Engineered Cell Monoclonal Screening and Monoclonality Verification (Post-print)

Authors: Jiang Yifan, Dong Jing, Wei Jingshuang

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Abstract

Monoclonality of engineered cells constitutes one of the critical factors ensuring product quality and has therefore garnered increasing attention. Presently, drug regulatory agencies require applicants to employ appropriate experimental methodologies to demonstrate the monoclonality of utilized cell lines. This review discusses approaches to ensure engineered cell monoclonality and the significance of maintaining monoclonality throughout biopharmaceutical manufacturing processes, by introducing the monoclonal selection process for production cell lines and commonly employed methods such as limiting dilution cloning, ClonePix, and flow cytometry.

Full Text

Preamble

Monoclone Selection and Monoclonal Verification of Engineering Cell Lines

Authors: Jiang Yifan*, Dong Jing, Wei Jingshuang
Affiliation: State Key Laboratory of Antibody Drug Development, New Drug Research and Development Company Limited, North China Pharmaceutical Corporation, Shijiazhuang 050015, China
Corresponding Author Email: jyifan@126.com

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Abstract

The monoclonality of recombinant cell lines is a critical factor ensuring product quality and has therefore received increasing attention from regulatory author-

ities. Currently, drug evaluation agencies require applicants to demonstrate the monoclonality of production cell lines through appropriate experimental methods. This review introduces the process of selecting monoclonal production cell lines and discusses common methodologies—including limiting dilution cloning, ClonePix, and fluorescence-activated cell sorting (FACS)—for ensuring monoclonality. We also examine the significance of maintaining monoclonal derivation throughout biopharmaceutical manufacturing.

Keywords: engineering cell line; monoclonality; single-cell cloning imaging system; limiting dilution

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1. Introduction

Biologics currently dominate the global pharmaceutical market. According to industry reports, eight of the top ten best-selling drugs worldwide in 2017 and 2018 were biologics, all produced by engineered cell lines expressing recombinant proteins [1,2]. The Chinese biologics market has grown correspondingly, from 62.7 billion RMB in 2012 to 152.7 billion RMB in 2016, representing a compound annual growth rate of 24.9% [2].

Compared to expression systems such as *E. coli* and yeast, mammalian cell expression systems—commonly used for large protein production—exhibit more extensive post-translational modifications and thus require higher quality homogeneity. The biopharmaceutical industry has reached consensus that therapeutic protein production cells must originate from a single progenitor cell, as monoclonality is essential for both product quality and expression stability [3,4]. This requirement is increasingly emphasized during regulatory submission processes. ICH Q5D states: "For recombinant products, the substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor" [5]. In 2011/2012, the U.S. FDA further required applicants to "submit data to the IND that provides assurance that this method resulted in derivation of a single cell clone or provide information on how you will go about generating these data and the timeframe for submission of the information" [6].

The three most common methods for obtaining monoclonal cell lines are: (1) limiting dilution cloning (LDC), (2) ClonePix, and (3) fluorescence-activated cell sorting (FACS) [4,7].

1.1 Limiting Dilution Cloning (LDC)

LDC is the traditional method for monoclonal selection. Its advantages include operational simplicity, minimal equipment requirements, and compatibility with imaging systems for documentation. However, its primary drawback is low efficiency, requiring screening of numerous 96- or 384-well plates to obtain desirable clones (typically selecting from no fewer than 1,000 clones).

LDC is highly dependent on manual operation, with variations in technique among different personnel affecting results. Additionally, differences in cell lines and cloning media across companies or research institutions introduce further variability. Therefore, organizations planning to use this method must establish standardized platform protocols and utilize fixed operators to minimize errors. Automated dispensing equipment can further reduce manual variation if available. For these reasons, LDC cannot provide a universal monoclonal formation rate, and it is generally accepted that a single round of LDC without imaging support is insufficient to confirm monoclonality. To achieve high monoclonality rates, at least two rounds of limiting dilution are recommended, with dilution ratios of 0.3–0.8 cells per well [8].

Given variations in cell lines, cloning media, and operational protocols among different laboratories—factors that directly affect single-cell viability (the percentage of single cells that grow into colonies)—and considering potential cell-cell interactions (such as clustering tendencies), simple Poisson distribution calculations are insufficient for determining monoclonal probability. We contend that the specific number of LDC rounds and optimal dilution ratios must be determined experimentally for each laboratory' s specific conditions.

Below, we describe two optimized experimental approaches for LDC using representative engineered cells after transfection and selection pressure.

Method 1: Integration with Monoclonal Imaging Systems
This approach involves plating cells at defined dilution ratios, then using an imaging system to photograph wells and record both the total number of colonies formed and the number confirmed as monoclonal (colonies originating from two or more initial cells where only one proliferates while others die or remain quiescent are counted as monoclonal). The monoclonal formation rate is then calculated.

Table 1 presents data from our laboratory using CHOK1 cells engineered to express a monoclonal antibody. Cells were plated at various dilution ratios, and the Solentim monoclonal imaging system was used to document colony formation and calculate monoclonal rates.

Table 1: LDC Combined with Monoclonal Imaging System to Calculate Monoclonal Rate

Dilution Ratio	Monoclonal Colonies	Total Colonies	Monoclonal Rate	Two-Round Monoclonal Rate*
0.3 cells/well	-	-	89.3%	98.9%
0.6 cells/well	-	-	76.4%	97.5%
0.8 cells/well	-	-	71.2%	96.9%

*Calculated values

The data demonstrate that in LDC selection, using a higher dilution ratio in the first round followed by a lower ratio in the second round yields more clones while maintaining high monoclonality rates. With an imaging system, monoclonality can be achieved and documented in a single LDC round, though optimal dilution ratios must be determined experimentally to maximize monoclonal yield per plate. For projects lacking early-stage monoclonality documentation, this method can provide supplementary evidence. It is important to note that inter-laboratory variations in cell lines, cloning media, and operational techniques result in different colony formation and monoclonal rates, making LDC data difficult to generalize across laboratories.

Method 2 [9]: Fluorescent Protein Mixing Without Imaging Systems
This approach involves transfecting host cells with red and green fluorescent proteins to create two separate cell pools. After selection pressure, equal numbers of cells from each pool are mixed 1:1 and plated at defined dilution ratios. The proportion of wells showing mixed red and green fluorescence is then observed under a fluorescence microscope.

The calculation formulas are as follows:

When considering only wells containing 1 or 2 cells, the single-round monoclonality rate (P1) is calculated by:
[Formula would be inserted here based on original text]

When wells contain 3 or more cells, the single-round monoclonality rate (P1) is calculated by:
[Formula would be inserted here based on original text]

The two-round monoclonal rate (P2) is calculated by:
[Formula would be inserted here based on original text]

Where:
N = total number of wells containing cells
N_0 = number of wells expressing both red and green fluorescent proteins

Method 1 can be applied to both LDC and FACS, while Method 2 is applicable to LDC, ClonePix, and FACS.

1.2 ClonePix for Monoclonal Selection

ClonePix is a high-throughput monoclonal screening device that operates in semi-solid medium with automated selection. Compared to LDC, ClonePix offers reduced manual intervention, higher throughput, and greater efficiency through fluorescence-based selection of high-producing clones. LDC uses liquid cloning media and ELISA-based screening, making it relatively more complex. However, optimized LDC media more closely resemble production culture conditions, potentially providing better predictive value for manufacturing performance.

ClonePix typically uses 6-well plates or dishes in semi-solid medium for monoclonal selection [10,11]. At the 2014 WCBP conference, Sarah Kennett noted that without reliable supporting data, single-round ClonePix selection achieves only 58–87% monoclonality [12]. However, recent studies demonstrate that with appropriate dilution ratios and optimized instrument parameters, high monoclonality rates can be obtained in a single round [9,13].

Literature reports provide calculation formulas and examples for determining monoclonal rates with ClonePix [13]. The formula is:

[Formula would be inserted here based on original text]

Where:
d(w) = well diameter
d(c) = colony diameter
n = number of colonies

For example, using a 6-well plate with 35 mm diameter and cell colonies of 0.75 mm diameter, the calculated monoclonal rate is 95.6% when n = 25 colonies [13].

Experimental studies have identified inter-colony distance as a key parameter (Table 2). With single-round selection, a colony distance of 1.0–1.2 mm yields 93% monoclonality, while distances >1.25 mm achieve >99% monoclonality. For applications requiring >99.5% monoclonality, multiple rounds are necessary. In practice, two consecutive ClonePix rounds or a ClonePix round followed by LDC subcloning are commonly used to increase monoclonal rates. Since ClonePix cannot directly integrate with imaging systems, LDC subcloning with imaging documentation is often employed to obtain reliable monoclonal evidence.

Table 2: Relationship Between Clonal Distance and Monoclonal Rate During ClonePix Selection

1.3 FACS for Monoclonal Selection

FACS selects single cells based on size, fluorescent dyes, and cell surface markers, and is widely used for single-cell isolation and rare cell sorting. This method can identify and isolate cells with high-level membrane protein expression [14]. When applied to monoclonal selection, FACS requires optimization to ensure one-to-one correspondence between sorted cells and individual wells. Cell morphology and condition affect sorting efficiency, and the FACS process itself may impact cell viability. Post-sorting colony formation rates are variable, necessitating optimized cloning media and robust cell lines.

FACS achieves high monoclonal formation rates and can be combined with imaging systems for verification, with reported monoclonality rates exceeding 99% when used together [5]. However, without imaging support, single-round FACS is generally considered insufficient, and subcloning via LDC is recommended to increase monoclonality rates.

2. Emerging Technologies for Monoclonal Selection

Recent years have seen the introduction of advanced equipment and methods for clone screening, including single-cell printers and Solentim VIPS, which significantly reduce manual dependency while improving efficiency and accuracy [9,16].

The single-cell printer deposits droplets from a cell reservoir through a nozzle, using high-resolution cameras to capture each droplet and confirm cell number. Droplets containing multiple cells or no cells are diverted to waste [17,18]. However, since this system does not image the well bottom, a separate monoclonal imaging system is required for documentation.

Solentim VIPS operates similarly to LDC but reduces droplet volume to approximately 30 nanoliters, achieving an average of far fewer than one cell per droplet. With up to 16 depositions per well, most wells receive a single cell (>80% single-cell wells, with some empty and some containing multiple cells). Cell identification is automated through AI-based software that analyzes 16 focal plane images of each droplet within 50 milliseconds to determine cell presence and number. Compared to manual LDC, VIPS yields more monoclonal colonies per plate while providing imaging evidence [19].

3. Regulatory Considerations and Supplementary Evidence

Monoclonality is fundamental to ensuring consistent product expression and quality. The advent of monoclonal imaging technology represents a significant advancement, providing direct visual evidence rather than probabilistic calculations. Imaging systems are currently the most reliable method for confirming monoclonality [20]. However, challenges remain: (1) achieving full-well imaging clarity, particularly at edges; (2) imaging only a single focal plane (typically the bottom), requiring cells to settle for clear visualization; and (3) accommodating non-planar well geometry through well-by-well autofocus. Modern automated systems integrated with barcode readers enable extended unattended operation while preventing manual handling errors such as misplacement or spillage [21].

For projects initiated before widespread imaging adoption, several approaches can strengthen monoclonality evidence:

1. **Calculate monoclonality rates** based on method parameters and conditions (as described above)
2. **Summarize stability data** across cell passages, evaluating growth characteristics, product quality, and expression consistency, as well as batch-to-batch product quality
3. **Generate a new master cell bank (MCB)** through subcloning, though this constitutes a major process change requiring feasibility assessment
4. **Employ next-generation sequencing** to demonstrate monoclonal derivation

5. **Apply fluorescence in situ hybridization (FISH)**, which detects consistency in chromosomal insertion sites to confirm MCB monoclonality [20,22,23]. FISH also screens out clones with unstable insertion sites or detrimental mutations that may pose risks during scale-up, even if derived from a single cell.

4. Stability Considerations

Engineered cells may undergo genotypic and phenotypic mutations during passaging, potentially introducing heterogeneity even in single-cell-derived banks. Heterogeneity arises from two sources: (1) non-clonal origin, or (2) mutations in a subpopulation derived from a single progenitor. While such sequence heterogeneity may not affect process consistency or robustness [24], some single-cell-derived clones remain unsuitable for manufacturing. Therefore, long-term stability studies are essential to evaluate molecular characteristics, growth profile, productivity, and product quality consistency beyond the anticipated production generation number [25].

Stability study duration should be determined based on projected manufacturing scale and corresponding passage numbers. Whether to maintain selection pressure during passaging depends on specific circumstances. While pressure-free passaging is recommended, most evidence suggests that maintaining copy number stability without selection pressure is challenging. Consequently, stability studies often include both pressure-free and pressure-maintained conditions, sometimes across multiple cell lines, to ensure identification of a stable production clone.

5. Conclusion

Ensuring high monoclonality rates mitigates risks to product quality homogeneity. This review summarizes common monoclonal selection methods, equipment, and strategies for achieving and documenting high monoclonality rates through experimental design, calculation, and statistical analysis. We hope these insights provide practical solutions for challenges encountered during cell line development and regulatory submission, offering valuable guidance to researchers in the field.

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Note: Figure translations are in progress. See original paper for figures.