ADVANCED INSTRUMENTS

Application Note

Development of a highly efficient, documented workflow for making clonal cell banks of gene-edited iPSCs

Philip D. Manos[1], Ian Taylor[2], Duncan Borthwick[2], Claire Richards[2]
1 - Evercell - 2 Advanced Instruments

Introduction

As cell therapy companies plan for future transfer from process development to clinical manufacturing, they look to adopt robust workflows that are designed to best manage consistency, quality of product and meet with appropriate regulatory requirements and manufacturing best practices.

Over the last decade, laboratories engaged in therapeutic monoclonal antibody production have engaged a 'clonally-derived' methodology which seeds individual cells from pools of cells thereby limiting heterogeneity. This practice, moreover, requires quality evidence of clonality, supporting the existence of a single cell post dispensing, for submissions to the medicines agencies.

Laboratories involved in cloning human induced pluripotent stem cells (hiPSC) cells will be familiar with the practice of limiting dilution (pipetting cells at a calculated <1 cell per well concentration) or using platforms such as fluorescently activated cell sorting (FACS). However, these methods are generally inefficient (low cell survival and low outgrowth) and moreover, do not support image-based evidence for purposes of assurance. In the case of FACS, this is also not conducive with a manufacturing environment.

Gene-editing steps in the process add additional challenges. Gene-editing methodologies often compromise cell health and survival. Depending on the complexity and type of approach utilized, this can dramatically change the scale of the project to find ultimately, a small number of cells that have survived the process, grown into colonies and then have, post genetic analysis, been found to contain the desired combination of edits. In our experience, for simple knockouts, 100-150 clones would need to be produced to generate 10 suitable clones for banking. For multiple knock- in gene edits, this requirement could be >1000 clones. At this scale, limiting dilution efficiencies are not a practical option.

We have previously reported on using a VIPS™ (Verified In- situ Plate Seeding) instrument (Solentim) and MatriClone™ a laminin-based matrix (Solentim) in a workflow for single cell seeding with parental hiPSC cell lines. VIPS offers both automated seeding and supporting clonality assurance through on-board imaging. With the additional numerical challenges of gene-editing workflows, we employed the same methodology here to examine the effectiveness of the VIPS + MatriClone workflow for gene-edited hiPSC cells.

The aims of this project were to show:

1. High seeding efficiency in 96 well plates (% of wells seeded with single cells).
2. High cloning efficiency (% outgrowth from single cell seeded wells).
3. Generation of sufficient number of clones (>150) which would provide enough suitable sequence-verified clones for banking.
4. Quality evidence in the form of images and documentation of clonality for regulatory submission.
5. Incorporation where possible of reagents compatible with clinical manufacturing practices and GMP environments.
6. Banked clones should at the end of this process, maintain stem-like characteristics including pluripotency and morphology.

Materials and Methods

VIPS is an automated single cell seeder that operates at very low pressures to dispense individual cells into 96 or 384 well plates. VIPS verifies the existence of a single cell in the well by way of a 20 layer z-stack of the nanolitre volume droplet, then applies artificial intelligence to identify the presence of a single cell, no cell or multiple cells. VIPS then automatically fills the wells with media after which it can obtain whole well images to support the existence of the single cell. This double time point of clonality (or double lock) is a key component for supporting future regulatory submission.

Using CRISPR-Cas9 via ribonuclearprotein (RNP) delivery we aimed to disrupt the EMX1 gene (codes for a transcription factor involved in neuronal patterning) locus in an hiPSC line. Following nucleofection, hiPSCs were seeded into 96 well plates as single cells via VIPS, or limiting dilution (LD) as a control, and then expanded in media containing MatriClone. Plates underwent daily whole-well imaging on the VIPS system to confirm clonal origin, and to track outgrowth of the colonies.

As shown previously in our publication (Manos et al.), VIPS demonstrated a 3-4 fold improvement in the number of colonies successfully derived from single cells per plate when compared to LD. In this study, from as few as three VIPS-seeded 96 well plates, 100 clonal colonies were generated, then selected and genotyped to confirm indel (insertion or deletion of bases) formation in the EMX1 locus. Two clones containing a confirmed indel were then expanded and underwent characterization.

1. Selection of target gene locus for editing, design and preparation of guide RNA (gRNA)

Selection of gene locus (EMX1) was based on previous publication (Ran et al., 2013) where CRISPR/Cas9 was utilized for indel formation. Briefly, the sequence of interest was inserted into Benchling's gene editing tool (Hsu et al., 2013) that resulted in a number of gRNA candidates. Three on-target gRNA's were selected (based on low off-target scores as determined by the probability of sequence mis- matched pairing).

2. Preparation and transfection of the cells

Prior to single cell seeding, the hiPSC line was adapted to MatriClone for a minimum of two complete passages. Cells were cultured via clump passaging technique, typically at a split ratio of 1:15 on 6-well plates pre-coated with MatriClone, prepared according to the manufacturer's instructions. The media used was mTeSR Plus (StemCell Technologies).

3. Pool screening and selection

On day 3 of growth post passage, the hiPSCs were single-cell dissociated using Accutase. A cell count was obtained and adjusted for delivery of the Cas9 and gRNA. Approximately 1 million cells were aliquoted into three separate tubes that were prepared with Cas9 and three different gRNAs identified in Step 1 above.

Cell and gene editing components were then delivered using the NEON electroporation unit according to manufacturer's manual. Each sample was then plated onto MatriClone-coated 6 well dishes in mTeSR supplemented with CloneR (StemCell Technologies) and allowed to expand.

On day 3, a sample from each dish delivered with a different gRNA was collected and assessed for efficiency using TIDE analysis. Briefly, multiple primers were first designed and screened for optimal PCR products from crude cell lysate. Then a portion of cells from each dish was taken from the expansion dish before then extracting the DNA and performing Sanger sequencing of the PCR product.

4. Seeding, seeding parameters and feeding

In day 5, the pool with the highest efficiency of indel formation obtained from TIDE analysis was gRNA#2, which was then selected for VIPS seeding (Figure 1). For the single cell seeding, hiPSC colonies were dissociated with Accutase into single cells and collected for viable cell counts using the Spectra (Nexcelom). Cell viability was 85% prior to seeding. Cell concentration was adjusted to approximately 10,000 cells/ml in mTeSR Plus for optimal seeding efficiencies. MatriClone in solution was added directly to the media in the cell reservoir which was then dispensed by the VIPS Cell Reservoir nozzle into each well. A total of 5 plates were seeded for a particular gRNA pool. Following single cell dispensing and immediate confirmation in the droplet by the VIPS, 125 µl of media with MatriClone and CloneR was added to each well via the secondary VIPS media dispensing system. Subsequently, the 96-well plates were fed by adding 50 µl to each well on day 1 and day 3 post- seeding, with a complete media change on day 5 post- seeding. This feeding regimen was repeated until day 12.

5. Expansion of clones

On day 12, hiPSC clones from VIPS seeded plates were selected based on hiPSC colony morphology obtained from the confluence detection algorithm provided by the VIPS (Figure 2). Using an EDTA-based dissociation reagent, individual clones from three separate 96-well plates were carefully clump-passaged in media containing CloneR (initial split only) into both a 96-well plate format and a single well of a 6-well plate (or equivalent) that was precoated with either MatriClone or Matrigel® (Corning). At the following passage (p+2 post seeding), ten hiPSC clones with confirmed indel formation were expanded into a minimum of a 6-well plate for cryopreservation and characterization.

6. Sequencing

Sequencing was performed by first collecting individual cell samples of the expanded clones and performing DNA extraction. Amplicons for the region of interested were then generated and used for Sanger sequencing before analyzing indels via TIDE analysis. Approximately 96 clones were selected for initial screening, of which 10 samples were found to have suitable gene-edited profiles and were continued for confirmation at a later passage.

7. Characterization of pluripotency marker expression, karyotype

Characterization was performed as described previously1. Briefly, two selected hiPSC clones were recovered from cryopreservation and seeded into Matrigel-coated 6 well plates before being transferred into new plates for further analysis. For ICC/IF, cells were split into imaging plates and stained with pluripotency markers Nanog and Tra-1-60. hiPSCs were karyotyped via g-banding.

Results

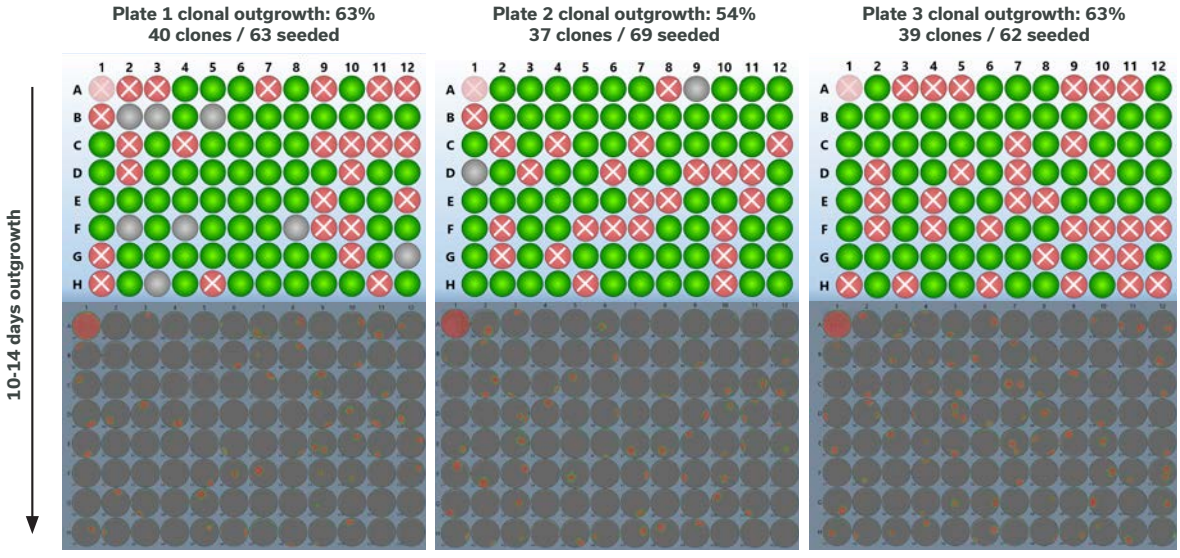

Figure 1. Single cell seeding and clonal outgrowth results from gRNA#2 seeded pool. Images from VIPS software.

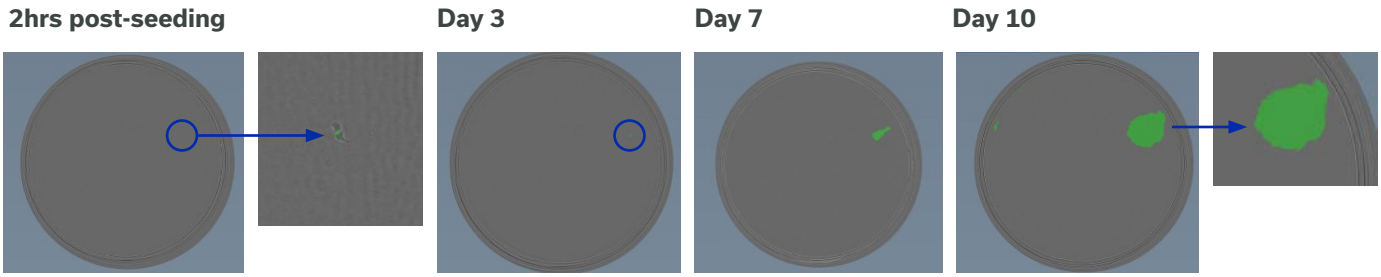

Figure 2. Colony morphology assessment on VIPS, utilizing a neural network algorithm to detect iPSC confluency.

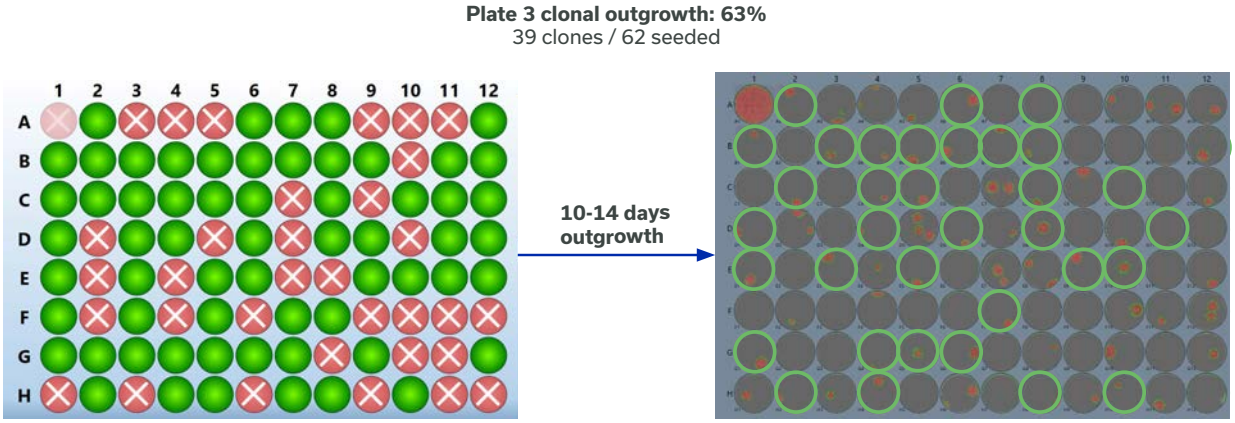

Figure 3. Example of seeding efficiency to clonal outgrowth on single seeding of gene edited iPSCs.

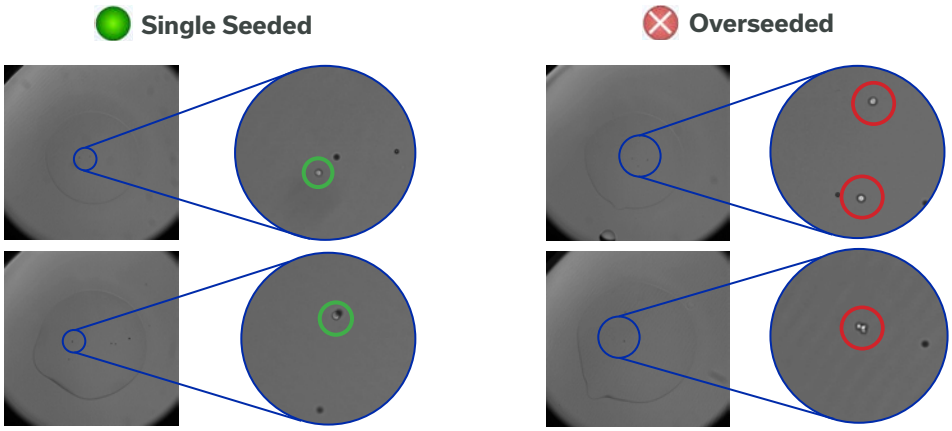

Figure 4. Automated image analysis of dispensed droplet can resolve individual cells from multiples, empty wells and cell debris, supporting evidence of clonality.

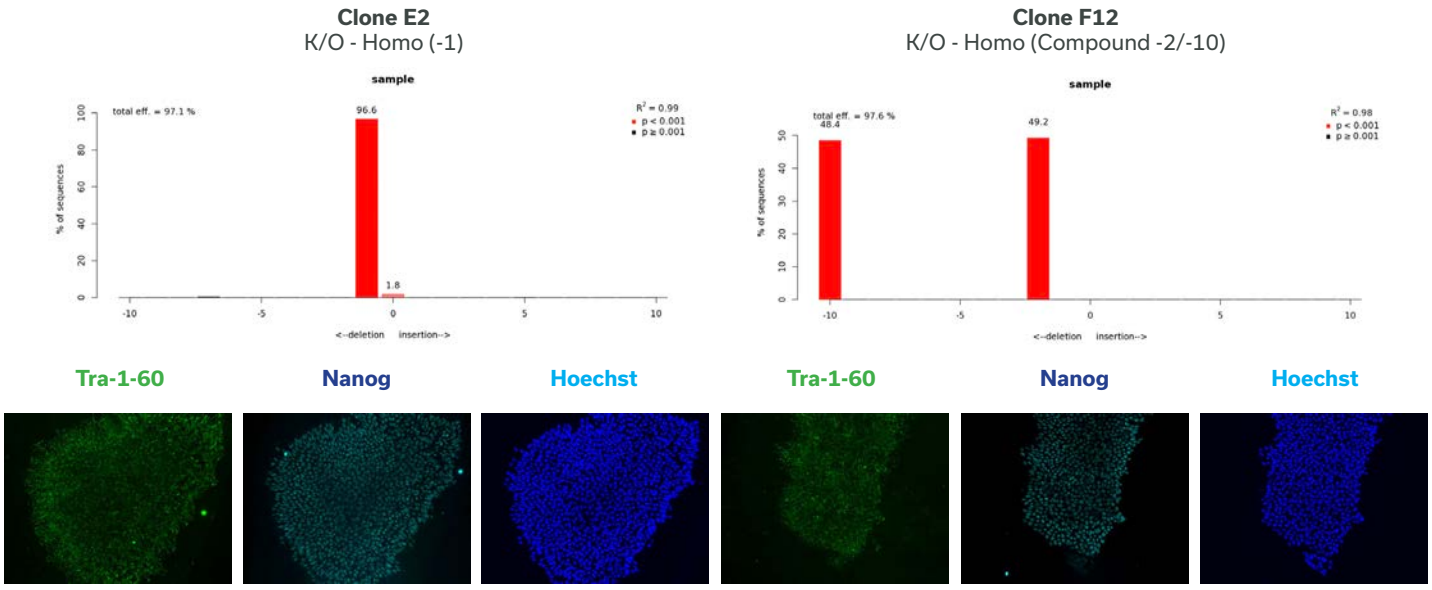

Figure 5. Characterization or indel formation and pluripotency marker expression in expanded hiPSC clones: Clone E2 a homozygous deletion with a single base deletion on both alleles; Clone F2 is a Compound Homozygous deletion with a 2 base pair deletion on one allele and 10 base pair deletion on the other.

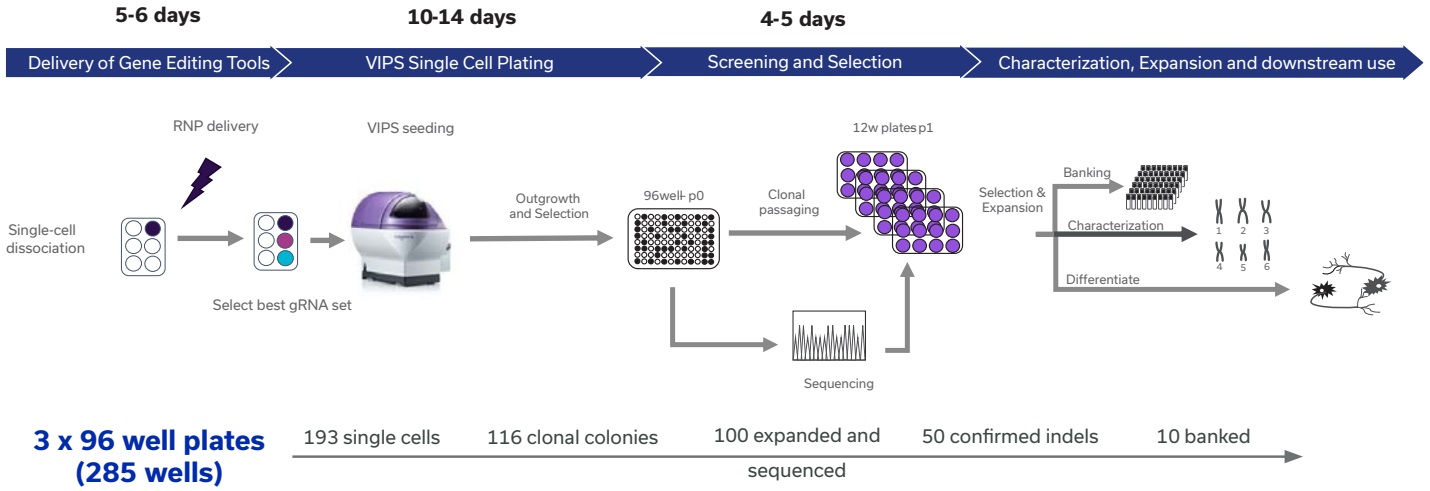

Figure 6. Design of automated workflow for single cell seeded, gene edited iPSC cells for banking with evidence of clonality.

Results and Conclusion

We previously demonstrated the significant improvement in utilizing VIPS and MatriClone in an iPSC single cell cloning workflow of a parental cell line, seeing a significant increase in the number of clonal colonies per 96 well plate compared with limiting dilutions[1].

Gene-editing of the hiPSCs adds a further level of complexity to this workflow and a higher stringency of selection for viable cells and resultant colonies. Probability calculations for edits and multiple edits, mean generating potentially many more clonal colonies per project. This requires more plates, a greater degree of automation and concurrently documenting the proof of the clones as they are printed such that this data can be submitted as part of an IND (Investigative New Drug) application.

In this study, we have demonstrated a representative gene-editing workflow for knockout of the EMX1 locus using the VIPS/MatriClone combination platform. Seeding efficiency using the VIPS averaged 65%, with cloning efficiency of 60% (or greater) across three fully plated 96 well plates, (Figure 3).

We successfully obtained 10 suitable clones from the 100 clones expanded. Furthermore, the VIPS system, in conjunction with its artificial intelligence based detection provides quality evidence of the existence of a single cell, accurately resolving a single cell from multiple cells and aggregates (Figure 4). An additional benefit of the VIPS system is the ability to capture daily whole well images (from Day 0 onwards) which enables tracking of colony outgrowth for clonal assurances, in addition to quality assurance. Importantly, we demonstrated that the hiPSC clones selected for banking maintained their characterization criteria of pluripotency marker expression and karyotype (Figure 5 – data shown for two of the clones).

Beyond the technical advantages of the VIPS platform, time requirements are often a critical factor. This can impact commercial occupancy time in the tissue culture suite for manufacturing. Importantly, the workflow process described can be completed in as little as 25 days (Figure 6) and requires plating of only three 96-well plates in order to generate 100 clones. By increasing efficiency and decreasing materials, we estimate up to a 50% reduction in time for typical gene-editing workflows. Therefore, by improving the overall efficiency, the VIPS platform allows for more complex editing projects. Moreover, the VIPS system provides quality data assurance and reporting that allows for real time assessment and regulatory compliance.

We should observe that for more challenging knock in editing projects, these will be much more inefficient meaning probably less colonies per plate and the need for many more plates to get the target number of colonies. We believe that the robustness of this workflow has immediate potential to impact standards, consistency, and confidence of clonality for anyone developing iPSC-derived cell therapies. Furthermore, the regulatory burden of translating this workflow from a process development towards clinical manufacturing environment is eased by way of GMP grade reagents and installation and operational qualification services around the instrument. It shouldbe noted here that clonally-derived Master Cell Banks for allogeneic cell therapies should be created under GMP conditions.

Further work will involve testing different dissociation reagents, ROCK inhibitors and medias for combinations compatible with GMP manufacturing environments. We believe this workflow will become a standard for quality cell therapy laboratories.

Acknowledgements

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Publication References

1. Manos et al. Standardizing the process for viable, high efficiency single cell cloning of hiPSCs whilst ensuring maintenance of phenotype. https://www.solentim.com/standardizing-the-process-for-viable-high-efficiency-single-cell- cloning-of-hipscs-whilst-ensuring-maintenance-of-phenotype/
2. Ran, F., Hsu, P., Wright, J. et al. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8, 2281–2308 (2013). https://doi.org/10.1038/nprot.2013.143
3. Hsu, P., Scott, D., Weinstein, J. et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31, 827–832 (2013). https://doi.org/10.1038/nbt.2647

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Two Technology Way/ Norwood, Massachusetts 02062, USA

800-225-4034 | 781-320-9000 | www.aicompanies.com