# No more manually scraping cells: An automated efficient solution to iPSC reprogramming

## Introduction

Patient-derived human induced pluripotent stem cells (hiPSCs) are a valuable resource for disease modeling, personalized therapies, and drug discovery. Donor somatic cells such as fibroblasts or blood cells are reprogrammed into hiPSCs using four key transcription factors (OCT4, SOX2, KLF4, and C-MYC). Once fully reprogrammed, the hiPSCs can be differentiated into target cells for therapeutic interventions or drug screening applications.

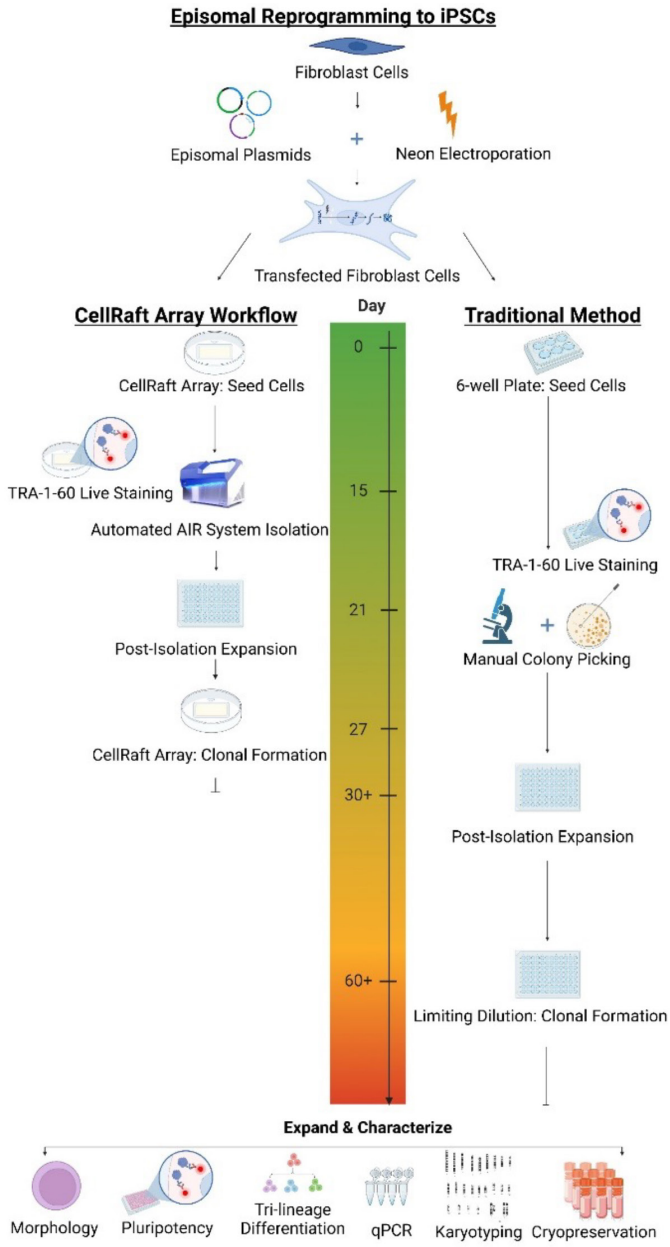
Despite the clinical potential of these cells, reprogramming is inefficient (<1%) and often leads to phenotypic and genomic instabilities. Furthermore, it is a laborious and time-consuming process involving manual workflows and requires additional screening to derive a monoclonal population. This traditional workflow often takes months to execute, necessitating multiple replicates due to poor success rates in obtaining an optimal number of stable clones (see Experimental Workflow). To robustly deploy hiPSCs in clinical settings, it is necessary to overcome these challenges. Here, we demonstrate how CellRaft AIR® Technology streamlines reprogramming by improving efficacy and efficiency compared to traditional methods.

### Key Highlights:

1. The CellRaft AIR System enables the automated isolation of hundreds of pluripotent colonies in half the time compared to manual scraping, pruning, or aspiration.
2. Reprogramming with the CellRaft Array generated thousands of individually segregated TRA-1-60 positive colonies.
3. Using the CellRaft Array, stable monoclonal iPSC cell lines can be derived and characterized up to four weeks sooner than the traditional method.

## Questions this RaftNote Answers

1. How can the efficacy and efficiency of episomal reprogramming be improved?
2. How can reprogrammed iPSC colonies be stably isolated without manually scraping and pruning?
3. How can monoclonal iPSC lines be derived without performing lengthy, tedious protocols?




**Experimental Workflow**: Comparative timeline of the traditional method vs. the CellRaft® Array workflow for reprogramming fibroblast into fully characterized clonal iPSCs.

## Materials and Methods

A 200µm Single CellRaft Array was prepared using standard manufacturer recommendations. A single well of a 6-well plate and the CellRaft Array were coated with 1% Geltrex™ (Gibco™ Cat. No. A1413301) at 37°C overnight in preparation for cell seeding. 1mL of fibroblast medium was dispensed into each well/reservoir ahead of electroporation. Electroporated fibroblast cells were resuspended in 1mL of fibroblast medium immediately following electroporation before being seeded dropwise into the pre-coated 6-well plate and CellRaft Array. On day one, fibroblast medium was replaced with N2B27 medium supplemented with fresh Basic Fibroblast Growth Factor (bFGF) (THERMO SCIENTIFIC Cat. No. PHG0264). From D1-D15, full medium changes were performed every other day using N2B27 medium supplemented with fresh bFGF. On D16, N2B27 medium was replaced with Essential 8™ medium (Gibco Cat. No. A1517001), and cells were cultured as iPSCs.

### *TRA-1 60 Staining*

On D15 of reprogramming, cells were live-stained in the 6-well plate and on the CellRaft Array using TRA-1-60 Alexa Fluor® 594 Conjugate Kit (THERMO SCIENTIFIC Cat. No. A24882) or Mili-Mark™ Anti-TRA-1-60 FITC conjugate (MILLIPORE™ Sigma Cat. No. FCMAB115F). For the TRA-1-60 Alexa Fluor 594 Conjugate Kit, 1mL of N2B27 medium was aspirated from the 6-well plate as well as the CellRaft Array. A 1:50 concentration of Anti-TRA-1-60 antibody was added directly into the medium. For the Anti-TRA-1-60 FITC conjugate, the entire 2mL volume of N2B27 medium was aspirated and a staining cocktail consisting of 1:100 concentration antibody, 1% Final Concentration Blocker™ BSA (10%) (Bovine Serum Albumin) (THERMO SCIENTIFIC Cat. No. 37525), in 1mL FluoroBrite™ DMEM (Gibco Cat. No. A1896701) was added to the plate and Array. For each staining method, the plate and Array were placed back into the 37°C incubator for 1.5 hours. After staining, the staining cocktail was aspirated, and the plate and Array were washed 2-3 times using FluoroBrite DMEM. Imaging was

performed in FluoroBrite DMEM medium. FluoroBrite DMEM medium was replaced with Essential 8 medium to continue expanding the cultures.

### *iPSC Isolation*

The CellRaft Array was stained using TRA-1-60 on D15 to identify undifferentiated cells for isolation. TRA-1-60-positive colonies on the Array were identified, counted, and mapped using the CellRaft Cytometry™ Software. 96-well collection plates coated with 1% Geltrex and filled with Essential 8 medium were loaded into the CellRaft AIR System. The CellRaft Array was topped up with Essential 8 medium to the isolation volume per standard manufacturer recommendation, and CellRafts were automatically isolated from the CellRaft Array into the collection plate using the CellRaft AIR System. The 6-well plate was manually scored for TRA-1-60-positive foci using a Nikon Microscope. Colonies identified using the microscope were manually scraped using 22-gauge needles to lift colonies off of the plate and a p200 pipette to transfer lifted colonies into a collection plate. Fragmented cell colonies were dispensed into 96-well collection plate coated with 1% Geltrex and filled with Essential 8 medium. Both 96-well collection plates from the CellRaft Array and the 6-well plate were returned to the 37°C incubator for expansion.

### *Monoclonal Colony Formation*

96-well collection plates from both the 6-well plate and CellRaft Array workflows were examined for outgrowth. Wells containing cells with iPSC-like morphology were dissociated using TrypLE™ Express (Gibco Cat. No. 12604013) and pooled for monoclonal formation. 50,000 cells from the CellRaft Array pool were seeded into a newly prepped and pre-coated 200µm Single CellRaft Array using 0.030mg/mL iMatrix-511 (Matrixome Cat. No. 892011) in mTeSR™ Plus medium (STEMCell™ Technologies Cat. No. 100-0276) with the addition of 1:10 CloneR™2 supplement (STEMCell Technologies Cat. No. 100-0691). 300 cells total from the manual scraping method of the 6-well plate were seeded into 3 x 96-well limiting dilution plates at 10 cell/mL pre-coated with the same concentration of iMatrix-511 in mTeSR Plus medium supplemented with CloneR2. The CellRaft Array was scanned daily to generate time course images using the CellRaft AIR System, and monoclonal iPSC colonies were automatically isolated. The limiting dilution plates were examined daily under the microscope to identify wells containing single colonies.

### *Trilineage Differentiation*

Monoclonal colonies generated from the CellRaft Array were expanded and dissociated into single cells using TrypLE. Single cells were seeded into pre-coated (0.030mg/mL iMatrix-511) 96-well plates for control, Endoderm, Mesoderm, and Ectoderm wells, while the rest of the cells were used for further expansion and cryopreservation. Replated monoclonal iPSCs were seeded in mTeSR Plus medium supplemented with 1:10 CloneR2 on D0. On D1, wells were differentiated using the STEMdiff™ Trilineage Differentiation Kit (STEMCell Technologies Cat. No. 05230). On D7, wells were live stained for markers of Endoderm (CXCR4 Alexa Fluor 594 conjugated, Bioss Antibodies Cat. No. bs-1011R-A594), Mesoderm (Anti-Human CD56/NCAM FITC conjugated, STEMCell Technologies Cat. No. 60021FI), and Ectoderm (CD133 FITC Conjugated, Novus Biologicals® Cat. No. NBP3-08969F).

### *qRT-PCR Genetic Analysis*

Monoclonal colonies generated from the CellRaft Array were also used for genetic analysis to identify abnormalities associated with Chr 1q, Chr 4p, Chr 8q, Chr 10p, Chr 12p, Chr 17q, Chr 18q, Chr 20q, and Chr Xp using the hPSC Genetic Analysis Kit (STEMCell Technologies Cat. No 07550). Samples were analyzed in triplicate using the Applied Biosystems Quantstudio™ 3 Real-Time PCR System.

## Results/Data

*Reprogramming Efficiency*

To compare the reprogramming efficiency of the traditional 6-well plate method to the direct to Array method (see experimental workflow), BJ fibroblasts at low passage (P3) were counted and electroporated using the Neon System to deliver the Epi5 reprogramming vectors. The cells were seeded immediately into a single well of a pre-coated 6-well plate or directly onto a pre-coated 200µm CellRaft Array. The 6-well plate was examined using manual microscopy over the course of 15 days to monitor the formation of colony foci (Figure 1 A-B). Over the same time period, the CellRaft Array was automatically scanned using the CellRaft AIR System, enabling time-course imaging of each CellRaft to analyze prospective colony formation (Figure 2). On day 15, both the 6-well plate and the Array were live-stained for TRA-1-60 to identify undifferentiated colonies, and the number of undifferentiated colonies was counted (Figure 1). A single well in the 6-well plate contained an average of 610 TRA-1-60-positive foci (n=4), while the CellRaft Array contained an average of 1106 individually segregated TRA-1-60-positive colonies (n=7) (Figure 3).

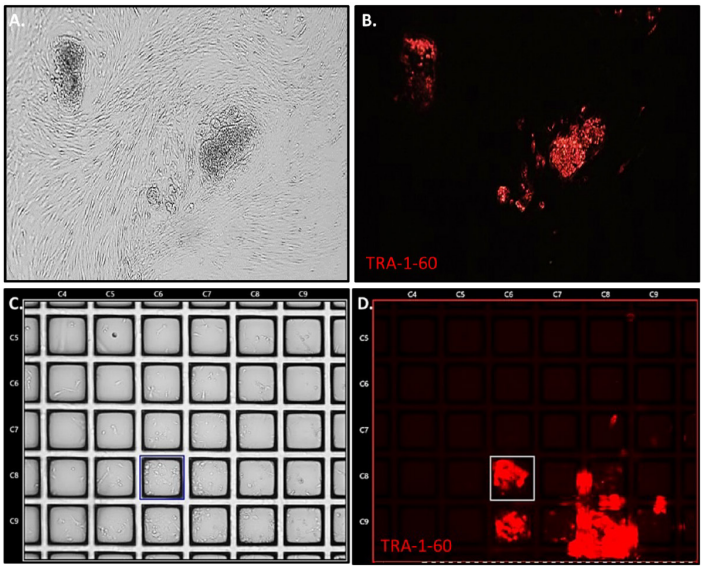



**Figure 1.** (A) 6-well plate containing reprogrammed BJ Cells in a lawn of non-reprogrammed BJ fibroblasts, 4X magnification. (B), same 6-well plate live-stained using TRA-1-60 Alexa Fluor 594 Conjugate Kit, 4X magnification. (C), 200µm CellRaft Array containing reprogrammed BJ Cells. (D), same 200µm CellRaft Array live-stained using TRA-1-60 Alexa Fluor 594 Conjugate Kit.

### *Colony Isolation*

After reprogramming, prospective hiPSC colonies were recovered from both the 6-well plate and the Array to compare the number of colonies that survived isolation. In the 6-well plate, hiPSC colonies were manually identified via brightfield microscopy and scraped into a 96-well plate using 22-gauge needles while on the microscope stage in a biosafety cabinet. The process of manually identifying and scraping 96 colonies required approximately 2.5 hours of intensive labor and resulted in 50 wells containing cell colonies after recovery (52%). In contrast, TRA-1-60-positive colonies in CellRafts were automatically isolated into two 96-well plates using the CellRaft AIR System in approximately 2 hours. Out of 192 isolations, 94 wells contained CellRafts that were successfully deposited into the 96-well collection plate, outgrew, and maintained stable undifferentiated colony formation with TRA-1-60-positive staining (Figure 4). Under brightfield observation, the hiPSC colonies isolated from the CellRaft AIR were substantially larger and more stable compared to the manual transfer, and 14 of the most desirable colonies were pooled for monoclonal hiPSC colony derivation.

### *Monoclonal Colony Derivation*

Once undifferentiated colonies from the CellRaft Array or 6-well plate workflows were generated, monoclonal populations needed to be derived to create a stable and reproducible iPSC line. To facilitate cloning, the surviving colonies from each plate type were pooled. The 50 wells containing cell colonies from the 6-well plate manual

isolation were pooled, counted using the Countess™ cell counter (33% viability), and used to seed a total of three limiting dilution 96-well plates (10 cell/mL). In comparison, the 14 wells containing colonies from the CellRaft Array were pooled, counted (60% viability), and 50,000 cells were seeded onto a new CellRaft Array. Clonality was monitored daily using manual microscopy on the 3 limiting dilution plates and daily via automated scanning of the Array on the CellRaft AIR system. For the limiting dilution plates, out of a total of 288 wells, only 18 wells (6.25%) contained surviving cells with varying morphologies (Figure 5), and only 8/18 wells contained cells that displayed iPSC-like morphology (2.8%). By day 11, only one well contained a single colony in a well that was presumed monoclonal (0.35%).

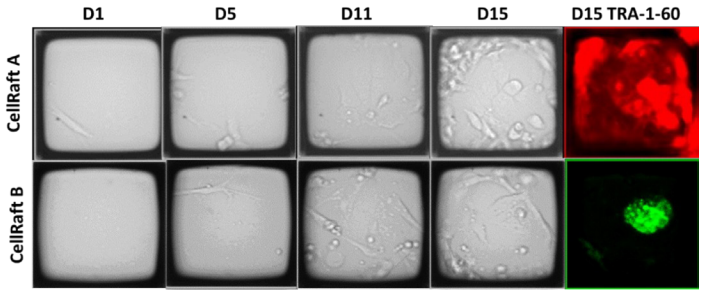



**Figure 2.** Representative CellRafts D1-D15 post-reprogramming. BJ Fibroblast Cells (ATCC) seeded on 200µm CellRaft Arrays post-transfection with Epi5 Episomal iPSC Reprogramming Kit (THERMO SCIENTIFIC). On Day 15, CellRaft Array A was live-stained using the TRA-1-60 Alexa Fluor 594 Conjugate Kit (THERMO SCIENTIFIC), and CellRaft Array B was live stained using Anti-TRA-1-60 FITC onjugate (Millipore Sigma).

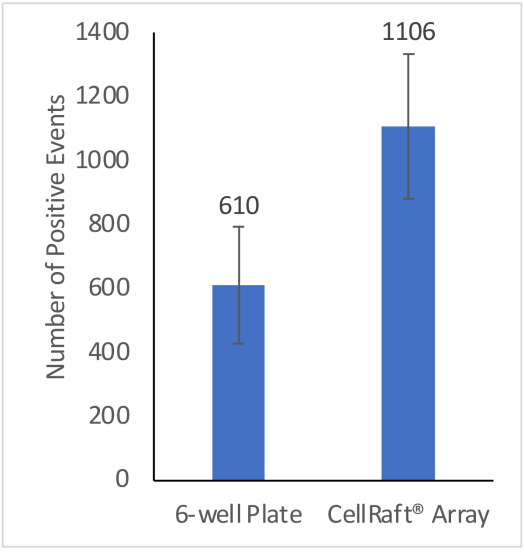



**Figure 3.** BJ Cells were transfected using the Epi5 Episomal iPSC Reprogramming Kit and cultured for 15 days in N2B27 reprogramming medium supplemented with fresh bFGF in a 200µm CellRaft Array or in a 6-well plate. On D15, cells were live-stained using TRA-1-60 Alexa Fluor 594 Conjugate Kit and positive events were calculated using CellRaft® Cytometry for the Array and manually counted and averaged n=3 counts for the 6-well plate. CellRaft Array n=7 and 6-well plate n=4.

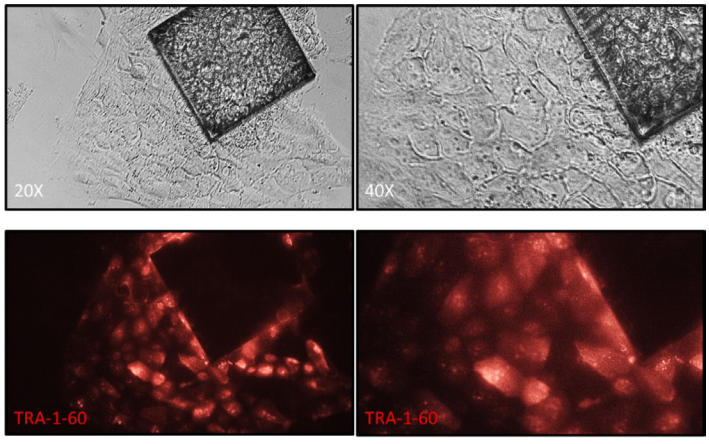



**Figure4.** Top, 200µm CellRaft containing cells with iPSC-like morphology isolated into a 96-well collection plate in 20X and 40X magnification. Bottom, same 200µm CellRaft live-stained using TRA-1-60 Alexa Fluor 594 Conjugate Kit at 20X and 40X magnification.

In contrast, in a single CellRaft Array, 336 monoclonal colonies were identified (Figure 6-7). A 96-well plate of undifferentiated monoclonal iPSC colonies was isolated using the CellRaft AIR System, yielding 69 wells containing cell colonies that continued to expand, and 13 wells from the 69 were identified to contain undifferentiated cells that had iPSC-like morphology (18.8%).

### *Trilineage Differentiation*

To demonstrate that the colonies isolated from the CellRaft Array were indeed pluripotent, 13 colonies from the CellRaft Array were expanded and cultured as iPSCs and plated for trilineage differentiation assessment, which is a gold-standard assay for reprogramming confirmation. Of the 13 putative iPSC colonies, all 13 were found to contain cells positive for markers of Endoderm (CXCR4), Mesoderm (NCAM/CD56), and Ectoderm (CD133) (Figure 8), confirming their pluripotency.

### *Genetic Analysis*

A common failure mode for reprogrammed iPSC clonal lines is genetic instability and the acquisition of abnormal karyotypes. To determine that the monoclonal iPSC lines derived from the CellRaft Array were karyotypically normal, 5 monoclonal colonies from the CellRaft Array were assessed for genetic abnormalities using qRT-PCR. All monoclonal colonies that were analyzed showed no significant abnormalities (Figure 9).

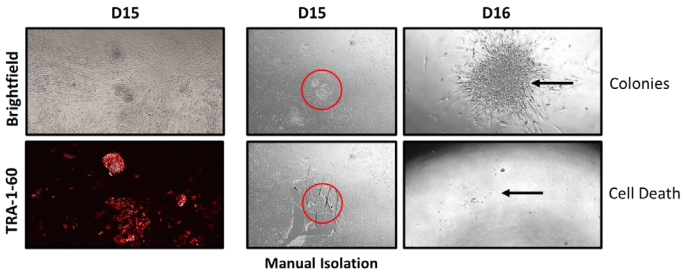



**Figure 5.** Left, 6-well plate image displaying reprogrammed iPSC colonies in a lawn of fibroblast. Right, manual isolation of 6-well plate using 22-gauge needles and transfer to 96-well collection plates a day later.

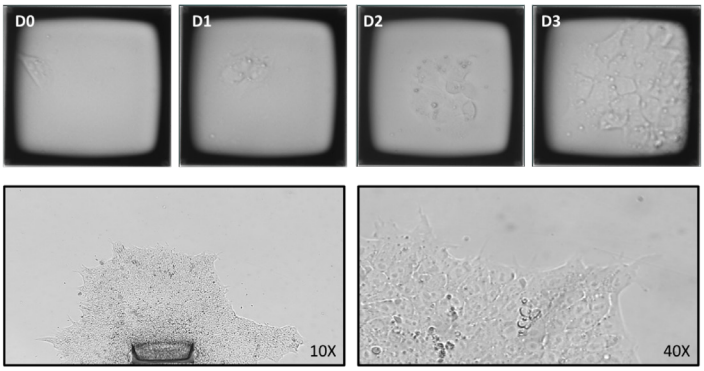



**Figure 6.** Top, Images of a CellRaft containing a clonal iPSC colony derived from bulk polyclonal reprogrammed cells with iPSC-like morphology (D0-D3 before being isolated into a 96-well plate). Bottom, post-isolation, the iPSCs grew off the CellRaft and a clonal colony emerged with iPSC-like morphology by D8.

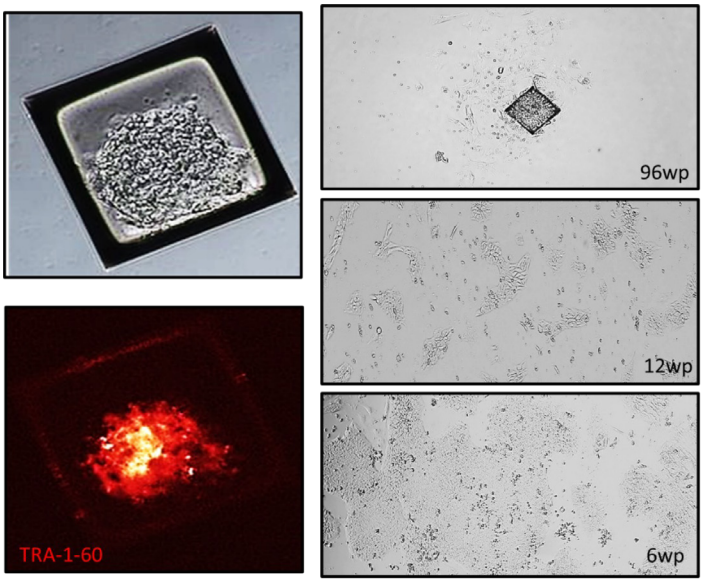



**Figure 7.** Left, outgrowth of iPSCs off 200µm CellRaft and live-staining with TRA-1-60 Alexa Fluor 594 Conjugate Kit at 20X magnification. Right, further passage and expansion of iPSCs for downstream characterization and biobanking at 4X.

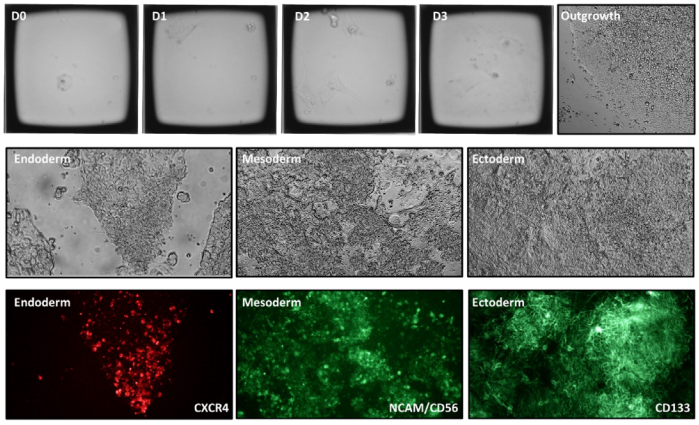



**Figure 8.** Top, CellRaft Array with monoclonal iPSCs isolated and expanded in a 96-well plate. Middle, iPSCs were replated for differentiation using the STEMdiff Trilineage Differentiation Kit (Stem Cell) and live-stained for markers of Endoderm (CXCR4 Alexa Fluor 594 conjugated, Bioss Antibodies), Mesoderm (Anti-Human CD56/NCAM FITC conjugated, Stem Cell), and Ectoderm (CD133 FITC Conjugated, Novus Bio), 20X magnification.

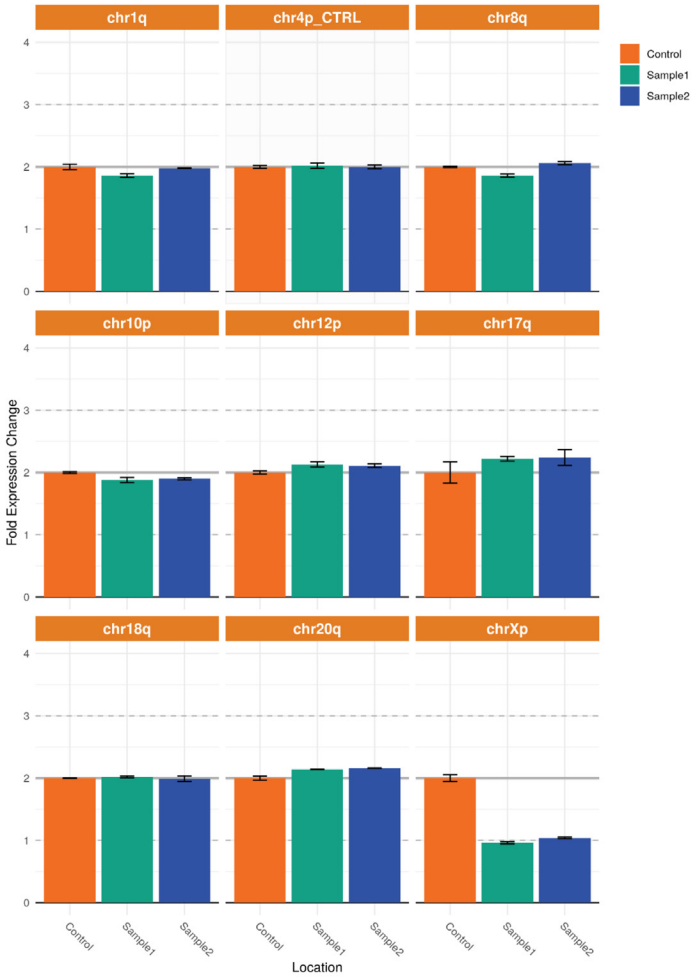



**Figure 9.** hPSC genetic analysis qPCR plot readout of Control (Female), Clone 1 (Male, clonal iPSC population in Figure 7), and Clone 2 (Male) using the hPSC Genetic Analysis Kit (StemCell).

## Discussion

### *Reprogramming Efficiency*

The CellRaft Array provided flask-like population cell culture conditions with the added benefit of single-cell segregation, allowing for interrogation of individual cells. Nearly twice as many TRA-1-60-positive, reprogrammed iPSC colonies were identified post-transfection on the CellRaft Array (CRA) as compared to the 6-well plate (6wp) method (1106 CRA vs 610 6wp). This doubled the initial reprogramming efficiency using the Epi5 Kit with the CellRaft Array as opposed to traditional methods and enabled the screening of a sufficient number of cells to ensure downstream success.

### *Automation*

A key bottleneck in the reprogramming workflow, in terms of both efficiency and efficacy, is the recovery of the putative iPSC colonies after reprogramming. The CellRaft AIR System eliminated the manual scraping aspect found in traditional workflows by providing a rapid and gentle solution for isolating reprogrammed iPSCs in half the time. While the initial recovery of colonies was comparable between methods (49% CRA vs. 52% 6wp), viability increased to almost double from the CellRaft Array compared with the 6-well plate method (60% CRA vs 33% 6wp). Manually dissecting undifferentiated colonies from the 6-well plate often resulted in large chunks of cells with contaminating fibroblasts still attached or wells with no cells. In addition, there were varying degrees of viability and differentiation seen in the wells containing manually scraped colonies. In comparison, wells containing CellRafts isolated using the CellRaft AIR System had fewer fibroblasts and differentiation, and the colonies were larger, more uniform, and displayed the canonical morphology that is the hallmark of proper iPSC growth.

### *Clonal iPSC line generation and confirmation*

With the ability to interrogate thousands of cells per consumable, a single CellRaft Array was able to screen more than 500X the cells per consumable than a 96-well plate, or more than 100X a 384-well plate. In a workflow that is plagued by the low frequency of events, the ability to scale the amount of cells that can be screened without increasing the amount of time, labor, or cost involved is paramount. The improvement from 0.35% of wells generating an iPSC colony via the traditional workflow compared to 19% with the CellRaft AIR workflow represents a 54% increase in success with far less effort.

In addition, after the colonies are derived, it is still necessary to phenotypically characterize them for clonality, pluripotency, and genomic stability. During the manual scraping or traditional limiting dilution methods, it is virtually impossible to ensure the transfer of a clonal colony or to confidently visualize a single iPS cell in a well. Thus, the cloning and stable cell line generation after reprogramming requires extensive validation as well as successive rounds of subcloning. In contrast, a key benefit of the CellRaft AIR Technology is the ability to automatically scan the CellRaft Array every day to generate an image-based chain-of-custody record of monoclonality. These time-based images bolster confidence that the colony arose from a single cell, and the resultant colony is TRA-1-60-positive and morphologically resembles an iPSC. Furthermore, the automated isolation of the colonies ensures that a single colony is transferred without needing to scrape, trypsinize, or otherwise contaminate the precious colony. These features provide a highly favorable growth environment that enables the transferred iPSC colonies to grow substantially faster, decreasing the amount of time required to generate a stable line.

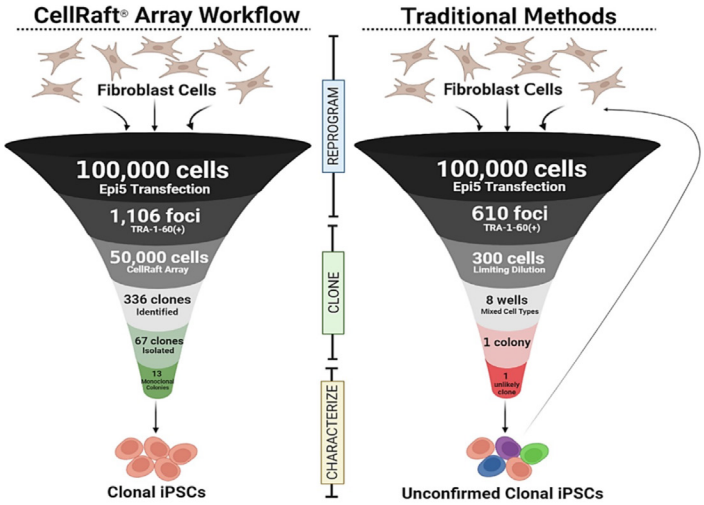



**Figure 10.** Comparison of the number of successful iPSC monoclonal lines that were generated by the CellRaft Technology workflow (left) compared to the traditional workflow (right).

## Conclusion

Reprogramming somatic cells to iPSCs is an incredibly inefficient workflow. With the CellRaft Array, we are able to alleviate the well-known pain points associated with reprogramming that make the workflow untenable for many labs and hamper therapeutic use. Overall, we demonstrated substantial improvements in efficiency, clonality, stability, and cell viability. Importantly, the entire process with the CellRaft Technology is image-guided, software-based, and automated, which helps to improve reproducibility (Figure 10), eliminate user error, and ensure consistency throughout the workflow. Finally, the CellRaft Array technology can decrease the time and effort required to derive clonal reprogrammed and/or differentiated populations of patient-derived somatic cells to aid in advancing drug discovery and personalized medicine.