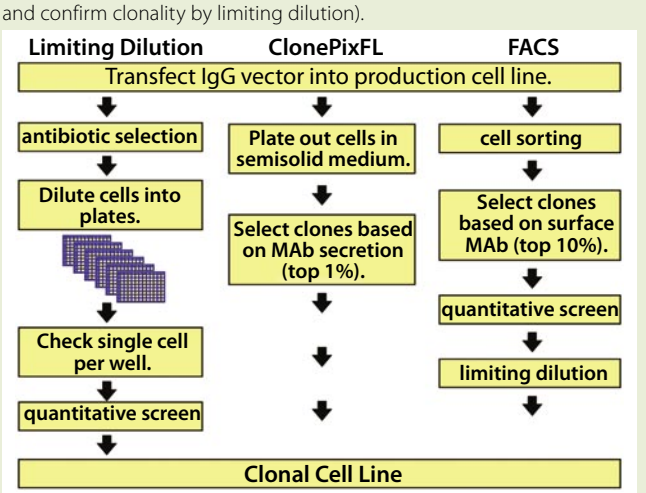


Figure 3: Selecting production cell lines — essentially as described in Figure 2 (timelines are similar in that limiting dilution takes ~42 days and ClonePixFL takes ~14 days to isolate a high producing cell line; FACS takes ~20 days to isolate an initial single cell line but another 20 days to screen and confirm clonality by limiting dilution).



Speeding the Process

There have been several attempts to automate the process of cell selection — from B-cell populations through to the selection of the expressing production cell lines. The principal driver has been eliminating the limiting-dilution method for single-cell selection and the hundreds to thousands of ELISA tests required to assay for appropriate immunoglobulins.

Primary Screens: Currently two principal methods have been validated to speed selection: the recently introduced ClonePix^{FL} technology from Genetix Ltd. and fluorescent-activated cell sorting using a FACS instrument. The former uses in-situ identification and measurement of cell-secreted proteins to identify and isolate appropriate cell colonies. The latter quantifies binding of antibodies to cell surfaces. Figure 2 compares both methods with limiting dilution.

The ClonePix^{FL} system takes advantage of the fact that cells can grow and divide while immobilized in a semisolid medium — in this case methyl cellulose. A cell population is plated in the semisolid medium and grown for 4–10 days. During that time, the amount of target protein secreted by those dividing cells is assessed by means of a fluorescently labeled antibody that passively diffuses through the medium and traps the secreted protein by complex formation. This system is powerful for highlighting the fact that "clonal" populations of cells are often highly heterogeneous in the amount of protein they secrete. The ClonePix^{FL} method quantifies secretion-associated fluorescence using a fluorescent camera system and selectively chooses colonies that secrete the most protein. Because the fluorescence detected is directly proportional to actual productivity, the system can rapidly generate high-producing established cell lines.

By comparison, standard FACS methods use a fluorescent antibody that binds to secreted MAbs during passage through the cell membrane (**3**). The principle behind this process is that the highest-secreting cell lines will have the most target protein in their cell membranes. In practice, FACS does not necessarily yield high-producing cell lines. One possible explanation is a lack of correlation between cell membrane proteins and secretion. For example, membrane passage may not be a rate-limiting step. Alternatively, highly fluorescent cells may be accumulating protein because of defective or reduced exocytosis, so they would not be high-secreting cell lines. Nevertheless, FACS is an efficient method for isolating single cells and establishing cell lines for protein production.

Production Cell Lines: Once users isolate a primary cell line that produces a MAb with the appropriate characteristics (such as immunoglobulin class, affinity, and secretion rate), they clone and transfect the associated genes into a production cell line. The traditional method requires multiple rounds of single cell cloning followed by assays such as ELISAs. That secondary screening step is common to all processes regardless of whether the primary cell line is a normal hybridoma, a fused "humanized" IgG expressed as a hybridoma, or a phage-encoded IgG.

Both ClonePix^{FL} and FACS can contribute to this step. Using identical methodologies to those described above, cells (typically CHOs) are selected using the in situ fluorescent secretion assay of ClonePix^{FL} or a FACS for single cell isolation (Figure 3).

One difference between these two processes is the amount of time taken for isolating a candidate production cell line. The ClonePix^{FL} gives rise to a candidate within four to six days, whereas FACS requires up to 20 days for establishment of a cell line. Secondary screening with ClonePix^{FL} is generally faster than primary screening because the expression levels of the secreted proteins are higher — both attributable to selection of the candidate molecule for secretion and to the nature of production cell lines.

Stability: MCBs and WCBs

Once an appropriate vector, gene, and host-cell combination are determined, a major factor in selection of an appropriate cell line is its genetic stability. The majority of cell lines for use in production are created by transfection, so expression levels can vary significantly based on the location of the integrated copies in the cellular genome. A further complication is that multiple (or partial) copies of integrated genes can recombine over time to cause variable levels of expression — thus becoming unsuitable for production scale-up.

The favored platform for production is a transfectant containing a single gene copy at one location. Even in such cases, cell lines can be subject to variations in production levels even if they are clonal — the principal mechanism being gene methylation. Some genes and some locations are more prone to methylation than others. Precisely what governs the degree of methylation is unclear, but variation within clones can be reduced by selecting the most stable cell lines. The traditional method of doing so

once again uses single cell cloning by limiting dilution. The process can be automated (Figures 4 and 5) with ClonePix^{FL} and FACS.

In the former case, an expressing clone is taken from a secondary screen in semisolid media at about five days (64-cell stage, or six generations). It is dispersed in one well of a 96-well plate and immediately plated in semisolid media containing a fluorescent antibody to measure secretion. After a further five days of growth, its fluorescence is measured. If a clonal cell line is stable, then all 64 clones growing up should fluoresce with the same intensity. If it is unstable, a range of fluorescence will be seen indicating high and low expression. A number of fluorescent clones can then be picked from the "stable" well and the process

Figure 4: Establishment of cell banks; production cell lines must have proven stability for antibody production, which can be determined by repeated rounds of limiting dilution and screening (~42 days by linking dilution or ~7 days by ClonePix^{FL} for each round of six generations); FACS can be used for rounds of single-cell isolation and screening from positive populations, with a time frame similar to that for limiting dilution.

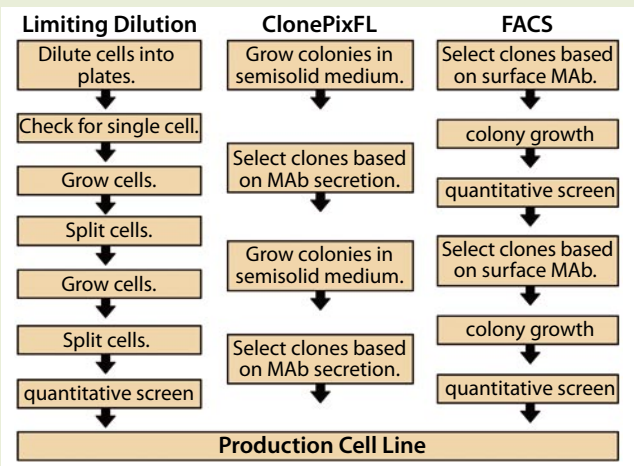
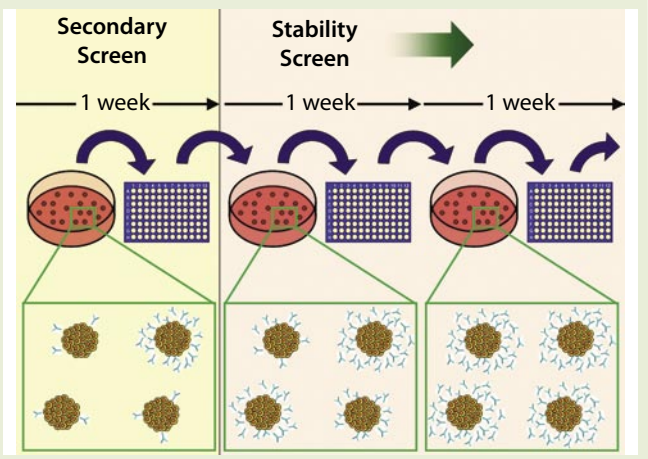


Figure 5: Schematic representation of the ClonePix^{FL} stability screen; the object is to obtain clones that uniformly express and secrete the same high yield of antibody (Y); clones are imaged, picked, and dispersed, and the operation is repeated until all daughter clones are identical and stable.



repeated for a further 6–10 generations (Figure 5).

The FACS system can also be used for finding stable cell lines by separating single cells from which clonal cell lines are derived. Cells are grown in wells and assayed by ELISA after each round of sorting and growth.

Growth Rate: The Other Dimension

Although antibody yield is of paramount importance for productivity measurements, another factor affecting production efficiency is the cellular growth rate. There has to be a compromise between growth and productivity. With limited-dilution and FACS-based systems, the growth rate of a cell line is measured late in their development. By contrast, the ClonePix^{FL} can measure the rates of both antibody secretion and cell growth almost simultaneously, the latter by measuring colony size using white light imaging.

The increasing number of MAb products on the market and in development is driving innovation in cell line engineering. A need to develop highly productive cell lines quickly is of paramount importance in getting therapeutic antibodies to market. As illustrated here, saving time and increasing productivity directly affect the costs of development and production. Improved time to market is vital for a billion-dollar blockbuster drug, for which every day lost in development can lose a company up to $5 million in potential revenue.

References

1 Werner RG. The Development and Production of Biopharmaceuticals: Technological and Economic Success Factors. *BioProcess International* 3(8, supplement) 2005: 6–15.

2 Hughes B. Co-Localization of Process Development with Manufacturing: A Critical Success Factor for Biopharmaceutical Production. *BioNet Conference* 17 December 2003, Dublin, Ireland. TecNet: www.tecnet.ie/pdf/brendanhughes.pdf.

3 Kenney JS, et al. Production of Monoclonal Antibodies Using a Secretion Capture Report Web. *Biotechnol.* 10(8) 1995: 787–790.

*Corresponding author **Julian Burke** is chief scientific officer, and **Chris Mann** is senior cell biologist at Genetix Limited, Queensway, New Milton, Hampshire BH25 5NN, UK; 44-1425-624626; julian.burke@genetix.com.*