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Accelerating Generation of Single Cell Clones by Using CellRaft AIR™ System Coupled With Fluorescence Activated Cell Sorting

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BACKGROUND

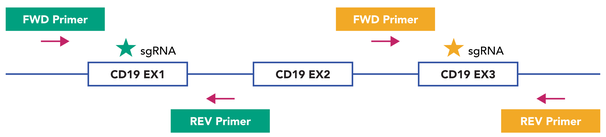
- Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome engineering of cell lines enables target discovery and serves to provide an exceptional disease model for preclinical research, including in the field of chimeric antigen receptor T-cell therapy
- The bottleneck for engineering cells continues to be single-cell clone generation
- Current processes vary from manual limiting dilution (preferred for adherent lines), to using a variety of high-speed cell sorters to a combination of both (bulk sort and plate them into single cells)

OBJECTIVE

- In order to increase the frequency of single-cell clone generation, we looked at various technologies available at disposal including several varieties of cell sorters and an image-based cell isolation system, CellRaft AIR™ by Cell Microsystems

METHODS

Figure 1. sgRNA and Primer Design



Stars on figure represent sgRNA edit sites.
EX, exon; FWD, forward; REV, reverse; sgRNA, single-guide ribonucleic acid.

- Single-guide ribonucleic acids (sgRNAs) and primer sets were designed for the gene of interest (**Figure 1**)

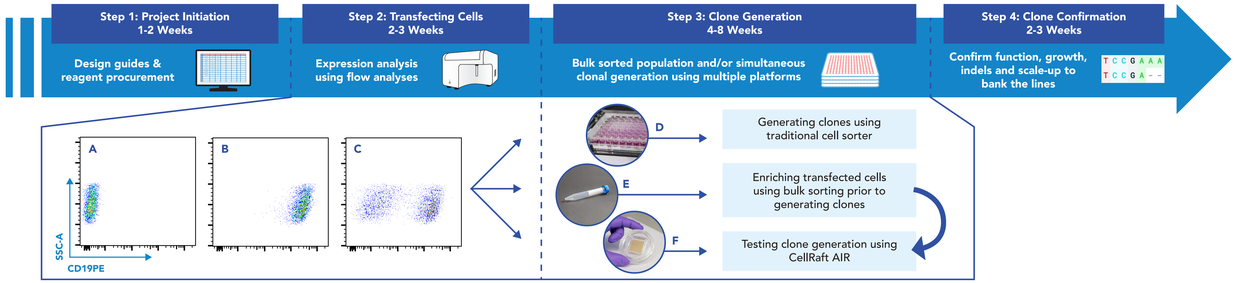
Table 1. CD19 sgRNAs Primer Sequences

CD19 sgRNA	Design ID from IDT	Primers Flanking sgRNA Region
EX1: 5'CTCGGGCCTGACTTCCATGG3'	Design ID: CD.Cas9.FSVH8562.AO	FWD: Primer 1 REV: Primer 1
EX3: 5'CTAGGTCCGAAACATTCCAC3'	Design ID: Hs.Cas9.CD19.1.AA	FWD: Primer 2 REV: Primer 2

EX, exon; FWD, forward; sgRNA, single-guide ribonucleic acid; REV, reverse.

- Guide RNAs were custom designed from Integrated DNA Technologies based on their highest possible on/off target scores (**Table 1**)
- Primer sets were designed on either side of the sgRNAs for the target region amplification and to confirm the indel formation by Sanger sequencing

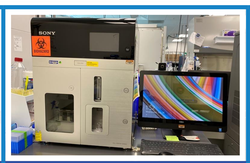
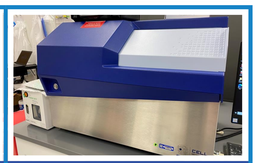
Figure 2. Timeline for Generating Engineered Cell Lines



Steps 2 and 3 are shown in greater detail to illustrate the workflow design phase. A,B. Unstained and stained Raji parental cells. C. Transfected cells with loss of CD19 expression nearly in 50% of the cell population; D-F. Methods to generate clones, either by sorting into multiple plates, or bulk plate sorting cells onto CytoSort® Arrays.

- The biggest bottleneck in the timeline was generating clones from transfected or bulk sorted cell lines (**Figure 2**)
- In order to shorten the timelines and make the process more efficient, we tested many cell sorters as well as a new product from Cell Microsystems, the CellRaft AIR™

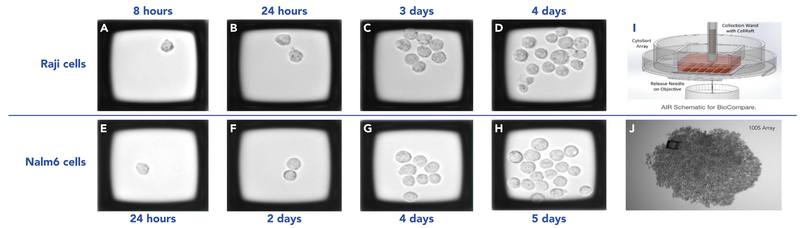
Table 2. Platforms Used for Single-Cell Clone Generation

Objective	BD FACS Aria Fusion	SONY SH800 Sorter	ThermoFisher Bigfoot Spectral Cell Sorter	Cell Microsystems' CellRaft AIR Imaging and Cell Isolating System
				
Post sort viability (bulk)	Almost 90% viable for suspension and 50% viable adherent lines	Almost 90% viable for suspension and 50% viable adherent lines	Almost 90% viable for suspension and adherent lines not tested	Instrument not designed for bulk sort
Clone forming efficiency (per 96-well plate)	Minimum 6-12 plates sorted, and 25-30 wells formed colonies (~30%) after 2-3 weeks based on cell type. Similarly, adherent cells plated and obtained on 3-5 clones from each plate (3-5%)	Similar results observed from SONY sorter. Around 30% for suspension, 3-5% for adherent	Similar results from Bigfoot sorter Around 30% for suspension, Adherent cells not tested	Almost 400 clones form per each array isolated into 3×96-well plates. Each plate will give rise to 50% single cells growing into colonies
Cell sort revival and colony growth	3-4 weeks for suspension, 3-6 weeks for adherent	3-4 weeks for suspension, 3-6 weeks for adherent	3-4 weeks for suspension, adherent cells not tested	3-4 weeks for suspension, adherent cells not tested
Instrument's ease of use (start-up, shut-down times, and maintenance)	40-60 minutes instrument start-up time (stabilizing stream based on nozzle used). Shut-down takes 15 minutes	20-minute instrument start-up time (stabilizing stream based on nozzle used). Shut-down takes 5-10 minutes; however, needs regular overnight shut-downs on monthly basis	15-minute instrument start-up time (stabilizing stream based on nozzle used). Manufacturers still optimizing the system to make it easier for users to work with	User-friendly imaging system works by simply switching on to scan, monitor cell growth, select better clones, and isolate (transfer) into 96-well plates

- We compared the conventional sorters, BDFACSAria, SONY SH8000, and Thermofisher Bigfoot, with an imaging and single-cell isolation system, Cell Microsystems' CellRaft AIR™, using several parameters, including ease of use, and start-up and shut-down processing times (**Table 2**)

RESULTS

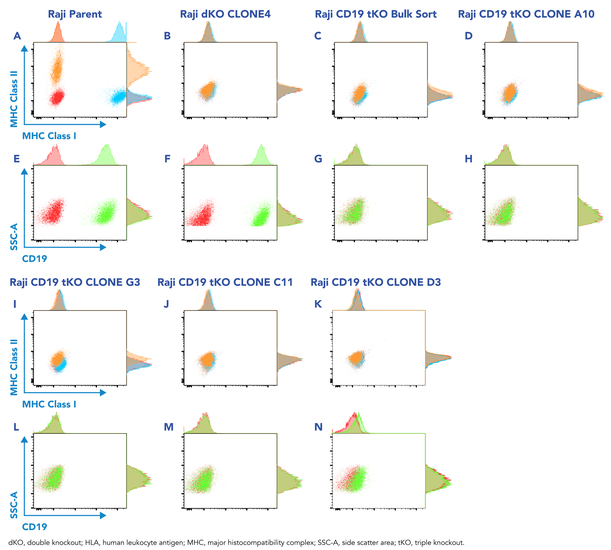
Figure 3. Images from Cell Microsytems CellRaft AIR™



A-D. Doubling of the Raji cell line. E-H. Doubling of the Nalm6 cell line. I. Cartoon image of collection wand selecting desired raft as specified by the user. J. A small square-shaped raft with 16 cells grown into a colony in one of the 96 wells.

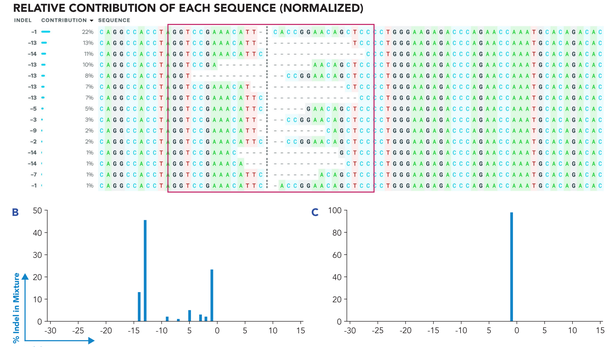
- Images from Cell Microsytems CellRaft AIR™ show doubling of Raji cell line (**Figures 3A-D**) versus Nalm6 cell line (**Figures 3E-J**), with sequential growth from single cell to ~16 cell stage
- The CellRaft AIR™ also has fluorescent imaging capability (not shown here) that can help identify specific population based on fluorescent parameters
  - Our lab is still working on using this capability to move directly from transfection to CellRaft AIR™ without the need for cell sorting

Figure 4. Bulk sort and clones analyzed for loss of protein expression by flow cytometry



dKO, double knockout; HLA, human leukocyte antigen; MHC, major histocompatibility complex; SSC-A, side scatter area; tKO, triple knockout.

Figure 5. Indel Formation and Analysis



Sanger sequence results for bulk sorted sample and single-cell clone G3. A. Multiple sequences represent different indels formed in bulk sorted sample. B. The indel plot display the inferred distribution of indels in the entire edited population. Each bar indicates the insertion or deletion of nucleotides along with percentage of the genome that contains (bulk sort). C. Single bar indicate homogeneous population (single-cell sort).
PAM, protospacer adjacent motif.

- Parent Raji cells (wild-type) show high expression for major histocompatibility (MHC) human leukocyte antigen Class I, Class II, and CD19 genes (**Figures 4A, 4E**)
- Double knockout clone 4 shows loss of MHC Class I and II (**Figure 4B**) and high expression before infection with CD19 sgRNA (**Figure 4F**)
- Bulk sorted cells show no expression of MHC Class I/II or CD19 (**Figures 4C, 4G**)
- Clones derived from bulk (A10, G3, C11, D3) show complete loss of expression of MHC Class I/II (**Figures 4D, 4I-4K**) and CD19 (**Figures 4H, 4L-4N**)

CONCLUSIONS

- We have observed 30% or less outgrowth of single cells from 96 well plates sorted from BDFACSAria, SonySH8000, and Thermofisher's Bigfoot sorters
- With Cell Microsystems' CellRaft AIR™ imaging and isolating system, we collected nearly 400 rafts containing single-cell–derived clones from each array. From those, more than 60% have potential for outgrowth. However, these kinetics will change based on cell type and transfection efficiency will determine number of clones to need to be tested
- Several adherent lines tested in the lab do not survive sorting at high pressure and their outgrowth is poor when sorted singly in plates. Manually limiting dilution was previously the only process for obtaining clones; however, we can now utilize the CellRaft AIR™ system for adherent lines as well
- Additionally, CellRaft AIR system allows us to image and monitor the cell growth from single cell to at least 32 cells before transferring them into 96 well plates. We observed better growth and survival rate of isolated cells when compared to other systems
- Moreover, the instrument does not require start-up and shut-down time. Additionally, this system will minimize several steps required to carefully consolidate clones from multiple plates
- The benefits we observed by incorporating CellRaft AIR™ system into our cell engineering workflow has led to shortened timelines and resulted in greater efficiency. To utilize all the features of CellRaft AIR™, our next steps involve staining and utilizing fluorescent parameters for selecting clones
- Additionally, we plan to incorporate automation for hit-picking, PCR, and flow cytometry analyses to further improve our timelines

ACKNOWLEDGMENTS

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DISCLOSURES

ST: employment with, stock or other ownership in, and research funding from Kite, a Gilead Company. **AA:** employment with and research funding from Kite, a Gilead Company; stock or other ownership in Gilead. **JH:** no relevant financial relationships to disclose. **RS:** employment with and travel support from Kite, a Gilead Company; stock or other ownership in and patents from Amgen; stock or other ownership in Gilead. **BR:** employment with, stock or other ownership in, and research funding from Kite, a Gilead Company.

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