§ ENGINEERED CELLS · EC: IMMORTALIZED

Limiting dilution & clonal expansion for immortalized cells.

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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 cells at the right confluence, limiting dilution should take 1 day and clonal expansion can take from 2 to 8 weeks depending on the doubling time of the immortalized cell line you are working with.
- 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.

Immortalized cells

This protocol can be used for suspension and adherent immortalized cell lines. The ease at which a clonal cell line can be obtained depends on the cell type. For most immortalized cell types, waiting 6 days after transfection is typically enough time for the cells to recover and stabilize. If working with frozen cells, incubate at 37°C and 5% CO_2 and ideally, passage cells once before plating for limiting dilution. If time is a constraint, incubate cells for at least 48 hours after thawing before starting limiting dilution. Please follow our EC: Immortalized Cell Pool Quick Start Guide for recommendations on how to thaw and expand EditCo Engineered Immortalized Cell Pools. Cell seeding density is highly cell type dependent; please follow the cell density recommendation from the corresponding vendor when thawing your cells.

In this protocol, cells from each edited population are diluted to 0.5 cells per well and plated on either a 96-well or a 384-well plate. However, if clonally expanding cells for the first time we recommend plating a higher number of wells (e.g., 2 × 96 or 2 × 384-well plates per edited target).

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
24, 96 or 384-well plates, TC-treated (for adherent cell types)	Multiple vendors (e.g., Thermo Fisher Scientific)
24, 96 or 384-well plates, untreated (for suspension cell types)	Multiple vendors (e.g., Thermo Fisher Scientific)
Complete cell growth medium	ATCC or other supplier recommended
Phosphate buffered saline without calcium and magnesium (DPBS)	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)
TrypLE™ cell dissociation reagent	Gibco™, Catalog # 100-0276 12605010

Material	Ordering information
Hemocytometer or automated cytometer	Multiple vendors
Trypan blue	Multiple vendors (e.g., Thermo Fisher Scientific)
Cell strainer (optional)	Multiple vendors (e.g., Thermo Fisher Scientific)

1. Protocol

1.1 Limiting dilution

> **NOTE**
>
> For adherent cell lines please follow all steps below. For suspension cell lines please skip steps 3 to 7.

1. Warm growth medium, TrypLE™ cell dissociation reagent and DPBS to room temperature.
2. Transfer 24-well plate containing transfected cells from the humidified CO_2 incubator to a biological safety cabinet.
3. Aspirate medium from cells across all conditions.
4. Carefully wash cells with DPBS.
    - 4.1 With a P-1000 micropipette, gently add 1 ml of DPBS to the cells. Gently tilt the plate from side to side to wash the cell surface, then aspirate the DPBS.
5. Add 250 µl of TrypLE™ (or other appropriate cell dissociation reagent) to cells (adjust volumes of TrypLE™ to the size of the cell culture vessel as needed).
    - 5.1 Incubate the cells for 5 minutes at 37°C and 5% CO_2 or until the cells detach.
    - 5.2 Transfer the 24-well plate back to the biological safety cabinet.
6. Neutralize the dissociation reagent by adding an equal volume of growth medium (250 µl) to that of the TrypLE™ to each of the wells.
7. Pipet 3–4 times (more if needed) with 1 ml pipette to break up any cell clumps. Cells can also be passed through a cell strainer.
8. Transfer the cells from each condition to a separate sterile 1.5 ml microcentrifuge tube.
9. Centrifuge the cells at 300 × g for 5 minutes.
10. Once the cells have been spun down, aspirate the supernatant.
11. Resuspend the cells in 1 ml of the corresponding complete culture medium.
12. Count the cells using a hemocytometer or automated cytometer.

1.2 Seed a 96- or 384-well plate with 0.5 cells/well

1. Calculate the concentration of cells (cells/ml) in the cell suspension.

2. Determine the number of 96 or 384-well plates for each pool.
3. Dilute cells in the complete culture medium to a concentration of 0.5 cells per well, transfer the cell suspension to a reservoir and dispense the cells with a multichannel pipette into the destination plate taking into account the plate format:
    - 3.1 1 × 96-well plate: 48 live cells total for a 96-well plate, 100 µl per well. Prepare at least 10 ml (96 × 100 µl) at 0.5 cell/100 µl.
    - 3.2 1 × 384-well plate: 192 live cells total for a 384-well plate, 25 µl per well. Prepare at least 10 ml (384 × 25 µl) at 0.5 cell/25 µl.
4. After all wells have been seeded, add 5 µl of cell suspension 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.
5. Repeat steps 11 and 12 in section 1.1 and section 1.2 for each edited population selected for clonal expansion.
6. Transfer plates to a humidified incubator at 37°C, 5% CO_2.
7. Expand and genotype clones (see below).

1.3 Image cells and change media

1. 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.
2. Image the cells on the 2nd day and monitor cell growth and attachment for adherent cells.
3. The cells should be imaged 5–7 days thereafter to monitor cell growth and established single colonies and close to hitpicking. This practice enables one to track cell growth over time and confirm that the final clones originated from a single cell.

> **NOTE**
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> Depending on the immortalized cell line you are working with, clonal isolation can take from 2 up to 8 weeks.

1.4 Select colonies and expand cells

1. Mark establishing colonies by circling the well on the lid and/or recording in a spreadsheet.

> **NOTE**
>
> Cells, including genetically modified cells, may grow at a different pace.

2. When colonies reach 70% confluence, prepare to transfer the selected clones from the culture plate into new plates for further expansion. For suspension cells, repeat all steps described in section 1.1 above (skip steps 3–7) with the adjusted volumes based on the plate formats. For adherent cells, follow all steps included in section 1.1, with the adjusted volumes for dissociation and rinse reagents to lift the cells from the old culture plate.

> **NOTE**
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> If you are using a 96-well plate, transfer to a 24-well plate; if you are using a 384-well plate, transfer to a 96-well plate. Follow the recommendations below depending on the well size.

Dish size	Dissociation reagent	Rinse reagent	Growth medium
One well in a 96-well dish	50 µl	50 µl	100 µl
One well in a 24-well dish	250 µl	250 µl	500 µl
One well in a 12-well dish	0.5 ml	0.5 ml	1 ml
One well in a 6-well dish	1 ml	1 ml	2 ml

3. During the transfer, take out a portion of cells from each colony and transfer all samples into a 96-well plate for genotyping.
4. Genotype the clones following Step 2 below.

1.5 Expand selected clones after genotyping

1. If further clonal expansion is desired, follow sections 1.1 and 1.4 according to the immortalized cell line you are working with (adherent or suspension) to transfer cells into bigger culture vessels. We recommend expanding selected clones from a 96-well plate to a 24-well plate, or from a 24-well plate to a 6-well plate.
2. To freeze your cells please follow our EC: Immortalized Cell 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.