Nov 19, 2019

Isolating a Monoclonal Cell Population by Limiting Dilution

Addgene the Nonprofit Plasmid Repository[1]

[1]Addgene

1 Works for me dx.doi.org/10.17504/protocols.io.4xvgxn6

Addgene the nonprofit plasmid repository
Addgene

ABSTRACT

This protocol is for isolating monoclonal cell population by limiting dilution. To see the full abstract and additional resources, please visit the Addgene protocol page.

Sample Data

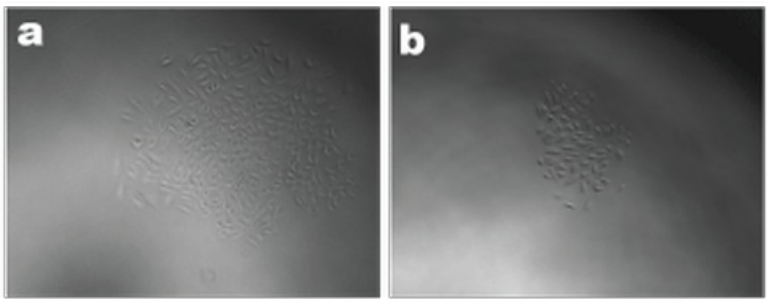

Figure 1: Generation of monoclonal cell lines from expansion of individual A549 cells stably expressing Cas9. A549 cells were transduced with lentiCas9-Blast[1] and then selected with 1 µg/mL blasticidin for 9 days. Single cells were then plated in individual wells of a 96-well plate and left undisturbed for 13 days. (a, b) Colonies formed in individual wells of a 96-well plate after expansion of single cells for 13 days. Note that growth rate differences can be inferred by the differences in colony size.

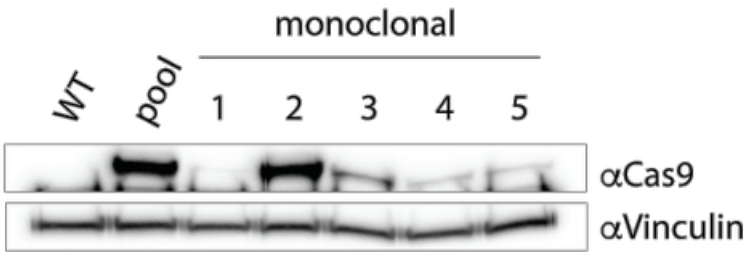

Figure 2: Cas9 expression in monoclonal cell lines generated from A549 cells transduced with lentiCas9-Blast[1]. A549 cells were transduced (MOI = 37) and selected with 1 µg/mL blasticidin for 9 days. Single cells from a stable cell pool were then expanded for an additional 3 weeks under blasticidin selection. Anti-Cas9 Western blotting was performed on whole-cell extracts from from the initial stable cell pool (pool) and from individual cell expansions (monoclonal, 1-5). Wild-type A549 cells do not express Cas9 and are included as a control (WT). Vinculin is a loading control.

[1]lentiCas9-Blast was a gift from Feng Zhang (Addgene plasmid #52962) and is described in *Improved vectors and genome-wide libraries for CRISPR screening. Sanjana NE, Shalem O, Zhang F. Nature Methods. 2014 Aug;11(8):783-4.*

EXTERNAL LINK

https://www.addgene.org/protocols/limiting-dilution/

GUIDELINES

Workflow Timeline

This protocol begins with a stable cell pool. For details, see our **protocol for generating stable cell lines with lentivirus**.

Day 0: (optional) Seed cells for the generation of conditioned medium

Day 1: Seed individual cells in a 96-well plate

Day 2-14: Monitor cells for growth and expand cells

Day 14-30: Analyze and expand monoclonal lines of interest

MATERIALS TEXT

Reagents

- Polyclonal stable cell pool
- DMEM high glucose (or appropriate cell medium)
- L-alanyl-L-glutamine (or alternative stable glutamine)
- Heat-inactivated FBS
- PBS pH 7.4
- Trypsin-EDTA
- Pipette tips
- Serological pipettes
- 96-well cell culture plates
- 40 µm cell strainer mesh (optional)
- 10 cm cell culture dishes (optional)
- 0.45 µm polyethersulfone (PES) filter (optional)

Equipment

- Biosafety cabinet
- Microscope
- Pipetman
- Pipettors
- Incubator
- Hemocytometer or other cell counter

Reagent Preparation

1. DMEM Complete: 10% v/v FBS and 200 mM L-alanyl-L-glutamine
   - To a **500 ml** bottle of DMEM high glucose, add **55 ml** of heat inactivated FBS and **11 ml** of 200 mM L-alanyl-L-glutamine. Store at **4 °C** .

2. Polyclonal stable cell pool: see our **protocol for generating stable cell lines with lentivirus**.

Pro-Tip
Because the polyclonal cell pool is always undergoing selection, it's best to generate monoclonal cell lines from the polyclonal pool as early as possible, though in theory a monoclonal line could be generated at any time.

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

BEFORE STARTING

Considerations Before You Start

- Some cell lines tend not to grow well in sparse/individual cultures due to lack of secreted growth factors, so not every cell type will be able to grow under the conditions described in this protocol.
- Conditioned medium (section one) can be used to enhance the growth of cells in individual cultures. Other methods for enhancing single cell growth, such as using culture medium with increased serum concentrations, may be optimal for different cell types.
- Before starting this procedure, be sure that the cell pool has been sufficiently selected with the appropriate antibiotic so that every cell in the culture has, in theory, been transduced. There are various schools of thought on using selective medium throughout subsequent experiments. When selective medium is used, it may have additional biological effects on the cell line. In the absence of selective medium, there is a chance that the integrated transgene could be lost through recombination. Some researchers choose to maintain cells in selective medium at a reduced antibiotic concentration (e.g., half the concentration that was used for the initial selection). Addgene recommends that the use of selective medium throughout experiments be determined empirically, and that the cell population be monitored regularly for transgene expression.
- This protocol has been optimized for adherent cells, but can be modified for suspension cells.
- The number of monoclonal lines generated using this protocol will vary based on several factors, including the growth properties of the cell lines, the effects of the transgene, and the specific conditions used. Each 96-well plate described in this protocol could, in theory, give rise to a maximum of 48 monoclonal cell lines. When this protocol was performed at Addgene on the A549 cells described in our sample data, each 96-well plate gave rise to 15-20 monoclonal lines.

Generating Conditioned Medium (optional)

1 Seed stable cells such that the next day they will be approximately 50-60% confluent. For Lenti-X 293T cells this is about 2×10^6 cells in a 10 cm dish. Each 10 cm dish should be seeded in **10 ml** DMEM complete, which will generate enough conditioned medium for one 96-well plate.

2 Allow the cells to grow overnight.

3 Replace the medium on the cells and collect the used (conditioned) medium.

4 Remove any cells from the conditioned medium by filtration through a 0.45 µm PES filter or centrifugation at > **500 x g** for **00:05:00** .

Do not use the medium if the cells are overly confluent or the medium is acidified (i.e., the medium appears orange/yellow if using a standard phenol red pH indicator) because the buildup of waste products could be suboptimal for cell growth. This conditioned medium will be used to make a cell solution later in this protocol.

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5 Isolate cells from the stable cell pool by trypsinization and break up any cell clumps into individual cells by passing several times through a serological pipet or by passing through a 0.40 µm cell strainer mesh. Quantitate the cell concentration in this homogenized cell solution with a hemocytometer or other cell counter.

Pro-Tip

Prepare a relatively dilute cell solution (<10^6 cells/mL) before counting the cells. When you ultimately transfer approximately 50-100 cells to the conditioned medium, it will be more accurate if a higher volume of homogenized cell solution is transferred.

6 Transfer cells from the homogenized cell solution into the conditioned medium prepared above to make a new cell solution at a concentration of 5 cells/mL. Prepare approximately **10 ml** of this cell solution for each 96-well plate you plan to seed.

This 5 cells/mL solution will be used to seed the 96-well plate.

If you chose not to use conditioned medium, prepare this cell solution in fresh complete medium.

6.1

Sample Calculation

First, use a hemocytometer to quantitate the homogenized cell solution.

Homogenized cell solution concentration: 4×10^5 cells/mL

To seed one 96-well plate, make 10 mL of a 5 cell/mL solution. Calculate the total cells needed:

Total cells needed: 10 mL × 5 cells/mL = 50 cells

Determine the volume of homogenized cell solution that corresponds to 50 cells:

Volume of homogenized cell solution needed: (50 cells)/(4×10^5 cells/mL) = 0.125 µL

Because this is such a small volume, first make 1 mL of a 1:100 diliution of the homogenized cell solution by adding 10 µL homogenized cell solution to 990 µL complete medium. Instead of transfering 0.125 µL, now transfer 100 times that volume, which is 12.5 µL.

To make the final 5 cell/mL solution, transfer 12.5 µL of the 1:100 dilution to 10 mL of conditioned medium.

7 Transfer **100 µl** of the 5 cells/mL solution into each well of a 96-well plate.

By doing this, you are seeding the plate at an average density of 0.5 cells/well. Seeding an average of 0.5 cells/well ensures that some wells receive a single cell, while minimizing the likelihood that any well receives more than one cell.

Pro-Tip

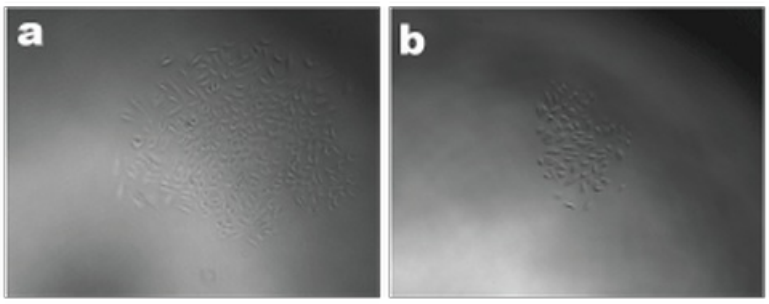
In each 96-well plate, seed one of the corner wells with ~1000 cells. This will help you focus the microscope later when scanning the plate for individual cells, which can be difficult to find.

8 Leave the cells undisturbed in an incubator for 7-14 days. After ~7 days, scan the plate for cell growth. If you do not observe any colonies, continue incubating the cells.

Pro-Tip
Use the well with 1000 cells to focus the microscope, then you can easily scan the plate for individual cells, which should be on the same focal plane.

9 Scan the entire plate and make note of each well in which you see growth. At this time, the cells will appear as colonies in the well (Figure 1). You will be able to tell if there was more than a single cell seeded in a well because you will observe more than one colony. Wells with more than a single colony do not contain a monoclonal population and should be discarded.

Pro-Tip
When scanning the plate, be sure to look thoroughly within each well, especially in the corner of the well as cells tend to settle there.



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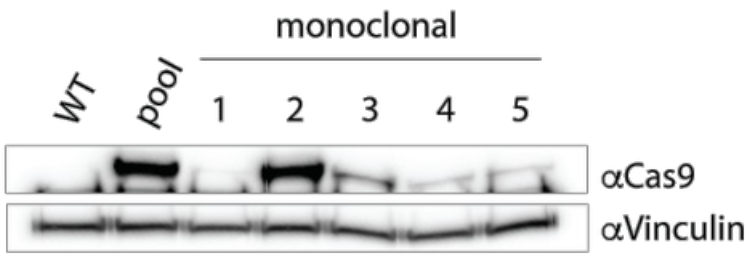
10 Once the cells have expanded but before they become over-confluent, trypsinize the cells and expand them to larger culture dishes, being sure not to cross contaminate the independent monoclonal lines.

Pro-Tip
When trypsinizing cells from the 96-well plate, use a microscope to ensure that the cells have detached from the plate and can be isolated. When trypsinized, detached cells will exhibit a rounded morphology.

11 After the monoclonal lines have been sufficiently expanded, screen the lines for transgene expression and/or other phenotypes. For example, perform Western blotting to screen for lines with the highest or lowest transgene expression (Figure 2).



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