Select high-value cell lines like a pro

Accelerate cell line development for recombinant proteins and monoclonal antibody production

eBook contents

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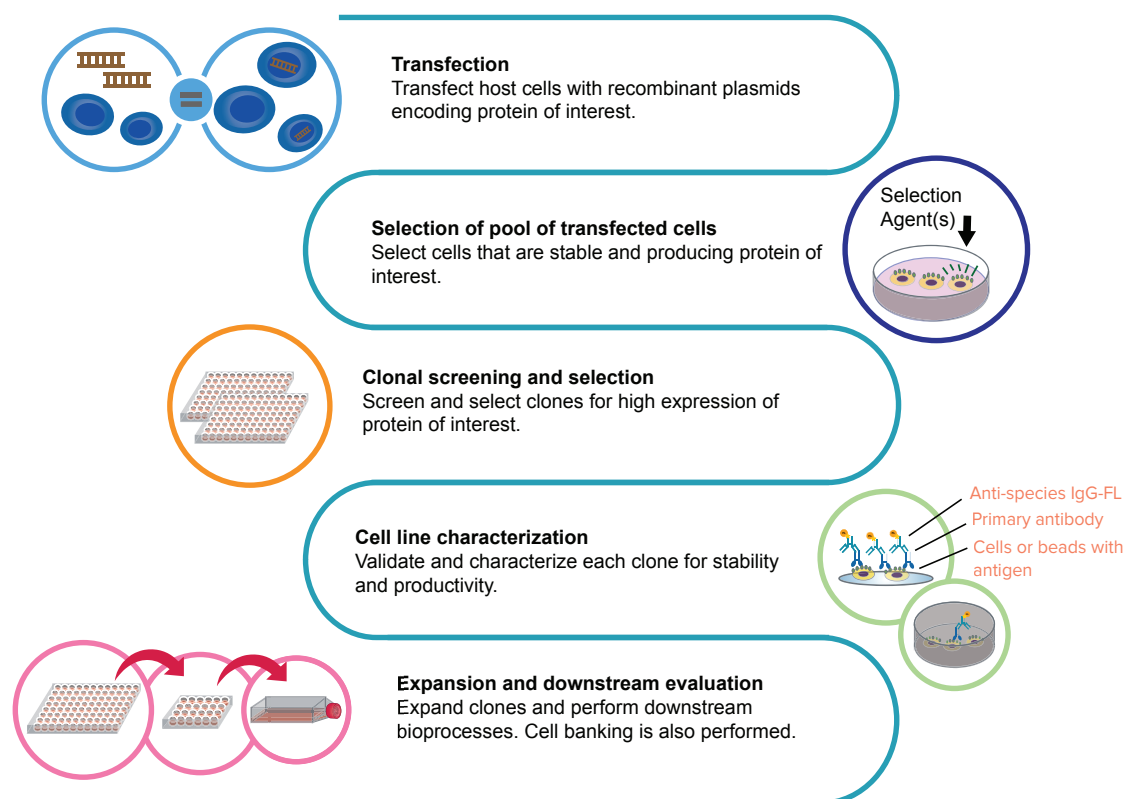moleculardevices.com

MOLECULAR DEVICES

Introduction

During the last decade, production of monoclonal antibodies (mAbs) and recombinant proteins using mammalian cells has led to a boom in the introduction of biotherapeutic proteins to the market. A total of 96 recombinant protein therapeutics have been approved between the first approval in 1986 and 2011.[1,3] The global market is growing at 12.5% from 2014 to 2019 and is expected to reach $3.96 billion by 2019.[2] Some of the key elements fueling the growth include the rising demand for monoclonal antibodies, introduction of innovative cell line technologies, and increasing vaccine production.[2] As growth continues, the need to bring down cell line development costs and shorten the time to market is more critical than ever. In this eBook, we present an overview of cell line development workflow as well as high-throughput solutions for accelerating the process, enabling easier and faster selection of high-producing mammalian cell lines.

Cell line development workflow

In order to generate high yields of recombinant protein products, stable cell lines such as hybridoma, CHO, or HEK 293 are typical vehicles of choice. The process of developing stable cell lines starts with transfecting host cells with recombinant plasmids. In the case of mAb production, the traditional approach is to generate mAb-producing cells (i.e. hybridomas) by fusing myeloma cells with desired antibody-producing splenocytes (e.g. B cells). These B cells are typically sourced from animals, usually mice. After transfection or cell fusion, large numbers of clones are screened and selected on the basis of expression (e.g. CHO cell lines) or antigen specificity and immunoglobulin class (e.g. hybridoma cell lines). Once candidate cell line clones are identified, each "hit" is confirmed, validated, and characterized using a variety of downstream functional assays. Upon completion, the clones are expanded or scaled up where additional downstream bioprocesses occur.

A

Cell line development workflow for recombinant proteins

Transfection
Transfect host cells with recombinant plasmids encoding protein of interest.

Selection of pool of transfected cells
Select cells that are stable and producing protein of interest.

Selection Agent(s)

Clonal screening and selection
Screen and select clones for high expression of protein of interest.

Cell line characterization
Validate and characterize each clone for stability and productivity.

Anti-species IgG-FL Primary antibody
Cells or beads with antigen

Expansion and downstream evaluation
Expand clones and perform downstream bioprocesses. Cell banking is also performed.

Figure 1. Workflow for generating recombinant proteins (A) and hybridoma cell lines (B) are presented.

1. Lai T, Yang Y, Ng S. Advances in Mammalian Cell Line Development Technologies for Recombinant Protein Production. *Pharmacueticals (Basel)*. 2013; 6(5):579-603.

2. Cell Line Development Market by Product (Equipment, Media, and Reagents), by Source, by Type, by Application (Bioproduction, Tissue Engineering & Regenerative Medicine, Toxicity Testing, Research, Drug Discovery) – Analysis & Global Forecast to 2019," MarketsandMarkets Inc., Sept. 2014.

3. Kim J, Kim Y, Lee G. CHO cells in biotechnology for production of recombinant proteins: current state and future potential. *Appl Microbiol Biotechnol*. (2012); 93:917-930.

Cell line development workflow for hybridomas

B
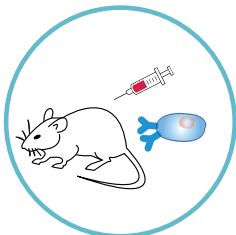
Immunization of mice & isolation of splenocytes
Mice are immunized with an antigen and later their blood is screened for antibody production. The antibody-producing splenocytes are then isolated for *in vitro* hybridoma production.
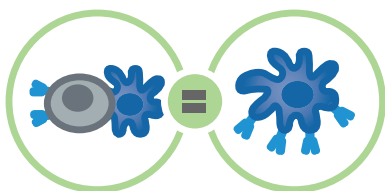
Preparation of myeloma cells
Myeloma cells are immortalized cells that, once fused with spleen cells, can result in a hybridoma capable of unlimited growth. Myeloma cells are prepared for fusion.
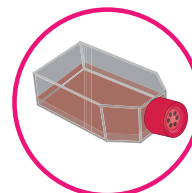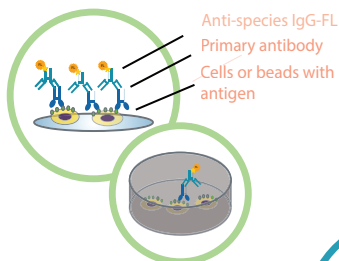
Fusion
Myeloma cells and isolated splenocytes are fused together to form hybridomas in the presence of polyethylene glycol (PEG), which causes cell membranes to fuse.

Clone screening and picking
Clones are screened and selected on the basis of antigen specificity and immunoglobulin class.

Anti-species IgG-FL
Primary antibody
Cells or beads with antigen

Functional characterization
Confirm, validate, and characterize (e.g. ELISA) each potentially high-producing colony.

Scale up and wean
Scale up clones producing desired antibodies and wean off selection agent(s).
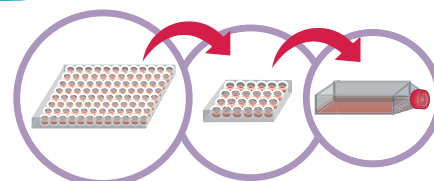
Expansion
Expand clones producing desired antibodies (e.g. bioreactors or large flasks).
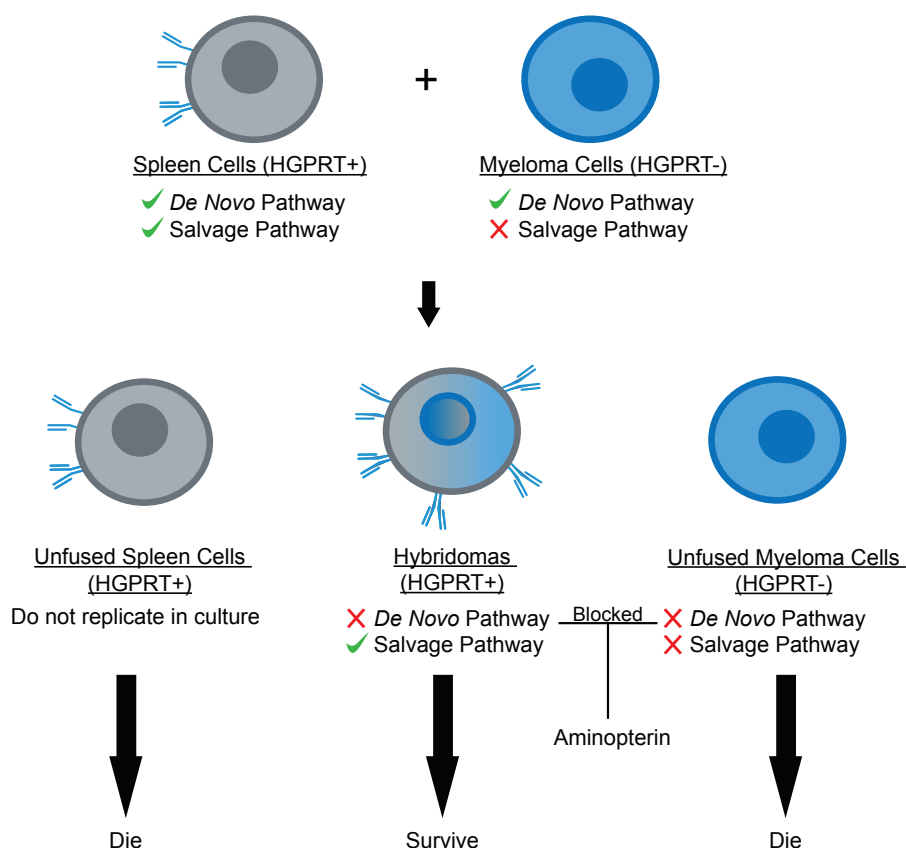
Hybridoma selection using HAT

Hybridomas are a specialized cell line created *in vitro* by the fusion of myeloma and spleen cells for the purpose of producing antibodies. The rationale behind fusing these two cell types together is that spleen cells produce the antibodies of interest but cannot replicate in culture. Thus, it is difficult to harvest antibodies from them. Myelomas, on the other hand, do not produce antibodies but do replicate in culture quite easily. Hybridomas take advantage of the properties of both cell types to mass produce antibodies of interest. During the fusion process, it is important to select for hybridomas over myeloma and spleen cells in order to maximize antibody yield.

Spleen cells are easily selected against since they do not replicate in culture. Myelomas are more challenging to select against, but it can be accomplished through the use of media containing hypoxanthine, aminopterin, and thymidine (HAT). In order to understand the rationale behind this approach, it is important to note that mammalian cells can synthesize DNA nucleotides using two different

pathways: the *de novo* and salvage pathways. Under normal conditions, mammalian cells will use the *de novo* pathway to replicate. When the *de novo* pathway is blocked, cells will then utilize the salvage pathway (only if hypoxanthine and thymidine are present) as an alternative means to replicate. The key to this approach is to use myeloma cells that are deficient in an enzyme (HGPRT) required for the salvage pathway. In this scenario, myelomas are unable to replicate because the *de novo* pathway is blocked by aminopterin and the salvage pathway is blocked by a deficiency in HGPRT. Hybridomas are able to replicate under these conditions, however, because they inherited a functioning HGPRT enzyme from the spleen cells. Following selection for hybridoma cells, aminopterin is no longer required as a selection agent. As cells recover from the selection pressures of aminopterin, they will still utilize the salvage pathway as a means to replicate. HT (hypoxanthine and thymidine) are maintained in the media until the hybridomas are fully recovered.

Spleen Cells (HGPRT+)
✔ *De Novo* Pathway
✔ Salvage Pathway

+

Myeloma Cells (HGPRT-)
✔ *De Novo* Pathway
✗ Salvage Pathway

Unfused Spleen Cells (HGPRT+)
Do not replicate in culture

Hybridomas (HGPRT+)
✗ *De Novo* Pathway — Blocked
✔ Salvage Pathway

Unfused Myeloma Cells (HGPRT-)
✗ *De Novo* Pathway
✗ Salvage Pathway

Aminopterin

Die

Survive

Die

Resources

• **Poster:** High-throughput hybridoma cloning and screening for antibody discovery

Clone Screening Technology

Cell colonies tend to be highly heterogeneous after recombinant cell cloning or hybridoma generation. Therefore, screening a large number of clones to identify high producers/expressors is a necessary step. Below is a table comparing some of the popular clone-screening methods.

Method	Description	Pros	Cons
Serial limiting dilution	Transfected cells are diluted sequentially on well plates to obtain an average of less than one cell per well. At this low concentration, the cell colonies derived are presumed to be originating from single cells.	• Low cost (before labor and consumables) • Relatively simple operation • Industry proven	• Time consuming, low throughput, and labor intensive • Single cells must be verified with additional methods like microscopes • Secondary screening assays are needed to identify high producers

Fluorescence-Activated Cell Sorter (FACS)	Uses an instrument that sorts cells one at a time based on the fluorescent characteristics of the cell. The instrument then applies a charge to the droplet containing the cell, forcing the cell to enter a specific collection tube.	• High throughput and shorter time to identify "hits"
		• As many as a dozen or more characteristics can be acquired for each cell
		• Sorting can occur at the single-cell level (depositing ~1 cell/well) or bulk level (e.g. top 5% of GFP+ cells)
			• Additional work needed to identify high producers
			• Cell viability is low due to stresses associated with disaggregation of cells into a single cell stream necessary for flow cytometry
			• Selection is based on immunodetection of proteins on the cell surface rather than actual secreted product
			  • Many proteins cannot be detected by this method
			  • This indirect method does not always correlate well with high-producing clones

ClonePix™ 2 System	Uses an automated colony picker capable of screening and selecting high-value clones from large heterogeneous populations of cells.	• Higher throughput than serial dilution; able to screen several thousand clones with minimal labor requirements • Clones can be selected based on user-defined characteristics (e.g. shape, size, proximity, and fluorescence intensity) • Able to screen for high-producing clones *in situ* using antibody detection reagents • Colonies are picked in a sterile environment • Industry proven • Select clones with extracellularly secreted proteins in semi-solid media	• Throughput is not as high as FACS, but protein expression is measured over time as clones proliferate

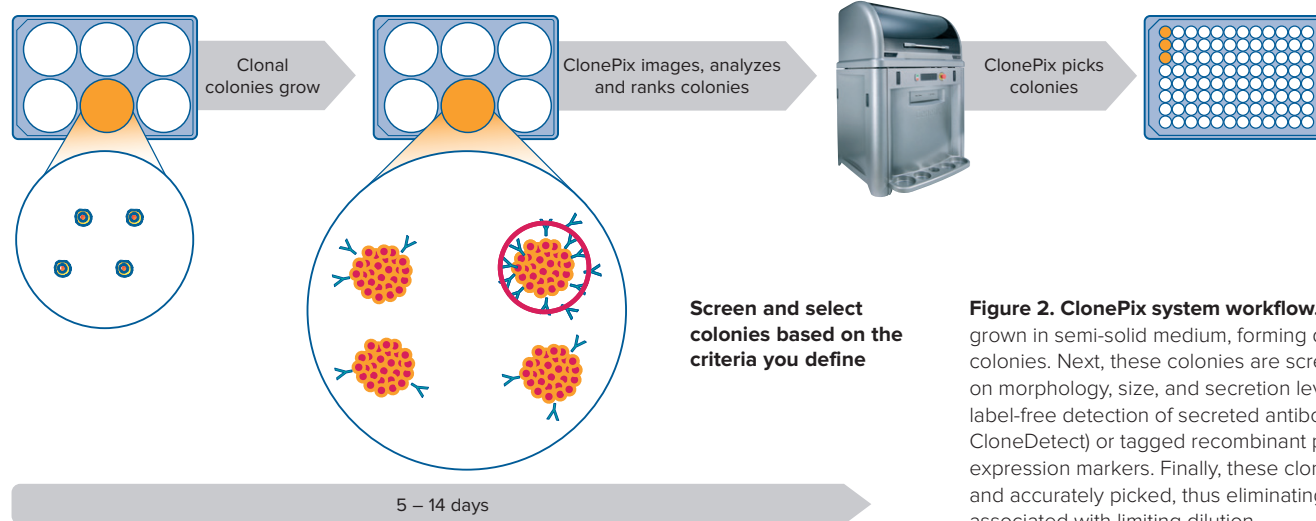# Automate fast, robust clone screening

Researchers are moving away from limiting dilution because it significantly prolongs the time to antibody discovery. Automation is needed to scale productivity, throughput, and reduce development time. One solution is to use the ClonePix 2 System, an automated colony picker capable of screening large numbers of clones from heterogeneous populations. The system requires mammalian cell lines to be cultured in semi-solid media (e.g. CloneMedia), enabling formation of discrete colonies, each originating from a single parent cell. Individual isolation of these discrete colonies with the system ensures high probability of monoclonality in a single step. In addition, reagents (e.g. CloneDetect) can be added directly into the semi-solid media to measure total protein secretion over time, or antigen specificity,

in situ. Heterogeneous populations of cells can then be rapidly screened using white light to detect colonies based on morphology and size (indicators of monoclonality and growth rates, respectively). Multiple fluorescent wavelengths can be used to quantify secreted proteins, cell surface protein expression, or antigen specificity *in situ*. The instrument's software also allows automatic colony ranking based on user-defined criteria such as fluorescence intensity, which is typically used to benchmark protein titer such as in a typical CHO cell line development process or target binding such as in hybridoma research workflow. The instrument can then accurately pick high-ranking colonies, eliminating errors associated with limiting dilution.

Select and pick with more accuracy and confidence

Cells plated into semi-solid medium

Select your colonies based on the system's automatic analysis and ranking

Clonal colonies grow

ClonePix images, analyzes and ranks colonies

ClonePix picks colonies

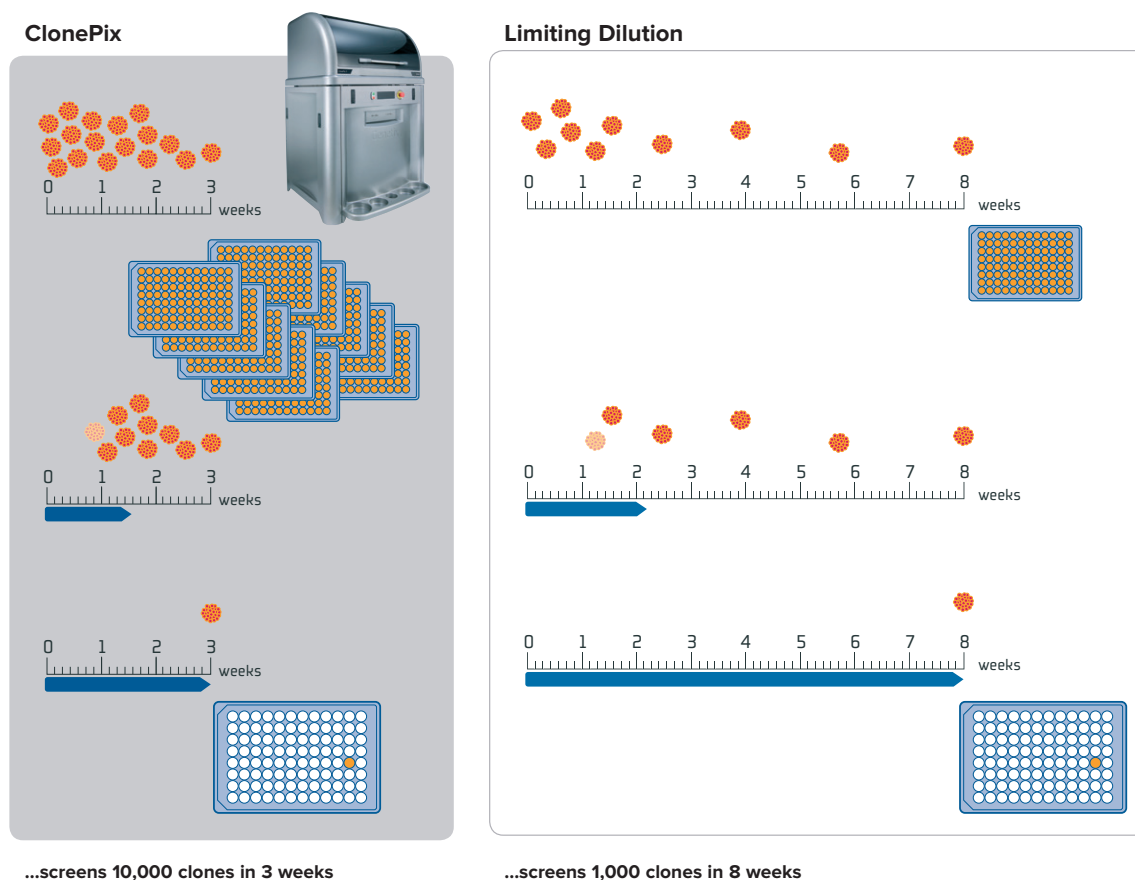**Screen and select colonies based on the criteria you define**

5 – 14 days

Figure 2. ClonePix system workflow. Cells are grown in semi-solid medium, forming discrete clonal colonies. Next, these colonies are screened based on morphology, size, and secretion level using label-free detection of secreted antibodies (such as CloneDetect) or tagged recombinant proteins and expression markers. Finally, these clones are ranked and accurately picked, thus eliminating errors associated with limiting dilution.

Figure 3. The principle of ClonePix technology. (A) Conceptual overview of secreted protein detection from individual clones grown suspended in a methycellulose matrix. **(B)** White light image of a clonal colony (in this case, antibody surrounding a growing hybridoma clone). Note the optical clarity of the image and spherical formation of the clonal colony (important for colony integrity) produced using CloneMedia medium. **(C)** Fluorescence image of secreted antibodies imaged on ClonePix system. **(D)** After automated picking of clones, the ClonePix system transfers the isolated target clones to destination plates.

A — Semi-solid media
Secreting clones
CloneDetect Reagent fluorescent probe
Secreting IgG

B

C

D
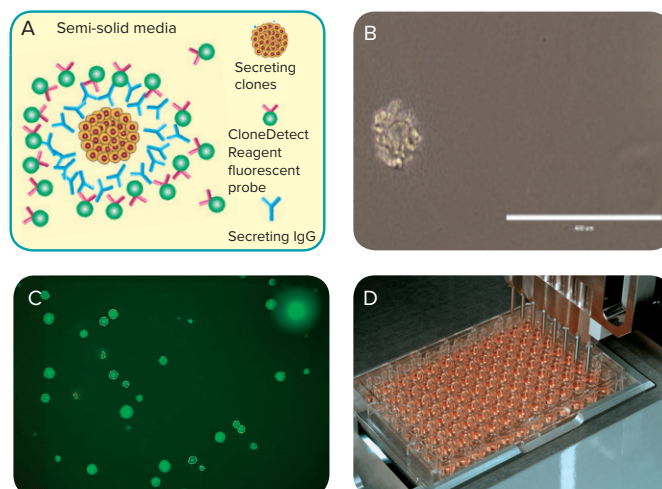
ClonePix vs. limiting dilution

ClonePix

Limiting Dilution

...screens 10,000 clones in 3 weeks

...screens 1,000 clones in 8 weeks

Figure 4. ClonePix system can shorten colony screening time significantly over traditional methods such as serial limiting dilutions. Cells are sequentially diluted into well plates at the concentration of less than one cell per well in the serial limiting dilution approach. Typically this method takes months to screen the desired colonies (e.g. only ~1,000 clones are screened in eight weeks). The ClonePix system, however, can shorten the time significantly (~10,000 clones in three weeks).

Resources

• **Publication:** Rapid automated selection of mammalian cell line secreting high level of humanized monoclonal antibody using ClonePix FL system and the correlation between exterior median intensity and antibody productivity

• **Poster:** Integration of automated colony picking into fast track cell line development process

• **Poster:** Rapid screening and selection of stable high producing clones

• **Brochure:** ClonePix 2

Monoclonal antibodies

Monoclonal antibodies continue to enjoy intense interest as potential therapeutics. Between 2008 and 2013, sales of monoclonal antibody products have increased 90% from ~$39 billion to $75 billion, while other recombinant protein therapeutics have only grown ~26%.[4] However, a major bottleneck in hybridoma cell line development is screening large heterogeneous populations of cells to find candidates yielding the highest amount of mAbs. This challenge can be overcome with automated technologies such as the ClonePix system, which enables *in situ* detection of secreted antibodies.

Here, we present a case study in which an optimally-producing cell line that secretes a highly specific, non-cross reactive monoclonal antibody against a double-stranded DNA (dsDNA) virus is identified.

Goal
To identify an optimal cell line that secretes a highly specific, non-cross-reactive monoclonal antibody to be used in biotherapeutic development against dsDNA viruses.
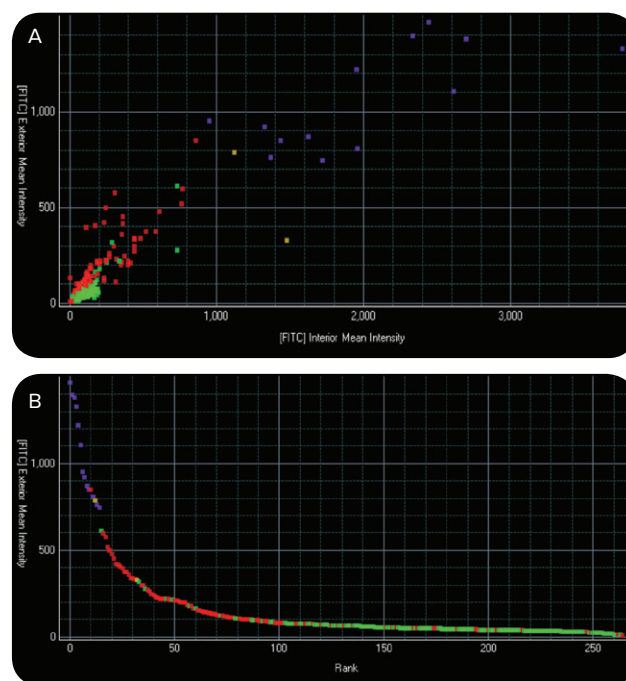
Background
Parental line was generated using limiting dilution method. Historical yield was less than 3mg/L of mAb, and quality of purified mAb was never uniform with varying amounts of aggregation observed in the preps.

Method
Parental hybridoma clones were expanded, followed by analysis and picking of the desired clones on the ClonePix system. A total of 480 clones were picked from source plate and imaged with the CloneSelect™ Imager for five days to determine cell growth. 146 top growers were isolated and screened for specificity.
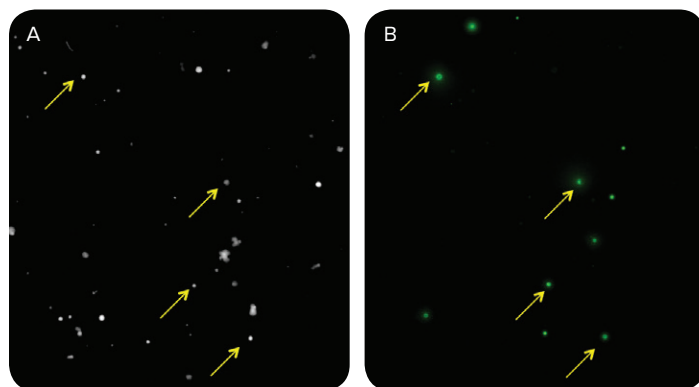
Figure 5. Visualization of IgG secretion in parental hybridoma cell line suggests only a small set (~7%) is producing dsDNA virus-specific antibodies. Hybridoma cells were plated in CloneMedia semi-solid medium. Fluorescently-conjugated CloneDetect agent (green) was added to enable *in situ* detection of secreted IgG. **(A)** To visualize all colonies in a single view, colonies were imaged on a ClonePix system in white light at 150 ms. **(B)** Colonies were imaged in fluorescence at 500 ms in order to determine which ones secrete higher amounts of IgG. Variability of FITC signal as quantitated by Interior Mean Intensity and Exterior Mean Intensity indicate an unstable, non-monoclonal hybridoma cell line.

Figure 6. Viable, high-secreting colonies are detected and selected using ClonePix Software analysis. (A) Scatter plot shows a linear correlation between Exterior and Interior Mean Intensity suggesting that the IgG is being secreted properly (otherwise immobilized on the cell surface with high Interior and low Exterior). The low IgG-yielding clones are due to a heterogeneous population of variable secreters (i.e. only a few hybridomas, 5-6% of the total population, are producing IgGs, while the majority of clones are growing without IgG expression). **(B)** Ranking plot shows clones selected and picked for further characterization in purple.

4. Ecker D, Jones S, Levine H. The therapeutic monoclonal antibody market. *mAbs*. (2015)7:1, 9-14.
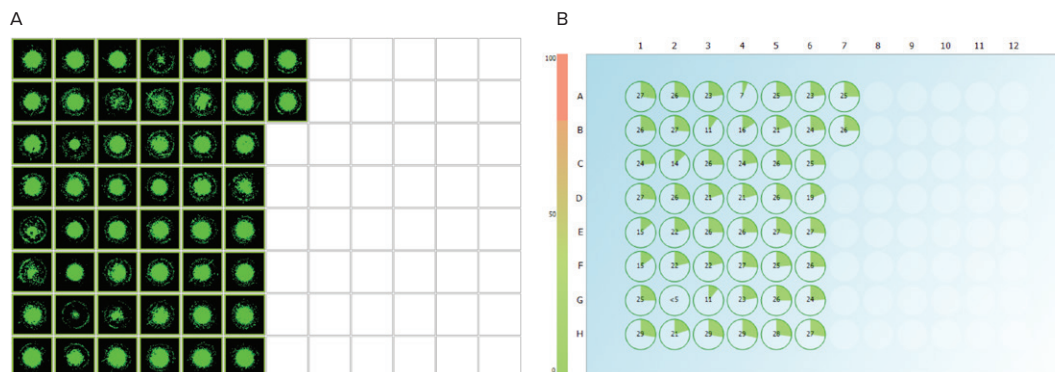
Figure 7. CloneSelect Imager software analysis enables identification of optimally growing clones. Clones were selected and picked using the ClonePix system and deposited into a 96-well plate. Cell growth was then assessed over a period of seven days with the CloneSelect Imager. **(A)** Image analysis performed with the CloneSelect Imager software on a subset of the selected clones. Total well coverage from day seven is displayed in green. **(B)** Colony growth is tracked using pie charts, which indicate cell confluency in each well on day seven.

Results

Two novel sub-clones showing optimal binding also showed a dramatic improvement in IgG production (17-25 mg/L) over historic yields of the parent clones (~1 mg/L). Hybridoma screening time was reduced by up to 50%.
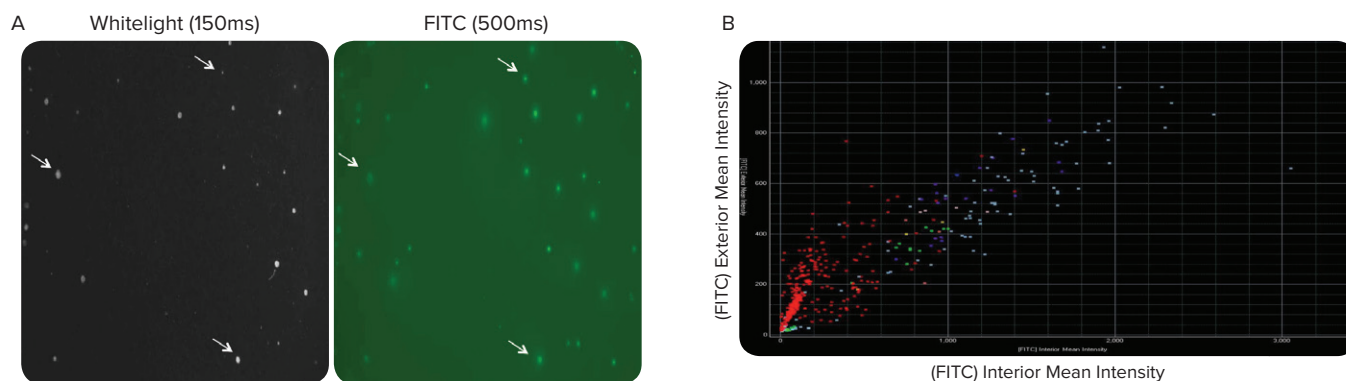
Figure 8. Sub-cloning of parental hybridoma by the ClonePix system results in uniform IgG secretion at higher yields. Subset data for one sub-clone is shown. **(A)** A significant improvement in percentage FITC-positive colonies as a result of sub-cloning is observed as compared to the parental clone (Fig. 5). IgG secretion was detected at seven days growth by 100 U/mL of CloneDetect reagent. **(B)** Scatter plot shows a linear correlation between Exterior and Interior Mean Intensity; the slope is shifted towards the Y-axis indicating greater uniformity, while more clones in the upper quadrants signify the presence of more highly FITC-positive clones.

Resources

- **Application Note:** Enhanced development of virus-specific hybridomas using ClonePix and CloneSelect Imager Technologies

- **Webinar:** Improved hybridoma discovery for immunodiagnostics using ClonePix and CloneSelect Imager technologies

- **Poster:** Rapid selective *in situ* screening of hybridomas in semi-solid medium

GPCRs

G protein-coupled receptors (GPCRs) are popular targets in drug discovery. However, the endogenous expression of GPCRs in mammalian cells is typically very low, with no more than 3,000 copies per cell. These levels are sufficient to maintain proper receptor function but present a challenge for GPCR drug discovery screening efforts. Most screening assays require a much higher concentration of functional GPCRs presented on the cell surface. Attempts to create expression systems in simple organisms have met with limited success due to inefficient folding (bacteria), low yield (yeast), or incorrect post-translation modification

(baculovirus). These challenges fuel the market need for mammalian expression systems capable of providing elevated GPCR expression levels required to support drug discovery efforts.
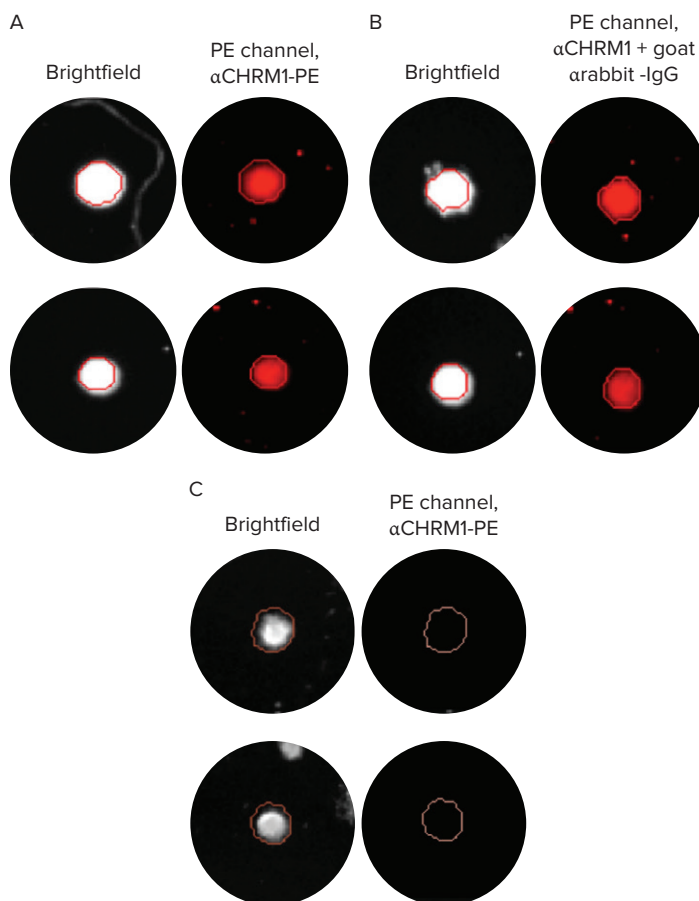
The ClonePix system helps tackle the challenge of locating and isolating rare clones from large heterogeneous pools of transfected cells. Utilizing both white and fluorescence imaging *in situ*, the system can quantitatively detect endogenous and cell surface protein expression levels of respective GPCRs.
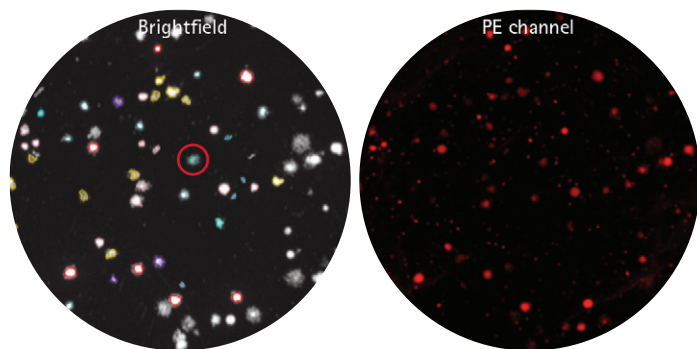
Figure 9. Detection of CHO-M1 cells on ClonePix system. A transfected CHO-M1 cell line expressing G protein-coupled muscarinic 1 cholinergic receptor (GPCR-M1) was chosen to demonstrate the feasibility of using the ClonePix system to detect cell surface proteins. CHO-M1 expressing clones were screened using anti-M1 antibody conjugated with PE (Phycoerythrin), selected based on fluorescence intensity, and picked using the ClonePix system. The system reveals diverse levels of fluorescent intensity with CHO-M1 cell line, demonstrating it can distinguish between various levels of expression of GPCR-M1 protein. Colonies recognized by the software are outlined in color under the brightfield channel. Fluorescence intensity is calculated based on the physical location of colonies.

Figure 10. Selection of GPCR M1-expressing clones with both direct-labeled antibody (A) and dual-labeled antibodies (B) approach are shown (brightfield and fluorescence images). The fluorescent intensity is proportional to M1 expression in the positive clones. **(C)** The negative control, CHO-K1 cell line demonstrates no fluorescence in the PE channel.

Resources

- **Application Note:** Rapid selection and development of GPCR expressing mammalian cell lines using novel ClonePix technology

- **Webinar:** Identification and selection of GPCR cell lines with ClonePix 2

- **Poster:** Rapid selection and development of GPCR expressing mammalian cell lines using novel ClonePix technology
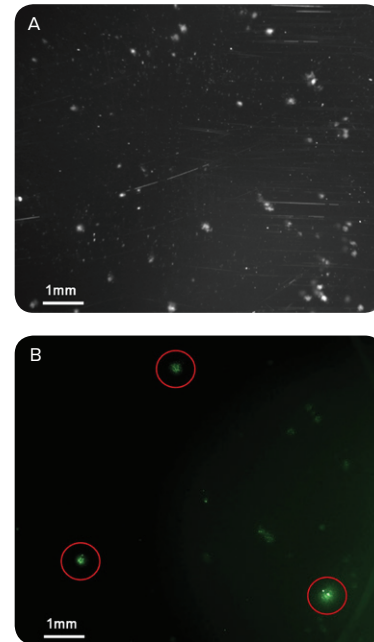
Other proteins

The underlying technology in the ClonePix system allows the system to screen and select high value cell lines beyond mAbs and GPCRs, such as tagged or untagged recombinant proteins and cell-surface proteins. Using target-protein specific, fluorescent-labeled antibodies combined with imaging and robotic processing/picking techniques, the ClonePix system can significantly improve efficiency while generating optimal cell lines that secrete the highest levels of the desired proteins. Some examples are shown below.
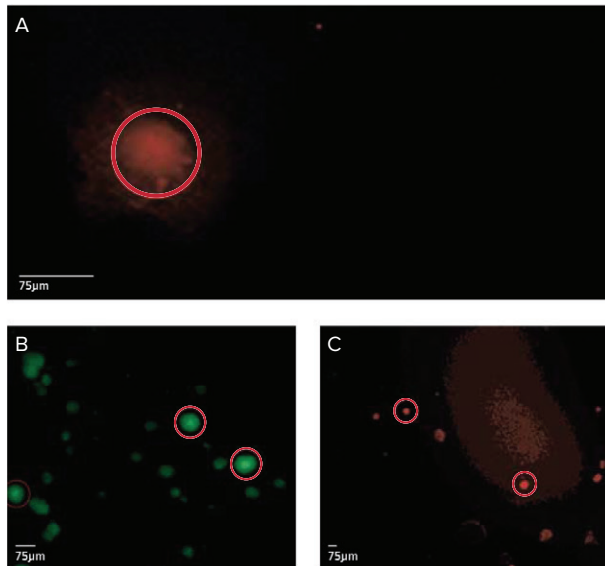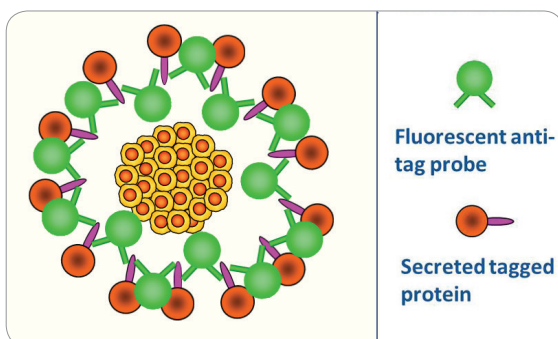

Figure 11. Selection of transfected, exogenous cell surface proteins. Detection and selection of colonies of cells expressing endogenous receptors (examples of selected colonies indicated with red circles); colorized images from the ClonePix system. **(A)** Jurkat cells grown in semi-solid medium with Red-Phycoerythrin (RPE) labeled antibody to the T-cell receptor CD3 at day 12 and the cells imaged and picked on day 14. **(B)** HEK 293 cells grown in semi-solid medium with FITC-labeled antibody to the ICAM-1 adhesion molecular added at day 12. The cells imaged and picked on day 14. **(C)** CGR8 mouse embryonic stem cells grown in semi-solid medium with RPE-labeled antibody to the pluripotency marker SSEA-1 added at day 9. The cells imaged and picked on day 10.

Figure 12. Principle for detecting tagged recombinant proteins. Principle of the technology in the ClonePix system for detecting tagged recombinant proteins. The secreted tagged protein is trapped in the vicinity of the colony and visualized using fluorescently-conjugated anti-tag antibody.
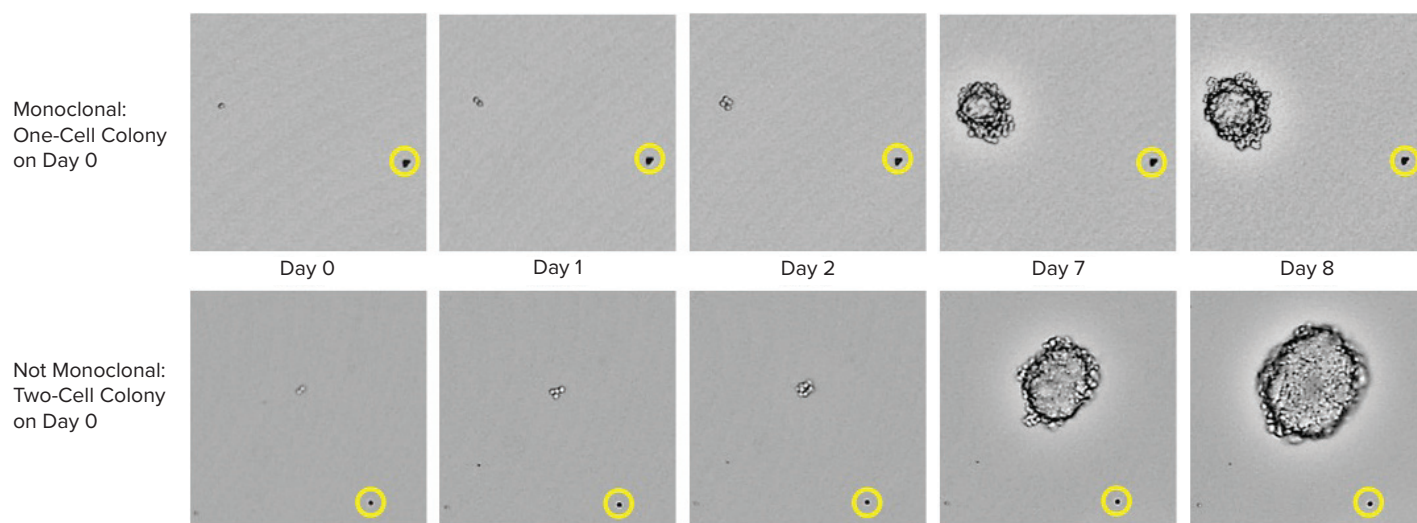

Figure 13. Detection and picking of cells producing tagged, non-mAb proteins. Tagged recombinant proteins can be detected by probing with fluorescently-conjugated antibody against tags. CHO cells were transfected with a construct encoding the protein of interest with the His6- and FLAG-tag sequences at the C-terminus of the protein and detected using a mix of anti-His and anti-FLAG antibodies. **(A).** White light image of adherent CHO colonies. **(B)** Fluorescent image of the colonies. They were grown as suspension in CloneMatrix-based semi-solid medium. Imaged with antibodies to His6 and FLAG, of which the anti-His6 antibody was conjugated to FITC to allow fluorescent visualization of the secreted protein.

Resources

- **Application Note:** Rapid automated selection of mammalian cell colonies by cell surface protein expression

- **Application Note:** Rapid and efficient selection of high producing mammalian cells secreting non-mAb proteins
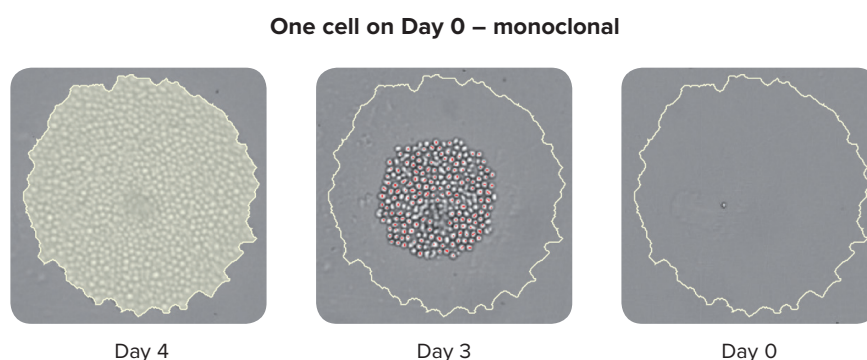
Monoclonality

When developing cell lines for biotherapeutics, it is crucial from a quality and regulatory perspective to ensure that the cell line originates from a single progenitor and is therefore monoclonal. Traditional cloning methods (e.g. limiting dilution and FACS) use statistical analysis to determine a confidence level for monoclonality. However, the documentation of monoclonality (a regulatory metric for biological cell lines) has driven the need for more robust technologies and methodologies in bioprocessing. Many researchers now routinely use imaging systems, such as the CloneSelect Imager, to verify monoclonality and monitor cell growth in cell culture media.

Monoclonal: One-Cell Colony on Day 0

Day 0 | Day 1 | Day 2 | Day 7 | Day 8

Not Monoclonal: Two-Cell Colony on Day 0

Figure 14. CHO-S cell growth in CloneMedia CHO Growth A semi-solid media. CloneSelect Imager was used to capture images from the 6-well plates at multiple time points. On Day 0, it is clearly observed on the top row that one cell is present while on the bottom row, two cells are observed. The yellow circles show the position of a bead that serves as a location reference to confirm that the same colony is imaged over time.

One cell on Day 0 – monoclonal

Day 4 | Day 3 | Day 0

Figure 15. Verification of monoclonality—viewing the origin of a colony. The growth (image) history of each well can be tracked back to its starting point—providing evidence of monoclonality.

Resources

• **Poster:** Rapid Monoclonality Verification Methods to Boost Cell Line Development

• **Brochure:** CloneSelect Imager

• **Application Note:** Confident identification of monoclonal CHO-S cells grown in semi-solid media using the CloneSelect Imager

Cell line development solutions

For detailed information, select the images or text.

ClonePix 2 System

CloneSelect Imager

XP Media and CloneMedia Complete Kit for Mouse Hybridoma Generation

CloneMatrix reagents and CloneMedia reagents

CloneDetect Reagent

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