CELL Microsystems®

CASE STUDY

High Efficiency Cell Line Development of a Density-Dependent Hepatic Cell Line

Introduction

Immortalized liver cell lines like Huh7, HepG2, and Hep3B are important tools for studying liver cancer, viral hepatitis, drug toxicity, and hepatic physiological processes. While obtaining a monoclonal population of cells is an essential step in many of these studies, most hepatic cell lines are notoriously difficult to clone from single cells. Common methods of single-cell cloning such as flow cytometry, single-cell dispensers, clone pickers, and limiting dilution leave a single cell in a well lacking growth factors secreted by neighboring cells. Hepatic cells are particularly dependent on these growth factors and often fail to proliferate in this biologically unfamiliar environment. Consequently, outgrowth efficiency is low and large amounts of media and cultureware are required to generate a relatively small number of clones that may not maintain desired phenotypes, especially if they had been subjected upstream to additional stressors like gene editing, viral infection, or drug treatment.

Hep3B, a human hepatocellular carcinoma line, is widely used in hypoxia studies for its ability to secrete erythropoietin (Epo) in response to hypoxia. An academic laboratory focused on hypoxia-related pathways needed a monoclonal population of Hep3B cells but found that seeding cells at low densities in traditional single cell cloning workflows permanently altered hypoxia-induced Epo expression. CellRaft® Technology overcomes the limitations associated with single cell cloning by allowing single cells to proliferate in shared media on the CellRaft Array while maintaining single cell spatial separation. Once colonies have reached desired confluence, CellRafts containing monoclonal colonies can be automatically isolated for further expansion and analysis using the CellRaft AIR® System. This Case Study presents an efficient, integrated workflow for cell line development of Hep3B cells.

Key Highlights:

1. Limiting dilution failed to generate monoclonal populations of Hep3B cells that maintained hypoxia-induced erythropoietin expression.
2. Hep3B clones were successfully propagated and isolated on the CellRaft AIR® System, and CellRaft Cytometry™ was used to identify hundreds of monoclonal colonies on one CellRaft® Array culture dish.
3. Clonal Hep3B colonies were isolated into 96-well plates and successfully expanded for downstream analysis.

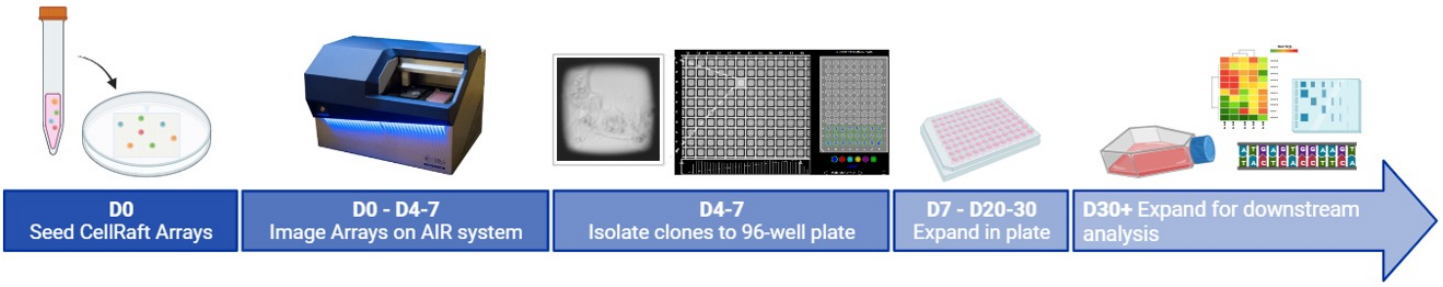
Scientific Problem

To study hypoxia-induced gene expression, researchers at an academic lab needed to generate monoclonal populations of Hep3B human hepatoma cells. However, when cells were plated in limiting dilution, populations that arose from a small number of cells (1-3 cells) exhibited a permanently altered hypoxia response. The researchers turned to the Cell Microsystems Clone Challenge to leverage CellRaft Technology, a novel platform for high-efficiency single cell cloning.

Experimental Design

Parental Hep3B cells were seeded on a 100S and 200S CellRaft Array in complete culture media. The Arrays were imaged on the CellRaft AIR System every 24 hours to monitor viability, clonality, and morphology. CellRaft Cytometry™ was used to identify CellRafts containing a single cell at the first scan and a monoclonal colony at the final scan. On day 4 for the 100S Array and day 7 for the 200S Array, two 96-well collection plates were pre-loaded with 100 µL conditioned or unconditioned complete culture media and CellRafts containing monoclonal colonies were automatically isolated into the collection plates. Conditioned media, was prepared by harvesting media from cells in log-phase growth in a tissue culture flask and filtering through a 0.2 µm sterile filter. Expansion off-Raft in the collection plate was visually confirmed. Clones that expanded were maintained in the collection plate until large colonies formed, then trypsinized and transferred to new wells of a 96-well plate to prevent overgrowth at the center of the colony. Clones were then expanded stepwise up to T-75 culture flasks and cryopreserved for future downstream analysis.

To measure Epo induction under hypoxic conditions, expanded clones were cultured in normoxia or hypoxia (1% oxygen) for 8 hours. RNA was harvested after the 8-hour exposure, and Epo fold-induction of hypoxia-induced Hep3B clones was measured by RT-PCR of cDNA.



CellRaft AIR Workflow: On day 0, Hep3B cells are seeded onto a CellRaft Array. The CellRaft Array is scanned on the CellRaft AIR system 4 hours post-seeding and every subsequent 24 hours until monoclonal colonies reach a desired size. CellRaft Cytometry is used to identify CellRafts containing monoclonal colonies, and desired CellRafts are automatically deposited into a collection plate using the CellRaft AIR System. From the collection plate, clones can be expanded for downstream analysis.

Results

Hep3B single cells proliferated into monoclonal colonies on both types of CellRaft Arrays, and growth from single cell to colony was tracked with daily scans on the CellRaft AIR System (Figure 1). CellRafts containing monoclonal colonies were identified using preset populations in CellRaft Cytometry (Figure 2), and hundreds of monoclonal colonies were available for isolation by day 4 from the 100S Array and day 7 from the 200S Array (Figure 3). Isolated clones successfully expanded off-Raft in 96-well plates (Figure 4). Colonies expanded off-Raft with very low efficiency (0-3% of isolated rafts) when isolated into unconditioned media. However, isolation into conditioned media significantly increased outgrowth compared to unconditioned media from 3% to 26% from the 100S Array and 0% to 43% from the 200S Array (Figure 5).

Time to complete the entire workflow from seeding single cells on the array to a 96-well plate of expanding cells with confirmed clonality was less than two weeks requiring less than two hours of hands-on time. As the doubling time of Hep3B cells is longer than most transformed cell lines and was heterogeneous between clones, total time for expansion from 96-well plates to T-75 flasks ranged from 35-61 days. Expanded clones exhibited a more homogeneous morphology compared to the parental polyclonal population (Figure 6). Hep3B clones tested by RT-PCR for hypoxia-induced Epo expression demonstrated a >30-fold induction of Epo in six out of the thirteen total clones tested (Figure 7).

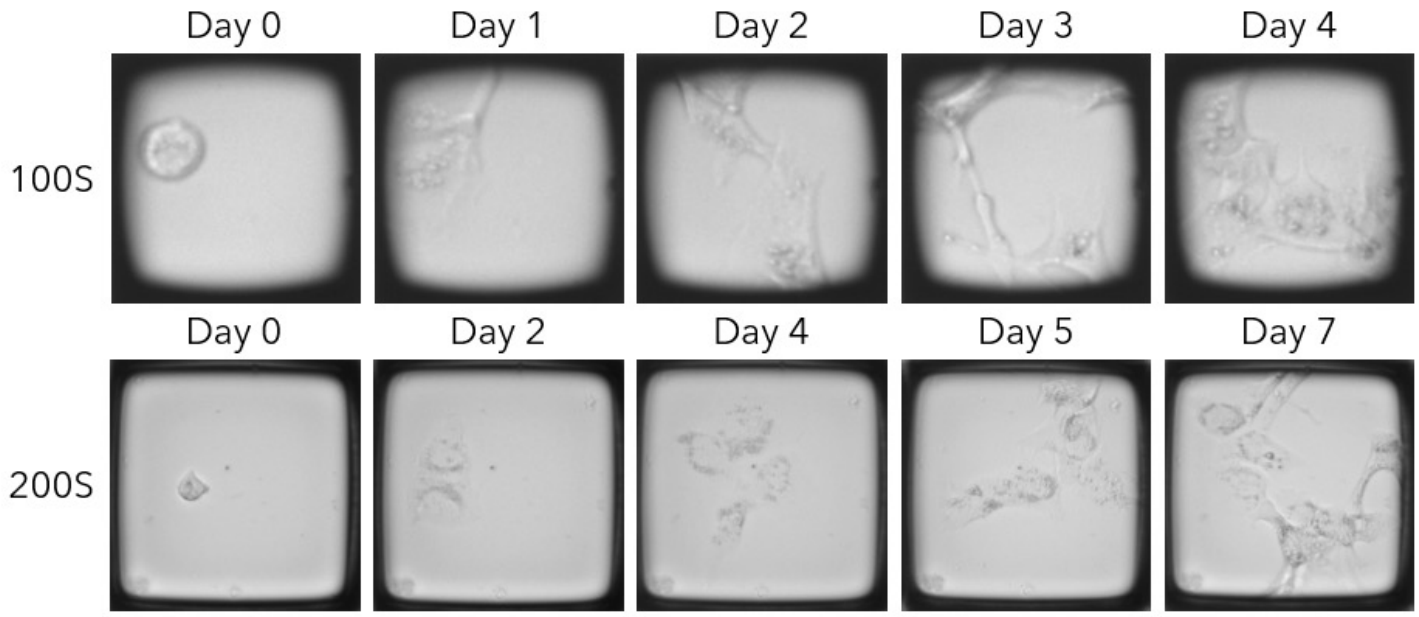


Figure 1. Representative time course images of Hep3B cells proliferating from a single cell to a monoclonal colony on 100S and 200S CellRaft Arrays. Hep3B cells were seeded on each CellRaft Array and serially scanned every 24 hours to monitor clonality, viability, and morphology. CellRafts of interest were automatically isolated on the CellRaft AIR System after 4 days from the 100S Array and 7 days from the 200S Array.

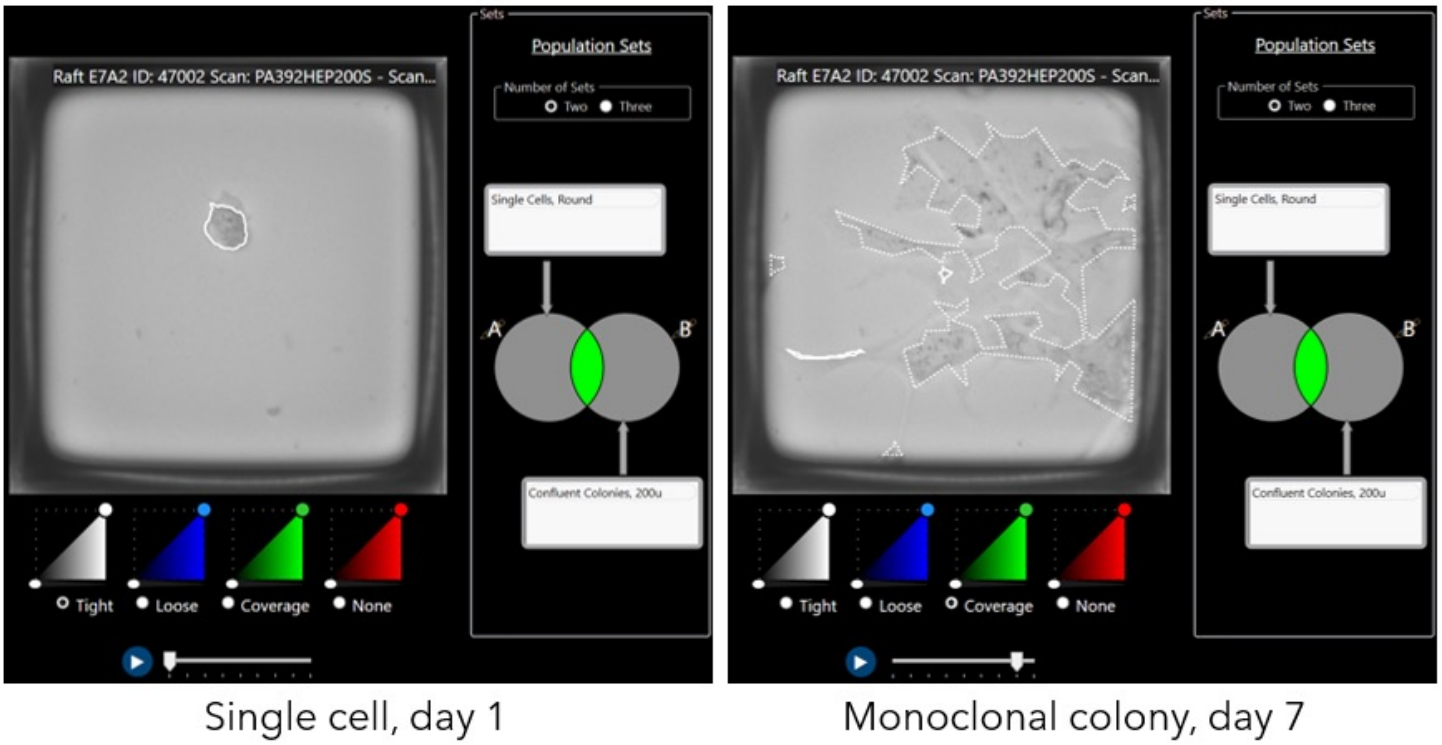


Figure 2. Representative example of the use of CellRaft Cytometry to identify CellRafts containing monoclonal colonies on the 200S CellRaft Array. Two population sets, single cells on day 0 and colonies on day 7, were overlayed to locate CellRafts containing monoclonal colonies.

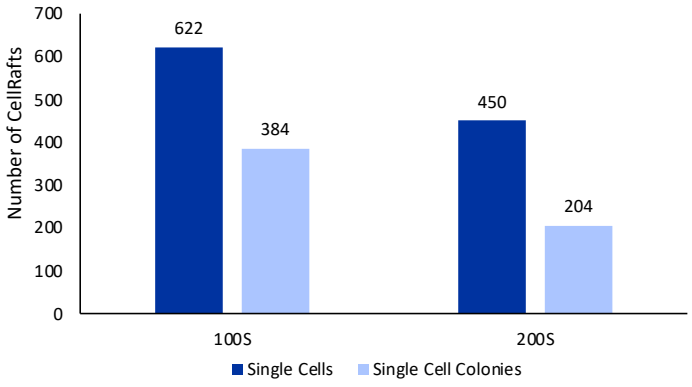


Figure 3. Number of single cells on day of seeding and single cell-derived colonies at day 4 (100S) or day 7 (200S) post-seeding on 100S and 200S CellRaft Arrays. Hundreds of monoclonal colonies were available for isolation and further expansion from each Array.

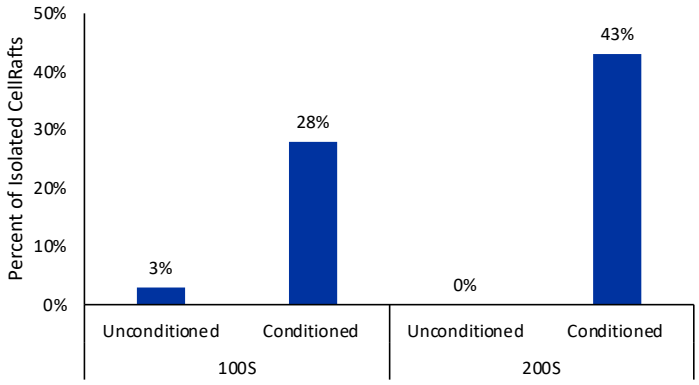


Figure 4. Outgrowth of Hep3B cells isolated from the CellRaft Array into conditioned or unconditioned culture media. Conditioned media resulted in significantly higher outgrowth efficiency from both Array types. Optimal outgrowth was obtained from the 200S CellRaft Array isolated into conditioned media.

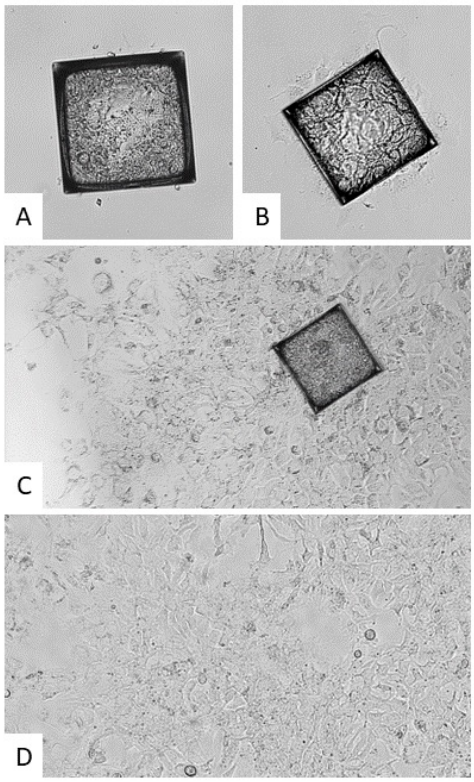


Figure 5. Hep3B clones post-isolation from 200S Array in conditioned or unconditioned culture media. (A) Hep3B clone in unconditioned media 9 days post-isolation failed to expand off-Raft (20X magnification). (B-D) Hep3B clone expanding off-Raft in conditioned media at 8 days post-isolation (20X magnification (B), 15 days post-isolation (10X magnification) (C), and expanded to a T-75 culture flask (10X magnification) (D). At 15 days post-isolation, the colony reached a sufficient size to expand to a new well of a 96-well plate to prevent contact inhibition at the center of the colony. The clonal population was then expanded stepwise to T-75 culture flasks and cryopreserved. Cells isolated into conditioned media were maintained in conditioned media until expansion to 6-well plates.

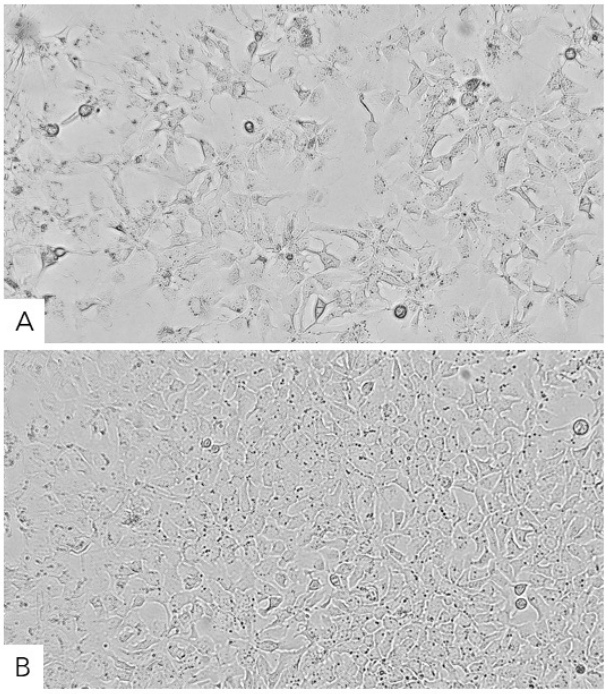


Figure 6. Parental polyclonal Hep3B population (A) and monoclonal population (B) of Hep3B cells in T-75 culture flasks (10X magnification). Monoclonal populations exhibited more homogeneous morphology compared to the parental polyclonal population.

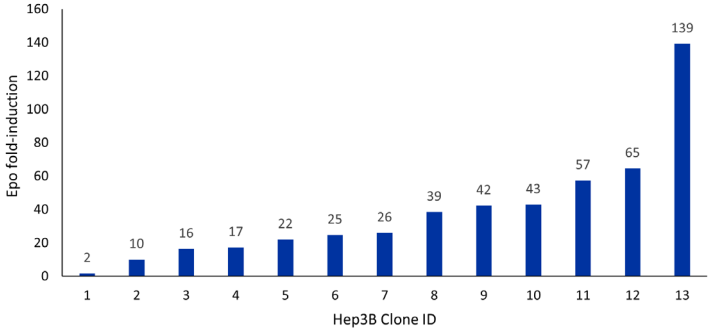


Figure 7. Epo fold-induction of hypoxic Hep3B clones (1% oxygen, 8 hours). Expected induction is 30-50 fold based on previous work with polyclonal Hep3B cells.

Discussion

The generation of homogeneous, monoclonal populations of cells that can provide high quality, reproducible data is an essential step in many cell biology workflows. While there are several established methods for single cell cloning, each one leaves a single cell in a well lacking growth factors from neighboring cells. For some cell types, this biologically unfamiliar environment can result in a lack of clonal proliferation and a loss of expected phenotypes. This struggle was exemplified in the case study presented here where attempts to generate single cell clones of Hep3B cells that maintained hypoxia-induced Epo expression failed as seeding cells at low densities caused permanent cellular differentiation.

A unique advantage of the CellRaft Technology over other workflows is the ability to grow single cells to monoclonal colonies in a shared media environment before isolation of an entire colony, rather than a single cell, to a 96-well plate. While over 100 cell lines have been validated on the CellRaft Technology platform with significantly increased efficiency over limiting dilution and without the use of conditioned media or growth factor supplementation, Hep3B colonies, still failed to expand off-Raft when isolated into fresh media. Isolation of colonies into conditioned media significantly increased outgrowth from 3% to 28% from the 100S Array and 0% to 43% from the 200S Array. Of 68 total clones that expanded in the original collection plate, 18 clones were fully expanded to T-75 flasks and banked for future analysis. RT-PCR of hypoxia-induced Hep3B clones revealed a >30-fold induction of Epo in six out of thirteen clones tested, a phenotype that was previously lost when seeding cells at low densities in traditional single cell cloning workflows. The generation of clonal populations, from seeding the CellRaft Array to expansion of clones in 96-well plates, required less than 3 weeks of total workflow time and less than 2 hours of hands-on time.

Conclusions

In this case study, CellRaft Technology was used to generate monoclonal populations of Hep3B cells that retained hypoxia-induced Epo expression, a phenotype that was previously lost due to differentiation of cells seeded at low densities during traditional single cell cloning workflows. While limiting dilution failed to generate Hep3B clones that retained Epo expression, a single CellRaft Array generated hundreds of colonies that retained this phenotype while requiring significantly less media, cultureware, and hands-on time than traditional single cell cloning workflows like limiting dilution.

References

1. Biorender. Retrieved March 28, 2023 from https://biorender.com/.

For more information on the presented data or CellRaft Technology, visit cellmicrosystems.com

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