CELL Microsystems®

OLS OMNI Life Science

CASE STUDY

Identification of a rare double positive clone

Collaborative case study in the use of a new technology platform for fast and easy cell line development

Monoclonal cell line development for NIH-3T3 Cells Expressing EBFP2 and HER-2

Developing novel cell lines and disease models is a cornerstone and a necessary tool for scientific discovery. Typically, this process involves creating a vector such as a plasmid, virus, doggybone DNA, or transposon to alter gene expression, either by overexpression or deletion. The construct is then transfected or infected into cells of interest, and successful insertion of the transgene is identified by flow cytometry, microscopy, or antibiotic selection **(Figure 1).** After a positive population has been recovered, a suitable clone with the desired phenotype needs to be generated to ensure homogeneity in downstream applications. Methods to achieve a clonal population include fluorescence activated cell sorting (FACS), low-pressure dispensers, clone pickers, and limiting dilution. However, developing cell lines using these methods often: a) negatively impacts cell viability, resulting in poor colony outgrowth from isolated clones b) incurs high cost of operation, c) requires large quantities of consumables, d) is inefficient and labor intensive to confirm clonality, and e) requires a high degree of operator expertise and troubleshooting.

The case study presented here exemplifies the above-mentioned failure modes and highlights the utility of a novel technology/platform that was leveraged to help a research lab at Duke University (Durham, NC, USA) accelerate their science. The experiment necessitated generating a NIH-3T3 cell line that expressed both human epidermal growth factor receptor 2 (HER2) and blue fluorescent protein (EBFP2) after two separate transductions. Per the lead investigator, previous attempts to generate a double positive line using FACS failed to yield a pure polyclonal population, necessitating the generation of a viable, stable monoclonal line. We leveraged the CellRaft AIR® System as a method of choice for identifying clonal NIH-3T3 cells that were double positive for both transgenes.

Key Highlights

1) Flow sorting failed to generate a stable double positive cell population, even after multiple rounds of sorting.
2) Rare double positive clones were able to be identified using on-array antibody staining and analysis with the CellRaft AIR System's software guided imaging.
3) The CellRaft AIR System successfully isolated 100% of the double positive clones with over 80% of the clones forming viable colonies for downstream applications.

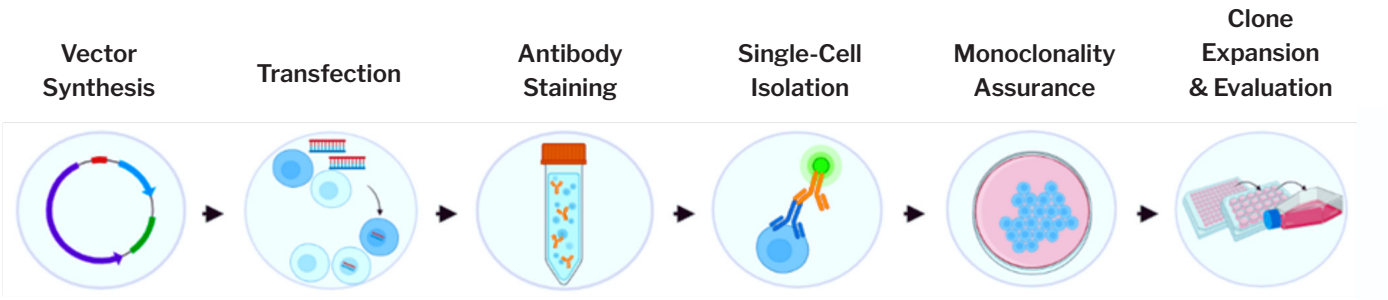

Figure 1: Cell Line Development Workflow. Traditional workflow involves synthesizing a vector with a gene of interest, transfecting cells with new genetic information, bulk staining a heterogenous population with an antibody of interest, isolating single cells with genes of interest, validating clonality, and expanding cells before transferring to cryovials for cryopreservation.

Scientific Problem

A scientist engineered NIH-3T3 fibroblast cells with HER2 to be a model for HER2 driven breast cancer. Since the HER2 transgene itself was not fluorescently tagged, the HER2 engineered cells were then transduced with EBFP2 for fluorescence visualization. Transduced cells were sorted twice using FACS in an attempt to derive a stable double positive population. However, during standard cell culture passaging, the population of cells appeared to be drifting in the expression of both EBFP2 and HER2 due to the population being heterogeneous and the normal (non-transduced) cells outcompeting the transduced cells. The team of researchers turned to *Cell Microsystems* for the use of the CellRaft AIR System to overcome these limitations and derive a suitable clone.

Experimental Design

A bulk heterogenous population of NIH-3T3 cells expressing EBFP2 and HER2 was seeded on the CellRaft® Array. Over several days, this population of cells was imaged on the CellRaft AIR System and monitored for cell viability, morphology, fluorescence, and clonality. First, a population of single cells was selected using software algorithms. From the single cells, the blue fluorescence channel on the CellRaft AIR System allowed for EBFP2 positive cells to be identified. Anti-HER2 with FITC conjugation was used to live-stain on-array to identify the HER2 expressing cells on day 6. Using CellRaft Cytometry™, populations were created for single EBFP2+ cells on day 1 and EBFP+/HER2+ on day 6, and the intersect of those two populations was used to identify the monoclonal double positives. The double positive clones were isolated into 96-well plates using the CellRaft AIR System, expanded, stained again for verification of HER2 expression off-array, and cryopreserved.

CellRaft AIR Workflow

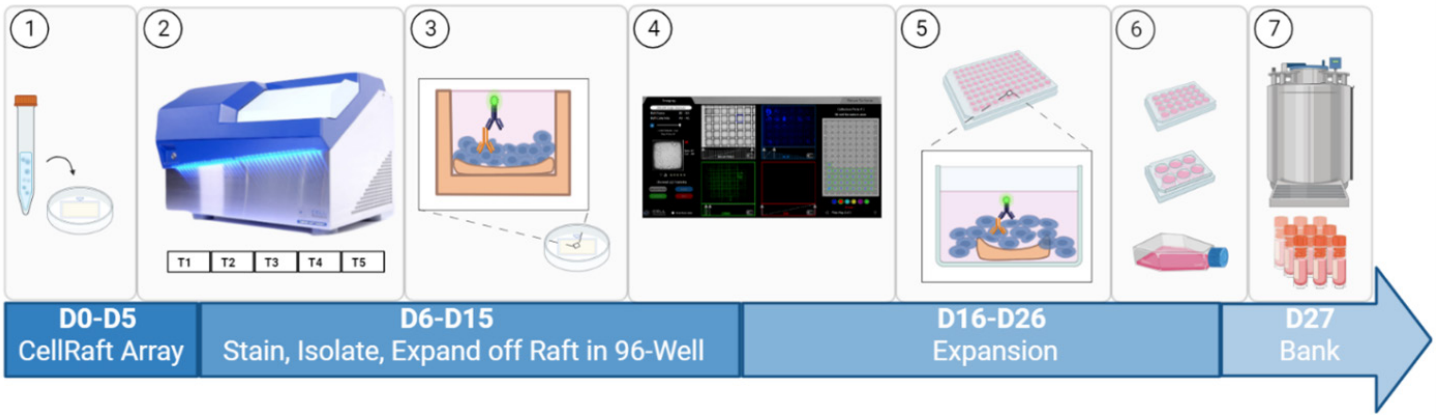

Figure 2: Cell Microsystems Workflow. (1) Heterogeneous cell mixture seeded onto a 200uM Single CellRaft Array. (2) CellRaft Array scanned on CellRaft AIR System over several timepoints. (3) CellRaft Array is live stained with Anti-HER2. (4) CellRaft Cytometry identifies rafts of interest containing the population of cells double positive for selected markers of interest. (5) CellRafts are isolated into 96-well collection plates and expanded into a sizable colony. (6) Monoclonal colonies are expanded and transferred into T-flasks. (7) Expanded clones are transferred to cryovials for cryopreservation as a master cell bank.

Results

After an initial FACS sort, approximately 10% of the total population of transduced NIH-3T3 cells were double positive for EBFP2 and HER2 **(Figure 3A).** The 10% double positive population was expanded post-sort and subsequently re-sorted, yielding an overall 89% EBFP2+/HER2+ population **(Figure 3B).** Despite the 89% purity reported via FACS, after thaw and passage it appeared that the expression of the two transgenes was not stable, with HER2 expression drifting throughout passage. To determine the percentage of double positive cells, the bulk population was stained with FITC conjugated-Anti-HER2. While the majority of cells visibly expressed EBFP2 **(Figure 4B),** the actual HER2 positive population was less than 10% **(Figure 4C).** To obtain a monoclonal EBFP+/HER2+ clone, the heterogenous population was seeded on a CellRaft Array, and after cell attachment, the arrays were imaged using the CellRaft AIR System over the course of 6 days **(Figure 5-6).** Using CellRaft Cytometry, CellRafts containing the desired clones of interest could easily be identified. As shown in **Figure 5**, CellRafts that contained a single EBFP2+ cell at timepoint 1 and a homogeneous EBFP2+/HER2+ colony at timepoint 6 were the rare double positives. In contrast, polyclonal CellRafts could also easily be identified and discounted, thereby ensuring both gene expression and clonality through image-based phenotyping **(Figure 6).**

Of the 6,000 cells screened on a single CellRaft Array, there were only 76 double positive clones, compared to over 400 EBFP2+/HER2- clones **(Figure 7).** We isolated 33 of the CellRafts containing the rare double positive clones with 100% isolation efficiency, and over 80% outgrowth efficiency. Thus, the CellRaft AIR system could successfully identify and isolate rare double positive clones that accounted for less than 1.5% of the total population. Importantly, once isolated and expanded, the clones were re-tested to confirm HER2 expression, and all clones were indeed double positive for both EBFP2 and HER2 **(Figure 8).**

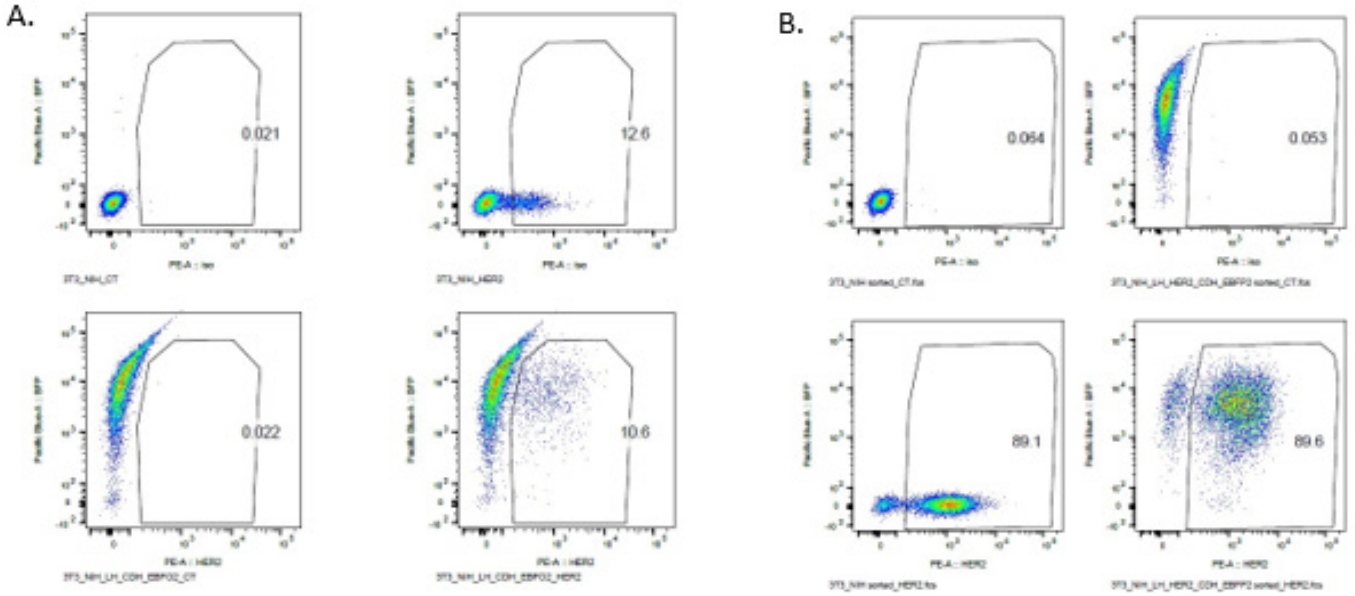

Figure 3: FACS analysis of NIH-3T3 HER2 EBFP2 cells NIH-3T3 cells were stained for HER2 expression and sorted for EBFP2 and HER2 double positivity. After an initial bulk sort (A), 10% of the population was double positive, and a re-sort (B) of that pre-sorted 10% yielded a population that was 89% double positive.

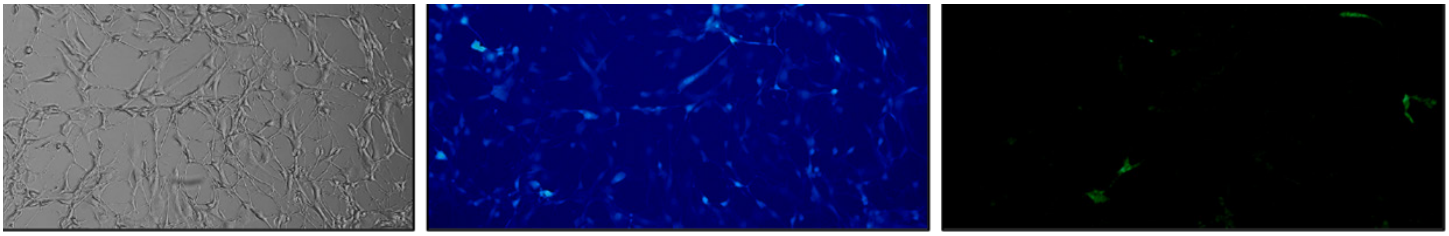

Figure 4. NIH/3T3 EBFP2+ HER2+ cells stained with Anti-HER2-FITC. (A) Brightfield, (B) EBFP2 expression (blue), and (C) HER2 expression (green). 10X Magnification.

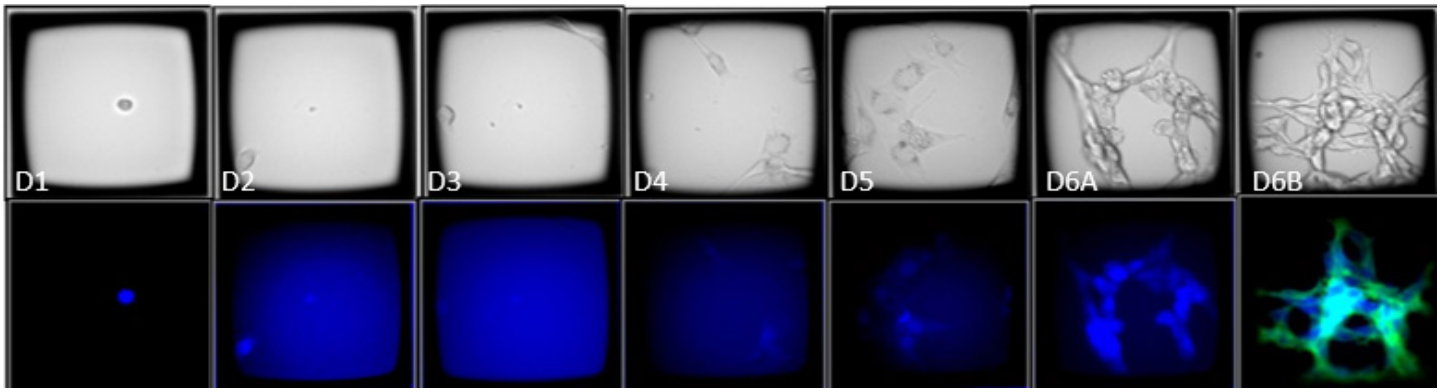

Figure 5: Time course images of a CellRaft containing an EBFP2+/HER2+ clone. The CellRaft arrays were imaged in brightfield and fluorescence 4 hours post-seeding and every 24 hours until colony formation. On day 6, the array was stained with Anti-HER2-FITC to visualize HER2 expression (green). 10X Magnification.

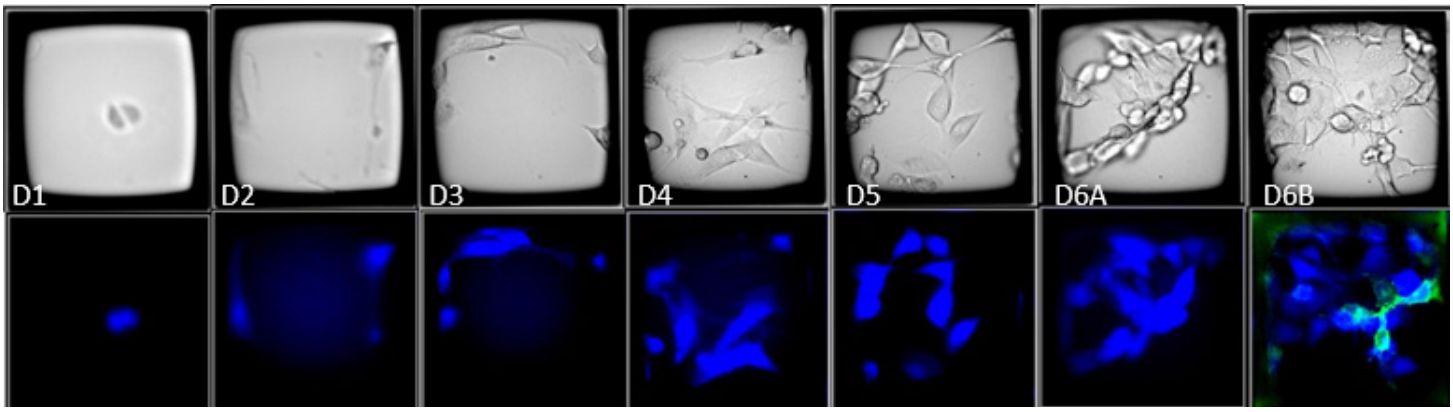

Figure 6: Time course images of a CellRaft containing polyclonal EBFP2+/HER2- and EBFP2+/HER2+ cells. The CellRaft arrays were imaged in brightfield and fluorescence 4 hours post-seeding and every 24 hours until colony formation. On day 6, the array was stained with Anti-HER2-FITC to visualize HER2 expression (green). 10X Magnification.

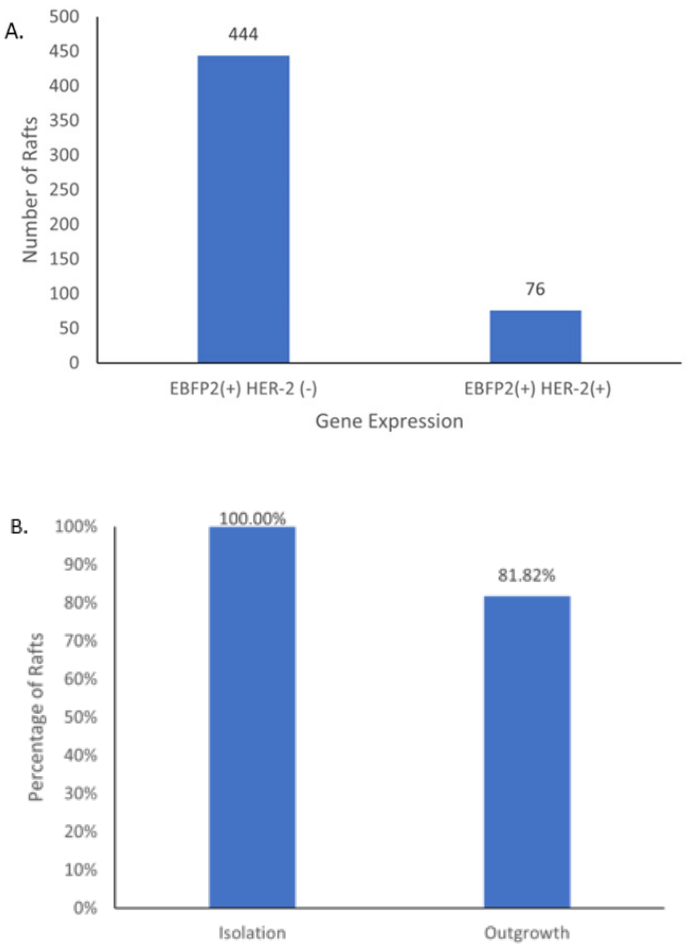

Figure 7: Identification and Isolation of NIH-3T3 EBFP2+/HER+ clones (A) Using CellRaft Cytometry, CellRafts containing single-cell derived colonies that were EBFP2+ and HER2+ were identified (B) CellRafts containing EBFP2+/HER2+ clones were isolated using the CellRaft AIR System and isolation and outgrowth efficiencies were determined by manual observation.

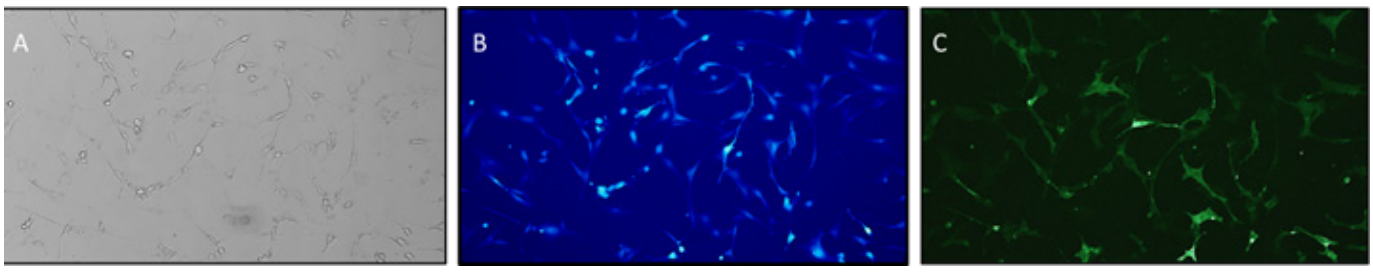

Figure 8: NIH-3T3 EBFP2+/HER2+ clone expanded off the CellRaft. After isolation and expansion, the double positive clones were stained with Anti-HER2-FITC to confirm gene expression. (A) Brightfield, (B) EBFP2 expression (blue), and (C) HER2 expression (green). 10X Magnification.

Discussion

A persistent challenge for cell line development is the generation of stable cell lines that maintain gene expression throughout thaw, passage, and experiment. This is especially true when multiple transgenes are being transduced simultaneously, and the expression of the individual genes is not correlated. Attempts to generate stable populations using methods such as FACS often yield heterogeneous populations of cells expressing varying degrees of the genes of interest, as well as
wild-type cells, which can ultimately obscure or dilute downstream analysis. In addition, this heterogeneity leads to an inherent competition for survival in the population, leading to drift and inconsistency in results. This struggle was clear in the case study data presented above. The NIH-3T3 EBFP2+/HER2+ cell line that was originally generated was only 10% double positive after transduction, and after successive rounds of FACS cleanup, only 89% purity of the polyclonal line was achieved. However, characterization of the cell line by immunofluorescence revealed that after expansion, roughly 1.5% of the population was actually double positive. Identifying, recovering, and single-cell expansion of this small percentage using FACS or limiting dilution would prove incredibly challenging, requiring the screening of millions of cells with a low success rate.

Conclusion

Given the critical need for rapidly developing genetically edited analytical cell lines to advance drug and disease research, the research community needs solutions for overcoming the bottlenecks preventing simple and efficient development of clonal cell lines.

Using the CellRaft AIR System, we were able to screen thousands of cells in a single consumable to identify the rare double positive clones, in a matter of weeks, not months. In total, the time from cell seeding to clonal outgrowth was only 16 days, and viable cell banks of the verified clones were cryopreserved in 27 days. Thus, the CellRaft AIR® Technology enables successful monoclonal cell line development for even rare or challenging cells faster and more efficiently than traditional methods.

> "The generation of these double positive clonal lines saved my lab of a lot of time and effort, allowing the rapid generation of multiple highly stable clones that had persistent expression for in vitro and in vivo assays. This proved superior to our flow-based methodologies, which were never as transgene positive or stable (in terms of expression) over multiple passages."
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> **Zachary Hartman, Ph.D.** Director, Center for Applied Therapeutics. Associate Professor, Departments of Surgery, Pathology, and Immunology, Tumor Immunology and Immunotherapeutics Laboratory. Duke University. Review of the CellRaft Air System.

References

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2. Molecular Devices. (2019, September 20). Optimize your high-value cell lines. San Jose.