High efficiency sorting and outgrowth for single-cell cloning of mammalian cell lines

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Research Article

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Abstract

To simplify single-cell cloning with the ability of sorting specific target populations of interest with a higher cell outgrowth, several cell lines were sorted onto 96-well plates containing cloning media formulations to enhance single cell survival. Cell lines that were tested included suspension cells (CHO ES, Expi293F, and Jurkat) and adherent cells (MCF7, A549, and HEK293). In parallel with the single-cell sorting experiments, limiting dilution at 0.5 cells/well was used as a control for viability relative to sorting and to demonstrate the increased efficiency of using the WOLF and N1 single-cell dispenser for cell line development. The combined use of the cell sorter and optimized cloning medium resulted in greater than 50% cell outgrowth. The WOLF with the N1 achieves cell line development goals by sorting specific cells with gentle sorting resulting in high probabilities of monoclonal outgrowth.

Introduction

Single-cell cloning has numerous applications, such as therapeutic protein production, drug screening, and gene therapy (Soitu et al. 2019). Therapeutic protein production has depended on using Chinese hamster ovary cells (CHO) and human embryonic kidney cells (HEK293). CHO cells are the most commonly used mammalian cell line and are responsible for the commercial production of 60-70% of the therapeutic proteins available in the market (Zhu et al. 2017). In addition, HEK293 is also used to produce biopharmaceuticals with the advantage of being of human-origin (Abaandou et al. 2021). Drug screening requires cell models for diseases such as MCF7, A549, and Jurkat. Additionally, single-cell cloning can play an important role in understanding cell function and disease mechanisms for insight into discovering treatments for gene or cell therapy (Bode et al 2021). An important component for therapeutic applications is to ensure monoclonality. Regulatory agencies require that the cell lines expressing the desired therapeutics for commercial manufacture are to be derived from a single progenitor cell (Chen et al 2019). A lack of monoclonality can result in discrepancies in product quality, inaccurate reproducibility of results, stability of recombinant proteins, and poor outgrowth. Challenges of single cell cloning can be mitigated by the cloning method and the media used to improve monoclonal outgrowth. The cloning method used should be capable of discriminating cells of interest from a heterogeneous population and be able to deposit a single cell per well for the colony to be monoclonal (Castan et al. 2018). Challenges with single cell cloning can be addressed by using the WOLF Cell Sorter alongside the N1 Single-Cell Dispenser (NanoCellect Biomedical, San Diego, USA) (Fig. 1A). Single cell deposition has been validated with 15µm Dragon Green (DG) Beads and CHO cells with an average efficiency of 91.7 ± 1.3% (Fig. 1B). Using the WOLF increases productivity in cell line development while maintaining high cell viability due to its capability of gently sorting one cell per well. In combination with optimized cloning media, there is an improvement in outgrowth of colonies that originated from a single cell.

Materials And Methods

Cell Lines

The following cell lines were single-cell sorted to determine the outgrowth with optimized cloning medium combinations to ensure single cells can survive and grow into well-established colonies. The cell lines used were CHO ES (Expression Systems, Davis, USA), HEK293 GFP (GenTarget Inc, San Diego, USA), HEK 293 GFP-RFP (GenTarget Inc, San Diego, USA), Expi293F (Gibco, USA), CHO-K1 (ATCC CCL-61), Jurkat Clone E6-1 (ATCC TIB-152), MCF7 (ATCC HTB-22), and A549 (ATCC CCL-185). A549 was transduced in-house with pre-made GFP (CMV, Bsd) lentiviral particles (GenTarget, San Diego, USA). Meanwhile, HEK293, CHO-K1, and HEK293 GFP-RFP were purchased as stable cell lines that can express fluorescent proteins.

Growth Medium

CHO ES were grown in ESF SFM Mammalian Cell Culture Medium (Expression Systems, Davis, USA). Expi293F were grown in Expi293 Expression Medium (Gibco, USA). Both these suspension cell lines were grown in 125mL Erlenmeyer shaker flasks with no added supplements. Adherent cells were grown in T75 tissue-treated culture flasks. HEK293 and MCF7 were grown in Dulbecco's modified Eagle medium (DMEM) (Genesee Scientific, San Diego, USA). Meanwhile, A549 and CHO-K1 were grown in Ham's F-12K (Kaighn's) medium (Gibco, USA). Jurkat is a suspension cell line that was grown in RPMI-1640 (Gibco, USA) in T75 suspension cell flask. The basal medium used to grow these cells was supplemented with 10% fetal bovine serum (FBS) (Genesee Scientific, San Diego, USA) and 1% antibiotic-antimycotic (Invitrogen, USA).

Sample and Sheath Buffer Preparation

Cells that were known to re-aggregate, such as MCF7, HEK 293, and A549, were sorted using a sheath buffer composed of 1X phosphate-buffered saline (PBS) (Genesee Scientific, San Diego, USA), 0.5% bovine serum albumin (BSA) (Thermo Scientific, USA), 5mM EDTA (Invitrogen, USA), and 12.5mM HEPES (Gibco, USA). For Jurkat, a sheath buffer composed of 1X PBS, 0.5% BSA, and 25mM HEPES was used. Finally, the serum-free suspension cell lines, used the media they grow in as sheath buffer supplemented with 12.5mM HEPES. The sheath buffer was also used as sample buffer when preparing the cells for sorting. Buffers were filtered with 0.2µm syringe filters prior to use.

Cloning Medium Preparation

Specific cloning media were prepared for the type of cell line used (Table 1).

Table 1 Preparation of Cloning Media to Optimize Single Cell Outgrowth: Better cell outgrowth was obtained when using cloning media that contained nutrients to aid in the survival of cells at a single cell level

Cell Line	Cloning Medium
CHO K1	Ham's F-12K (Kaighn's) medium, 10% FBS, 1% anti-anti
CHO ES	80% EX-Cell CHO, 20% ESF SFM, 6mM L-GlutaMAX, 1X MEM NEEA, 1:80 ClonaCell-CHO ACF Supplement
Expi293F	HE150 Medium
HEK293	FluoroBrite DMEM, 10% FBS, 4mM L-GlutaMAX
MCF7	DMEM, 10% FBS, 1% anti-anti
Jurkat	RPMI, 10% FBS, 10% conditioned medium
A549	FluoroBrite DMEM, 10% FBS, 4mM L-GlutaMAX

HEK293 and A549 used Fluorobrite DMEM (Gibco, USA) with 10% FBS and 4mM L-GlutaMAX (Gibco, USA). Expi293F was grown in HE150 Medium (GMEP Cell Technologies, Japan) that was supplemented with 6mM L-GlutaMAX. CHO ES was grown in a cloning medium composed of 20% ESF SFM Expression Medium with 80% EX-Cell CHO medium (Millipore Sigma, Temecula, USA) that was supplemented with 6mM L-GlutaMAX, 1X MEM non-essential amino acids (Gibco, USA) and 1:80 ClonaCell CHO ACF Supplement (StemCell Technologies, Vancouver, Canada). MCF7 cells were grown in DMEM with 10% FBS. CHO-K1 were grown in basal medium composed of Ham's F-12K (Kaighn's) medium with 10% FBS and 1% antibiotic-antimycotic. Lastly, Jurkat cells were grown in RPMI-1640 supplemented with 10% FBS and 10% conditioned medium.

Single-Cell Sort

The tested cells were analyzed on the WOLF (NanoCellect Biomedical, Inc., San Diego, USA) to acquire a scatter plot of FSC and BSC and create a singlets gate to remove doublets from the sort. CHO ES, Expi293F, and Jurkat cells were sorted without viability dyes or markers . HEK293, CHO-K1, and A549 cells were sorted based on GFP fluorescence. HEK293 cells that were dual-expressing GFP and RFP were sorted based on being able to express both fluorophores. MCF7 cells were sorted based on viability dye staining, such as DRAQ7 (BioStatus, Leicestershire, United Kingdom). Prior to sorting, the cells were prepared to have a final concentration of 1.0×10^5 cells/mL in the same buffer used for the sheath and were filtered using 35µm filter cap tubes (Genesee Scientific, San Diego, USA). Using a sterile cartridge, the cells were sorted into three 96-well plates that were pre-filled with 200 µL of cloning medium specific to each cell line to enhance outgrowth. CHO-K1, CHO ES, HEK293, MCF7, Jurkat, and A549 were grown in Tissue Culture 96-well plates (CellTreat Scientific Products, USA). While Expi293F were sorted in ultra-low attachment 96-well plates (Crystalgen, Inc, New York, USA)

Limiting Dilution

Additional 96-well plates were prepared by limiting dilution to function as a control. The cells were diluted to deposit 0.5 cells/well into 96-well plates that were pre-filled with 200µL of the optimized cloning media for the specific cell line. For the CHO-K1 GFP cell line, the sample used for limiting dilution was prepared at a 3:1 ratio with a non-GFP expressing wild type CHO-K1 (ATTC CCL-61).

Analysis of Cell Outgrowth

Plates were maintained in a 37°C incubator, 5% CO_2, and analyzed on Day 7 and Day 14 using the Celigo (Nexcelom Bioscience LLC, Massachusetts, USA). CHO-ES, Jurkat, Expi293F, and MCF7 cells that did not express a fluorescent protein were analyzed using only the brightfield channel. Meanwhile, CHO-K1 GFP, A549, and HEK293 GFP were analyzed with the brightfield channel alongside the green channel (483/536 ex/em). Finally, HEK293 GFP+RFP were analyzed with the brightfield channel, green channel (483/536 ex/em), and red channel (531/629 ex/em) channel. Monoclonal outgrowth was calculated by counting the number of wells that contained a single colony and comparing and confirming that the visualized colony is monoclonal by analyzing the 96-well plates on Day 7 and Day 14 (Fig. 2). The experiments were performed in triplicate for each cell line tested.

Results And Discussion

Traditional methods for single cell cloning have included techniques such as limiting dilution and fluorescence activated cell sorting (FACS). The gold standard method of liming dilution is based on diluting cells to low concentrations to obtain a single cell per well, based on Poisson statistics. Limiting dilution can result in multiple wells having more than one cell and therefore not being monoclonal. Because of this, cells of interest require multiple rounds of cloning to assure monoclonality and increasing the time it takes to develop a new cell line. In addition, FACS is a method that can analyze millions of cells and is able to isolate cells of interest based on fluorescent or scatter-based markers. It is capable of discriminating doublets; increasing the probability that one cell is deposited per well. However, traditional sorting systems rely on high pressure that results in cells encountering mechanical stress from the large changes in pressure and the shear forces generated at the nozzle. Moreover, many systems require high maintenance to maintain sterility and prevent contamination (Yim and Shaw 2018).

While droplet-based sorting and the WOLF can both select for cells of interest, the WOLF has a low sorting pressure of less than 2 psi, which is gentle compared to conventional droplet based sorters. Also, the WOLF uses sterile, single-use cartridges to prevent microbial or cross contamination. Therefore, the WOLF is optimal to select for the cells of importance while depositing a single cell per well resolving the challenge of using traditional methods for single cell isolation.

When analyzing the 96-well plates on Day 14, total outgrowth differs across cell lines with the number of wells that contained a single colony ranging from 50-90% depending on the cell line (Fig. 3). On average, they were 66.7 ± 15.9% wells containing a single colony when sorting with the WOLF compared to an average of 23.8 ± 6.8% of colonies obtained from limiting dilution. Therefore, the WOLF can reduce the number of cloning rounds needed to ensure monoclonality or the amount of plates to prepare to increase the number of available clones to be used for any downstream application.

Using the WOLF with its WOLFViewer, the applicable gating strategies allowed for the selection of specific target populations increasing that the sorted cell is the one of interest leading to a more productive cell line development workflow. Fluorescent markers or antibody dyes were not always needed to perform sorting with higher monoclonal outgrowth. CHO ES, Jurkat, and Expi293 used a label-free method of sorting and relied on a scatter gate followed by a singlet gate to isolate a single cell per well (Fig. 4A). CHO ES had a monoclonal outgrowth of 91.0 ± 1.0% compared to 30.2 ± 3.7% when using limiting dilution. Expi293F had 59.8 ± 3.9% monoclonality compared to 20.3 ± 6.4% from limiting dilution. Lastly, Jurkat had a monoclonal outgrowth of 45.4 ± 3.4% compared to 31.6 ± 7.3% from using limiting dilution. Overall, label-free sorting had a two-fold increase or higher in the number of wells that had one colony compared to limiting dilution.

Furthermore, limiting dilution does not select for target populations of interest. For example, when performing limiting dilution using the 3:1 mixture of non-GFP expressing CHO-K1 Wild Type with CHO-GFP, there was an average total outgrowth of 32.5 ± 9.8% where 13.5 ± 4.4% of the colonies were monoclonal and were expressing GFP while the remainder of the wells contained the non-GFP expressing wild type CHO cells. This demonstrated that limiting dilution lacks the ability to select the target population leading to more rounds of cloning to obtain the cell of interest. When sorting CHO-K1 GFP, a sample gate, followed by a singlets gate, and a GFP-positive gate was used and resulted in 87.0 ± 3.3% monoclonal outgrowth of pure GFP-expressing CHO Cells compared to the previously mentioned GFP-expressing monoclonal outgrowth using limiting dilution (Fig. 4B). The sorting strategy to isolate GFP-positive cells was also used for A549 and HEK293 GFP resulting in monoclonal outgrowth of 63.1 ± 5.7% and 73.8 ± 2.9%, respectively. Meanwhile, when using the WOLF to sort the HEK293 GFP-RFP cells, it resulted in monoclonal outgrowth of 57.2 ± 7.04% based on a bivariate plot gating the cells that expressed GFP and RFP (Fig. 4C). Cell lines that are prone to undergo rapid cell death and changes, such as MCF7, benefited from using a viability dye to exclude dead cells from the sorting (Fig. 4D). MCF7 had monoclonal outgrowth of 56.6 ± 2.3% when using a viability dye compared to 17.2 ± 3.2% when using limiting dilution where a viability dye cannot be used to select for the live cells only.

A second challenge resolved was to ensure cell survival at a single cell level. Cell outgrowth was strongly dependent on the type of cloning medium used to grow each cell line and requires optimized formulations to mimic a greater number of cells to support cellular growth (Castan et al. 2018). Cloning media requires supplements that are needed to support cell growth at low cell densities. Traditionally, FBS

has been used due to being rich in nutrients and growth factors, but regulations are trying to steer away from using FBS due to having batch-to-batch variations and introduction of adventitious agents (Lim and Shaw 2018). Other supplements have included the use of conditioned media, which is a collection of proteins that contain signal peptides that have been processed by the endoplasmic reticulum and Golgi apparatus composed of proteins that include enzymes, growth factors, cytokines, and hormones that are important for cell growth (Dowling and Clynes 2011). Fully defined media requires growth factors such as insulin growth factor-1 (IGF-1), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), albumin, fibronectin, thrombospondin-1, laminin, clusterin, decorin, cathepsin B, legumain, and protease inhibitors (Lim and Shaw 2018).

The importance of media optimization was observed during preliminary experiments. For example, initial testing for the outgrowth of suspension CHO ES cells was using basal growth medium instead of the optimized cloning medium. These initials tests resulted in no cells growing in the sorted 96-well plates nor in the plate prepared via limiting dilution (data not shown). However, once the cloning medium was supplemented to support single cell growth, outgrowth increased to 91.0 ± 1.0% of the plate containing colonies while 30.2 ± 3.7% outgrowth was observed when using limiting dilution. Another initial test consisted of growing A549 with basal growth medium, which resulted in an outgrowth of 50.0 ± 4.54%. Once the cloning medium was changed to DMEM, the outgrowth increased to 63.1 ± 5.7%, which is a percent increase of 23.2%. The 96-well plate were also important to enhance cell survival leading to a better outgrowth. For example, initial testing with Expi293F used TC-treated culture plates with its optimal cloning medium resulting in 51.2 ± 4.8%. The monoclonal outgrowth increased by 15.5% once the cells were sorted into ultra-low attachment 96-well plates.

Conclusions

Using the WOLF alongside the N1 Single Cell Dispenser is an ideal platform that enhances outgrowth due to combining the ability to select specific cells of interest, gently sort these cells, and depositing one cell per well in different cloning media that will further support cell line development workflows. The capability for the system to deposit a single cell per well with high fidelity can also decrease the time it takes to develop new cell lines since there is no need to perform several rounds of cloning to ensure monoclonality. In addition, there will not be a need to prepare multiple plates via limiting dilution to match the amount of plates obtained when you sort with the WOLF. Therefore, the WOLF is the method that has resolved the challenge of what method to use to ensure high levels of monoclonality.

Declarations

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Author Contributions JM contributed to the experimental design and AM performed sample preparation, experiment operation, and data analysis. All authors have read and approved the final manuscript.

Conflict of Interest Authors Adonary Munoz and Jose Morachis are employees of NanoCellect Biomedical Inc

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Figures

Figure 1

WOLF and N1 Single-Cell Dispenser Setup. A 96-well plates or 384-well plates were easily placed into the N1 dispenser to facilitate single cell dispensing. B The ability to deposit a single cell per well has previously been validated and resulted in an dispense efficiency of 92.3 ± 9.6% for DG beads and 90.8 ± 4.3% for CHO cells in 96-well plates. Meanwhile, the dispense efficiency when using 384-well plates was of 93.3 ± 8.76 % for DG beads and 90.6 ± 3.9% for CHO cells, which demonstrates that a well will contain a colony originating from a single cell.

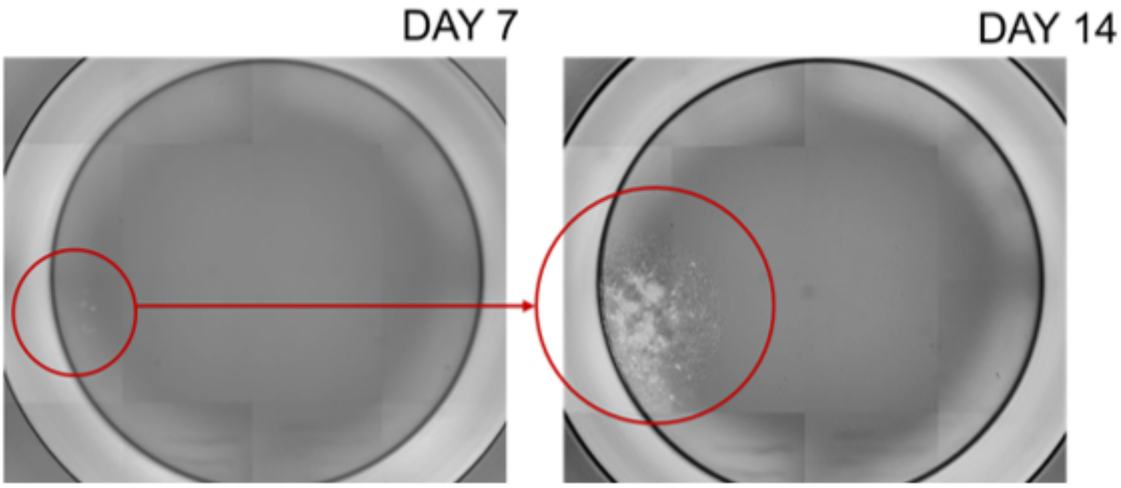

Figure 2

Representative images of the cell lines visualized on Day 14 at 10X magnification on the Celigo. Colonies stayed in the same position of the well for Day 7 and Day 14 allowing the identification that the observed colony is the same for both days of analysis

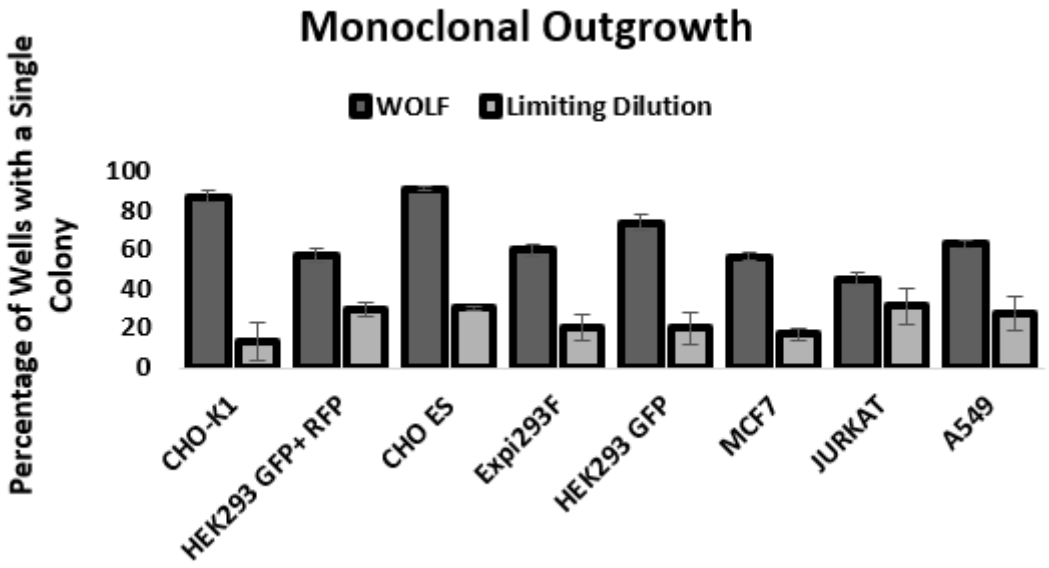

Figure 3

Percentage of Wells Containing One Colony per Well on Day 14. Using the WOLF for cell line development resulted in more colonies available for cell line development compared to limiting dilution without the need to perform multiple rounds of cloning.

Figure 4

Gating Strategies for Specific Single Cell Isolation. When using the WOLFViewer, it is easy to gate the population of interest to ensure that the target cell of interest is isolated during the sort. A Gating strategy for cells without the need of any dyes was used to sort CHO ES, Jurkat, and Expi293F. Gating strategy for the cells that expressed fluorescence proteins such as B only GFP or C cells that dual-express GFP-RFP. D Gating strategy for cells that were stained with a viability dye such as when sorting MCF7.