Limiting Dilution & Clonal Expansion

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ABSTRACT

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 cell line with a particular edit or knockout, clonal expansion of individual cells is necessary. This protocol describes how to isolate single cells from a CRISPR-edited pool, and expand and genotype the subsequent clonal populations.

The isolation of single cells from a knockout cell pool can be accomplished through limiting dilution. While there are more sophisticated means to accomplish single cell plating (e.g., flow cytometry), limiting dilution is a technique that can be carried out with standard pipetting tools. The obvious drawback to this technique is that it relies on statistical probability (based on a Poisson distribution) to deposit a single cell per well. A typical concentration used for limiting dilution is 0.5-1 cells per 100 µl aliquot because it maximizes the probability of obtaining a single cell per well while minimizing 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. It is much less difficult to exclude wells without cells than to sort out heterogeneous populations arising from multiple cells.

In this protocol, cells from each edited population are diluted to 0.5-1 cells per 100 µl and plated on at least two 96-well plates (100 µl/well). However, if clonally expanding cells for the first time we recommend plating a higher number of cells (e.g., 4 x 96-well plates per condition).

To control for potential health/growth defects caused by the editing, we recommend including mock and/or negative control for single cell dilution, although these do not require multiple 96-well plates. The *RELA* positive control, however, does not need to be plated out for clonal expansion as it serves mainly as a positive control for transfection and editing efficiency.

EXTERNAL LINK

https://www.synthego.com/resources/Limiting-Dilution-&-Clonal-Expansion-Protocol

Limiting Dilution and Clonal Expansion.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working.

GUIDELINES

Important Considerations

The ease at which a clonal cell line can be developed depends on the cell type. It is critical that the cells are healthy and have reached their respective doubling rate before limiting dilution is attempted. For most cell types, waiting 6 days after transfection is typically enough time for the cells to stabilize. Ideally, limiting dilution and clonal expansion should be conducted after 1 passage. Clonal expansion from a single cell can take anywhere from 2-8 weeks depending on the cell type. It is important to continually monitor the health and growth of

clones during expansion.

It is important to consider that edits do not necessarily equate to knockouts. The insertion or deletion (indel) of a number of nucleotides that is not divisible by three will cause a frameshift mutation in the target gene. The resultant protein will most likely be nonfunctional.

Pools with the highest indel frequencies generate clonal cell lines with desired edits most efficiently. Synthego Knockout Cell Pools guarantee a genotypic and protein knockout of at least 50%.

Synthego's Inference of CRISPR Edits (ICE) tool can accurately characterize edited cell pools and clones. Learn more about ICE here.

The protocol workflow is as follows:

1. Limiting Dilution (1 Day)
2. Clonal Expansion (2-8 weeks)
3. Genotyping Clones
4. Storage & Additional Analyses

Additional Information

For an up-to-date list of all Synthego Protocols and other resources, please visit synthego.com/resources

For technical assistance, contact our Scientific Support Team:
Ph: 888.611.6883
Email: support@synthego.com

MATERIALS

NAME	CATALOG #	VENDOR
1.5 ml microcentrifuge tubes		
10 ml sterile reagent reservoir		
Multichannel pipette or similar		
96-well cell culture plates (up to 25)		
Normal growth medium		
Phosphate buffered saline without calcium and magnesium (PBS)		
TrypLE™ cell dissociation reagent	12605010	Life Technologies
Hemocytometer or automated cytometer		
Trypan blue		
Cell strainer (optional)		

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

Limiting Dilution

1 Warm medium at 37°C in a water bath.

37 °C water bath

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 PBS.

5 Aspirate PBS.

6 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 if cells have been passaged).

250 µl TrypLE

7 Incubate the cells for 5 minutes at 37°C and 5% CO_2 or until the cells detach.

37 °C 5% CO2 Incubation

00:05:00 5% CO2 Incubation

8 Transfer well plate back to the biological safety cabinet.

9 To neutralize dissociation, add an equal volume of medium (250 µl) to that of the TrypLETM to each of the wells.

250 µl medium

10 Pipet 3-4 times (more if needed) with 1 ml pipette to break up cell clumps. Cells can also be passed through a cell strainer.

11 Transfer the cells from each condition to a separate sterile 1.5 ml microcentrifuge tube.

12 Count the cells from each condition using a hemocytometer or automated cytometer.

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

14 Determine the number of 96-well plates for each pool. It is recommended to plate one or two 96-well plates for eachedited pool to increase the likelihood that at least one successful knockout clone is generated.

15 Dilute cells in a series of 10-fold dilutions to a final concentration of 0.5-1 cells/100 µl of medium (this will give around 1 cell/well in most of the wells in a 96-well plate) or 120 cells in 12 ml of normal growth medium. Scale according to the number of plates desired. For example, if 5 x 96-well plates are desired for a particular condition, dilute 600 cells in 60 ml of normal growth medium.

NOTE

The concentration of cells can be adjusted (0.5-5 cells/100 µl).

16 Transfer the diluted cell suspension to a large sterile reservoir.

17 Dispense 100 µl/well of the diluted cell suspension to each well using a multichannel pipette.

18 Repeat steps 13 to 17 for each edited population selected for clonal expansion.

go to step #13 **Repeat step 13 to 17**

19 Transfer plates to a humidified incubator at 37°C, 5% CO_2. Change medium as needed.

37 °C **5% CO2 Incubator**

20 Expand & genotype clones (see below).

Clonal Expansion

21 Visually screen plates using a microscope for established single colonies for about two weeks. If a clone imager is available, we recommend imaging each of the 96-well plates 2-4 hours 02:00:00 - 04:00:00 after plating the single cell dilution (before the cell can divide). The cells should be imaged 5-7 days thereafter. This practice enables one to track cell growth over time and confirm that the final clones originated from a single cell.

Mark establishing colonies by circling the well on the lid and/or record in a spreadsheet. Note that different cells (or possibly genetically modified cells) may grow at a different pace. When colonies reach 70% confluence, transfer them to a 24-well plate. During transfer, take out a portion of cells from each colony for genotyping (see below). By continuing to expand only colonies with confirmed edits, one can reduce the burden of culturing and future screening assays. Maintain a few colonies that grow from the Mock or Negative Control conditions to be used as an isogenic wild type comparison to the knockout cell line.

Genotyping Clones

22 To genotype your clones, isolate genomic DNA, PCR-amplify the edited region, and sequence the amplicons via Sanger sequencing. Synthego's Inference of CRISPR edits (ICE) tool can be used to analyze the sequence data.

NOTE

A clone can be reported as wild type (unedited), containing a homozygous edit, or containing a heterozygous edit. A homozygous edit is when both alleles of a chromosomal pair have the same mutation. These colonies should be saved and marked as potential knockouts. A heterozygous edit is when one allele has a mutation and the other does not (wild type), or when both alleles have different mutations (i.e., compound heterozygous). The heterozygous edit can be easily identified by

the overlapping peaks in the chromatogram centered around the region targeted by the guide. Double-stranded breaks repaired by the NHEJ pathway often result in heterozygous edits. This is especially true when editing hyperploid cell lines. Because compound heterozygous mutations may inactivate genes, such colonies should be marked as potential knockouts.

NOTE

Whether the edits are of homo- or heterozygous nature, 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.

Storage & Additional Analyses

23 After identifying putative knockouts by genotype, the cells should be split and expanded for cryopreservation while performing further analysis to functionally validate the knockout. Functional validation of a knockout can be achieved using immunoblot, ELISA or flow cytometry analysis or an appropriate activity assay when available.