Accelerating genome engineering of induced pluripotent stem cell lines for disease modeling by implementing automated workflows

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ABSTRACT

Using state-of-the-art genome editing tools, such as Cas9 protein and synthetic guide RNAs, the genome of human induced pluripotent stem cells (hiPSCs) can now be easily edited to introduce genetic defects related to disease. Genome edited hiPSCs can then be differentiated into eg. cardiomyocytes or neurons, which can be implemented to model disease *in vitro* for basic research or drug discovery. While in general genome editing of hiPSCs has become standard practice, higher throughput, larger scale and consistent generation of genome edited hiPSCs lines remains challenging due to the complex nature of hiPSC culture conditions. Furthermore, manual picking of hundreds of colonies to identify clonal lines carrying the desired genomic change remains labor intensive. We therefore implemented automation to standardize and facilitate key steps covering the majority of the genome editing workflow in hiPSCs.
Of the required steps to generate a clonal, genome edited hiPSC line, we so far achieved full automation of single cell seeding, expansion and consolidation, simply relying on available hiPSC products, a cell sorter, whole well imaging scanner and a simple liquid handler. Using SNP introductions to model disease as a genome editing example in hiPSCs, we demonstrate, using these automated workflows, that clonal genome edited hiPSC lines can be derived reproducibly across multiple hiPSC backgrounds, with high first-time-right rates that drive throughput and scale. These methods did not affect the karyotype of the generated hiPSC lines, which furthermore maintained their typical pluripotency characteristics and potential to differentiate into specialized cells, allowing us to study the biology of the genomic changes made.

INTRODUCTION

Since the availability of feeder-free culture systems for hiPSCs, cell handling has become easier and now allows manipulations of hiPSCs, including at the genomic level via CRISPR/Cas9 or TALEN. While high editing efficiencies can now be reliably achieved for the majority of genomic targets, the downstream process of isolating pure clonal hiPSC lines with the genomic edit of choice remains a labor intensive process, as many clones typically need to be generated, isolated and screened (**Figure 1**). Common methods for isolation of single cell clones include limited dilution cloning in 96-well plates (LDC) or limited dilution plating (LDP) in large culture dishes. While with LDC you can at least automate the cell plating, neither method ascertains that a clonal population has been obtained and often requires several rounds of cloning. In addition, obtained clones typically need to be manually picked from the 96-well plates or the larger dishes, which depending on the number of clones to be screened (and heavily depends editing efficiencies) can be a daunting task (**Figure 1**).

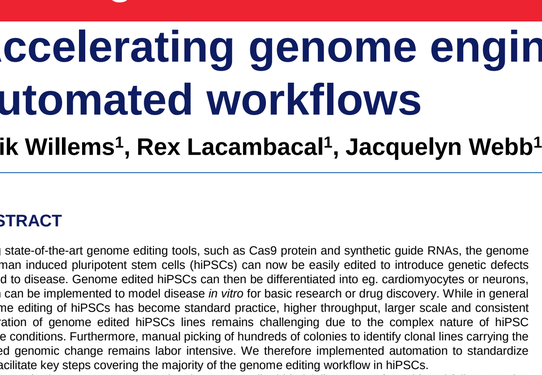


Figure 1. Overview of the common genome editing workflow for genome editing in a standard hiPSC line. *Current methods for all steps of the workflow are shown, with an indication of throughput and/or limitations. Red stars indicate two main bottlenecks for the throughput and reproducibility of the genome editing workflow in hiPSCs and present an opportunity for development of automated workflows.*

RESULTS

The main pain points of the genome editing workflow we sought to solve were related to the isolation of clonal lines, which typically is a labor intensive process (**Figure 1, red stars**). Through the implementation of automation approaches, we were aiming to improve the reliability, throughput and timeline of the typical genome editing workflow in hiPSCs. We looked at the deposition of single hiPSCs into 96-well plates, clone feeding and clone consolidation into 96-well plates for downstream processing.
To allow reliable high-throughput isolation of single cells in 96-well plates on a fluorescence activated cell sorter (FACS) that allows plate seeding, we first implemented stringent gating strategies to ensure that single, viable and pluripotent cells were isolated (**Figure 2**). Factors that may improve survival after sorting were then tested to understand how the most efficient single cell cloning in feeder-free conditions could be achieved. Through extending the RevitaCell™ exposure window from 24h to 72h we were able to dramatically increase clone survival in Essential8™ when seeding low amounts of cells into a 96-well (**Figure 2, left histogram plot**). Clone survival from seeding one single cell was, however, limited and therefore the effect of matrix protein and media system was further investigated.

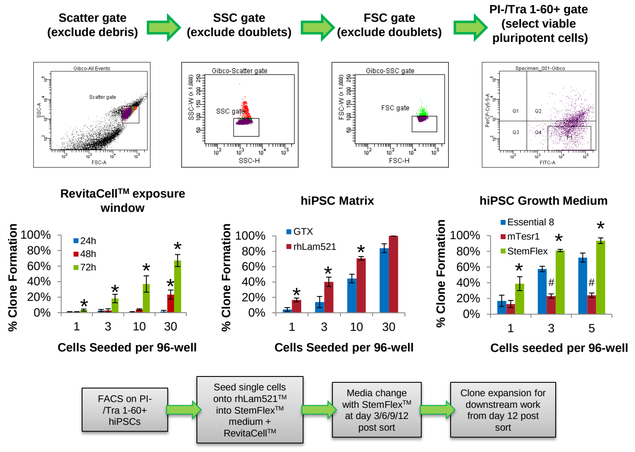


Figure 2: Improvements to increase the recovery of single cell clones isolated through automated single cell seeding via FACS. *A stringent gating strategy was used to identify single, viable and pluripotent hiPSCs which were seeded using a cell sorter as indicated. Effects of RevitaCell™ exposure window, hiPSC matrix protein and hiPSC growth medium were investigated. The novel workflow based on these results is diagrammed.*

Sorting cells on rhLaminin-521™ further increased clone recovery from single cells, up to 15% and this improvement was enhanced when sorting cells into StemFlex™ medium, yielding up to 40% clone survival from single cell seeds (**Figure 2, middle and right histogram plot respectively**). Clonal survival after single hiPSC deposition into 96-well plates using a cell sorter can thus be improved extensively by following the summarized post sort steps as indicated in the flow chart in **Figure 2,** facilitating automated workflows downstream. The FACS mediated plating method was found effective on multiple hiPSC lines, with clonal survival rates of at least 25% (**Figure 3**).
Once the cells were deposited into 96-well plates on rhLaminin-521™, StemFlex™ medium and RevitaCell™ medium was exchanged every 3 days using a standard liquid handler, ensuring slow liquid aspiration and dispensing speeds to avoid cell loss. Within 10-14 days clones can be observed and processed for screening (**Figure 3**). Using whole well-based image analysis, wells with clones can be identified and loaded into plate maps for automated clone consolidation. Using the culture conditions identified for single cell plating, we implemented automated cherry picking to consolidate clones into 96-well plates with a liquid handler that allows single channel manipulations (**Figure 4**). Generally, 2-3 plates containing clones can be consolidated into 96-wells with a post manipulation recovery of ~95%. Consolidated clones recover and proliferate normally, yielding 80% confluency in 3-4 days and have a normal pluripotent morphology (**Figure 4**).

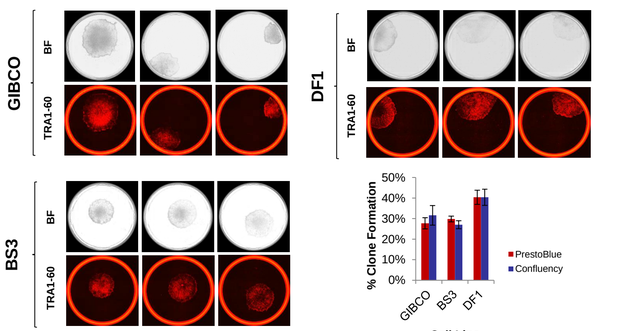


Figure 3. Single cell clone generation from different hiPSC lines. *Individual clones are shown and stained for TRA 1-60 to demonstrate their pluripotent nature. Cloning efficiency measured by confluency or PrestoBlue® for the different lines is also indicated.*

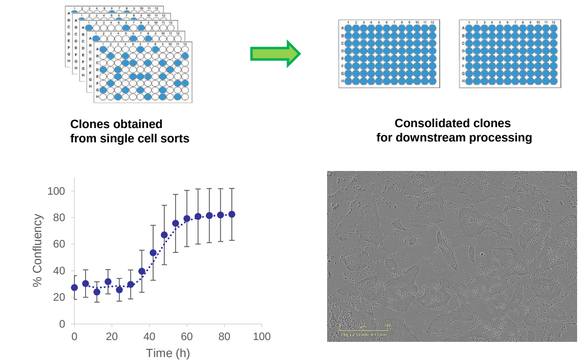
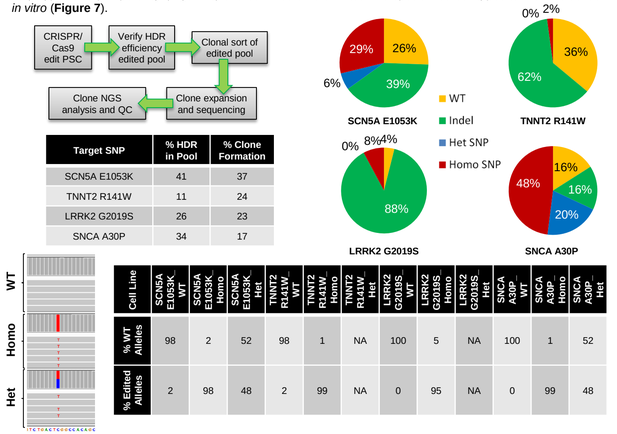


Figure 4: Automated cherry picking of clones to facilitate clone screening. *Using plate maps generated via image based identification of wells containing a clone, cherry picking using a simple liquid handler can be used to consolidate single cell clones into a few 96-well plates. This can be achieved with a ~95% success rate using the culture conditions identified in Figure 2, and hiPSCs expand normally over the next 2-3 days (growth curve of hundreds of clones and representative hiPSCs are shown).*

These automated steps were then tested during introduction of SNPs into different genomic loci in hiPSCs. After delivery of the editing tools we obtained 11-41% homology driven repair or SNP introduction in the pools (**Figure 5**). Single cell clones were then isolated from each pool using the automated approach and yielded 17%-37% surviving clones, which were then screened by Sanger sequencing for the presence of homozygous and heterozygous SNPs as well as indels (**pie charts Figure 5**). Isolated single cell clones were further analyzed by next generation sequencing to understand if the isolated clones were truly derived from a single cell. Ratios of 100% WT or SNP are expected for unedited or homozygotes, whereas for heterozygotes, both the WT and SNP allele should be represented by equal amounts. Across all for targets and all the derived clonal lines, this is indeed the case (**Figure 5, bottom table**), demonstrating that a single round of automated isolation and expansion is sufficient to obtain single cell derived clonal lines, which has a positive impact on effort and timelines. Furthermore, the genome edited hiPSCs generated through the automated workflow had unaffected karyotypes and pluripotency (**Figure 6**) and could be differentiated into specialized cell types to model disease *in vitro* (**Figure 7**).



Target SNP	% HDR in Pool	% Clone Formation
SCN5A E1053K	41	37
TNNT2 R141W	11	24
LRRK2 G2019S	26	23
SNCA A30P	34	17

Cell Line	SCN5A E1053K WT	SCN5A E1053K Homo	SCN5A E1053K Het	TNNT2 R141W WT	TNNT2 R141W Homo	TNNT2 R141W Het	LRRK2 G2019S WT	LRRK2 G2019S Homo	LRRK2 G2019S Het	SNCA A30P WT	SNCA A30P Homo	SNCA A30P Het
% WT Alleles	98	2	52	98	1	NA	100	5	NA	100	1	52
% Edited Alleles	2	98	48	2	99	NA	0	95	NA	0	99	48

Figure 5. Generation of disease models in Cas9-expressing hiPSCs. *Cas9 hiPSCs were used to introduce SNPs known to be associated with Parkinson's (LRRK2 G2019S, SNCA A30P) or cardiac disease (SCN5A E1053K, TNNT2 R141W). HDR efficiency was target dependent and homozygous SNP clones were identified for all targets. After expansion and consolidation clones were analyzed by NGS for allele ratios to demonstrate that clonal lines can be established with one round of clonal isolation.*

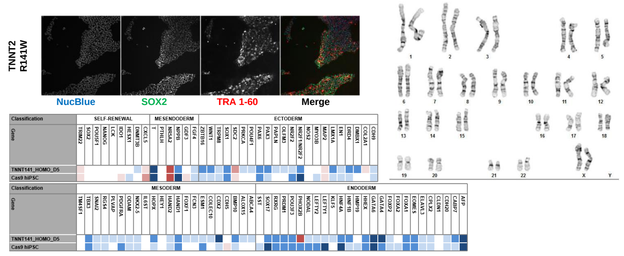


Figure 6: The genome editing workflow does not affect pluripotency or karyotype of hiPSCs. *Introduction of automation in the genome editing workflow did not affect pluripotency (ICC for pluripotent markers and hPSC Taqman Scorecard data are shown) or the karyotype of hiPSCs.*

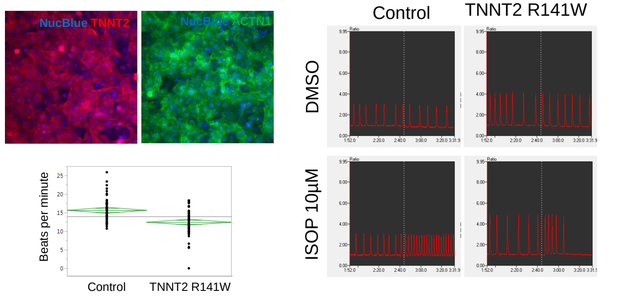
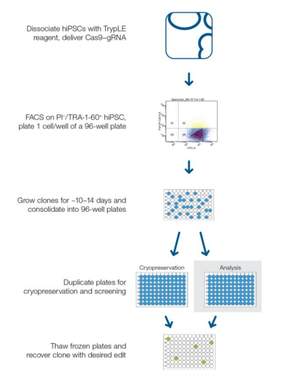


Figure 7: Analysis of an *in vitro* generated model of dilated cardiomyopathy generated through genome editing of hiPSCs. *Genome edited hiPSCs were generated and differentiated into cardiomyocytes to study the effect of the TNNT2 R141W mutation, which has been associated with dilated cardiomyopathy. Diseased hiPSCs formed cardiomyocytes normally (example ICC shown) and derived cardiomyocytes showed a significant difference in functionality under normal conditions. Furthermore, when challenged with Isoproterenol (ISOP), TNNT2 R141W cardiomyocytes stopped contracting.*

CONCLUSIONS

With hiPSC-based disease modeling at the forefront of research and drug discovery, the ability to generate disease models through genome editing has taken an important place in the field. However, achieving scale to produce large collections of genome edited hiPSCs has been challenging due to the labor intensive nature of the process.
We therefore attempted to fully automate the main bottlenecks of the genome editing workflow in hiPSCs, namely single cell isolation, expansion and consolidation. Relying on reagents that support hiPSC growth under stressful conditions, (rhLaminin-521™, StemFlex™ medium and RevitaCell™), FACS, a simple liquid handler and a whole well scanner, automation of clone isolation was implemented, which not only dramatically reduced hands on time and scalability, but also improved reliability and timelines of genome edited hiPSC line generation.
This achievement illustrates that hiPSC workflows can be easily automated, which allows the scale up of hiPSC cell line generation for a diversity of applications, including *in vitro* disease modeling for basic research and drug discovery.



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