§ ENGINEERED CELLS · EC: IPSC

Limiting dilution & clonal expansion for iPS cells.

DEVELOPED BY EDITCO BIO

Introduction

CRISPR-Cas9 can be used to generate edited cell pools for a variety of applications. These pools contain a heterogeneous mix of cells, some unedited and some with different genetic alterations at the targeted locus. In order to produce a monoclonal cell line that uniformly contains a single CRISPR edit at the intended target site, clonal isolation and expansion from a single cell is necessary. This protocol describes how to isolate single cells from a CRISPR-edited pool, expand, and genotype the subsequent monoclonal populations.

The isolation of single cells from a knockout cell pool can be accomplished through limiting dilution. While there are more sophisticated methods to do single cell plating (e.g., flow cytometry), limiting dilution, which relies on statistical probability, is a simple and straightforward technique that can be carried out with standard pipetting tools. A typical target cell concentration used for limiting dilution is 0.5 cells per well because it maximizes the probability of obtaining a single cell per well while it minimizes the probability of depositing multiple cells per well. At this concentration, one can expect about a third of the wells to contain a single cell and most wells to be empty, minimizing heterogeneous populations arising from multiple cells.

Important considerations

- Cells must be healthy and reach 50% to 80% confluency before single cell dilution is attempted. Monitor the health and growth of clones continually during expansion.
- When working with fresh healthy iPS cells at the right confluence, limiting dilution should take 1 day and clonal expansion should take from 2 to 8 weeks.
- Cell handling impacts cell health. Minimize rough pipetting by setting the pipettor on slow, and attach a 10 µl aspirating pipet tip to a 2 ml aspirating pipet, to aid precise aspiration in all steps.
- Pools with the highest indel frequencies generate clonal cell lines most efficiently.
- Obtain single cell clones in parallel from the mock transfected cells or negative control. This acts as a control for potential health/growth defects caused by the editing or single cell isolation process.

- Use the Inference of CRISPR Edits (ICE) tool to characterize edited cells of pools and clones for both KOs and KIs. See our ICE Knockout Analysis protocol or ICE Knock-in Analysis protocol for instructions on how to use ICE and interpret results.

iPS cells

iPSC recovery and culture maintenance require mTeSR™ Plus complete medium (containing supplement) and iMatrix. If working with frozen cell stocks, before preparing the cells for single-cell cloning, incubate the cells at 37°C and 5% CO_2 for 5–7 days after thawing until the cells reach a full growth rate. Please follow our EC: iPSC Pool Quick Start Guide for recommendations on how to thaw and expand EditCo Engineered iPS Cell Pools.

In this protocol, cells from each edited population are diluted to 0.5 cells per well and plated on a 384-well plate. However, if clonally expanding cells for the first time we recommend plating a higher number of wells (e.g., 2 × 384-well plates per condition).

> **NOTE**
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> iPS cells do not like being in single cells. Although CloneR is used in this protocol and helps with viability, the survival rate of single clones might be highly reduced during the clone isolation process. Depending on the editing efficiency and the number of clones you want, you might need to seed more than one 384-well plate. Alternatively, you can do manual colony picking — see The New York Stem Cell Foundation recommendations.

Materials required

Material	Ordering information
1.5 ml microcentrifuge tubes	Multiple vendors (e.g., Thermo Fisher Scientific)
10 ml sterile reagent reservoir	Multiple vendors (e.g., Thermo Fisher Scientific)
Multichannel pipette or similar	Multiple vendors
6, 12, 96 or 384-well plates, TC-treated	Multiple vendors (e.g., Thermo Fisher Scientific)
10 µl aspirating pipet tip	Multiple vendors (e.g., Thermo Fisher Scientific)
2 µl aspirating pipet	Multiple vendors (e.g., Thermo Fisher Scientific)
Phosphate buffered saline without calcium and magnesium (DPBS)	Multiple vendors (e.g., Thermo Fisher Scientific)
Hemocytometer or automated cytometer	Multiple vendors
Trypan blue	Multiple vendors (e.g., Thermo Fisher Scientific)

Material	Ordering information
Cell strainer (optional)	Multiple vendors (e.g., Thermo Fisher Scientific)
mTeSR™ Plus Medium	Stemcell Technologies, Catalog # 100-0276
Rock Inhibitor	Stemgent, Catalog # 04-2012
CloneR™2	Stemcell Technologies, Catalog # 100-0691
iMatrix511	Reprocell, Catalog # NP892-011
DMEM/F12	Thermo Fisher Scientific, Catalog # 10565018
StemPro™ Accutase™ Cell Dissociation Reagent	Thermo Fisher, Catalog # A1110501

1. Protocol

1.1 Prepare iPS cells for cloning

1. When cells are at ~50–70% confluency in the 6-well plate, prepare cells to a concentration of 0.5 cells/well.
2. Warm Accutase, mTeSR™ Plus medium, and DMEM/F12 medium to room temperature.
3. Thaw a 1 ml CloneR 10X stock from −20°C at 4°C.
4. Pre-treat the cells to be cloned with ROCK inhibitor for at least 1 hour.
    - 4.1 Mix 1 µl of 1000X ROCK inhibitor in 1 ml of complete mTeSR™ Plus medium in a microcentrifuge tube.
    - 4.2 Exchange the mTeSR™ Plus medium the cells are culturing in to that of fresh mTeSR™ Plus medium with ROCK inhibitor added. Incubate the plate at 37°C and 5% CO_2 for at least an hour.
5. Observe the cells under a microscope after ~1 hour of ROCK inhibitor treatment. Cells should be flattened on the surface and noticeably growing outwards from their colonies.
6. Aspirate the media from the wells and wash cells with DMEM/F12 medium or DPBS.
    - 6.1 With a P-1000 micropipette, gently add 1 ml of DPBS or DMEM/F12 medium to the cells. Gently tilt the plate from side to side to wash the cell surface, then aspirate the DPBS or DMEM/F12 medium.
7. Add 500 µl of Accutase to the wells and tilt the plate to evenly coat the cells.
    - 7.1 Incubate the plate at 37°C and 5% CO_2.
    - 7.2 Check the cells for detachment and singularization under a microscope every 5 minutes and try not to exceed 15 minutes of total incubation time. For single cell cloning, singularization is extremely important.
8. Neutralize the Accutase by adding 500 µl of DMEM/F12 medium to the cells.
    - 8.1 Mix once by pipetting up and down, then prepare 2 microcentrifuge tubes.
    - 8.2 Make two vials with cells: one “freeze” vial with 850 µl of the cell suspension and one “clone” vial with 150 µl of the cell suspension.

9. Centrifuge the cells from both vials at 300 × g for 5 minutes.
10. Prepare the final cloning media for the cells while they are spinning down.
    10.1 Mix together 9 ml complete mTeSR™ Plus medium + 1 ml CloneR + 12.5 µl iMatrix in a 15 ml conical tube.
11. Once the cells have been spun down, aspirate the supernatant from both tubes.
12. Resuspend the cells in the tube labeled “freeze” with 1 ml of NutriFreez cryopreservation medium.
    12.1 This will be a frozen backup vial.
13. Resuspend the cells in the tube labeled “clone” with 1 ml of mTeSR™ Plus medium.
14. Set aside 100 µl of the suspension in a microcentrifuge tube for counting.
15. Count the cells with a hemocytometer or other cell counting method.

1.2 Seed a 384-well plate with 0.5 cells/well

1. 192 cells total will be needed for a 384-well plate. Prepare at least 10 ml (384 × 25 µl per well) at 0.5 cell/25 µl in the final cloning media containing CloneR and iMatrix: 9 ml complete mTeSR™ Plus medium + 1 ml CloneR + 12.5 µl iMatrix.
2. Mix the conical tube containing cells by inverting gently 2 times, then pour its contents into a sterile 25 ml reservoir.
3. With a manual P-200 multichannel pipet, add 25 µl of the cell suspension in each well. Pipet gently to avoid bubbles.

> **NOTE**
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> If using an 8-channel multichannel pipette in a 384-well plate, each pipet tip will reach alternating wells in a row or column, so each row or column will need 2 dispenses total. Do not use an electronic pipet, as that may affect the even distribution of cells across the plate.

4. After all wells have been seeded, add 5 µl of cell suspension from the microcentrifuge tube containing the higher density of cells to the A1 well. This will deposit more cells in A1, which helps with focusing the microscope for image acquisition and provides a positive cell growth control.

1.3 Prepare the plate for image acquisition, image cells and change media

1. Centrifuge the freshly seeded plate at 100 × g for 1 minute to dislodge bubbles and to bring cells down to the bottom of the well.
2. Incubate the plate at 37°C and 5% CO_2 for at least 2 hours.
3. Obtain Day 0 images of each well, then place the plate back into the incubator for 48 hours. You can mark the lid of the wells that have more than 1 cell to discard these wells.
4. Image the cells on the 2nd day and monitor cell growth.
5. On the 3rd day, change the media from each well and replace with fresh mTeSR™ Plus medium.
   5.1 Carefully aspirate the old medium containing CloneR and iMatrix with a multichannel aspirator (CloneR can be removed from the culture after 72 hours). Change the tips after every well aspiration to prevent

cross-contamination between clones.

5.2 Dispense 10 ml of mTeSR™ Plus medium into a 25 ml reagent reservoir and carefully add 25 µl of media to each well. Pipet down towards the walls of each well and change tips if they touch the cell surface.

6. Image the cells and change mTeSR™ Plus medium every other day until day 9 or day 10. At this time, wells with single colonies should be about 60–80% confluent and ready to be manually picked and transferred to a 96-well plate.

1.4 Transfer cells to 2 × 96-well plates

1. Split each single colony into two 96-well plates: one plate for cell expansion and the other plate for genotyping.
2. Genotype the clones following Step 2 below.

1.5 Expand selected clones from 96-well to 6- or 12-well after genotyping

1. Once the clones have been genotyped, select the clones with the desired genotype and expand cells from the 96-well plate to a 12- or 6-well plate.
2. Prewarm complete mTeSR™ Plus medium, Accutase, and DMEM/F-12 to room temperature.
3. Aspirate media from the plate to be passaged and wash once with DPBS or DMEM/F12 medium.
4. Add pre-warmed Accutase to the plate and place the plate in a 37°C incubator (see suggested volume below). Observe intermittently to determine when cells begin to detach (5–8 minutes). Try not to leave cells in Accutase for longer than 10 minutes.

Dish size	Accutase	Growth medium
One well in a 12-well dish	0.5 ml	1 ml
One well in a 6-well dish	1 ml	2 ml

5. When cells are completely detached from the dish by themselves, add the same volume of pre-warmed DMEM/F-12 to the plate to dilute Accutase.
6. Pipette the cell mixture in the plate up and down once to dissociate any cell clumps (avoid making bubbles).
   - 6.1 Wash the bottom of the plate well to ensure detachment of cells.
   - 6.2 Transfer the cell suspension to a 15 ml conical tube.
   - 6.3 Count the cells using the cell counter.
7. Centrifuge tubes at 300 × g for 5 minutes.
8. For passage or expansion, aspirate the supernatant and re-suspend cells thoroughly in the complete mTeSR™ Plus medium containing iMatrix and ROCK inhibitor.
   - 8.1 Count the cells and seed them into new dishes. Please follow the indications below depending on the well size.

Component	6-well	12-well
iMatrix	4.8 µl (0.25 µg/cm^2)	1.75 µl (0.25 µg/cm^2)
ROCK inhibitor	2 µl (final working concentration of 10 µM)	1 µl (final working concentration of 10 µM)
mTeSR™ Plus	2 ml	1 ml
Cell density	0.2–0.5 × 10^6	0.1–0.3 × 10^6

9. Incubate at 37°C and 5% CO_2, and change media after 24 hours to complete mTeSR™ Plus medium without ROCK inhibitor. Change media every 1–2 days thereafter until 80–90% confluency.
10. To freeze your cells please follow our EC: iPSC Clone Quick Start Guide.

2. Genotyping clones

To genotype your clones, isolate genomic DNA, PCR-amplify the edited region, and sequence the amplicons via Sanger sequencing following our Genotyping Protocol. The Inference of CRISPR Edits (ICE) tool can be used to analyze the sequence data.

A clone can be reported as wild type (unedited), homozygous, heterozygous or compound heterozygous. When a clone (cell line or edited organism) carries two copies of the same edited allele for a given gene, it is said to be homozygous. When it carries one edited allele and one unedited (WT) allele, it is heterozygous. When it carries two different edited alleles, it is compound heterozygous. A heterozygous edit can be identified by the overlapping peaks in the chromatogram centered around the region targeted by the guide.

For knockout edits, it is important to consider that edits do not necessarily equate to knockouts. Whether the edits are homozygous, heterozygous or compound heterozygous, it is recommended to select colonies containing indels that give rise to frameshift mutations, ideally those that generate a premature stop codon. Indels that maintain the gene's reading frame or alter the coding of only a few amino acids towards the 3′ end of the exon may not lead to the loss of the protein's function.

3. Storage & additional analyses

After genotyping the clones, split the cells in two: one culture can be expanded for cryopreservation while the other can be used to validate the knockout by measuring protein abundance to confirm the desired knockout edit (e.g., western blot, flow cytometry, ELISA, immunostaining).

Additional information

For the current library of EditCo protocols and quick start guides, visit editco.bio/resources. For technical assistance, contact technicalsupport@editco.bio.

About EditCo Bio

EditCo Bio, Inc. is a gene-editing company headquartered in Redwood City, California, supplying reagents, kits, libraries, and engineered cell lines — including engineered iPSCs, T-cells, and validated clones — for biology R&D.