RaftNotes

Automated cloning for CRISPR workflows using the CellRaft AIR® System

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Abstract

Gene editing workflows require transformation of a large number of cells, followed by isolation of individual cells from the larger population to establish clonal colonies. Given the large number of gene edits required for contemporary research and the labor-intensive components of the workflow, there is an unmet need to automate post-transformation cloning. Here we present data and protocols describing the CellRaft AIR System as a fully automated cloning platform. Using an imaging-based sorting modality, single cells can be monitored for transformation-positive phenotypes as well as their retention of clonality as colonies begin to grow. Using the protocols described here, we also provide evidence of improved cell viability with relatively robust HEK293 cells showing a nearly 3-fold improvement in viability. Other laboratories have shown even more dramatic improvements in viability for more sensitive cells such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). Finally, the system generates clonal colonies in 4-5 days, a significant improvement over the months required in more traditional workflows.

The CellRaft AIR System and CytoSort Array

The AIR System is a bench-top instrument comprising an internal microscope with both brightfield and three-channel fluorescent imaging capabilities. The system employs the CytoSort® Array as a substrate for single cell imaging and culture, as well as the proprietary CellRaft Technology for the isolation and collection of single cells. The core technology comprises a disposable microwell array, the CytoSort Array, on which cells are seeded and imaged. To isolate single cells, a motorized needle penetrates the resealable elastomeric floor of the array to displace the individual CellRaft from its microwell. The CellRaft material is loaded with magnetic nanoparticles, allowing retrieval of the CellRaft and the attached cell using a magnetic wand. The AIR System relies on the same CellRaft Technology, automating fluorescent imaging, cytometric sorting and the mechanical release and collection operations to reduce hands-on time and dramatically improve throughput.

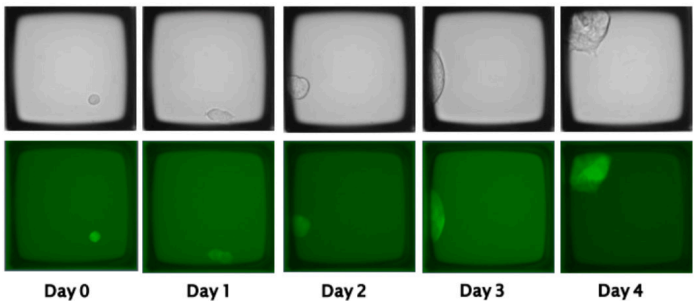

Figure 1: Cells transfected with a GFP-expressing plasmid can be seeded as single cells and tracked for the growth of clonal colonies on the CytoSort Array using the AIR System.

Cloning on the AIR System and CytoSort Array

The AIR System and CytoSort Array are ideally suited to generating clonal colonies for any transformation-based workflow, including gene editing. Single cells are seeded on the CytoSort Array after

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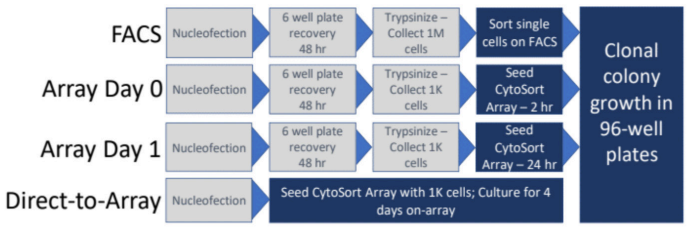


Figure 2: Schematic representation of the workflows evaluated for post-CRISPR cloning.

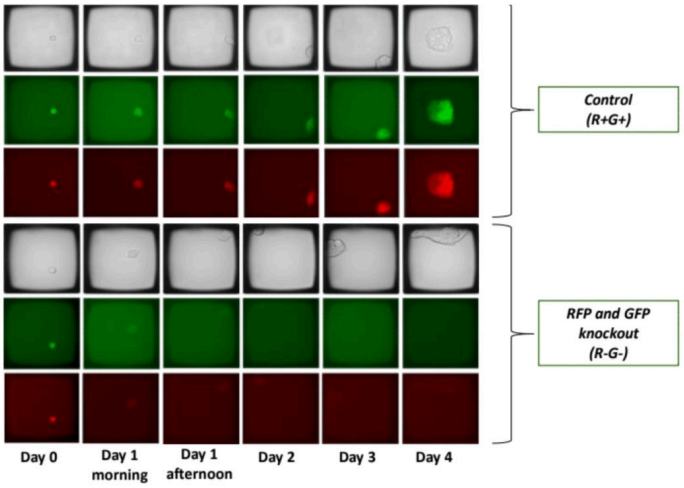


Figure 3: Single cell suspensions were seeded on the CytoSort Array and imaged on the AIR System each day for 5 days (Day 0 = day of transfection). Each scan required 15 minutes, and arrays were placed back into a standard laboratory incubator between scans. By imaging, colonies were monitored for inclusion of extraneous cells to ensure clonality. Presence of cells in each microwell was confirmed with brightfield imaging.

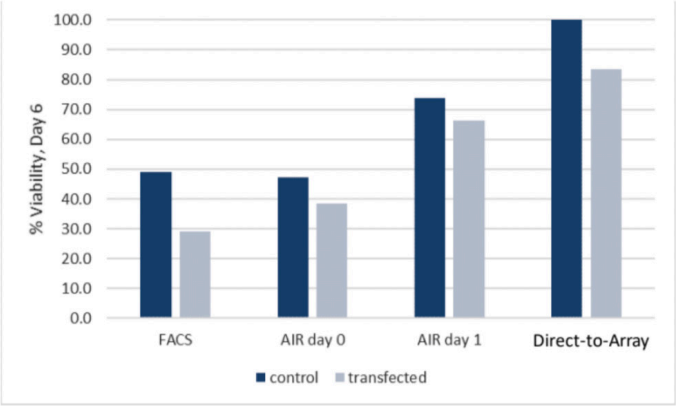
introducing transgenic or CRISPR/Cas9 elements. Cells are then imaged at various intervals to ensure the single cell in a given microwell of the CytoSort Array grows into a clonal colony. Figure 1 provides an example of HEK293 cells transfected with a GFP-expressing plasmid. Single cells were seeded on the array after transfection and clonal colony growth was monitored over the course of 5 days. Colonies-of-interest can then be released from the CytoSort Array using the CellRaft AIR System. After release from the microwell, the AIR System deposits the colony, still attached to the CellRaft, into a 96-well plate for continued expansion culture. Generally, approximately 90% of colonies are viable after transfer to a 96-well plate. In the remainder of this RaftNote, we provide additional details on this workflow specific to cells which have undergone CRISPR/Cas9 gene editing using a ribonucleoprotein (RNP)-based workflow, where the Cas9 protein is pre-complexed with a guide RNA prior to introduction into the cell.

Comparing cloning workflows (AIR vs. FACS)

To test the AIR System for automated CRIPSR cloning, a HEK293 cell line was generated which expresses both RFP and GFP. Guide RNAs were then generated against both transgenic fluorescent proteins. These guides were complexed with Cas9 using standard protocols provided by Integrated DNA Technologies (IDT) and introduced into cells using a Lonza Nucleofector. For post-CRISPR cloning, four workflows were evaluated as described below with schematics of each shown in Figure 2. The first (FACS in Figure 2) is a traditional flow sorting-based workflow with cells seeded in 6-well plates for a 48-hour recovery period prior to sorting and isolation on a BD FACS Aria at the UNC-Chapel Hill Flow Cytometry Core facility. In the second and third workflows, cells were also given a 48-hour recovery period in 6-well plates but were then re-plated on CytoSort Arrays and isolated after 2 hours (AIR Day 0 in Figure 2) or 24 hours of recovery (AIR Day 1 in Figure 2), respectively. In the final workflow (Direct-to-Array in Figure 2), cells were seeded immediately on the CytoSort Array, entirely omitting the 48-hour recovery period. In all cases, cells or colonies were isolated into 96-well plates and monitored for viability.

Monitoring Clonality on the AIR System

Ensuring that cells undergoing gene editing grow into clonal colonies is an essential component of the CRISPR workflow. Using the CellRaft AIR System, single cells can be imaged and tracked over the course of colony



Workflow	Control Cells	Transfected Cells
FACS	49%	29%
Array day 0	47%	38%
Array day 1	74%	66%
Direct-to-Array	100%	83%

Figure 4: Clonal colonies collected from the AIR System exhibit 2 to 3-fold greater viability compared to FACS-based workflows.

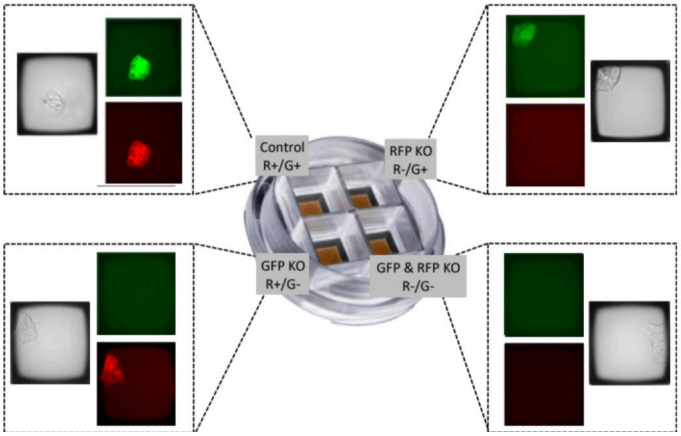


Figure 5 Use of the CytoSort QuadArray to isolate clonal colonies from four distinct CRISPR gene editing processes. Using the GFP/RFP-expressing model system, all four conditions were processed in parallel to generate both experimental and control gene editing cell lines in a single cell culture consumable. Given the 500 µL volume of each QuadArray well, reagent consumption was also reduced by 50-100 fold.

formation. Figure 3 provides an example of cells which have undergone simultaneous GFP and RFP knockout, with colonies emerging from a single cell over the course of 4 days. In addition, the knockout phenotypes (elimination of GFP and RFP signal) can be tracked over time which provides a unique capability for phenotypic CRISPR screening. Control cells which underwent transfection without the presence of a guide RNA are also shown, with no change in fluorescent protein expression. Imaging can therefore be used to characterize cellular phenotypes after gene editing, a growing requirement for multiplex genome edits for large-scale screening studies.

Improved clonal viability using the AIR System

Cell sorting and single cell culture often leads to high degrees of cell death. Even after single cells are isolated in a cell culture dish, their viability is often quite low. Typically, this is due to the inability of a single cell to "condition" its cell culture environment through secreted factors, exosome production or other factors. Single cell viability is improved on the CytoSort Array because the culture media volume is shared by thousands of cells across the array. The ability to render cells recoverable as individuals, while simultaneously allowing thousands of cells to condition the culture environment provides an ideal solution to cloning experiments, particularly with delicate cell types such as primary cells, iPSCs or hESCs. After monitoring colonies for clonality on the AIR System, colonies were isolated according the protocols described in Figure 2. A 96-well plate of either single cells (workflows 1-3) or clonal colonies (workflow 4) was collected (96 individual isolates from each workflow). Colonies were then monitored for viability over the course of a week. Viability data (Figure 4) indicates that the AIR-based workflows enhanced viability by 2-3 fold with the highest viability exhibited by those colonies recovered directly into 96-well plates after 48 hours on the CytoSort Array. We suspect the additional viability is due to omission of the multiple trypsin and re-plating steps associated with workflows 1-3.

Efficiency of cloning using the CellRaft AIR™ System

Genome editing with delicate cell types can take months to produce stable clonal colonies. Most service organizations indicate a 2-3 month turn-around-time even for robust cells. The workflow presented here requires approximately 4-5 days to generate stable clonal colonies which are up to 90% viable and verified for clonality via imaging-based tracking (Figure 5). Cell Microsystems has also developed the CytoSort QuadArray and HexaQuad Array, which allows 4 or 24 different gene edited cell lines to be developed in parallel, respectively. Also, because each reservoir of all the CytoSort Arrays requires as little as 500 µL of media, the consumption of reagents during the workflow is reduced 50-100 fold.

While the CytoSort QuadArray allows four cell lines to be processed in parallel, a new product from Cell Microsystems, the CytoSort HexaQuad Array, is effectively a 24-well plate with each reservoir containing its own microwell array, thereby allowing 24 gene editing experiments to be conducted simultaneously. For labs interested in maximizing throughput even further, multiple arrays can be prepared and imaged concurrently through the time course. As seen in Figure 6, the HexaQuad Array is based on a standard ANSI/SLAS labware footprint and is compatible for use with liquid handling systems to further enhance automation.

Figure 6: The HexaQuad Array which allows clonal colonies to be cultured and isolated from 24 distinct gene editing workflows simultaneously.

Acknowledgments:

This work was funded by NIH SBIR/STTR grants including: R41HG009467-01A1; R41GM131475-01; R44GM134993-02. We would like to thank our collaborators on these programs for their support and feedback, including William Buchser, PhD (Washington University – St. Louis); Michael McConnell PhD (U of Virginia) and William Marzluff, PhD (U of North Carolina at Chapel Hill).

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

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