ADVANCED INSTRUMENTS

Assurance of Clonality:

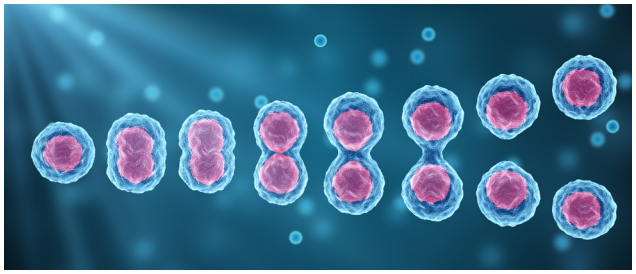
a key regulatory requirement for biotherapeutics

The clonality of Master Cell Banks (MCBs) for production cell lines is a key requirement in biotherapeutics manufacturing. New devices help optimize the cloning process.

Since the FDA's approval of the first recombinant protein in 1986, the number of approved biopharmaceuticals has grown constantly over the years, reflecting their increasing relevance in modern medicine.[1] By 2021, the FDA approved the 100th monoclonal antibody product[2] together with nine other new biologic drugs, accounting for 16% of the total number of drugs approved that year.[3] This 35-year pattern of steady growth in the biopharmaceutical market reflects how these drugs are one of the most effective clinical treatment modalities for a broad range of diseases; as a result, they are increasingly being used in almost all branches of medicine.[4]

Biotherapeutics are cutting-edge medicines which target specific molecules in the human body involved in the development of widespread diseases including cancer, immune and metabolic disorders.[5] Mammalian cell lines, such as Chinese Hamster Ovary (CHO) cells and Human Embryonic Kidney 293 (HEK 293) cells, are the most common platforms used to manufacture recombinant DNA (rDNA) derived human therapeutic proteins. These cell lines, engineered to carry the gene that encodes the desired protein, are the most important assets that a biotech company can have. Therefore, companies are required to ensure the safety, purity and potency of these

cell lines or cell substrates and to limit variability in the production process, in order to ensure a consistent final product.



One major factor that could potentially impact process consistency and product quality is the clonal derivation or clonality of the cell line.[6] "Clonality, in short, is basically a cell line that has started off from a single cell," explains Andrea Gough, Sr. Manager, Business Development at Advanced Instruments. "Whether you want to manufacture recombinant antibodies, viral vectors, vaccines or induced pluripotent stem cells (iPSC), it is recommended to begin with a homogeneous, clonally-derived production cell line to reduce both process variability and potential risks for patients."

Clonality: a key requirement for regulatory submission

A well-characterized clonally-derived cell line is not only a fundamental component of the biopharmaceutical manufacturing process but also a requirement for regulatory submission, most typically at the Investigational New Drug (IND) application stage. In fact, clonal derivation can impact a product's critical quality attributes.[7] Therefore, assurance of clonality in the production cell line is critical, and it should be confirmed early during cell line development. "Multiple regulators are becoming more stringent and require more detailed evidence of the clonality of the MCB," continues Gough. "As a result, companies aim to start with a clonal cell line early in the development process and capture the required evidence as soon as possible." In fact, as a part of the overall control strategy, regulatory agencies require that all biomanufacturing cell lines be "cloned from a single cell progenitor" to ensure safety and consistent product quality.[8] The United States (FDA) and the European Union (EMA) have different, though overlapping, guidelines for the manufacturing of biotherapeutics with regard to the clonal derivation of production cell lines (see table 1).

"The importance of clonality is related to the stable expression of the product and the potential risks for the patients if there are any supply issues following drug approval. Therefore, it is stressed in the regulatory requirements," says Gough. "Even though the CHO cell line is considered to be a very robust cell line and has been used in the

Guidelines for the manufacturing of biotherapeutics
The "ICH Q5D guidelines," issued by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) in 1998, states that "for recombinant products, the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor."[9]
The FDA guideline "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use," issued in 1997, notes that "the master cell bank (MCB) is defined as a collection of cells of uniform composition derived from a single tissue or cell."[10]
The EMA's "Guideline on development, production, characterisation and specification for monoclonal antibodies and related products," issued in 2016, states that "the cell substrate to be used for the production of the monoclonal antibodies should be a stable and continuous monoclonal cell line that has been developed by means of recombinant DNA and/or other suitable technologies."[11]
WHO's "Technical Report (TRS) 978," issued in 2013, states that "for proteins derived from transfection with recombinant plasmid DNA technology, a single fully documented round of cloning is sufficient, provided that product homogeneity and consistent characteristics are demonstrated throughout the production process and within a defined cell age beyond the production process."[12]

Table 1

manufacture of biotherapeutics since the 1980s, its genome is quite 'plastic' and can change over time, so starting with a single CHO cell can help reduce risks." In fact, this instability and chromosomal heterogeneity can affect cell growth, productivity and product quality.[13]

Clonal assurance reduces the potential of those heterogeneous population changes and thus the risks for supply problems and, ultimately, for patient health. "The worst scenario for a biotech/pharma company is having production issues after a drug is released," explains Gough. "If the company can't supply the drug, this can have major consequences for patients awaiting treatment. Minimizing this risk for patients' health is also an important consideration for regulatory agencies."

Limiting dilution: the classic approach to sub-cloning

In order to generate a clonally-derived cell line, a single precursor cell needs to be isolated, cultured and expanded. The traditional method to perform this subcloning step is limiting dilution (LD), which was developed at the beginning of the 1980s for cloning hybridoma cell lines in the production of monoclonal antibodies.[14] "Limiting dilution is the process of taking your cells and diluting them significantly in cell culture media down to a theoretical concentration of 0.5 cells per well before plating and expanding them," explains Gough. "This approach is based on probability: The reason you want such an extremely low cell concentration per well is to avoid having many wells with either two cells or no cells. This would indeed happen if you would start with a higher target concentration of, for example, one

Limiting dilution, example at 0.5 cells per well

Assumption 1 - Cells are distributed according to a Poissoin distribution.

Cells per well	0	1	2	3	4	5
% of wells	61%	30%	8%	1%	0%	0%

At a seeding density of 0.5 cells per well, 39% of wells contain cells, the rest will be empty.

Assumption 2 - All cells have an equal chance of survival and growth.

% of colonies derived from 1 cell	77%
% of colonies derived from more than 1 cell	23%

Of the 39% of wells containing cells, 77% will contain 1 cell and 23% will contain more than 1 cell.

Assumption 3 - A 2nd round of limiting dilution delivers a high probability of monoclonality.

Probability of clonality after 1 round	77%
Probability of clonality after 2 rounds	95%

The probability of clonality is derived from statistical analysis based upon work by Coller & Coller (ref. Hybridoma, 1983 2: 91-96) and is based on 3 assumptions.

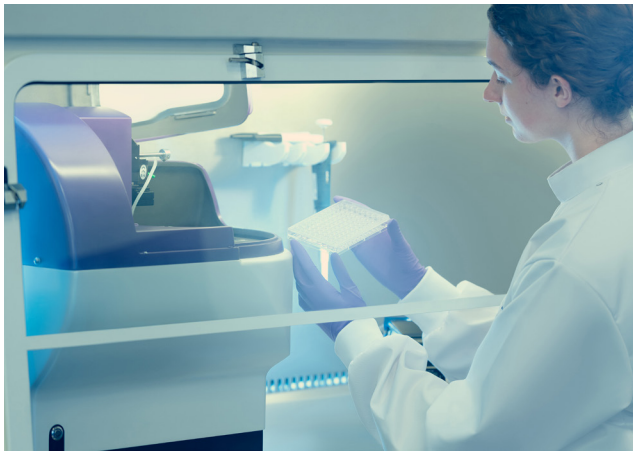
Table 2

cell per well." In fact, LD relies on a probabilistic approach which provides a relatively high statistical probability of "clonal" derivation of the resulting cell lines, especially when a second round of cloning is carried out (see table 2). Additionally, microscopic assessment can be used to visually confirm the presence of a single cell in a well. Though it is a relatively inexpensive process, LD is very labor intensive, time consuming, slow and low in throughput; it is also highly variable between scientists and does not allow for documentation from a manual microscope. Moreover, the underlying statistical considerations are based on studies carried out with beads instead of cells and do not take into account the fact that cells do not behave like beads, as they tend to stick together. Therefore, even when performing a double round of cloning, complete assurance of genetic homogeneity may not be achieved.

Alternative cloning technologies: optimizing the isolation of single-cells

Recent technological advances have significantly improved the cloning selection system. In modern cloning approaches, specialized instruments are used to ensure that a single cell is seeded into each well of a microtiter plate. Flow cytometry (FACS), which was originally developed as a research tool, now allows the detection and selection of cells based on the expression of defined cell surface markers.[15] "FACS is a well-known technology," says Gough. "It is a highly efficient, high-throughput method because it can screen millions of cells in a relatively short time. However, it requires high technical expertise, and cells have to endure high mechanical stress as they are pushed through a very small hole in the sorting mechanism at an extremely high speed and pressure." Indeed, due to this shear stress, many of the selected clones do not survive the selection procedure, and outgrowth rates are therefore low. Moreover, in order to be detected, cells need to be tagged with a fluorescent dye which could negatively influence the growth and expansion of the selected cell clone and also have undesired side effects in vivo.

The need for gentler fluidics and the challenges associated with the LD method have driven the development of new low-pressure automated seeding methods. Depositing devices, such as Advanced Instruments VIPS™, use image-based cell detection to deposit a single cell per well and immediately confirm these single cells by imaging them in situ in the well. Such devices achieve a seeding efficiency of 70-85%, varying with cell type, which is much higher than LD (30%) (see table 3).



VIPS™

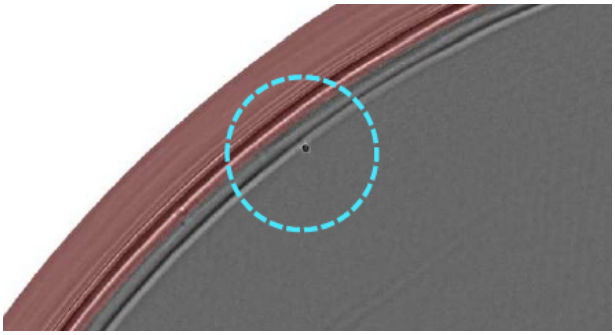
	Limiting Dilution	FACS	VIPS™
Seeding efficiency	30%	99%	70-85%
96-well plates necessary to screen 1000 cells	50	10	10
Efficiency	Low	High	High
Outgrowth probability	High	Low	Low

Table 3

VIPS™ offers an efficient alternative to both LD and FACS," remarks Gough. "This depositing device, which has integrated imaging, allows scientists to take pictures of the single cells deposited in the wells. It is gentler than FACS and does not damage the selected cells, which can then grow and expand."

A new level of clonality assurance: whole well imaging devices

Due to the large volume of detailed information requested by regulators to evaluate the cloning process, biotech and pharma companies need to provide stringent proof of clonality. Therefore, they need valid data, such as images, to show what happened during cell bank generation. "Microscopic imaging of well plates at deposition, post-deposition - within 24 hours - and during subsequent colony expansion are valuable for assuring the clonal derivation of a cell line," explains Gough. "The VIPS™ device couples the cell seeding process with a droplet image and day 0 whole well image. The in-well imaging of the droplet in the dry well gives evidence of successful single cell isolation." This visual evaluation of the technique's "success" in real time also contributes to eliminating additional laboratory work, significantly enhancing process efficiency.

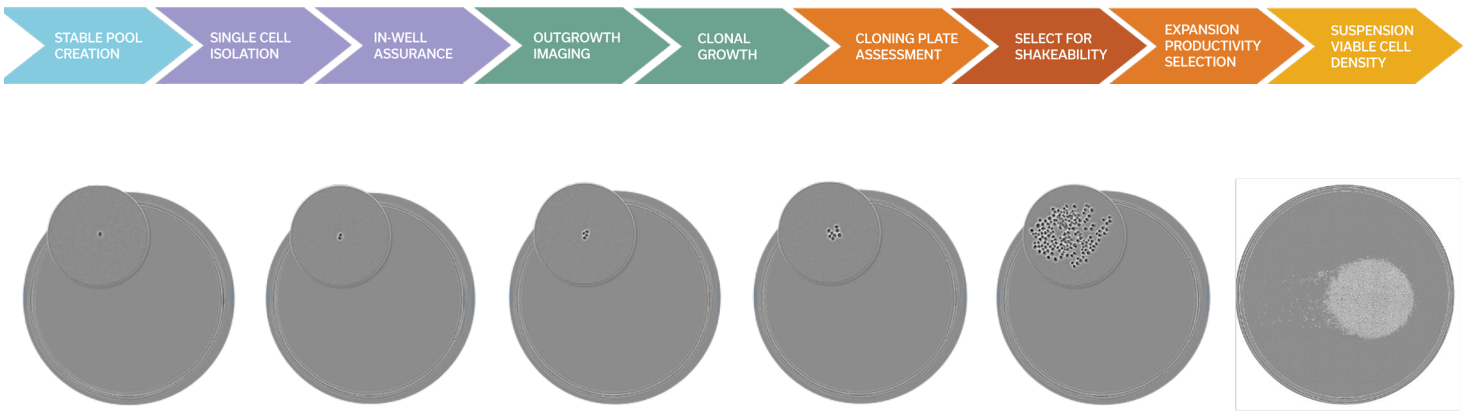


Scale: 200 µm

In addition, modern imaging technologies allow the monitoring of cell line outgrowth, which usually takes between 14 and 21 days for simple molecules like immunoglobulines and up to 28 days for more complex molecules. Solentim Cell Metric® provides whole well, high contrast imaging of the media-filled well to confirm the presence of a single cell at day 0 and daily imaging thereafter during the entire culture period. "Imaging devices capture an image of the whole well on day zero, so you can see the single cell," says Gough. "Then they take pictures at regular intervals, allowing you to monitor the growth of that single cell into a colony. This procedure allows you to collect image-based evidence for clonality without interfering with cell growth."

When using imaging systems it is important to consider that their accuracy may be affected by

Cell Line Development (CLD)



Daily whole well imaging as quality evidence of clonality

factors such as calibration, focus, illumination, focal depth and resolution issues. In fact, since these devices have a limited depth of focus, cells need to be on the bottom of the well to avoid the appearance of false negative colonies days after seeding. Moreover, the bottom of each well is at a different height in the plate due to plastic molding tools in the manufacturing processes. It is also beneficial to determine the probability that the imaging system will see a cell at the edge of the well.

Technological progress has allowed the development of more efficient cloning procedures. Such accurate and efficient analytical procedures are needed by the rapidly growing biopharmaceuticals market. In fact, profound knowledge and control of the manufacturing processes have become essential, considering that at the end of 2019, more than 1,000 clinical trials were in progress worldwide[16] to understand how biotherapeutics can improve treatment of many of today's chronic diseases.

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We can help you choose the right instrument based on your process and regulatory requirements.

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