

Thank you for attending the Allen Institute for Cell Science Stem Cells and Gene Editing Workshop. This booklet of guidelines and best practices has been compiled by our scientists to provide a reference for you to use in the future, and to share with others in your labs or scientific community.

The mission of the Allen Institute for Cell Science is to create dynamic and multi-scale visual models of cell organization, dynamics and activities that capture experimental observation, theory and prediction to understand and predict cellular behavior in its normal, regenerative, and pathological contexts. Our vision is to create new directions and opportunities in cell science through mission-oriented, large-scale, multi-disciplinary team science. We want to better explain and predict the behaviors of cells, whether healthy or diseased. Our first project will be to understand how the parts of the cells integrate to determine diverse cellular behaviors as revealed through a dynamic and animated virtual model of the cell.

As a result of our efforts to achieve these goals, we have identified methods and workflows for endogenously tagging expressed and silent genes in human induced pluripotent stem cells. In this workbook, we introduce key points for each main section, provide supplemental example data or workflows where applicable, and describe our full standard operating procedures for selected applications. A full list of Allen Institute for Cell Science methods can be found at our website, www.allencell.org/methods-for-cells-in-the-lab. Additionally, a series of videos highlighting some of these methods can be found on our website at www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.

The guidelines provided in this booklet were developed using the WTC-11 hiPSC line generated by the Gladstone Institutes, and are considered by the Allen Institute for Cell Science to be “best practice” to our knowledge at the time of this publication. Certain assays or protocols may need to be adapted or optimized for other cell lines, cell types, and applications. Throughout this workbook, the terms crRNA and gRNA or guide RNA are used interchangeably.

For follow up questions or comments, we recommend visiting the Allen Institute for Cell Science Forum (allencell.org/forum).

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mEGFP was a gift from Karel Svoboda (Addgene plasmid # 18696 ; <http://n2t.net/addgene:18696> ; RRID:Addgene_18696)

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Section 1a. Designing crRNAs for endogenous tagging or other large knock-in with CRISPR/Cas9

Key points:

- Know your genome. If available, cell line-specific variant information is useful when designing crRNAs and homology arms.
- Know your cell line. If available, transcript abundance data (ex. RNA-Seq) can help you decide what isoform(s) to tag based on transcript levels in your cell line.
- Design more than one crRNA per target while balancing off-target effects with proximity to desired cut site.
- The crRNA cut site is not necessarily the same as the knock-in insertion site!

Tool/Resource	Application	Notes
Geneious, Benchling, Ugene	Software for viewing, annotating, and editing sequences	Geneious requires subscription; Benchling, and Ugene are free alternatives.
https://www.ncbi.nlm.nih.gov/gene/	Access and download sequences from NCBI in genbank, fasta, and other formats	Search using official gene symbols (ex. TTN instead of titin) whenever possible; make sure you are looking at the right species and paralog; download sequences in genbank format because they include annotations.
https://ensembl.org/Homo_sapiens/Info/Index	Access and download sequences from ensembl in genbank, embl, and other formats	Alternative to NCBI
https://www.fpbases.org/	Database of fluorescent proteins	Contains links to original manuscripts and outside resources
https://www.addgene.org/fluorescent-proteins/	Resource for fluorescent protein plasmids	
http://crispor.tefor.net/	CRISPOR design tool for CRISPR guides	Calculates off-target scores and cutting efficiency scores
https://genome.ucsc.edu	UCSC genome browser has pre-computed CRISPR tracks for human and mouse genomes (10Kb around each exon)	There may be differences in parameters between the UCSC tracks and the main CRISPOR site. The CRISPOR webserver is the most up-to-date version.
http://www.rgenome.net/cas-offinder/	Cas-OFFinder webserver for finding CRISPR off-targets	Can search for up to 9 mismatches and off-targets with DNA and RNA bulges

General steps for designing crRNAs

1. Download sequence for the genomic region you want to edit in genbank format or another format that includes both the sequence and gene model/isoform annotations
2. Use CRISPOR to search for crRNAs within 50 bp on each side of your desired insertion site
3. Select crRNAs with few off-targets that are near your insertion site. We generally use targets within 50 bp, but we have had successful editing with targets up to 100 bp away from the desired insertion site.
 - a. crRNAs should have a high specificity score (MIT specificity score >50) and no off-targets with 2 or less mismatches unless mismatches are in the seed region (10 bp proximal to

PAM). CRISPOR will find off-targets with up to 4 mismatches with an NGG, NAG, or NGA PAM.

- b. once you have selected a few candidate crRNAs, use Cas-OFFinder to search for off-targets with bulges (RNA or DNA 1nt in length); avoid crRNAs that have off-targets with a single bulge in the non-seed and no other mismatches in the seed region

Resources:

Bae, S. et al. *Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases*. Bioinformatics 2014. <https://doi.org/10.1093/bioinformatics/btu048>

Graham, D. et al. *Resources for the design of CRISPR gene editing experiments*. Genome Biology 2015. <https://doi.org/10.1186/s13059-015-0823-x>

Haeussler, M et al. *Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR*. Genome Biology 2016. <https://doi.org/10.1186/s13059-016-1012-2>

Notes:

Section 1b. Designing a donor template for endogenous tagging or other large knock-in with CRISPR/Cas9 for genes that are expressed in hiPSCs

Key points:

- Homology arm length should be proportional to the insertion; for mEGFP (~700 bp) knock-in, we routinely use a 1 kb homology arm on each side
- N-terminal tag with “standard” first exon: 5’HA-endogenous start codon-FP/Tag- Linker-3’HA
- C-term tag: 5’HA-Linker-FP/Tag-endogenous stop codon-3’HA
- Linkers can be determined from literature (often cDNAs) or based on charge/size (see resources, below)
- Optimize codons in the linker for cell line species
- Donor template sequence can be used for gene synthesis, donor plasmid cloning, etc.

General steps for designing donor template:

1. Define insertion site, cell-line specific variants in the region, and crRNA binding sites (Section 1a)
2. Define homology arms
3. Create a new template that includes the linker and FP/Tag in the proper order (linker first for C-term, FP/Tag first for N-term), flanked by the appropriate 5' and 3' homology arms
4. Check the crRNA binding sites (protospacers) for disruption by the FP/Tag
5. Introduce mutations to disrupt CRISPR/Cas9 binding at in-tact protospacers making synonymous mutations in coding regions, avoiding the use of rare codons, and avoiding introducing mutations in highly conserved regions
 - a. Mutations to the PAM, if possible without disrupting coding regions or conserved non-coding regions
 - b. Two mutations to the protospacer seed region, if possible without disrupting coding regions or conserved non-coding regions
 - c. Three mutations to the protospacer with at least 1 in the seed region, if possible without disrupting coding regions or conserved non-coding regions
 - d. If none of the above are possible, consider using a different crRNA
6. Confirm that entire design is in-frame

Resources:

Haupt et al. *Endogenous Protein Tagging in Human Induced Pluripotent Stem Cells Using CRISPR/Cas9*. Journal of Visualized Experiments (JoVE). <https://www.jove.com/video/58130/endogenous-protein-tagging-human-induced-pluripotent-stem-cells-using> ©2018 JoVE

Allen Institute for Cell Science Lab Plasmids
https://www.addgene.org/The_Allen_Institute_for_Cell_Science/

Chen, Zaro and Shen. “Fusion protein linkers: property, design and functionality.” *Adv Drug Deliv Rev*. 2013. (<https://doi.org/10.1016/j.addr.2012.09.039>)

Paix et al. *Precision genome editing using synthesis-dependent repair of Cas9-induced DNA breaks*. PNAS. 2017.

Roth et al. *Reprogramming human T cell function and specificity with non-viral genome targeting*. Nature Letters. 2018. <https://doi.org/10.1038/s41586-018-0326-5>

Example codon frequency table for human: <https://www.genscript.com/tools/codon-frequency-table>

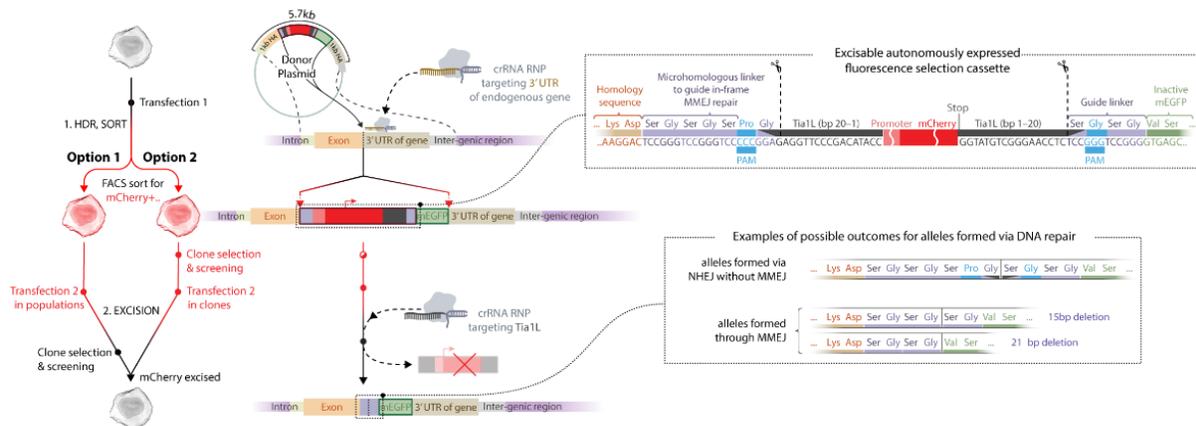
Notes:

Section 1c. Designing a donor template for endogenous tagging or other large knock-in with CRISPR/Cas9 for genes that are not expressed in hiPSCs

The guidelines above for crRNA design (Section 1a) and donor template design (Section 1b) still apply. For genes that are not expressed in hiPSCs, an additional selection cassette is included in the initial edit. Using the strategy described below, a second application of CRISPR/Cas9 with a specifically designed crRNA will induce the excision of the selectable cassette resulting in a subset of clones with an in-frame mEGFP insertion with a customizable linker and no other genomic “scar”. The fusion protein will only be expressed when cells are differentiated to a cell type where the endogenous protein is naturally present. The assays for identifying genetically correct clones described in Section 5 also apply to this editing method.

Key points:

- A strong promoter not likely to be silenced in hiPSC should be used to drive the expression of the selectable cassette, such as CAGGS
- Selection can be performed by FACS enrichment or by drug selection, if desired and the selection cassette contains the necessary fluorescent protein or drug resistance
- Excision of the selectable cassette can be highly efficient with the use of optimized reagents such as Thermo Fisher Invitrogen TrueCut Cas9 protein v2 (Cat#A36497) and Synthego modified sgRNAs (>70% in WTC-11)
- Clones can be generated and screened for precise editing either before or after excision, but clones screened before excision should be re-cloned and re-analyzed for precision after the excision has been performed
- Due to excision by NHEJ or MMEJ, a variety of linker sequences may be present in the population after excision- Sanger sequencing should be done to verify clones with in-frame, desired linkers



General workflow for tagging silent genes in hiPSC. Schematic of tagging approach with MYL2 donor plasmid as an example. Tia1L protospacers are orientated “PAM-out.” Scissor icons indicate positions of anticipated Cas9 cleavage. Options 1 and 2 reflect possible workflow variations. Adapted from Fig. 1 Roberts et al. Stem Cell Reports, 2019.

Resources:

Roberts et al. *Fluorescent gene tagging of transcriptionally silent genes in hiPSCs*. Stem Cell Reports, 2019 (in press); also on BioRxiv: <https://doi.org/10.1101/342881>

Notes:

Section 2. Ribonucleoprotein (RNP) transfection of hiPSC using electroporation

Key points:

- RNP method uses short-lived reagents that may play a role in reducing off-target effects
- Start with healthy cells- reference Section 8
- Pre-treat hiPSC with ROCK inhibitor 1-3 hours before transfection to promote survival after transfection
- When using the Neon Electroporation device be sure to prepare an excess of reagents to ensure no bubbles occur in the electroporation tip
- Add donor template at the very last opportunity, right before transfection- this may prevent potential unwanted cutting of the donor template by the RNP complex

Resources:

Haupt et al. *Endogenous Protein Tagging in Human Induced Pluripotent Stem Cells Using CRISPR/Cas9*. Journal of Visualized Experiments (JoVE). <https://www.jove.com/video/58130/endogenous-protein-tagging-human-induced-pluripotent-stem-cells-using> © 2018 JoVE

Roberts and Haupt, et al. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Molecular Biology of the Cell*, 2017.
<https://www.molbiolcell.org/doi/abs/10.1091/mbc.e17-03-0209>

DeWitt, M. A., Corn, J. E. & Carroll, D. Genome editing via delivery of Cas9 ribonucleoprotein. *Methods*. 121-122 9-15, (2017).

Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research*. 24 (6), 1012-1019, (2014).

Allen Cell Methods: Gene editing: RNP transfection for gene-editing hiPSCs (video)
<https://www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.html>
or https://www.youtube.com/watch?time_continue=6&v=jGwXMWNLB9g

Standard operating procedure downloads:

SOP: RNP transfection

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

Notes:

Section 3. Enrichment of edited cells via FACS

Key points:

- Talk to your flow core early in your experimental planning
- Pre-treat hiPSCs with ROCK inhibitor to promote survival
- Endogenously tagged genes can give a very dim signal. Use an untagged control to establish the FP+ gate.
- Sort cells in bulk into a well(s) of a 96-well plate (<2500 cells/well), or a well(s) of a 24-well plate (10,000-40,000 cells/well)

Before you transfect, talk to your flow core:

- Tell them you're sorting iPSCs. If they have stem cell expertise, follow their recommendations. If not, request the following (applicable to an Aria):
 - A 130 μm nozzle (100 μm is also acceptable). As a rule of thumb, the nozzle size should be at least 4x the diameter of the cell. We use a 130 μm nozzle to be extra gentle on the cells.
 - If the core's sorter is usually run with a smaller nozzle, they will need extra time to setup the sorter with a large nozzle and different instrument configuration.
 - Stream stability will be easily affected by air leaks, microbubbles, and debris.
 - Low sample pressure (typically 10 psi for a 130 μm nozzle)
 - Preservative-free sheath fluid. If your core does offer this, they will need extra time to switch the sheath fluid and flush out the old sheath.
 - Sorting into tissue culture plates to minimize cell loss (preferable to sorting into tubes).
 - If sorting into tubes, use polypropylene and coat the walls with media.
 - We recommend Eppendorf tubes for better pelleting, rather than round-bottom FACS tubes.
- Tell them what fluorophores you're using:
 - These include fluorescent proteins, conjugated cell surface antibodies, nucleic acid stains, and viability stains.
 - If there are spectral compensation issues, your core will tell you what single-color controls to provide. **You may need to do additional transfections to make these single-color controls.**
- Reserve your sorting day/time.
 - In our lab, **sorting iPSCs typically begins at 12pm or later.** This allows 1 hour for instrument startup and QC, 2 hours for the stream to "settle down", and a ½ hour for setting up the drop delay, deflection plates, and sorting template.
 - We budget 15 min to look at controls and set gates, 30-45 minutes of sorting per experimental sample, and 15 min for troubleshooting and **recalculating drop delay if needed (we recommend this after 10 pixels of drift).**

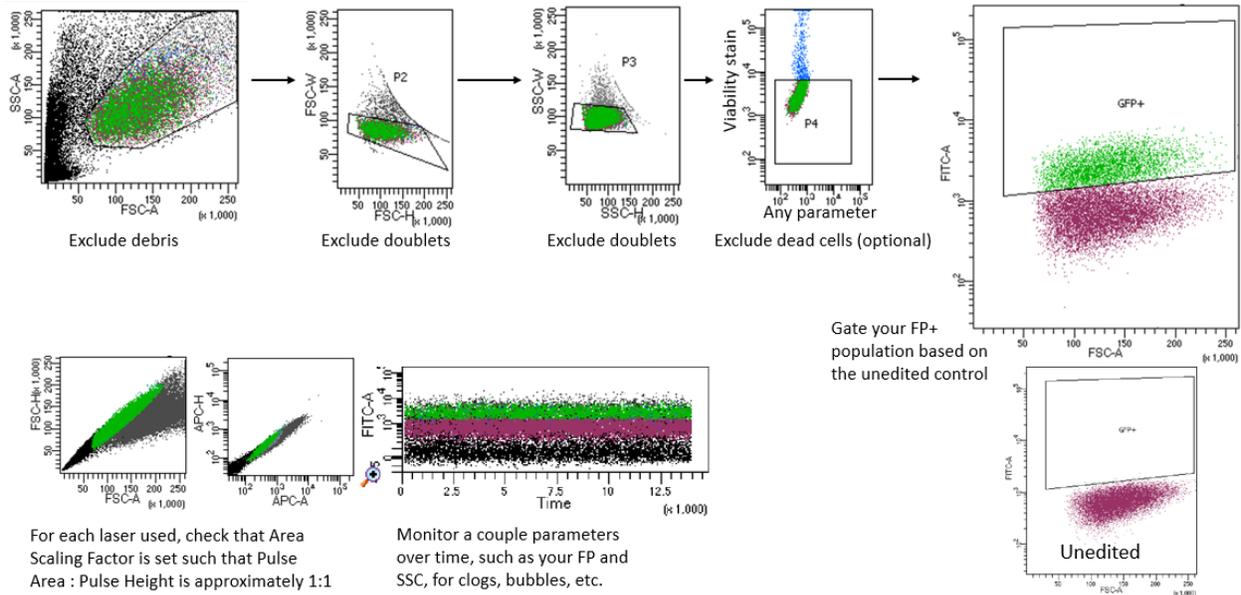
The day of sort:

- Pretreat the cells with ROCK inhibitor for 2-24 hours (if pre-treating overnight, use a 0.5X dose of ROCK inhibitor).
- If possible, dissociate and sort samples individually, rather than dissociating all samples at the same time.
- Filter cells with a 35 μm mesh-capped FACS tube to remove aggregates.
- Storage temperature (room temperature or ice) is cell line-dependent. We keep WTC-11 at room temperature in mTeSR supplemented with ROCK inhibitor.
- Bring cells, viability stain (if using), collection plate (Matrigel-coated tissue culture wells filled half full with mTeSR+ROCK inhibitor), and extra mTeSR+ROCK inhibitor to the sorter.
- Confirm that the calculated drop delay matches the current break off point.
- Gate out doublets.
- Set a low flow rate (We aim for <400 events/sec on flow rate 1).

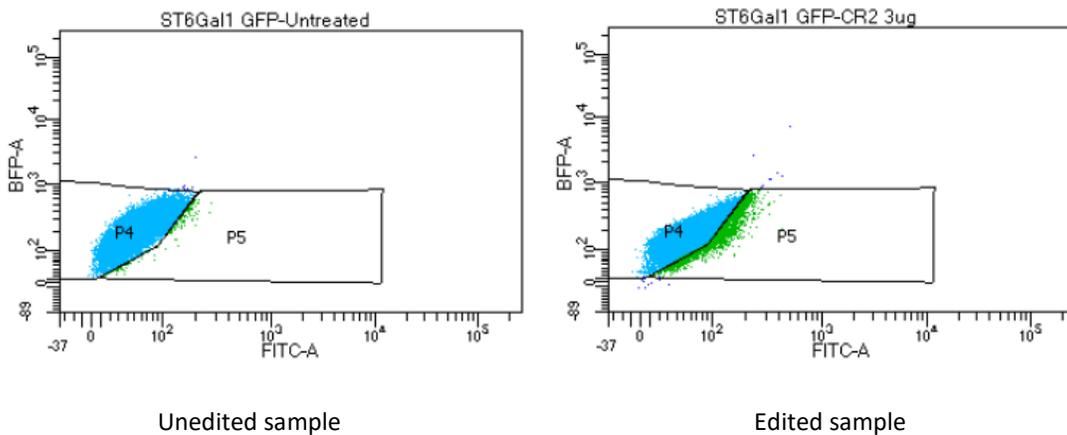
Care of cells after sort:

- Top up the collection well(s) with mTeSR+ROCK inhibitor and place in incubator.
- At ~24 hours, feed cells mTeSR with a 0.5X dose of ROCK inhibitor.
- At ~48 hours, feed cells plain mTeSR.
- If very few cells are collected (i.e. <500 cells/well in a 96 well plate):
 - Top up the collection well(s) with mTeSR+ROCK inhibitor and place in incubator.
 - Skip a media change.
 - At ~48 hours, feed cells mTeSR with a 0.5X dose of ROCK inhibitor.
 - At ~72 hours, feed cells plain mTeSR.
 - If very few colonies form, split the colonies 1:1 to promote expansion.

Example sort plots:



There may be cases where the edited population does not have a clear separation from the parent population. This case (shown below) demonstrates the importance of establishing your FP+ gate by analyzing an unedited control sample. In this example, cells in the gate “P5” were sorted, expanded and confirmed to be 50% GFP+ by microscopy.



Resources:

Haupt et al. *Endogenous Protein Tagging in Human Induced Pluripotent Stem Cells Using CRISPR/Cas9*. Journal of Visualized Experiments (JoVE). <https://www.jove.com/video/58130/endogenous-protein-tagging-human-induced-pluripotent-stem-cells-using> © 2018 JoVE

Roberts and Haupt, et al. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Molecular Biology of the Cell*, 2017.
<https://www.molbiolcell.org/doi/abs/10.1091/mbc.e17-03-0209>

Notes:

Inspector - Global Sheet1

General Headers and Footers: Show and Print Page Numbers, Print Headers and Footers

Title: Diva Software Version Number

Left: None Selected, Right: None Selected

Status Parameters

Name	Delay	Area Scaling	Ratio
VIOLET	-31.98	0.77	
BLUE	0.00	0.65	
YEL_GR	-80.27	0.73	
RED	-58.10	0.62	

Window Extension: 4.00, FSC Area Scaling: 0.45

Acquisition Dashboard

Current Activity: CR1, Stopping Gate Events: 0 evt, Elapsed Time: 00:00:00

Threshold Rate: 0 evt/s

Basic Controls:

Acquisition Setup: Stopping Gate: 20000 evt, Stopping Time (s): 0, Storage Gate: 50000 evt, Flow Rate: 1.0

Acquisition Status: Processed Events: 0, Threshold Count: 0

Electronic Abort Rate: 0, Electronic Abort Count: 0

Hardware: Voltage: 17.45, Waste Drawer: 17.45, Drop Delay: 17.45, Auto Delay: 0

Global Worksheet - Global Sheet1

Stream: Sweet Spot

Amplitude: 13.8, Frequency: 12.0, Drop 1: 228 230, Gap: 15 15

SSC-A vs FSC-A

FSC-W vs FSC-H (1)

SSC-W vs SSC-H (2)

DAPI BFP-A vs FSC-A

MEGFP FITC-A vs FSC-A

MEGFP FITC-A vs SSC-A

MEGFP FITC-A vs SSC-A

MEGFP FITC-A vs SSC-A

Global Sheet1: Sort Layout_001

Device: 96 Well - Falcon, Precision: Continuous, Target Events: Continuous, Save Sort Reports: Ask User, Save Conflicts: Index Sorting

Channel	Sort	Target	Index
C	2	3	4
D	5	6	7
	8	8	8

Sort Rate: NA, Conf. Cnt: NA, Conf. Rate: NA, Efficiency: NA

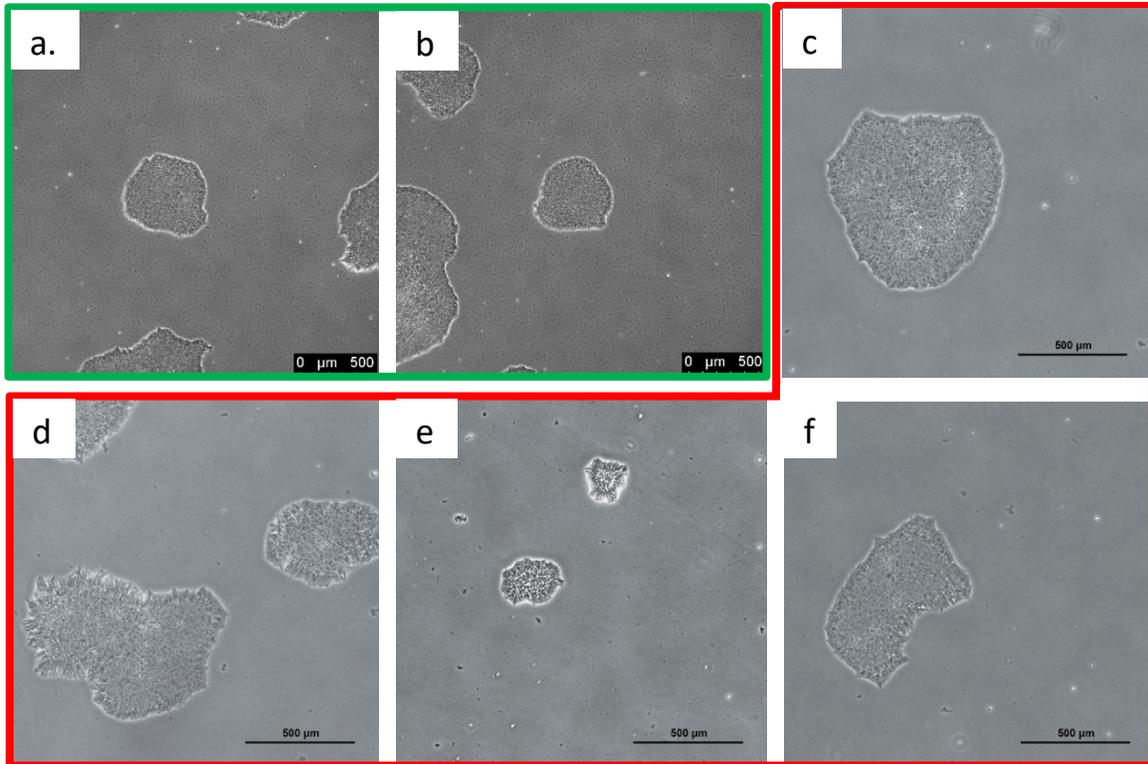
Section 4. Generating clonal hiPS cell lines from an edited population

Key points:

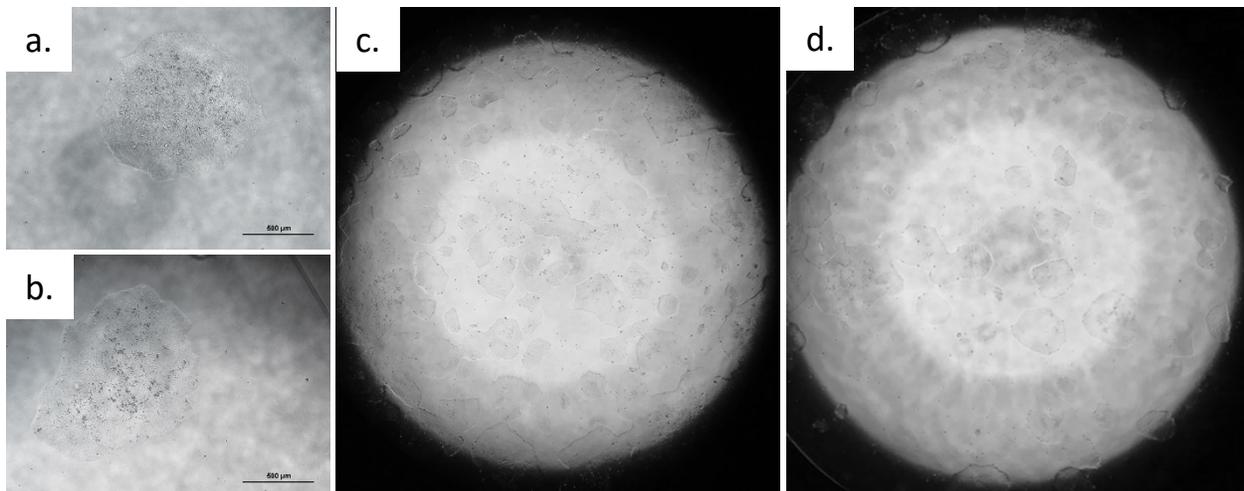
- Seeding density of 10,000 cells per 10 cm plate is recommended for getting well separated colonies - leave cells in ROCK inhibitor for 48 hours after seeding this plate to promote single cell colony growth
- Colonies are large enough to pick when they are visible by eye (~500 μm), usually after 5-7 days
- When aspirating the supernatant from cell pellets in a 96-well V-bottom, manually aspirate using a multi-channel pipette and transfer the aspirated liquid into an extra plate in case too many cells are disrupted and need to be recovered (as opposed to using a suction aspirator)
- Use ratio passaging as an opportunity to collect leftover cells for genomic DNA isolation (see example timeline, below)
- CryoStor CS 10 (Sigma-Aldrich Cat. #C2874-100ML) is recommended freezing reagent, but standard freezing media (10% DMSO, 30% KSR, 60% mTeSR1) has also been shown to work
- If passaging multiple columns at once, limit the amount of columns in Accutase such that no cells are in Accutase for more than 6 minutes

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1		Plate cells at 10,000 cells in a 100 mm plate					
Week 2	Pick clones into a 96-well plate			When clones have about tripled in size, passage at a 1:1 ratio into a fresh 96-well plate			
Week 3	When the majority of clones show mature morphology and are 10-80% confluent, identify individual ratios for splitting each clone to "normalize" cell number in a fresh 96-well plate *			When the majority of clones are 60-80% confluent, freeze in 2 sister 96-well plates at -80°C. Don't forget to take notes on clone morphology!			
Week 4	Harvest gDNA, do genomic analysis on clones to identify those that are precisely edited						
Week 5	Thaw clones that are precisely edited into a 24-well plate			When clones show mature morphology and/or are 60-80% confluent, passage into a 6-well plate			
Week 6	When clones show mature morphology and/or are 60-80% confluent, passage into a 100 mm plate			Cryopreserve clones in liquid nitrogen, continue passaging for downstream assays or use, etc.			

Example timeline for passaging clones in 96-well format. * Indicates the passage that leftover cell suspension is pelleted for gDNA preparation. Alternatively, a sister plate could be plated for the purpose of harvesting for gDNA preparation. This workflow allows for a temporal break in cell culture while the cells are in cryostorage to perform genomic analysis. Note: hiPSCs should always be fed fresh mTeSR daily.



Example images of colonies 5-7 days post-plating at 1×10^5 cells/10cm dish. Colonies should display good morphology and be isolated enough to prevent clone mixing when picking unique colonies. **a and b, green box)** Good examples of colonies that are ready to pick and have good morphology and appropriate colony size. **c-f, red box)** Bad examples of colonies; the colony shown in **c)** has good morphology, but its size indicates it may have originated from more than one cell; **d)** shows poor morphology as well as merging of two colonies into one; **e)** shows a colony that is too small and immature for colony picking; **f)** shows a colony that has an odd shape and size, suggesting that it came from more than one cell originally (non-clonal). Scale bars are as shown.



Example clone cultures that are ready to passage. **a,b)** Clones are ready for 1:1 passaging in 96 well plate. These clones display good morphology and have approximately tripled in size from the time they were picked. Scale bars are 500 µm. **c)** Shown here is a well of a 96-well plate ready to be passaged at a ratio of approximately 1:10 for 3-day culture, or 1:20 for 4-day culture. Shown in **d)** is a well of a 96-well plate where the clone could be passaged at approximately 1:5 for a 3-day culture, or 1:8 for a 4-day culture. All examples display suitable morphology.

Resources:

Haupt et al. *Endogenous Protein Tagging in Human Induced Pluripotent Stem Cells Using CRISPR/Cas9*. Journal of Visualized Experiments (JoVE). <https://www.jove.com/video/58130/endogenous-protein-tagging-human-induced-pluripotent-stem-cells-using> © 2018 JoVE

Notes:

Section 5. Quality Control: Identifying precisely edited clones

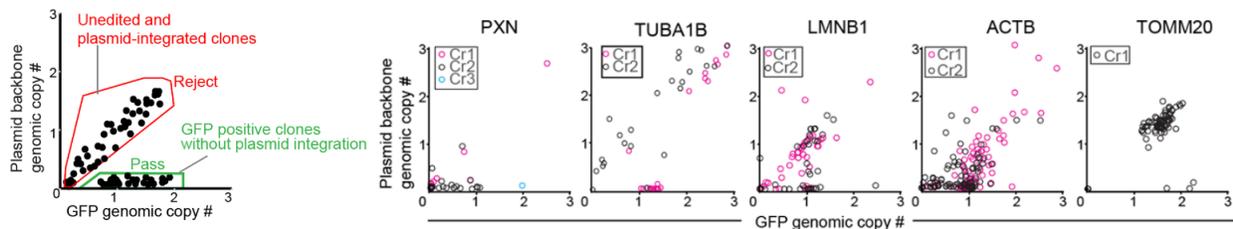
The following section covers methods for identifying precisely edited clones. As a general reference for all topics in this section, we refer you to **Roberts and Haupt, et al. *Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization*. Molecular Biology of the Cell, 2017. <https://www.molbiolcell.org/doi/abs/10.1091/mbc.e17-03-0209>**

In general, the following PCR-based assays require genomic DNA (gDNA) from the clones of interest. One way to obtain the gDNA is to take a split (or cells leftover from passaging) during clone passaging in 96-well plates (see Section 4). A 96-well gDNA isolation kit is recommended, and many of these kits can be automated for high throughput. Make sure that whichever kit you use has chemistry compatible with downstream applications, particularly sensitive ddPCR assays, if using. We recommend Macherey-Nagel NucleoSpin 96 Tissue kits, prepped with a vacuum manifold. For PCR assays, we have had success with Takara PrimeSTAR® HS DNA Polymerase with GC Buffer (Cat# R044B). We also typically optimize our PCR primers using a touchdown cycling method. See MBoC reference for more details.

Section 5a. Droplet digital PCR (ddPCR) for identifying precisely edited clones without on- or off-target plasmid backbone integration

Key points:

- Amplicon Design
 - Amplicon should be 80-150 bp. Longer amplicons can lead to less efficient amplification and can decrease separation of positive and negative drops, making gating difficult.
 - Probe should have a melting temperature 1-2°C higher than primer pair
 - Be sure to check amplicon specificity with PrimerBlast or equivalent
 - If multiplexing two amplicons ensure that different color probes are used for each amplicon and that PCR cycle conditions are optimized for both reactions.
- Ensure that PCR reaction is not overloaded with template, we load between 10-80 ng of gDNA
- Ensure droplet counts are above 12000/well
- Identify clones with on- or off-target plasmid integration using a primer/probe set designed to amplify the plasmid backbone
- Assay can easily be done in high throughput for screening clones



Using ddPCR to identify precisely edited clones. Right cartoon illustrates hypothetical clones that would pass or fail based on GFP copy number (want 1 or 2 copies/genome) and plasmid backbone genomic copy number (want <0.2 copies/genome). Left are examples of ddPCR screening data from experiments representative of the range of outcomes observed. Each data point represents 1 clone. Clones with GFP genomic copy number of ~1 to ~2 and plasmid backbone genomic copy number <0.2 were considered for further analysis. Figure adapted from Roberts et al. *Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization*. MBoC. 2017.

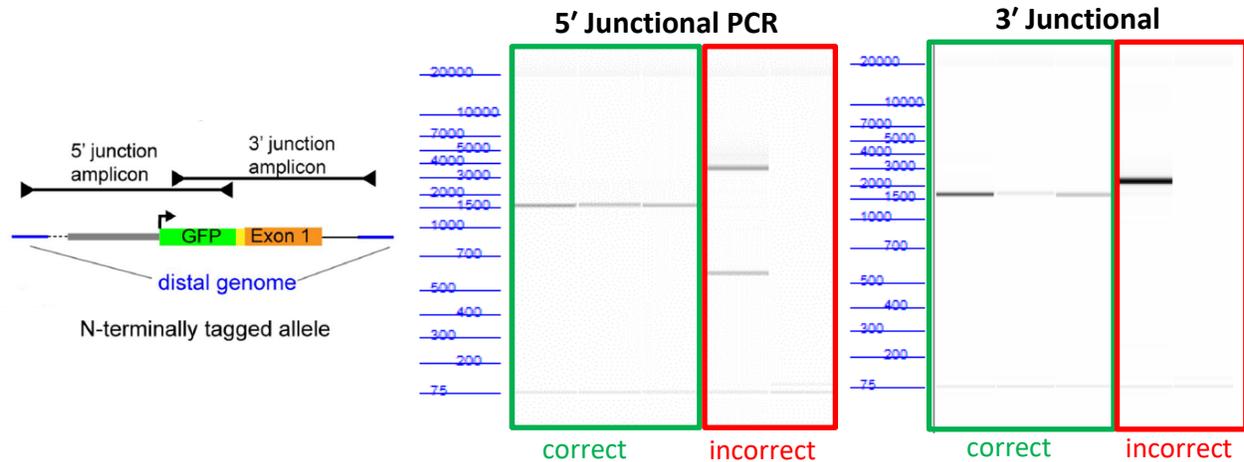
Additional Resources:

SOP: ddPCR CNV assay for screening clones
<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

Section 5b. Junctional PCR identifies precise on-target editing

Key points:

- At least one primer for each assay should bind the genomic DNA outside of the region used in the donor homology arm
- Sanger sequence the amplicons to look for small changes not detected by size analysis on a gel or Fragment Analyzer
- Untreated gDNA, plasmid spike-in, and no template control reactions should not show any amplification products when junctional PCR primers are used



Example data for a 5' and 3' junctional amplicons. Cartoon on left illustrates the location of junctional amplicon primers (one inside the introduced tag, and one outside of the genomic region used for the homology arm for each side). Example data middle and right: amplicons are expected to be ~1500 bp. Incorrect junctional PCR products either vary from the expected size or are not amplified at all. Junctional PCR products should be Sanger sequenced to verify they are free from small indels or mutations not detected by the resolution of gel electrophoresis.

Notes:

Section 5c. PCR and Sanger sequencing identify heterozygous clones with an un-edited wild-type allele

Key points:

- Because it can be costly, it is recommended to perform Sanger sequencing on only <48 clones already identified to pass the ddPCR and Junctional PCR assays outlined in the sections above
- It's okay if these primers bind to the genomic DNA in a region used for the donor homology arm(s), just keep this in mind during analysis
- Untreated control will show amplification of wildtype product; plasmid spike-in will show amplification of insert product (if primers bind within both homology arm regions), no template control should not amplify
- Gel purification is not necessary; mutations and indels should be detectable in the mixed Sanger read using “manual” interpretation or an automated TIDE or ICE tool like ice.synthego.com



Example data to identify clones with undamaged wild type allele. In this example, crRNA1 was used to introduce an mEGFP tag to the N-terminus of the gene. A dark blue bar annotates the expected mEGFP insertion site, between the start codon (M) and first codon (S). For both examples, a PCR amplification product containing an amplicon the expected size of the wildtype sequence, and an amplicon the expected size of the insertion sequence was sent for Sanger sequencing without gel purification. In the example clone on the top, both Sanger sequencing from the 5' side (top trace), and Sanger sequencing from the 3' side (bottom trace) show the expected wildtype sequence up to the point of mEGFP insertion. Deconvolution of the mixed regions of the traces would reveal both the mEGFP sequence and the wildtype sequence, as expected for a clone with no NHEJ damage to the wildtype allele, because both amplicons are present in the Sanger sequencing reaction. In the example clone on the bottom, Sanger sequencing from the 5' side (top trace) is correct up to the point of insertion, but the Sanger sequencing from the 3' read (bottom trace) shows a deletion before the expected insertion site. The deletion detected in the example clone on the right corresponds to the region of dsDNA break induced by Cas9 paired with crRNA1.

Notes:

Section 5d. Identifying Cas9 induced off-targets

Key points:

- Because this assay is a bit more time consuming and less amenable to high-throughput screening, this assay is recommended for a subset of clones (10-20) that have already been shown to be genetically correct.
- Use guide design and off-target search tools such as CRISPOR to identify potential off-target sites for the crRNA used and use PCR and Sanger sequencing to specifically amplify and genotype these regions
- *Careful up-front crRNA design and use of RNP can reduce the risk of off-target mutations*

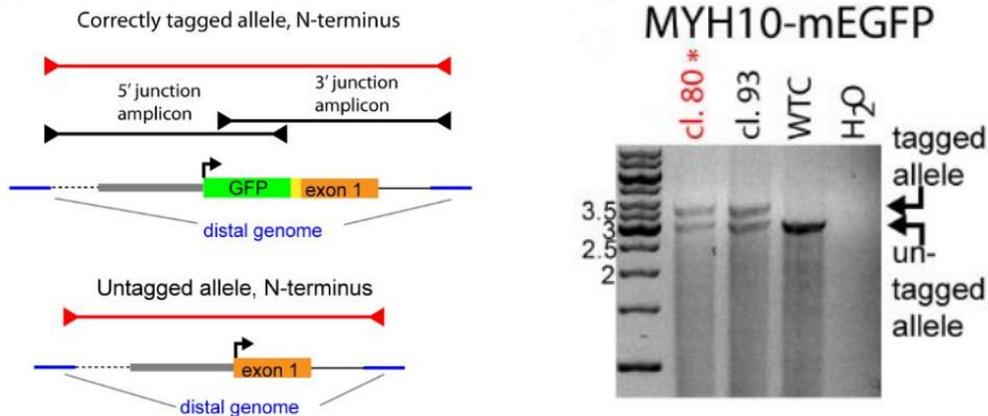
If you introduce your tag with guides that have predicted off-target sites with perfectly matching seed regions and less than 3 mismatches in the non-seed, consider Sanger sequencing these sites in edited clones.

1. Design primers to amplify a region at least ~150 bp around each predicted cut site.
2. Amplify and Sanger sequence this region in your edited clones and in an unedited control sample so that you can exclude any cell line specific variants.
3. Non-homologous end joining is the most likely repair outcome after Cas9 double stranded breaks, so look for indels near the predicted cut site.

Section 5e. Full allele amplification and sequence verification

Key points:

- Because this assay is a bit more time consuming and less amenable to high-throughput screening, this assay is recommended for a subset of clones (5-8) that have already been shown to be genetically correct.
- Primers should be outside of the donor template homology arm.
- The PCR amplification for heterozygous clones should result in 2 amplicons, one corresponding to the untagged allele, and the other corresponding to the tagged allele.
- Gel-purification and Sanger sequencing of each individual band is recommended



Amplification of complete junctional (non-tiled) PCR products to demonstrate presence of the allele anticipated from tiled junctional PCR product data. Left cartoon: Junctional PCR primers complementary to sequences flanking the homology arms in the distal genome (also used in tiled junctional PCR assays, shown in black), were used together to co-amplify tagged and untagged alleles (red). N-terminal tag shown as an example. Right gel image: example data. Molecular weight markers are as indicated (kb). Two final clones (indicated by “cl. #”) are represented for each experiment. Asterisk indicates the final clone chosen for distribution and imaging. Figure adapted from Roberts et al. MBoC 2017.

Notes:

Section 6. Identifying clones with proper fusion protein localization

Clones that have been identified to be genetically correct are highly likely to express the expected fusion protein, but protein localization must be verified in the cell. The following sub-sections outline the recommended assays for identifying clones that are expressing a fusion protein with proper localization and protein size. Whereas most assays outlined in Section 5 can be performed at relatively high throughput (96-384 clones), the assays in this section require more resources and are recommended to be performed on 3-5 clones proven to have precise editing. Additional protein-specific assays may be needed, depending on the protein of interest, and are not discussed here.

Section 6a. Imaging confirms expected localization in live cells

Key points:

- Cells should be plated on glass bottom multi-well plates with German high performance #1.5 cover glass, see SOP below
- Seeding density may need to be optimized for each cell line, but generally 2500 cells per well of a 96-well plate grown for 4 days results in colonies of appropriate size and maturity for imaging
- Because endogenously tagged proteins can be dim, we recommend reducing background fluorescence by using phenol-red free Matrigel and phenol-red free mTeSR1. See protocol below for catalog numbers.

Resources:

Numerous examples of live cell imaging can be found on allencell.org, including live cell imaging examples of each of the released Allen Cell Collection cell lines in the Cell Line Catalog <https://www.allencell.org/cell-catalog.html>

SOP: Cell plating for imaging
<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

Notes:

Resources:

Roberts and Haupt, et al. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Molecular Biology of the Cell*, 2017.

<https://www.molbiolcell.org/doi/abs/10.1091/mbc.e17-03-0209>

<http://www.bdbiosciences.com/us/applications/research/intracellular-flow/intracellular-buffers-and-ancillary-reagents/permwash-buffer/p/554723>

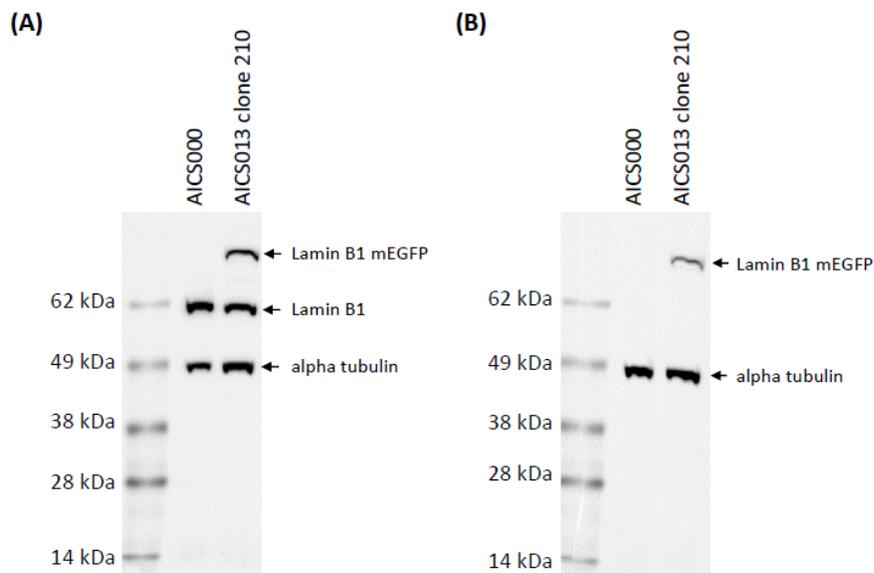
<https://www.thermofisher.com/order/catalog/product/S36936>

Notes:

Section 6c. Western Blot (WB) confirms expected size and relative abundance of tagged and untagged protein

Key points:

- When possible, we choose antibodies that recognizes the untagged terminus of the protein to avoid potential interference of the FP tag with antigen recognition and binding of the antibody
- We optimize our Western blots by running a preliminary experiment using a range of protein amounts. We calculate the relative total levels of proteins for all protein amounts and select a protein amount which is within the linear range of the signal intensity for our actual experiment
- We have a loading control (e.g. beta-actin or alpha-tubulin) and ensure that similar amounts of protein are loaded for all samples so we can quantify relative protein levels
- We use both a protein-specific and a FP-specific antibody. This allows us to confirm that the higher molecular weight, protein-specific band is the FP-tagged protein. In addition, we can determine whether unbound FP as well as untagged and/or tagged degradation products are present in the edited cells.



Whole cells lysate was extracted from AICS000 and AICS013 cells using urea sample buffer. 10 μ g whole lysate was separated on 4-12% Bolt Bis-Tris Plus gels and transferred onto 0.45 μ m nitrocellulose membranes. Untagged and GFP-tagged Lamin B1 was detected using (A) rabbit polyclonal anti-Lamin B1 antibody (abcam #ab16048; 1:2,000 in PBS-T with 5% milk, overnight at 4°C) and (B) mouse monoclonal anti-GFP antibody (Sigma-Aldrich #11814460001; 1:250 in PBS-T with 5% milk, overnight at 4°C). Alpha tubulin was used as loading control (mouse monoclonal anti-alpha tubulin; ThermoFisher #62204; 1:10,000 in PBS-T with 5% milk in PBS-T, overnight at 4°C). A goat polyclonal anti-mouse Alexa 647-conjugated secondary antibody and a goat polyclonal anti-rabbit Alexa 647-conjugated antibody were used (1:10,000 in PBS-T with 5% milk, 2 hrs at room temperature). Blots were imaged at different exposure times using the ChemiDoc MP system (BioRad). Appropriate exposure times were used for semi-quantification of protein levels. Multiple clones of AICS013 were analyzed on the same blot and the blot was cropped to only show the final clone of AICS013.

	AICS000	AICS013 clone 210
relative levels of total Lamin B1	1.00	0.80
relative levels of mEGFP-tagged Lamin B1 in AICS013 clone 210	N/A	0.33

Resources:

Roberts and Haupt, et al. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Molecular Biology of the Cell*, 2017.
<https://www.molbiolcell.org/doi/abs/10.1091/mbc.e17-03-0209>

Additional example Western Blots available on Allencell.org

Notes:

Section 7. Identifying clones that have good stem cell morphology and have maintained desired stem cell qualities

Having a precisely edited hiPSC clone is only useful if the clone has good stem cell morphology and maintains stem cell characteristics such as pluripotency and an ability to differentiate. The following subsections outline the recommended assays for identifying clones that are most likely to be a renewable resource and lead to successful differentiation experiments. Whereas most assays outlined in Section 5 can be performed at relatively high throughput (96-384 clones), the assays in this section require more resources, and are recommended to be performed on 3-5 clones proven to have precise editing.

Section 7a. Stem cells should be monitored for a stable karyotype

Key points:

- Stem cells have a propensity to acquire trisomy of chromosomes 12 and 17, as well as partial duplications of chromosome 1
- G-banding karyotypes should be able to identify most common chromosomal rearrangements
- qPCR-based options with sensitivities similar to G-banding exist in the marketplace, and can be adapted for ddPCR

References:

Baker et al. "Detecting Genetic Mosaicism in Cultures of Human Pluripotent Stem Cells". Stem cell Reports, 2016. DOI 10.1016/j.stemcr.2016.10.003

Codner et al. "Aneuploidy screening of embryonic stem cell clones by metaphase karyotyping and droplet digital polymerase chain reaction". BMC Cell Biology, 2016. DOI 10.1186/s12860-016-0108-6

Notes:

Section 7b: Stem cells should maintain expression of key stem cell pluripotency markers

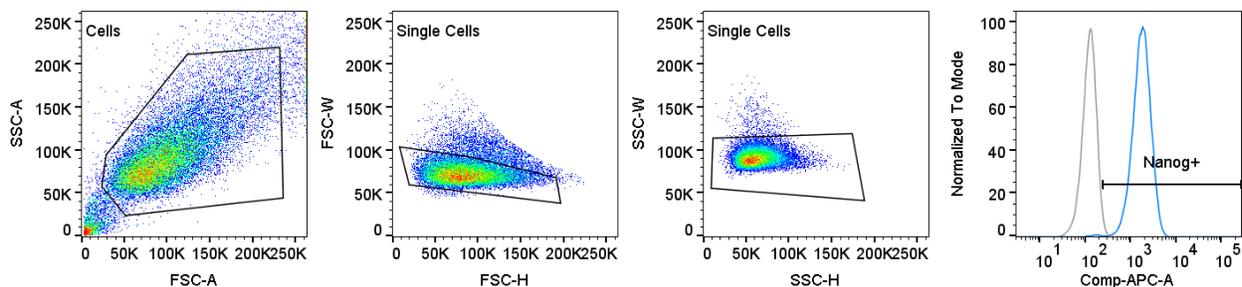
Key points:

- Stem cells should meet the following criteria for stem cell marker expression:
 - SSEA1: <15% positive
 - OCT3/4, Nanog, Sox2, TRA160, SSEA4: >85% positive
- Flow cytometric quantification of pluripotency markers *cannot* confirm the ability of iPSCs to differentiate into the three germ layers.

Laser	BP	LP	Intracellular Panel	Surface Panel
405	525/50	505LP	Oct3/4 BV510 (antibody switch in progress)	TRA160 BV510 (antibody switch in progress)
	450/50		Sox2 V450	SSEA1 BV421
488	610/20	595LP		
	540/30	525LP		
	530/30	505LP	GFP	
561	670/30	635LP		
	610/20	595LP	mCherry	
	586/15		RFP, mTagRFP	
637	780/60	750LP		
	730/45	695LP		
	670/30		Nanog AF647	SSEA4 AF647

Example flow cytometry panel (*improvements in progress*): These are the panels we currently use on fixed cells for analysis on our FACSAriaIII SORP. The antibodies are from BD Biosciences. *We are currently in the process of improving our flow panels. We are moving our surface staining panel to live cells to decrease technical variation. We are changing the BV510 antibodies to different colors due to compensation with GFP.*

We fix our cells with 1X BD Cytofix, wash with PBS, then wash with FACS buffer. For surface markers, we incubate cells in antibody cocktail for 30 minutes, then wash with FACS buffer. For intracellular staining, we permeabilize cells with 0.1% TritonX-100 in FACS buffer for 10 minutes, dilute antibodies in 1X BD Perm/Wash, add the antibody cocktail to permeabilized cells, incubate for 30 minutes, wash with BD Perm/Wash, then wash with FACS buffer.



Example gating scheme using an FMO control to gate Nanog+ cells

Notes:

Section 7c. Stem cells should maintain the ability to differentiate into other cell types from all 3 lineages (endoderm, ectoderm, and mesoderm). Applying a directed differentiation protocol can confirm commitment potential (e.g. cardiomyocytes)

As part of our gene editing QC workflow, edited lines undergo a directed cardiomyocyte differentiation. If your own research necessitates differentiation into a particular cell type, you may consider testing differentiation potential into that cell type using established protocols. If differentiated cell types will not be specifically evaluated in your research, we still recommend performing a trilineage potential assay to confirm stem cell pluripotency after gene editing.

Key points for cardiomyocyte differentiation:

- Cell density and growth rate can significantly impact the success of a differentiation experiment.
- Undifferentiated stem cells must maintain ideal health and morphology prior to beginning differentiation experiments.
- Each cell line may require optimization of the directed cardiomyocyte differentiation protocol, including seeding density and/or confluency at the initiation of differentiation, as well as small molecule concentration(s).

Resources:

SOP: Cardiomyocyte differentiation methods

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

Allen Cell Methods: Setting up the WTC-11 parental line and our gene-edited cells for cardiac differentiation

<https://www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.html>

or <https://www.youtube.com/watch?v=cCVUrKGRINs>

Allen Cell Methods: Single cell passaging human iPS cells

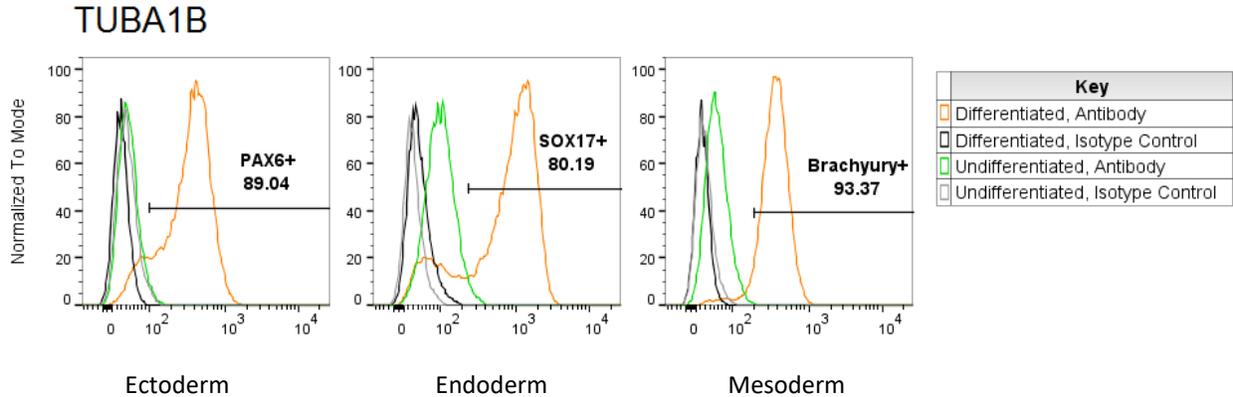
<https://www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.html>

or <https://www.youtube.com/watch?v=wao2UcMFPMc>

Notes:

Key points for trilineage potential assays:

- There are commercially available kits for directing stem cells into the three different lineages; we have used STEMCELL™ Technologies Trilineage Differentiation Kit (Cat# 05230); this kit enables an abbreviated directed differentiation using three lineage-specific medias (Ectoderm, Mesoderm, and Endoderm) where cells can be analyzed in 5-7 days post differentiation.
- Cells can be analyzed for early markers of the three germ layers by various methods. We have done this with antibody staining and analysis by flow cytometry, and by using ddPCR to detect increased expression of the same early differentiation markers.



Sample Trilineage data by flow cytometry analysis: We use goat anti-Brachyury APC and goat anti-Sox17 APC from R&D and mouse anti-Pax6 AF647 from BD Biosciences. Mesoderm and Endoderm samples are permeabilized with BD Perm/Wash buffer, while Ectoderm samples are permeabilized with 0.1% TritonX-100 in FACS buffer.

Resources:

Roberts and Haupt, et al. Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Molecular Biology of the Cell*, 2017.
<https://www.molbiolcell.org/doi/abs/10.1091/mbc.e17-03-0209>

Notes:

Section 7d. Gene edited stem cells should grow at similar rates to unedited controls

Key points:

- Compare the growth of edited clones to that of unedited cells that are approximately the same passage number
- Choose a method that is both repeatable and can be appropriately high throughput for your needs- we use CellTiter-Glo.
- Additional considerations for CellTiter-Glo:
 - Measure the output at approximately the same time of day at the timepoints 0 hr, 48 hr, 72 hr, and 96 hr
 - Cell density should be optimized such that 4 days of growth results in most cell lines being 60-80% confluent; plate all lines being tested at the same density
 - Consider that differences in measured growth rate may be affected by the number of cells in each well that actually attach to the matrix and grow, in addition to the actual rate of growth.

Resources:

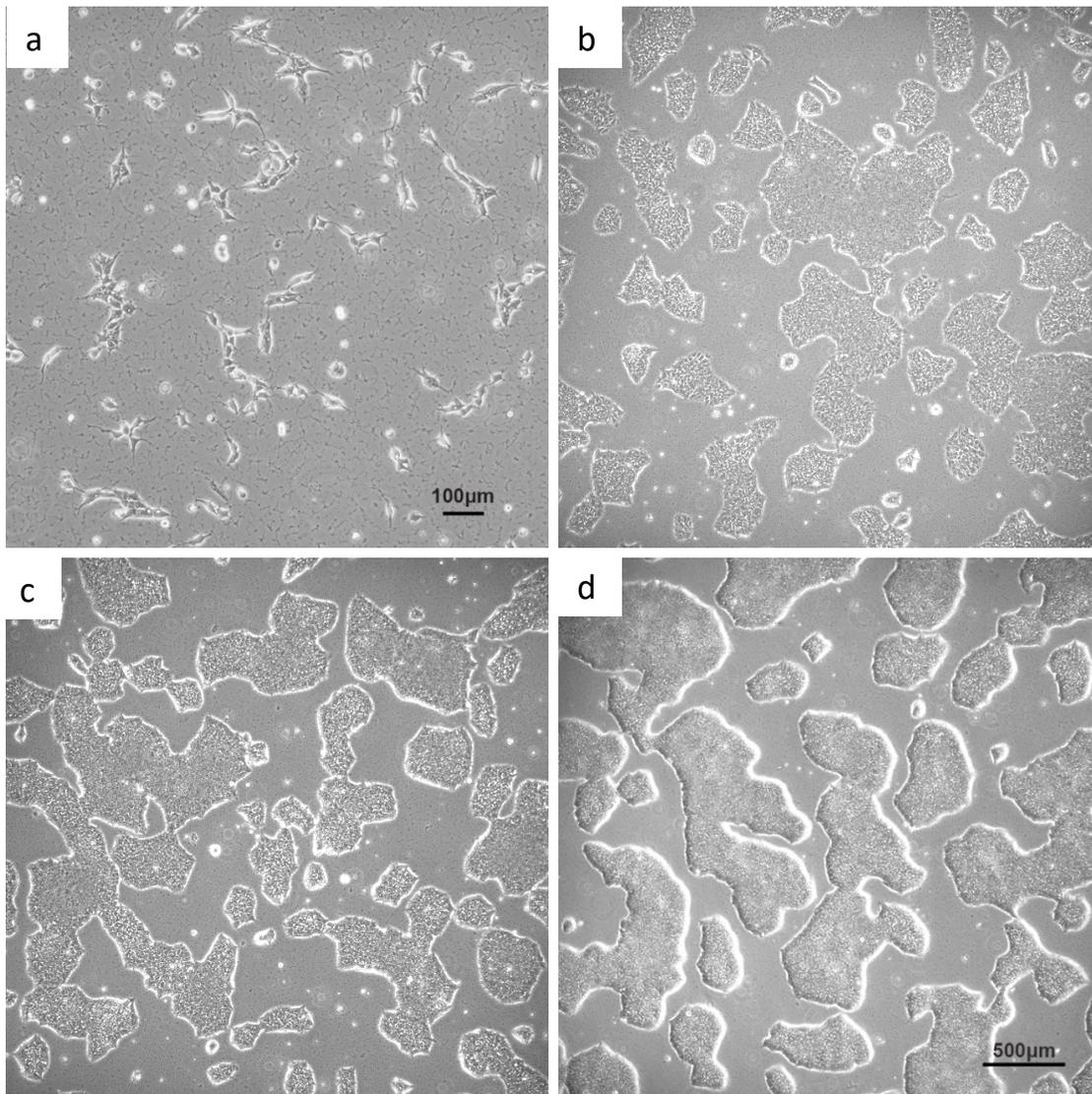
https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-luminescent-cell-viability-assay/?catNum=G7570

Notes:

Section 8. hiPSC Morphology Guide (WTC-11)

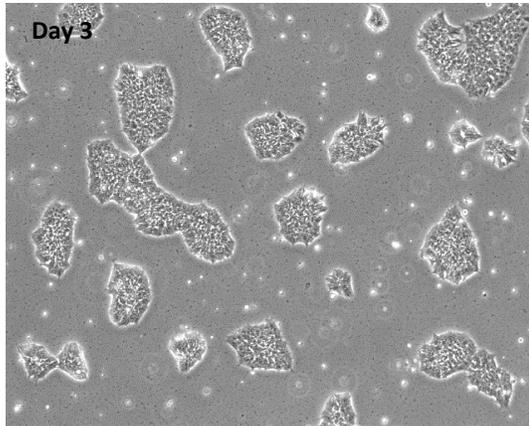
Key points:

- Colonies with a high degree of stemness will show well defined edges, and a uniform/smooth “lake-like” middle; avoid cultures with spontaneous differentiation in > 5% of the plate
- Loss of stemness can be confirmed by testing for low levels of human stem cell markers (Oct3/4, Nanog, SSEA4) by flow or immunostaining
- Seeding density and confluence are critical for maintaining stemness; avoid passaging over-confluent monolayers- aim for 70-85% confluence
- Proper trituration is key for achieving consistency when passaging hiPSCs in single cell suspension by resulting in colonies of uniform size and maturity
- Monitor cultures for hiPSC maturity; aim for a majority (>60%) of mature colonies with well packed, small, smooth cells in the middle at d3-d4 after plating

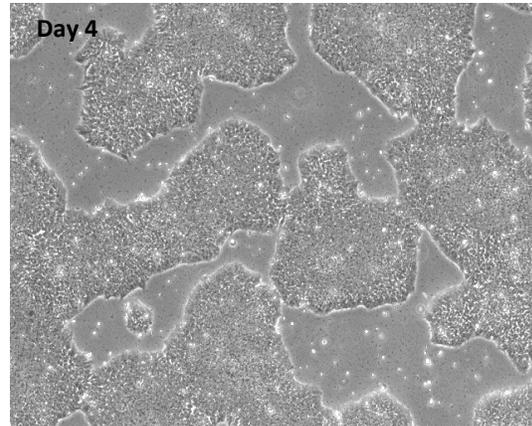


Examples of hiPSCs with good morphology. a. Cells from an ideal single cell suspension 24h after plating; cells are still in ROCK inhibitor media. Even single cell suspension should lead to uniform mature colonies in 3-4 days. 10x magnification; scale bar is 100 μm. **b.** Same cells at 3 days of growth **c.** Healthy stem cell culture with some smaller, immature colonies. **d.** Good, mature morphology and is at an ideal density to be passaged. **b-d.** Scale bar is 500 μm.

A. 500K cells seeded

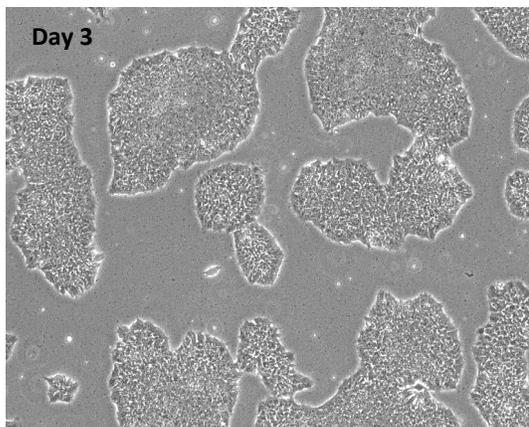


Not ready for passaging - ~50% confluency

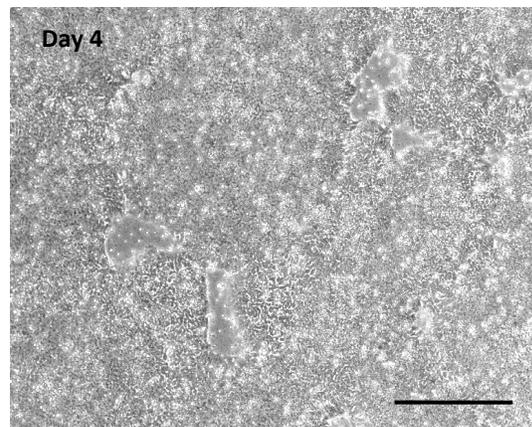


Ready for passaging - ~85% confluency

B. 1 million cells seeded



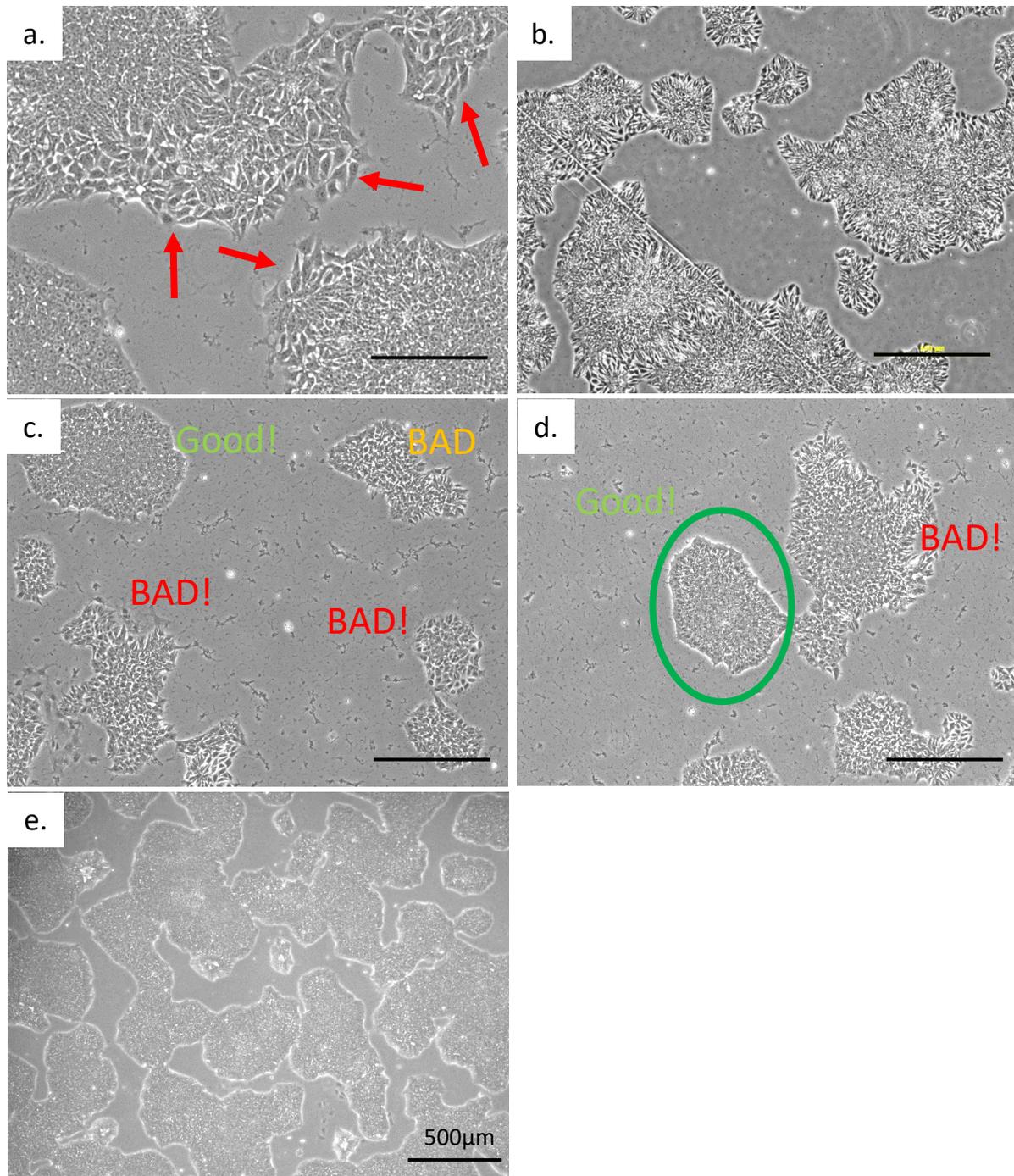
Ready for passaging - ~70% confluency



Over-confluent - Do not use

Documenting confluency based on seeding density. **A.** Cells plated at 500k in 10cm plates are generally ready for passage in 4 days. **B.** Cells plated at 1×10^6 in 10cm plates are ready for passage on day 3.

Note: It is very useful to plate out multiple densities for an unfamiliar cell line. For Allen Institute for Cell Science cell lines, please refer to the seeding density recommendations in the "Certificate of Analysis" provided with each cell line.



Examples of hiPSCs with bad morphology. **a.** Colonies have elongated cells around the edges. **b.** Cells not fed for one day; observe these warning flags/signs of stress: spiky, flat, different morphology on all borders. **c and d.** Mixed cultures: Differentiation shown through presence of flatter, different-looking cells. **e.** Crater-like features among nice, mature stem cell colonies. Scale bars are 500 µm.

Notes:

Section 9. Single-cell passaging of hiPSC (WTC-11)

Key points for single-cell passaging:

- Cells should start with good morphology (see Section 8)
- Ensure all reagents are warmed and prepared in the hood before starting to passage
- To achieve good single cell suspension, monitor the cells under the microscope during triturations
- Apply gentle, even forces with the pipette, and avoid forcefully “squishing” cells between the pipette and the plate when trituring
- When passaging more than one cell line at a time, stagger the timing of the Accutase addition so that no cells are in Accutase for longer than 5 minutes
- To achieve even distribution of cells in plates after seeding, rock plates front to back and side to side several times after placing plates in the incubator (see video link for demonstration)
- While we recommend Accutase be warmed at 37°C, it can also be used at room temperature

Resources:

SOP: WTC-11 Culture

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

Allen Cell Methods: Single cell passaging human iPS cells

<https://www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.html>

or <https://www.youtube.com/watch?v=wao2UcMFPMc>

An excerpt from our protocol (Figures and tables available in the full protocol in the last section of this notebook):

Passaging and Maintaining AICS Cells on Plastic Tissue Culture Treated Vessels

Warm Accutase in a water bath at 37°C or to RT on bench; mTeSR1 must only be warmed to RT on the bench. AICS cells are grown in a standard 5% CO₂ incubator at 37°C and must be fed fresh mTeSR1 once every day.

1. When cells reach 70-85% confluency, passage the cells. See Figure 1.
2. Aspirate and discard old medium.
3. Gently add RT DPBS. Do not dispense DPBS directly onto cells, but rather introduce DPBS at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
4. Aspirate and discard DPBS wash.
5. Add pre-warmed Accutase (see Table 1 for volumes) and incubate in 37°C incubator for 3-5 min. Note: if using 37°C pre-warmed Accutase, expect shorter incubation times (3-4 minutes). RT Accutase incubations can be expected to take longer (4-5 minutes).
6. Check for detachment by gently tilting vessel and/or observing under the microscope. If all cells have not detached in 3-5 minutes, incubate an additional 2 min and check again. Continue to incubate and check as necessary, only until cells are no longer attached to the plate surface, although cells may remain in large clumps.
Note: Avoid incubating cells with Accutase for longer than is necessary to detach from plate surface. Most clonal lines derived from WTC will be fully detached from vessel surface in less than 8 min.
7. Add RT DPBS to dilute out the Accutase in the vessel as follows (see Table 1 for volumes):
 - a. Add DPBS to vessel and very gently triturate cell suspension by aspirating and then gently rinsing the cells across the vessel surface 6-10 times (ex. in a continuous zig zag or windshield wiper motion) to the point where all cells have been released from the vessel surface and the suspension is homogenous - all without creating bubbles. Do this using adequate but not excessive force. Note: We recommend trying this technique with either a 5 or 10mL serological pipette to achieve optimal single cell suspension; results will vary by individual and manufacturer of pipettes used.
 - b. Check that single cell suspension has been achieved in >90% of the suspension using microscope. If single cell suspension is not achieved, triturate 4-5 more times (as described above in step 7a) and check again.
Note: Cells can be passaged in small clumps, but cell counting may not be accurate. Single cell suspension is especially important for certain downstream applications such as transfection, cell sorting by FACS, or plating at low density for clone picking.
8. Transfer cell suspension to 15 mL conical tube.
9. Rinse the vessel one time with DPBS for final wash (see Table 1 for volumes). Add to conical. Alternatively, you may skip the wash step and add DPBS directly to conical if maximal cell recovery is unnecessary for your application.

10. Spin down cells at 1000 rpm (RCF= 211 x g) for 3 min at RT.
11. Carefully aspirate and discard DPBS/Accutase supernatant from cell pellet and re-suspend in desired volume of mTeSR1 + Ri. See Table 1 for recommended re-suspension volumes for most accurate counts based on source vessel size.
12. Cells can be seeded directly into new vessels at a specific cell count (3-4 days to confluency) depending on day needed. See Table 1 for recommended cell plating densities based on vessel size. We recommend counting cells and plating specific numbers for most reproducible culturing conditions.
Note: Cells in suspension settle quickly. After counting, we recommend gently re-suspending cell suspension before seeding by triturating the entire volume three times using a serological pipette. Cells should be mixed approximately every 2-3 min when seeding multiple vessels.
13. Matrigel-coated vessels cannot be re-used, so always seed cells onto fresh Matrigel coated vessels prepared with RT mTeSR1 + Ri.
14. After seeding, slide vessels front-to-back and side-to-side to ensure even cell distribution across vessel surface at least 2-4 times and keep level for even cell attachment.
Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it's important to place plates on a level surface as quickly as possible.
15. Always record the correct date, passage number and identifier on vessel(s).
16. Incubate cells at 37°C and 5% CO₂.
17. Observe 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri).
18. Change media every 24 h with mTeSR1.

Notes:

Section 10. Thawing hiPSC (WTC-11) from liquid nitrogen

Key points for single-cell thawing:

- Cells should start with good morphology (see Section 8)
- Thaw in a 37°C water bath until a small ice pellet is visible; work carefully and quickly at this step
- Once cells are thawed, slowly add media to cells (avoid mixing as this could damage the fragile cells)
- Total time from cells thawed to cells re-suspended should be less than 10 minutes (for this reason, only thaw 1-2 vials at a time)
- Always handle cells gently during freezing and thawing as they are sensitive when exposed to DMSO at room temperature- pipette slowly and use the largest aperture pipette as is reasonable for the activity

Resources:

Allen Cell Methods: Thawing hiPSCs

<https://www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.html>

or <https://www.youtube.com/watch?v=wAHTh9kHKIM>

SOP: WTC-11 Culture

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

An excerpt from our protocol (Figures and tables available in the full protocol in the last section of this notebook):

Thawing AICS Cells

1. Prepare a 15 mL conical tube with 5 mL of RT mTeSR1 + Ri.
2. Carefully remove vials from LN₂ storage, being sure to vent cap to relieve any LN₂ trapped inside, then re-tighten cap.
3. Quickly thaw the frozen vials using 37°C water bath until only a small ice pellet is visible. Limit the amount of time cells are in Freezing Media at RT to 10 min or less.
4. Fully rinse outside of closed vial with 70% EtOH and dry with Kimwipe before transferring vial to sterile hood.
5. Using a 5 or 10 mL serological pipette, slowly add 1 mL of RT mTeSR1 + Ri to the vial with the cells. Do not mix. Using the same pipette, slowly aspirate the now ~ 1.5 mL volume of diluted cells from the vial. Work carefully to remove all the liquid without creating extensive bubbles. Transfer the diluted cells from the vial into the prepared 15 mL conical tube from step 1 for a total of 5.5 mL. Do not mix; avoid disrupting the cells.
6. Spin cells at 1000 rpm (RCF= 211 x g) for 3 min at RT in a swinging bucket centrifuge.
7. Aspirate and discard supernatant, then re-suspend pellet in 3-5 mL RT mTeSR1 + Ri.
8. Seed cells in Matrigel-coated vessels; see chart for plating densities. Generally, a vial of 1 x 10⁶ cells thawed into a 10 cm plate will be ready for passaging 3 days later.
9. After seeding, slide vessel front-to-back and side-to-side to ensure even cell distribution across vessel surface at least 2-4 times and keep level for even cell attachment.
Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it's important to place plates on a level surface as quickly as possible.
10. Incubate cells at 37°C and 5% CO₂.
11. Observe 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri).
12. Change media every 24 h with mTeSR1.

Section 11. Cryopreservation of hiPSC (WTC-11) in vials for long-term storage in liquid nitrogen

Key points for cryopreservation:

- Cells should start with good morphology (see Section 8)
- Maintain ~ 1 x 10⁶ cells per 0.5 mL of freezing media; more cells per volume increases survival
- Cells recover from freezing in cryovials best when in single cell suspension at time of cryopreservation
- Freezing media comprised of 10% DMSO, 30% KSR, 60% mTeSR is recommended when cryopreserving WTC-11 in single cell suspension in cryovials
- Efficient cell handling promotes cell survival
 - Minimize time cells are in freezing media at RT to 10 minutes or less
 - Strive for even distribution between multiple vials
 - If freezing large batches, make smaller aliquots to help minimize time in DMSO

Resources:

SOP: WTC-11 Culture

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

SOP: Cell line scaleup and banking

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

An excerpt from our protocol (Figures and tables available in the full protocol in the last section of this notebook):

AICS Cell Freezing (Cryo-preserving)

1. Prepare fresh Freezing Media. Freezing media should be made 10 min-6 h in advance of use and discarded thereafter.
 - a. mTeSR1 with 30% KSR, and 10% DMSO
e.g. for a 10 mL volume: 6 mL mTeSR1 + 3 mL KSR + 1 mL DMSO
Note: No Ri is used in the Freezing Media.
Per manufacturer's instructions, KSR is stable for up to 4 weeks at 2°C to 8°C, protected from light. Working volumes can be aliquoted and stored at -20°C to -5°C. Thaw aliquots as needed. Avoid additional freeze-thaw cycles.
2. Prepare Mr. Frosty freezing containers by adding room temperature isopropanol per manufacturer's instructions.
3. Label CryoVials as appropriate.
4. When cells are 70-80% confluent, detach and pellet using Accutase per Passaging and Maintenance protocol, and re-suspend final pellet in mTeSR1 + Ri (see Table 1 for recommended re-suspension volumes for most accurate counts based on source vessel size).
5. Count cells.
6. Calculate the mL of cell suspension needed using the following equation:
$$\text{mL cell suspension to spin down} = \frac{\# \text{ vials desired} \times 1 \times 10^6}{\frac{\# \text{ cells}}{\text{mL}}}$$

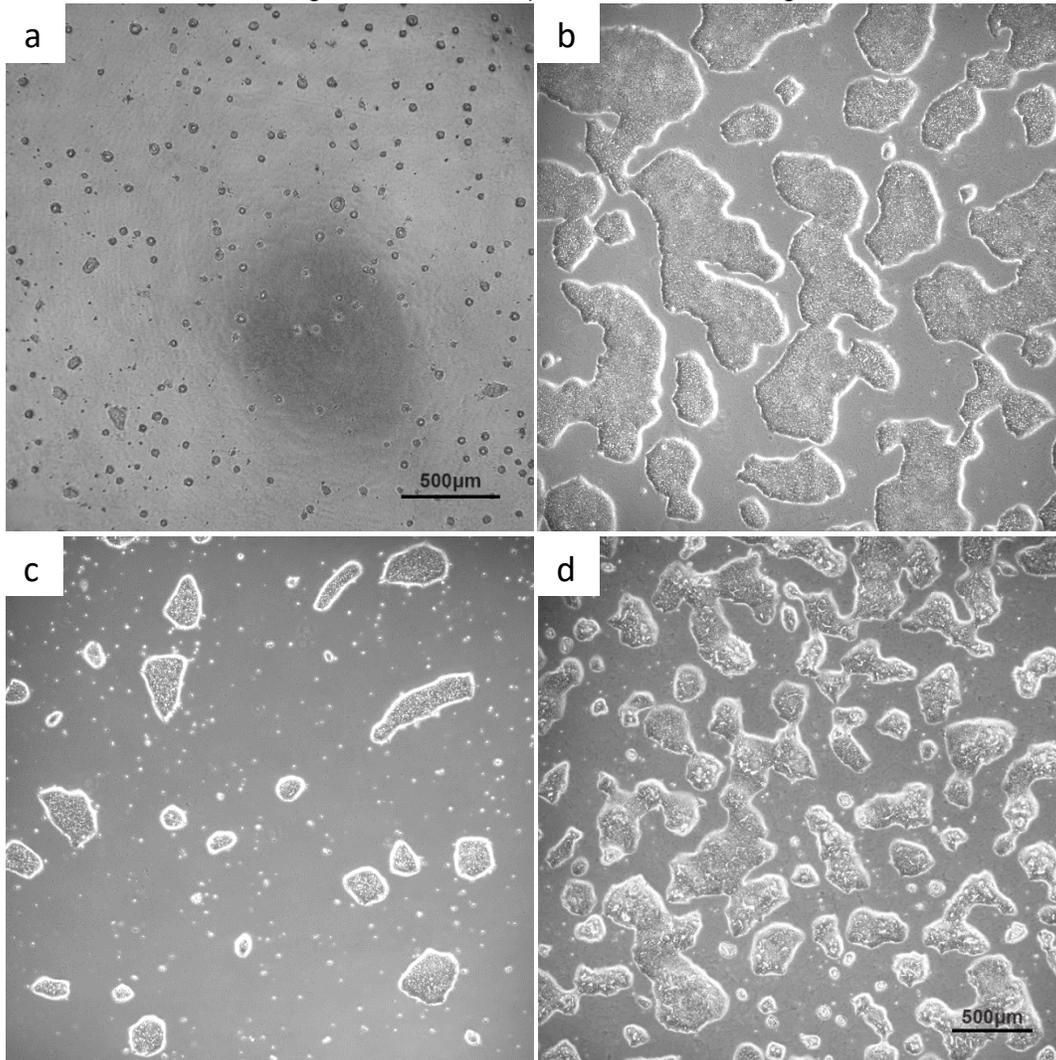
Note: calculate enough cells for at least 1 extra CryoVial for pipetting error.
7. Pellet cells to be frozen in a 15 mL conical by spinning at 1000 rpm (RCF= 211 x g) for 3 min at RT.
8. Re-suspend cells in appropriate volume of fresh freezing media (0.5 mL Freezing Media per vial of 1x10⁶ cells) and aliquot 0.5 mL volumes of cells in freezing media into pre-labeled and prepared CryoVials.
Note: use a 5 mL serological pipette to dispense 0.5 mL aliquots into each CryoVial. Work quickly so cells don't settle toward bottom of pipette.
9. Tightly close vial caps.
10. Quickly transfer CryoVials to room temperature Mr. Frosty containers and place containers in -80°C freezer. Limit the amount of time cells are in Freezing Media at RT to 10 min or less.
11. After 24 h, transfer CryoVials to LN₂ storage. Do not leave cells in Mr. Frosty containers in the -80°C freezer for longer than 96 h.

Notes:

Section 12. Matrigel and Media

Key points for Matrigel:

- Store vials of Matrigel at -80°C long term, or -20°C short term
- Matrigel should be thawed only at 4°C , never thaw Matrigel in a water bath or at RT
- Aliquot Matrigel in 1 mL units into 50 mL conical tubes
 - Keep Matrigel and receiving tubes on ice during aliquotting
 - Use a pre-chilled pipette
- Use phenol red free DMEM/F12 to dilute concentrated Matrigel 1:30
- To get a uniform suspension of diluted Matrigel, mix the full volume 3-5 times
- Lot Testing: We perform lot testing on all Matrigel products used at our institute by passaging the WTC-11 line 5 consecutive times on a test lot, tracking cell growth throughout. At the conclusion of passaging, we analyze the stem cells by flow cytometry for stem cell markers. Alternatively, Stem Cell Qualified Matrigel is available for purchase from Corning.



Matrigel concentration can affect morphology Different Matrigel concentrations will affect plating of the stem cell and colony growth behavior. Images were taken at day of expected harvest. **a.** Nearly all of colonies appear “balled up” or “donut-shaped” = Matrigel likely too concentrated. 5x magnification; scale bar is 500 μm . **b.** Colony growth is as expected = Matrigel is at correct concentration. **c.** Colonies are growing slower than anticipated, and colony edges are “severe” = Matrigel likely too dilute. **d.** Matrigel is likely too concentrated, but not as concentrated as in a; some balled up colonies are present and other colonies appear raised with crater-like areas. c-d: Scale bar 500 μm .

Resources:

SOP: WTC-11 Culture

<https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>

Allen Cell Methods: Techniques while working with Matrigel:

<https://www.allencell.org/videos-and-tutorials.html>

or <https://youtu.be/spAMz5riLRE>

Notes:

Key points for mTeSR1 Media:

- mTeSR1 comes from STEMCELL™ Technologies as a 2-part kit with one component stored at 4°C and the other at -20°C. Thaw the frozen bottle at room temperature or at 4°C – do not thaw in a water bath
- To prepare mTeSR1 complete media:
 - Sterile filter and add penicillin/ streptomycin (1% final V/V)
 - Use within 2 weeks
 - Warm before use to room temperature or in a 30°C water bath. Do not warm in a 37°C water bath.
- Alternative medias include StemFlex by Thermo Fisher

Notes:

Culture and Freezing Methods for WTC-11 Derived AICS hiPSC Lines

Required Reagent List:

- Complete mTeSR1 culture media, referred to in this protocol as simply “mTeSR1”: 400 mL basal media with provided 100 mL 5X supplement (catalog # 85850, STEMCELL™ Technologies) with added 5 mL (1% v/v) Penicillin/Streptomycin (catalog # 15140-122, Gibco) *Refer to page 16 of the STEMCELL™ Technologies technical manual about preparation, storage and shelf life of this media.*
- Growth Factor Reduced (GFR) Matrigel® (catalog # 354230, Corning)
- DMEM/F12 media, phenol red-free (catalog # 11039-021, Gibco Life Technologies)
- ROCK inhibitor (Ri) [10mM]_{stock} reconstituted in DMSO per manufacturer’s instructions (Y-27632, catalog # 72308, STEMCELL™ Technologies)
- DPBS, without Ca⁺⁺ or Mg⁺⁺ (catalog # 14190-144, Gibco Life Technologies)
- StemPro® Accutase® (catalog # A11105-01, Gibco Life Technologies)
- Treated plastic Tissue Culture dishes and/or plates (see Table 1 for appropriate catalog numbers)
- 0.22 µM Sterile Media Filter, Stericup™ 500 mL, or similar (catalog # SCGPU05RE, Millipore)
- DMSO (catalog # D2650, Sigma)

Additional for Freezing:

- Knock Out Serum Replacement (catalog # 10828-028, Gibco Life Technologies), referred to in this protocol as “KSR”
- CryoVials with rubber gasket and internal threading (catalog # 12-565-167N, Fisher Scientific)
- Mr. Frosty Freezing container (catalog # 5100-0001, Fisher Scientific)
- Isopropanol (catalog # 19516-500ML, Sigma)

Recommended Equipment:

- All work described in this protocol should be performed in a sterile Bio Safety Cabinet (Nuair Class II Type A2, or similar) using proper sterile technique.
- Tissue culture incubator capable of maintaining an environment with 5% CO₂ and 37°C (ThermoFisher Scientific Heracell™ VIOS 160i, or similar)
- Swinging bucket centrifuge capable of reaching speeds of 211 x g (Eppendorf 5810R, or similar). *Throughout protocol, RPM values are directly applicable to Eppendorf S-4-104 rotor (18.9 cm radius).*
- Cell Counter (Beckman Coulter® Vi-CELL™, or similar)
- Phase-contrast microscope with 4X and 10X objectives (Nikon Eclipse TS100, or similar)
- Serological pipettes (5-25 mL) and pipet-aid (Drummond Pipet-Aid, or similar)
- Standard size pipette set capable of pipetting 2-1000 µL (Rainin LTS, or similar)

Steps before starting:

1. If passaging, check that the morphology of your cells is consistent with known, good hiPSC morphology (Fig. 1). Ideally, cells should be at ~75% confluency for passaging and freezing, and cells should be fully recovered from previous passage. Some dead cells in the media is normal, but this should not be more than 1-5%.
2. If necessary, prepare fresh mTeSR1 media:
 - a. Thaw 5X supplement at room temperature (RT) for ~4-6 h, or at 4°C overnight. Do not thaw 5X supplement at 37°C.
 - b. Combine 5X supplement with 400 mL mTeSR1 and 5 mL Pen/Strep.
 - c. Sterile filter media with a 0.22 µM media filter before first use.
3. Bring mTeSR1 media to RT on the bench. Do not warm mTeSR1 in a 37°C water bath.
4. Pre-warm Accutase in a 37°C water bath or allow to come to RT.

5. Label vessel(s) (culture dish or CryoVials, etc.) with cell line name, clone, passage number, date, and attach barcode (as applicable).
6. Prepare mTeSR1 + ROCK inhibitor (Ri) media. mTeSR1 + Ri should always be used with cells for 24 h after they are treated with Accutase to promote cell survival.
 - a. Dilute Ri at 1:1000 in mTeSR1 media.
 - b. Mix well by pipetting.
e.g., for 100 mL mTeSR1, add 100 μ l Ri.
Note: Lyophilized Ri stock is reconstituted in DMSO at 10 mM, per manufacturer's instruction. We recommend making 250 μ L aliquots in 1.5 mL Eppendorf tubes and storing at -20°C for up to 6 months. Minimize freeze/thaw of aliquots.

Table 1: Vessel Formats

Vessel formats	Vol. (mL/well) for Matrigel coating	Vol. (mL/well) for media	Vol. (mL/well) for Accutase	Vol. (mL/well) of DPBS for trituration	Vol. (mL/well) of PBS for final wash	Vol. (mL/sample) of media to re-suspend pellet in for accurate counting	Cell Plating Density/ approx. days to 70% confluency ^A
10 cm dish^B (cat. 353003, Corning)	5	10-12	3	3-7	7-8	10	500K-1M / 3-4 days
6 well plate (cat. 657-160, Greiner Bio-One)	1.5	2-4	1	1-4	2-3	3-4	50-100K / 3-4 days
24 well plate (cat. 662-160, Greiner Bio-One)	0.5	1-2	0.5	1	1	n/a	20-40K / 3-4 days
96 well plate (cat. 353072, Falcon)	0.1	0.15- 0.2	0.04	0.170	n/a	n/a	2.5K-4K / 3-4 days
T25 flask (cat. 353014, Falcon)	4	5	2	2	4	5-7	350K / 3-4 days
T175 flask (cat. 159920, Nunc)	10	25- 40	10	20	20	25	1.5M-3M / 3-4 days

^ACan be cell line-dependent. Please see recommended seeding densities in AICS catalog. ^BWe recommend culturing in 10 cm dishes for most purposes, especially when more than 1×10^6 cells are needed for downstream applications.

Methods:

Matrigel Coating Plastic Tissue Culture Vessels (Matrigel:DMEM/F12= 1:30)

1. Prepare Matrigel coated vessels as needed. Per manufacturer's instruction, coated vessels are only good for 14 days, Matrigel should be stored at -80°C long term or -20°C for short term, and should be thawed **only** at 4°C overnight. **Never thaw in a water bath or at RT.** Freeze-thaws should be avoided, therefore we recommend making aliquots of Matrigel. See Step 2, below.
Note: We do not test our Matrigel to make sure cells cultured show expected morphology and expression of stem cell markers over 3-5 passages.
2. To aliquot Matrigel:
 - a. From a 10 mL glass vial of thawed 4°C Matrigel, aliquot 1 mL units into individual 50 mL conical tubes.
 - i. Keep 10 mL source glass vial and 50 mL conical tubes on ice or at 4°C while working.
 - ii. When making aliquots, we recommend using 5 mL serological pipettes that have been pre-chilled at -20°C to prevent Matrigel from gelling inside pipette. Make sure to change pipette between uses (maximum of 2 minutes at RT) to a fresh pre-chilled pipette at -20°C.
 - b. Store aliquots at -20°C for up to 3 months.
3. To prepare 1:30 Matrigel dilutions for vessel coating:
 - a. Thaw Matrigel aliquot in a 4°C fridge until frozen pellet is no longer visible (~ 2 h or overnight for 1 mL).
 - b. Dilute 4°C Matrigel 1:30 by adding 29 mL of ice-cold (4°C) DMEM/F12 media to the 1 mL of Matrigel in a 50 mL conical tube.
 - c. Ensure diluted Matrigel is homogeneously mixed by carefully, but thoroughly, pipetting the full mixture up and down 3-5 times using a 25 mL pipette.
 - d. Diluted Matrigel should be used to coat vessels immediately and should not be re-frozen. If spending more than 5 minutes coating vessels from a prepared dilution of Matrigel, keep the dilution on ice (or at 4°C) while working.

4. Transfer enough 1:30 diluted Matrigel into each well or vessel to coat bottom (see Table 1). If preparing 96 well plates, we recommend transferring the diluted Matrigel into a boat and using a multi-channel pipette to quickly dispense Matrigel into each well. An electronic repeater multi-channel pipette is ideal for fastest dispensing. Ensure that the entire vessel surface is coated with liquid-- if necessary, rock or tap vessel to do this.
5. Choose either the "Fast" or "Storage" Protocol, below
 - a. Fast Protocol (for same day use of coated plastic vessels):
 - i. Incubate at least 1 h, or up to 6 h, at RT.
 - ii. Tip plate to a 45° angle and aspirate and discard all excess liquid by aspiration. Gently add fresh RT mTeSR1 + Ri. Work quickly so that wells do not dry out. Try to limit time between aspirating and dispensing media to < 30 s.
 - iii. Seed cells within 10 min of adding media. If not seeding plate within 10 min, store prepared plates with media for up to 1 h in a tissue culture incubator at 5% CO₂ and 37°C until ready to seed.
 - b. Storage Protocol (for use next day or up to 2 weeks later):
 - i. Wrap vessels with Parafilm to prevent evaporation and store on level surface in 4°C fridge for up to 2 weeks.
 - ii. When ready to use, remove from fridge and allow plates to come to room temperature in hood or on bench top.
 - iii. Tip plate to a 45° angle and aspirate and discard all excess liquid by aspiration. Gently add fresh RT mTeSR1 + Ri. Work quickly so that wells do not dry out. Try to limit time between aspirating and dispensing media to < 30 s.
 - iv. Seed cells within 10 min of adding media. If not seeding plate within 10 min, store prepared plates with media for up to 1 h in a tissue culture incubator at 5% CO₂ and 37°C until ready to seed.

Thawing AICS Cells

13. Prepare a 15 mL conical tube with 5 mL of RT mTeSR1 + Ri.
14. Carefully remove vials from LN₂ storage, being sure to vent cap to relieve any LN₂ trapped inside, then re-tighten cap.
15. Quickly thaw the frozen vials using 37°C water bath until only a small ice pellet is visible. Limit the amount of time cells are in Freezing Media at RT to 10 min or less.
16. Fully rinse outside of closed vial with 70% EtOH and dry with Kimwipe before transferring vial to sterile hood.
17. Using a 5 or 10 mL serological pipette, slowly add 1 mL of RT mTeSR1 + Ri to the vial with the cells. Do not mix. Using the same pipette, slowly aspirate the now ~ 1.5 mL volume of diluted cells from the vial. Work carefully to remove all the liquid without creating extensive bubbles. Transfer the diluted cells from the vial into the prepared 15 mL conical tube from step 1 for a total of 5.5 mL. Do not mix; avoid disrupting the cells.
18. Spin cells at 1000 rpm (RCF= 211 x g) for 3 min at RT in a swinging bucket centrifuge.
19. Aspirate and discard supernatant, then re-suspend pellet in 3-5 mL RT mTeSR1 + Ri.
20. Seed cells in Matrigel-coated vessels; see chart for plating densities. Generally, a vial of 1 x 10⁶ cells thawed into a 10 cm plate will be ready for passaging 3 days later.
21. After seeding, slide vessel front-to-back and side-to-side to ensure even cell distribution across vessel surface at least 2-4 times and keep level for even cell attachment.
Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it's important to place plates on a level surface as quickly as possible.
22. Incubate cells at 37°C and 5% CO₂.
23. Observe 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri).
24. Change media every 24 h with mTeSR1.

Passaging and Maintaining AICS Cells on Plastic Tissue Culture Treated Vessels

Warm Accutase in a water bath at 37°C, while mTeSR1 must only be warmed to RT on the bench. AICS cells are grown in a standard 5% CO₂ incubator at 37°C and must be fed fresh mTeSR1 once every day.

19. When cells reach 70-85% confluency, passage the cells. See Figure 1.
20. Aspirate and discard old medium.
21. Gently add RT DPBS. Do not dispense DPBS directly onto cells, but rather introduce DPBS at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
22. Aspirate and discard DPBS wash.

23. Add pre-warmed Accutase (see Table 1 for volumes) and incubate in 37°C incubator for 3-5 min. Note: if using 37°C pre-warmed Accutase, expect shorter incubation times (3-4 minutes). RT Accutase incubations can be expected to take longer (4-5 minutes).
24. Check for detachment by gently tilting vessel and/or observing under the microscope. If all cells have not detached in 3-5 minutes, incubate an additional 2 min and check again. Continue to incubate and check as necessary, only until cells are no longer attached to the plate surface, although cells may remain in large clumps.

Note: Avoid incubating cells with Accutase for longer than is necessary to detach from plate surface. Most clonal lines derived from WTC will be fully detached from vessel surface in less than 8 min.
25. Add RT DPBS to dilute out the Accutase in the vessel as follows (see Table 1 for volumes):
 - c. Add DPBS to vessel and very gently triturate cell suspension by aspirating and then gently rinsing the cells across the vessel surface 6-10 times (ex. in a continuous zig zag or windshield wiper motion) to the point where all cells have been released from the vessel surface and the suspension is homogenous- all without creating bubbles. Do this using adequate but not excessive force. Note: We recommend trying this technique with either a 5 or 10mL serological pipette to achieve optimal single cell suspension; results will vary by individual and manufacturer of pipettes used.
 - d. Check that single cell suspension has been achieved in >90% of the suspension using microscope. If single cell suspension is not achieved, triturate 4-5 more times (as described above in step 7a) and check again.

Note: Cells can be passaged in small clumps, but cell counting may not be accurate. Single cell suspension is especially important for certain downstream applications such as transfection, cell sorting by FACS, or plating at low density for clone picking.
26. Transfer cell suspension to 15 mL conical tube.
27. Rinse the vessel one time with DPBS for final wash (see Table 1 for volumes). Add to conical. Alternatively, you may skip the wash step and add DPBS directly to conical if maximal cell recovery is unnecessary for your application.
28. Spin down cells at 1000 rpm (RCF= 211 x g) for 3 min at RT.
29. Carefully aspirate and discard DPBS/Accutase supernatant from cell pellet and re-suspend in desired volume of mTeSR1 + Ri. See Table 1 for recommended re-suspension volumes for most accurate counts based on source vessel size.
30. Cells can be seeded directly into new vessels at a specific cell count (3-4 days to confluency) depending on day needed. See Table 1 for recommended cell plating densities based on vessel size. We recommend counting cells and plating specific numbers for most reproducible culturing conditions.

Note: Cells in suspension settle quickly. After counting, we recommend gently re-suspending cell suspension before seeding by triturating the entire volume three times using a serological pipette. Cells should be mixed approximately every 2-3 min when seeding multiple vessels.
31. Matrigel-coated vessels cannot be re-used, so always seed cells onto fresh Matrigel coated vessels prepared with RT mTeSR1 + Ri.
32. After seeding, slide vessels front-to-back and side-to-side to ensure even cell distribution across vessel surface at least 2-4 times and keep level for even cell attachment.

Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it's important to place plates on a level surface as quickly as possible.
33. Always record the correct date, passage number and identifier on vessel(s).
34. Incubate cells at 37°C and 5% CO₂.
35. Observe 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri).
36. Change media every 24 h with mTeSR1.

AICS Cell Freezing (Cryo-preserving)

12. Prepare fresh Freezing Media. Freezing media should be made 10 min-6 h in advance of use and discarded thereafter.
 - b. mTeSR1 with 30% KSR, and 10% DMSO
e.g. for a 10 mL volume: 6 mL mTeSR1 + 3 mL KSR + 1 mL DMSO
Note: No Ri is used in the Freezing Media.
 Per manufacturer's instructions, KSR is stable for up to 4 weeks at 2°C to 8°C, protected from light. Working volumes can be aliquoted and stored at -20°C to -5°C. Thaw aliquots as needed. Avoid additional freeze-thaw cycles.
13. Prepare Mr. Frosty freezing containers by adding room temperature isopropanol per manufacturer's instructions.
14. Label CryoVials as appropriate.

15. When cells are 70-80% confluent, detach and pellet using Accutase per Passaging and Maintenance protocol, and re-suspend final pellet in mTeSR1 + Ri (see Table 1 for recommended re-suspension volumes for most accurate counts based on source vessel size).
16. Count cells.
17. Calculate the mL of cell suspension needed using the following equation:

$$mL \text{ cell suspension to spin down} = \frac{\# \text{ vials desired} \times 1 \times 10^6}{\frac{\# \text{ cells}}{mL}}$$

Note: calculate enough cells for at least 1 extra CryoVial for pipetting error.
18. Pellet cells to be frozen in a 15 mL conical by spinning at 1000 rpm (RCF= 211 x g) for 3 min at RT.
19. Re-suspend cells in appropriate volume of fresh freezing media (0.5 mL Freezing Media per vial of 1×10^6 cells) and aliquot 0.5 mL volumes of cells in freezing media into pre-labeled and prepared CryoVials.
Note: use a 5 mL serological pipette to dispense 0.5 mL aliquots into each CryoVial. Work quickly so cells don't settle toward bottom of pipette.
20. Tightly close vial caps.
21. Quickly transfer CryoVials to room temperature Mr. Frosty containers and place containers in -80°C freezer. Limit the amount of time cells are in Freezing Media at RT to 10 min or less.
22. After 24 h, transfer CryoVials to LN₂ storage. Do not leave cells in Mr. Frosty containers in the -80°C freezer for longer than 96 h.

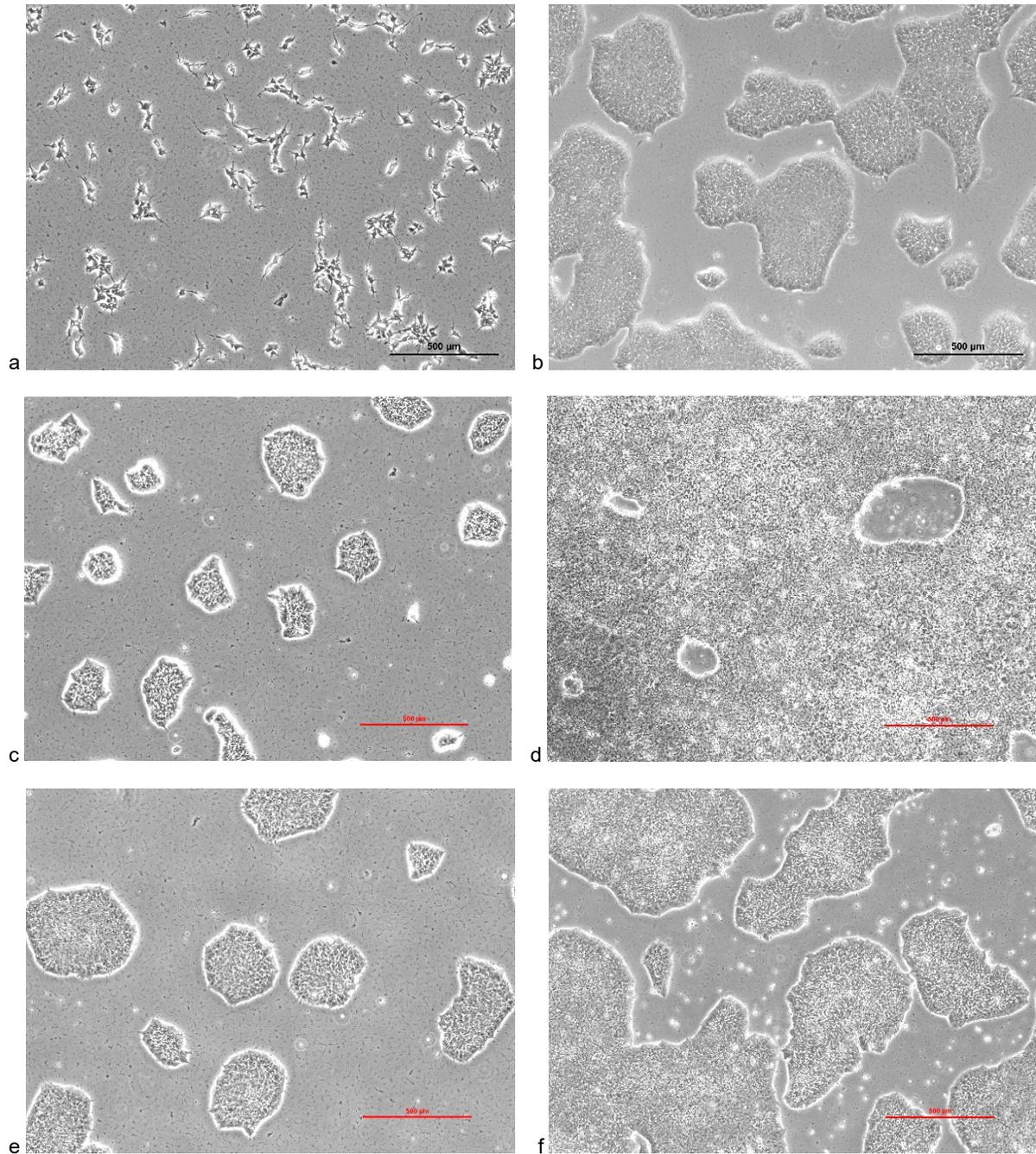


Figure 1. Examples of Confluency: (a) 1×10^6 cells plated in 10 cm dish, 24 h after seeding. Cells maintain a “spiky” morphology due to RI treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. (b) Same cells from (a) after 3 days of growth. Cells have good mature stem cell morphology and are at an ideal density to be passaged again. (c) Immature colonies have a slightly spikey edge and are loosely packed in colony interior. Passaging immature cells should be avoided. (d) Overgrown culture that is too confluent to continue to use. Future genomic integrity and/or morphology may be compromised. (e) Mature colonies at low density, can be picked as individual colonies or passaged. (f) Similar to (b), another example of a culture that has good mature stem cell morphology and is at an ideal density to be passaged again.

RNP Transfection in WTC-11s

Required reagent list:

- Complete mTeSR1 culture media, referred to in this protocol as simply “mTeSR1”: 400 mL basal media with provided 100 mL 5X supplement (catalog # 05850, STEMCELL™ Technologies) with added 5 mL (1% v/v) Penicillin/Streptomycin (catalog # 15140-122, Gibco) *Refer to page 16 of the STEMCELL™ Technologies technical manual about preparation, storage and shelf life of this media.*
- Matrigel (catalog # 354230, Corning)
- DMEM/F12 media, phenol red-free (catalog # 11039-021, Gibco Life Technologies)
- ROCK inhibitor (Ri) [10mM]_{stock} reconstituted in DMSO per manufacturer’s instructions (Stemolecule™ Y27632, catalog # 04-0012-10, Stemgent)
- DPBS, without Ca⁺ or Mg⁺⁺ (catalog # 14190-144, Gibco Life Technologies)
- StemPro® Accutase® (catalog # A11105-01, Gibco Life Technologies)
- Tissue Culture Treated 6-well Plates (catalog #657-160, CellStar)
- crRNA (custom synthesis from Dharmacon, or other vendor, unique to each target site)
- tracrRNA (constant sequence of RNA used with any Dharmacon crRNA, catalog #U-002000)
- PCR tubes (preferred brand)
- 1.5 mL Eppendorf DNA LoBind Microcentrifuge Tubes, or similar (catalog #13-698-791, Fisher Scientific)
- Duplex Buffer: 100 mM Potassium Acetate; 30 mM HEPES, pH 7.5
- TE pH 7.5 (catalog #11-01-02-02, IDT)
- Cas9 Protein (Berkeley QB3 MacroLab, <http://qb3.berkeley.edu/macrolab/>)
- Donor plasmid maxi-prep, eluted in Endotoxin-Free Water
- Neon 100 µL Kit (catalog #MPK10096, Fisher Scientific)

Required instruments:

- Thermocycler or heat block capable of reaching 95°C
- Neon Electroporation Device
- Micro-centrifuge
- Equipment necessary for sterile cell culture of hiPSC, see “Culture and Freezing Methods for WTC-11” protocol

Steps before starting:

7. Check that the morphology of your cells is consistent with known, good hiPSC morphology (Fig. 1). Ideally, cells should be at ~75% confluency, and should be fully recovered from previous passage. Some dead cells in the media is normal, but this should not be more than 1-5%. **Take a 4x and 10x image of cells prior to transfection; add this to your cell line documentation excel file.**
8. If necessary, prepare fresh mTeSR1 media:
 - a. Thaw 5X supplement at room temperature (RT) for ~4-6 h, or at 4°C overnight. Do not thaw 5X supplement at 37°C.
 - b. Combine 5X supplement with 400 mL mTeSR1 and 5 mL Pen/Strep.
 - c. Sterile filter media with a 0.22 µm media filter before first use.
9. Bring mTeSR1 media to RT on the bench. Do not warm mTeSR1 in a 37°C water bath.
10. Pre-warm Accutase in a 37°C water bath or bring to RT on the bench.
11. Label vessel(s) (culture dish or CryoVials, etc.) with cell line name, clone, passage number, date, and attach barcode (as applicable). Bring any Matrigel-coated vessels to room temperature.
 - a. If necessary, prepare Matrigel-coated vessels (refer to “Culture and Freezing Methods for WTC-11” protocol)
12. Prepare mTeSR1 +ROCK inhibitor (Ri) media. mTeSR1 + Ri should always be used with cells for 24 h after they are treated with Accutase to promote cell survival.

- a. Add Ri at 1000X dilution to mTeSR1 media.
- b. Mix well by pipetting.
e.g., for 100 mL mTeSR1, add 100 μ l Ri.
Note: Lyophilized Ri stock is reconstituted in DMSO at 10 mM, per manufacturer's instruction. We recommend making 200 μ L aliquots in 1.5 mL Eppendorf tubes and storing at -20°C for up to 6 months.

Method: Pre-complex crRNA and tracrRNA (to be done 2.5 h- 72 h in advance of transfection)

2. Reconstitute crRNA and tracrRNA to 100 μ M in TE. Alternate vortexing 30 sec and pulse-spinning for a minimum of 3 times to ensure all lyophilized RNA is reconstituted.
3. Combine crRNA and tracrRNA in a sterile PCR tube so that each is at a final concentration of 40 μ M in a volume appropriate for the experiment. Use Duplex Buffer as the diluent.
(eg. 8 μ L 100 μ M crRNA, 8 μ L 100 μ M tracrRNA, and 4 μ L Duplex Buffer).
4. In a thermocycler or heat block, heat the crRNA:tracrRNA mixture(s) from step 2 to 95°C for 5 min.
5. Remove from thermocycler or heat block and allow tube(s) to cool on the bench top for a *minimum* of 2 h.
6. Store duplexed crRNA:tracrRNA in PCR tubes at -20°C long-term, or keep on ice if complex is to be used the same day. Always thaw on ice or at 4°C from -20°C storage.

Method: Transfection with Neon Electroporation Device

Steps before starting:

1. Pre-treat cells to be transfected with mTeSR1 + Ri media for 2-6 hrs prior to transfection to promote cell survival. Cells should appear to have spikey edges around colonies with Ri treatment.
2. Prepare all plate layouts and calculations prior to starting (example of calculations given in Figure 2).
Note- It is recommended to plan for 2-3 replicates per transfection. For example, if transfecting 3 crRNAs and doing 2 replicates of each, you'll want to plan for 6 experimental transfections, plus a transfection for each control. You will need to ensure you have enough cells, duplexed diluted crRNAs, and diluted Cas9 (see Figure 2).
3. See Table 1 for recommendations on transfection controls included with each experiment.
4. Label 1.5 mL Eppendorf tubes for each reaction preparation.
5. Label 1.5 mL Eppendorf tubes for cell aliquots.
6. Prepare 10 μ M working concentrations of crRNA:tracrRNA duplex by diluting each in TE.
 - a. Keep dilutions and stocks on ice.
 - b. Working concentrations of crRNA:tracrRNA duplex can be stored at -20°C for up to 2 weeks; avoid multiple freeze-thaw cycles (<3 recommended).
7. Prepare 10 μ M working concentrations of Cas9 by diluting in TE.
 - a. Use a freshly thawed aliquot of Cas9 whenever possible.
 - b. Thaw Cas9 from -80°C storage on ice or at 4°C until no ice pellet is visible (~2-5 min).
 - c. Keep dilutions and stocks on ice.
 - d. Working concentrations of Cas9 protein can be stored at -20°C for up to 2 weeks; avoid multiple freeze-thaw cycles (<2 recommended).
8. Prepare working concentrations of donor plasmid at 1 μ g/ μ L by diluting in TE. Higher concentrations of donor plasmid can be used, but lower concentrations should be avoided (<500 ng/ μ L). We recommend 1 μ g/ μ L for easy calculations and pipetting.
9. Prepare the destination 6-well plate by aspirating and discarding the excess Matrigel liquid, and adding 4 mL of RT mTeSR1 + Ri media to each well. Keep plate with media in the incubator at 37°C and 5% CO₂ until ready to plate cells after the transfection procedure (plate should not be stored in incubator for longer than 2 h before use).

Transfection:

1. Set up the Neon device in the hood, per manufacturer's instructions.
2. Passage cells according to "Culture and Freezing Methods for WTC-11" protocol, proceeding until cells are in suspension and have been counted.
3. Prepare cell aliquots for each transfection reaction (room temperature):

- a. Aliquot cells in mTeSR + Ri into separate 1.5 mL Eppendorf tubes at numbers required for all transfection reactions, including excess
e.g. if cell suspension is at a concentration of 1×10^6 cells/mL and 1.2×10^6 cells are required per transfection reaction, then aliquot 1.2 mL cells/tube.
4. Prepare Ribonucleoprotein (RNP) Complex tubes for each transfection reaction (room temperature):
 - b. Add the required volume of 10 μ M working solution of crRNA:tracrRNA duplex per reaction to the pre-labeled 1.5 mL Eppendorf tubes for each reaction preparation.
 - c. Add the required volume of 10 μ M working solution of Cas9 protein per reaction to the pre-labeled 1.5 mL Eppendorf tubes already containing crRNA:tracrRNA duplex from step 4a by slowly adding the Cas9 and then gently pipetting up and down three times.
 - d. Incubate RNP complex at room temperature for a minimum of 10 min and no longer than 1 h prior to addition of cells in step 7, below.
5. Pellet cell aliquots from step 4 as they are ready to be used (we recommend 1-3 reactions at a time), so as to not allow cells to sit pelleted for longer than 5 min. Pellet cells by spinning in a micro-centrifuge or swinging bucket centrifuge at 211 x g for 3 min at RT.
6. Gently aspirate and discard supernatant with a pipette and re-suspend cell pellet in the required volume of Buffer R from the Neon Transfection Kit.
Note- when aspirating, it is okay to leave behind a *small* (<10 μ L) residual volume of mTESR + Ri with the cell pellet to ensure the pellet is not disturbed.
7. Transfer re-suspended cells in Buffer R into the tube containing the RNP complex to be transfected. Mix the entire volume gently 3 times by pipetting.
8. Add the required volume of plasmid to the cell-RNP mixture. The mixture is now ready for electroporation.
9. Using the Neon pipette and pipette tip provided with the kit, *gently* pipette the cell-RNP-plasmid mixture 2 times before aspirating the 100 μ L necessary for the reaction, being careful not to introduce bubbles.
10. Load the tip containing the cell-RNP-plasmid mixture into the Neon chamber, making sure the tip fully engages with the Neon chamber (should click in). Apply the voltage. We recommend setting 8 for WTC-11 RNP transfection: 1300 V, 30 ms, and 1 pulse.
Note- BUBBLE ALERT! Visually inspect the Neon tip for any bubbles that were introduced during the loading process. If bubbles appear, re-aspirate cell-RNP-plasmid mixture into tip without ANY bubbles and try again, Even very tiny bubbles can cause arcing, and result in failed transfection. When applying the voltage, watch for tiny bubbles to appear near the Neon electrode at the bottom of the chamber, and listen for a “popping” noise to indicate that the voltage has been properly applied.
11. Quickly unload the tip from the Neon chamber and gently dispense cells into the prepared 6-well plate containing mTeSR + Ri. Gently agitate plate to evenly distribute cells.
12. Repeat Steps 5-11 for each reaction. When all reagents are properly prepared ahead of time, the electroporation process (steps 5-11) for 5-6 samples should take less than 30 min.
13. Incubate cells for 24 h before changing media to mTeSR1 without Ri, then feed daily as usual.
14. Cells can be FACS sorted or used in downstream applications once they reach a healthy confluency and maturity, usually in 3-4 days.

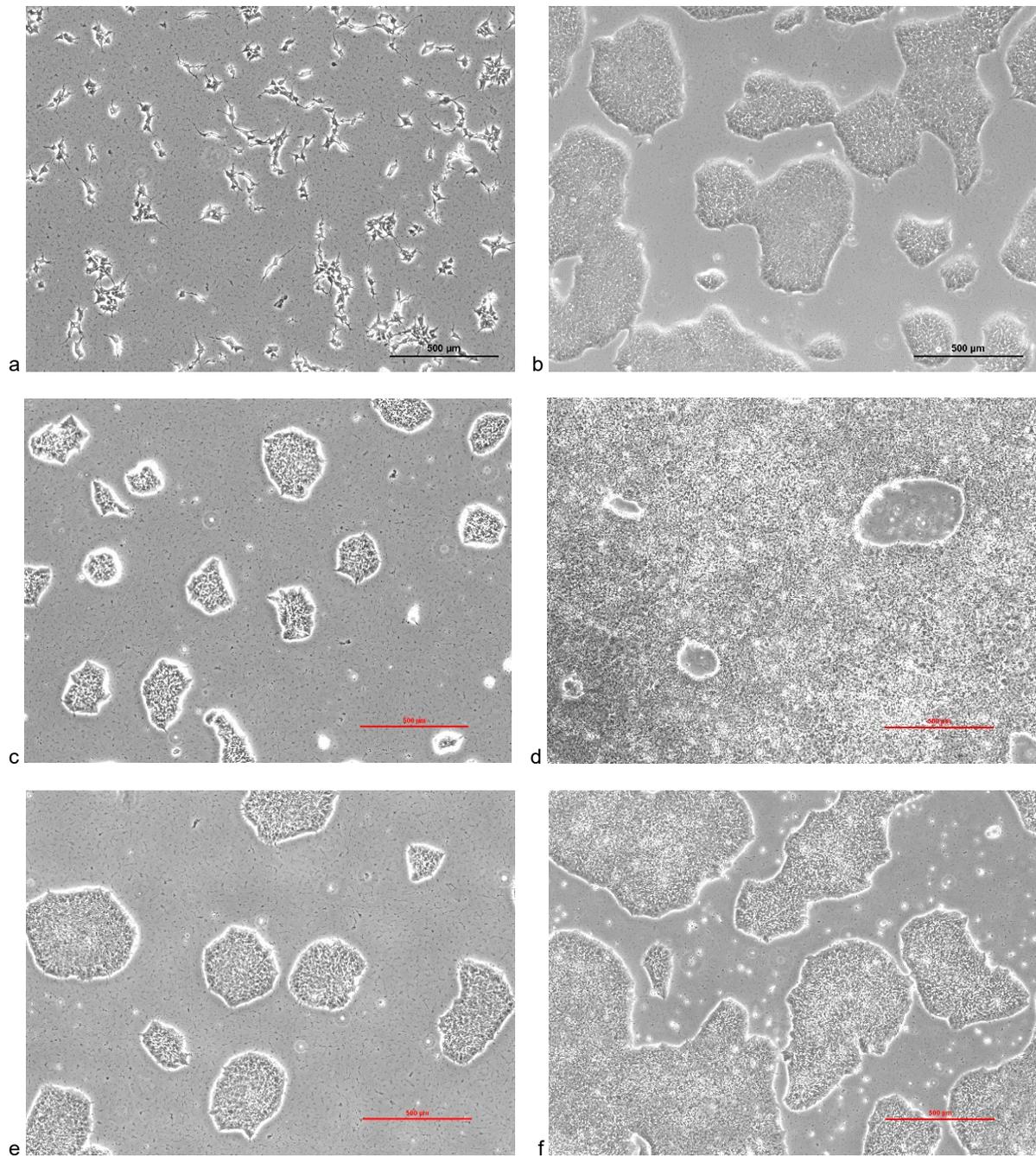


Figure 1. Examples of Confluency: (a) 1×10^6 cells plated in 10 cm dish, 24 h after seeding. Cells maintain a “spiky” morphology due to RI treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. (b) Same cells from (a) after 3 days of growth. Cells have good mature stem cell morphology and are at an ideal density to be passaged again. (c) Immature colonies have a slightly spikey edge and are loosely packed in colony interior. Passaging immature cells should be avoided. (d) Overgrown culture that is too confluent to continue to use. Future genomic integrity and/or morphology may be compromised. (e) Mature colonies at low density, can be picked as individual colonies or passaged. (f) Similar to (b), another example of a culture that has good mature stem cell morphology and is at an ideal density to be passaged again.

Example Reaction (rxn) Volumes		
	Vol per rxn	x # rxns (1.3)
crRNA:tracrRNA Duplex(10 μ M)	1.25	1.63
Cas9 Protein (10 μ M)	1.25	1.63
Plasmid (1 μ g/ μ L)	2.00	2.60
Constant Reaction Volume (μ L)	4.50	5.86
	800,000	cells/rxn
	1.3	rxns
	1,040,000	cells/aliquot
	124.1	μ L Buffer R/aliquot

Figure 2. Example calculations for a reaction using 2 μ g plasmid and 2 μ g Cas9 protein with a 1:1 molar ratio of Cas9:RNA. crRNA:tracrRNA duplex and Cas9 Protein are at a working concentration of 10 μ M for ease of pipetting, while still keeping the overall reaction volume less than 10 μ L (a requirement per Neon manufacturer's protocol that the reagent be < 10% of total volume). All volumes given in μ L. For ease of use of Neon tips, a minimum of 1.3x reaction volume is recommended. In grey-shaded cells: Row 1 is the desired number of cells transfected, Row 3 is Row 1 multiplied by the number of reactions calculated for excess (shown in Row2), and Row 4 is the amount of Buffer R required to re-suspend cells based on a 100 μ L reaction at 1.3x excess and takes into account the reaction volume of RNP complex.

Table 1. Recommended Controls.

Positive Control	pMax-GFP + RNP with target crRNA sequence	pMax-GFP or other constitutively expressed FP delivered as a plasmid. Confirms transfection was successful in the presence of RNP. Sample can be preserved for T7 Assay (or similar) to determine % editing of crRNA sequence.
Negative Control 1	Donor Plasmid + RNP with non-targeting crRNA sequence	Controls for viability and will account for any toxicity associated with the donor plasmid preparation since the crRNA is non-targeting and therefore should not cause DNA-cutting related toxicity.
Negative Control 2	Buffer only	Cells are re-suspended in Buffer R and transfected with the Neon device but are not treated with RNP or plasmid. Accounts for toxicity associated with electroporation method or buffer.

Droplet Digital PCR (ddPCR) Copy Number Variation Assay for GFP-Edited Cells Using Manual Droplet Generation

Required Reagent List:

- DG8 Cartridges (catalog # 1864108, Bio-Rad)
- Primers mEGFP-(FWD-GCCGACAAGCAGAAGAACG; REV-GGGTGGTCTGCTGGTAGTGG)
- Probe mEGFP-(/56-FAM/AGATCCGCC/ZEN/ACAACATCGAGG/3IABkFQ/)
- Primers AmpR-(FWD-TTTCCGTGTCGCCCTTATTCC; REV-ATGTAACCCACTCGTGCACCC)
- AmpR Probe-(/5HEX/TGGGTGAGC/ZEN/AAAAACAGGAAGGC/3IABkFQ/)
- ddPCR 2X Master Mix for Probes, no dUTP (catalog # 1863024, Bio-Rad)
- HindIII-HF (20,000 U/mL) (catalog # R3104S- NEB)
- 20X PrimePCR RPP30 Reference Assay (catalog # 1863024, Bio-Rad)
- Gaskets (catalog # 186-3009, Bio-Rad)
- 96 well Plates (catalog #12001925 , Bio-Rad)
- Pierceable Foil Heat Seals (catalog# 1814040, Bio-Rad)
- Droplet Generator Oil for Probes (catalog 1863005, Bio-Rad)
- Droplet Reader Oil (catalog 186-3004, Bio-Rad)
- DG8™ Cartridge Holder (catalog 186-3051, Bio-Rad)
- Molecular Grade Water
- RNase Away (catalog, #10328011, Fisher)

Required Instruments or Equipment:

- Thermal Cycler with Deep Well Block for 96-well plates
- 96 Well Cold Block
- X200 Droplet Generator
- X200 Droplet Reader, with Plate Holder
- Thermal Plate Sealer

Steps Before Starting

7. All Reactions should be set up in a dedicated, decontaminated PCR hood to avoid contamination of reaction with aerosolized plasmid and/or gDNA.
 - a. Decontaminate PCR hood by applying RNase Away as per recommended instructions to all surfaces and pipettes.
 - b. Following RNase Away treatment clean all surfaces and pipettes with 70% EtOH and allow to dry.
 - c. Finally, treat hood with UV light for 15 min.
8. In cleaned PCR hood prepare 20X Primer Probe Assay Master Mix for the GFP and AmpR assays. 20X Primer Probe Assay Master Mix can be premade up to six months prior to experiment and stored at 4°C.

Note: RPP30 20X Primer Probe Assay Master Mix is purchased directly from BioRad, see Reagent List.

 - a. Prepare 20X GFP Primer Probe Assay Master Mix with final concentrations as follows: 18 μM GFP FWD Primer, 18 μM GFP REV Primer, and 5 μM GFP Probe
 - i. For 96 reactions worth of Assay Master Mix, combine 21.6 μL of 100 μM GFP FWD Primer, 21.6 μL 100 μM GFP REV Primer, and 6 μL of 100 μM GFP

- Probe, then bring the final volume up to 120 μL in Molecular Grade H_2O . This will be your 20X Primer Probe Assay Master Mix for the GFP Assay.
- b. Prepare 20X AmpR Primer Probe Assay Master Mix with final concentrations as follows:
 - i. For 96 reactions worth of Assay Master Mix, combine 21.6 μL of 100 μM AmpR FWD Primer, 21.6 μL 100 μM AmpR REV Primer, and 6 μL of 100 μM AmpR Probe and bring the final volume up to 120 μL in Molecular Grade H_2O . This will be your 20X Primer Probe Assay Master Mix for the AmpR Assay.
9. Bring all reagents to room temperature (RT) and vortex well before use.
 10. Prepare gDNA using Purelink gDNA kit (Invitrogen) at final concentration of 2-20 $\text{ng}/\mu\text{L}$, eluted in Molecular Grade H_2O .
 11. Recommended Controls:
 - a. GFP+/AmpR- (gDNA from a known GFP+ cell line with no AmpR integration)
 - b. GFP+/AmpR+ (gDNA from a known GFP+ and AmpR+ cell Line)
 - c. WT gDNA with Donor plasmid added
 - d. Wild type or untreated gDNA
 - e. No Template Control (NTC)
 12. Generate an experimental template using the QuantaSoft Software provided with the Droplet Reader that is specific to your experiment.

Method: ddPCR Reaction Set Up

1. Prepare Reaction Master Mixes: prepare all reactions at RT in PCR hood
 - a. GFP-FAM/RPP30-HEX Duplexed Master Mix

12.5 μL	2X ddPCR Supermix for Probes, No UTP.
1.25 μL	20X Primer Probe Assay Master Mix for GFP
1.25 μL	20X PrimerPCR® RPP30 Reference Assay
0.30 μL	HindIII HF (20,000U/mL)
30 ng	Template gDNA
Up to 25 μL	Molecular Grade H_2O

- b. AmpR-HEX Master Mix

12.5 μL	2X ddPCR Supermix for Probes, No UTP.
1.25 μL	20X Primer Probe Assay Master Mix for AmpR
0.30 μL	HindIII HF (20,000U/mL)
30 ng	Template gDNA
Up to 25 μL	Molecular Grade H_2O

2. Vortex mixes to ensure homogenous distribution of reagents.
3. Pipette 22 μL of GFP-FAM/RPP30-HEX Duplexed Master Mix into a standard 96 well plate in only the odd numbered columns (1,3,5,7,9,11- or a subset if running fewer than 48 samples/plate).
4. Pipette 22 μL of AmpR-HEX Master Mix into the even numbered columns (2,4,6,8,10,12- or a subset if running fewer than 48 samples/plate).

Note: a multi-channel pipette or electronic pipette is recommended for pipetting master mixes.
5. Pipette 3 μL of template gDNA into each reaction well: 1 for GFP-FAM/RPP30-HEX and 1 for AmpR-HEX. Be sure to change tips between wells.

Note: 8 wells should be dedicated for controls (4 for GFP and 4 for AmpR, see "Steps Before Starting" for recommended controls). Each 96-well reaction plate can analyze 44 samples and 4 controls.
6. Mix reaction plate by gently pipetting 20 μL up and down 5 times, avoiding bubbles as they drastically inhibit droplet formation.

	01	02	03	04	05	06	07	08	09	10	11	12
A	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U GFP U RPP30	COLUMN 6 A ABS U AMP
B	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U GFP U RPP30	COLUMN 6 A ABS U AMP
C	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U GFP U RPP30	COLUMN 6 A ABS U AMP
D	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U GFP U RPP30	COLUMN 6 A ABS U AMP
E	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	GFP+/RPP30+... A ABS + RPP30	GFP+/RPP30+... A ABS - AMP
F	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	GFP+/RPP30/A... A ABS + RPP30	GFP+/RPP30/A... A ABS + AMP
G	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	NTC A ABS N RPP30	NTC A ABS N AMP
H	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	NTC A ABS N RPP30	NTC A ABS N AMP

Figure 1. Example Plate Layout. If preparing a full 96 well plate, pipette 3 μ L of gDNA from column 1 of 96 well gDNA plate into columns 1 and 2 of reaction plate, ensuring to change tips between wells. Do this across the plate leaving 4 wells in columns 11 and 12 empty for controls.

Method: ddPCR Droplet Generation

***Before starting!** Be sure to have all reactions set up before starting droplet generation as droplets are not stabilized before cycling and should not be left at room temperature for more than 15 min. If using multiple cartridges one can leave the reactions on ice for up to 45 min.

2. Insert a DG8 Cartridge into a DG8 Cartridge Holder.
3. For droplet generation, transfer 20 μ L of the 25 μ L reaction prepared in the above steps into the middle row of a DG8 Cartridge, labeled "Sample". Each DG8 Cartridge (as shown below) can hold 8 samples. For 96 samples, 12 DG8 cartridges can be pre-loaded with sample and oil prior to droplet generation in step 4, below.



4. Load 70 μ L of Droplet Generation Oil for Probes into the bottom wells of the DG8 Cartridge, labeled "Oil".



5. Once both Sample and Oil are loaded, attach a Gasket across the top of the cartridge and place into the Q200 Droplet Generator and close lid. Droplet formation will start automatically and will take \sim 90 s per cartridge. Only one cartridge can be loaded at a time, therefore 12 cartridges will take approximately 35-45 min.



6. After droplet formation is complete, the resulting droplets will be available in the top row of the cartridge, labeled "Droplets", and should appear cloudy. Using a multi-channel P200 pipette set to 40 μ L, slowly aspirate up the droplets and deposit into a fresh Bio-Rad 96 well plate kept in a 96 well cold block, or ice bucket.



7. Once all droplets have been generated, and the 96 well plate has been loaded with droplets, place pierceable foil seal on top of plate with the red band facing up. Seal the plate in a thermal plate sealer at 180°C for 5 s.
8. Once the plate has been sealed, immediately place the plate into the deep well thermal cycler and run the PCR cycling program. **Never spin droplet plate.**

a. Cycling Conditions for ddPCR Assays

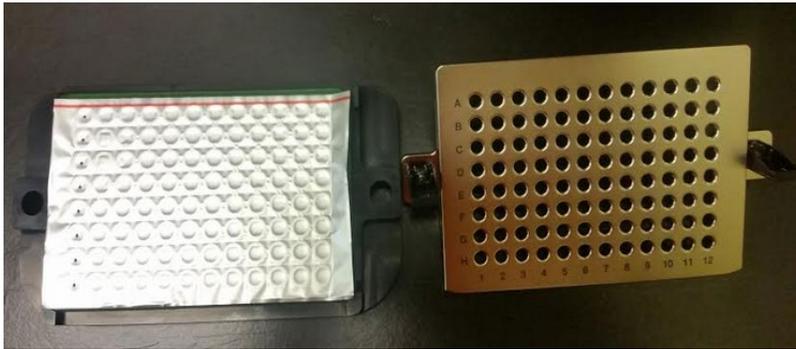
95°C	10 min	
94°C	30 s	X 39 cycles
60°C	20 s	
72°C	15 s	
98°C	10 min	

*After PCR cycling, droplets are stable at 4°C for up to 3 days

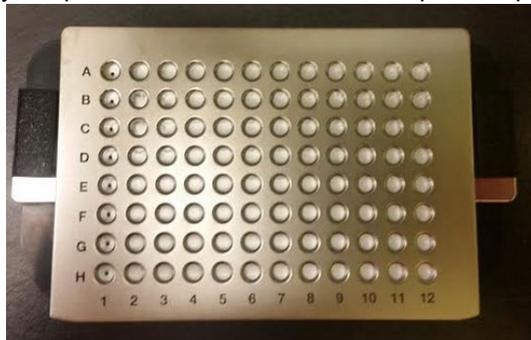
Method: ddPCR Droplet Reading

(A full 96 well plate will take ~ 2.5hrs to read on the QX200 Droplet Reader)

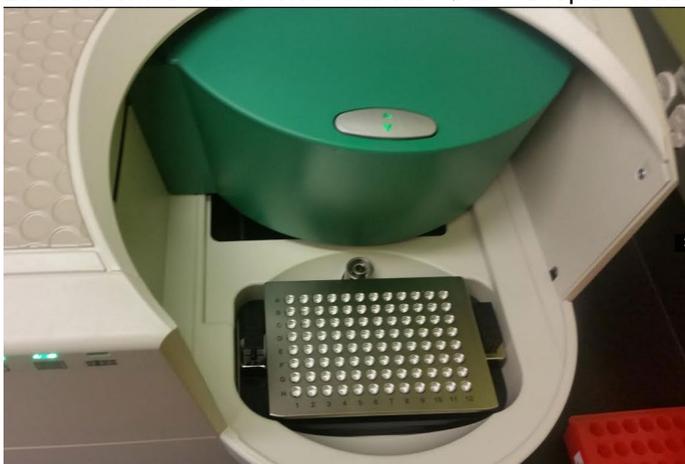
1. After cycling is complete, load plate in proper orientation into the QX200 Droplet Reader Plate Holder, as shown below.



2. Securely snap the metal lid shut over the top of the plate, as shown below.



3. Load the entire Plate Holder into the QX200 Droplet Reader.



4. Launch the QuantaSoft software and load your prepared template. Click “run” to start the droplet reading process.
5. Results can be analyzed using the QuantaSoft program or exported as an Excel worksheet.

- Once threshold gates have been set, use the calculated copies/ul to further calculate copy number/genome

Well	E	Experiment	Sample	T	Target	Status	Cono(copies/μL)	Supermix
A01	A	ABS	5k	U	GFP	Manual	268	ddPCR Super
A01	A	ABS	5k	U	AMP	Manual	278	ddPCR Super

- To calculate the final copy number of GFP per genome calculate as the ratio of $[(\text{copies}/\mu\text{L}_{\text{GFP}}) - (\text{copies}/\mu\text{L}_{\text{nonintegratedAmpR}})] / (\text{copies}/\mu\text{L}_{\text{RPP30}})$, where a ratio of 0.5 is equal to 1 integrated copy/genome and a ratio of 1 is equal to 2 integrated copies/genome.
- The AMP or KAN signal is determined to be from residual non-integrated/background plasmid when the ratio of AmpR/RPP30 or KAN/RPP30 falls below 0.2 copies/genome, ratios above 0.2 copies/genome could indicate a non-clonal line with at least 1 copy of the backbone stably integrated.
- For primary screening $[(\text{copies}/\mu\text{L}_{\text{GFP}}) / (\text{copies}/\mu\text{L}_{\text{RPP30}})] * (\text{copies of RPP30/genome}) = \text{GFP copy\#/genome}$.

