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Generating Stable Cell Lines with Lentivirus

Addgene the Nonprofit Plasmid Repository[1]

[1]Addgene

1 Works for me

dx.doi.org/10.17504/protocols.io.4xtgxnn

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ABSTRACT

This protocol is for generating stable cell lines with lentivirus. To see the full abstract and additional resources, please visit the Addgene protocol page.

Sample Data

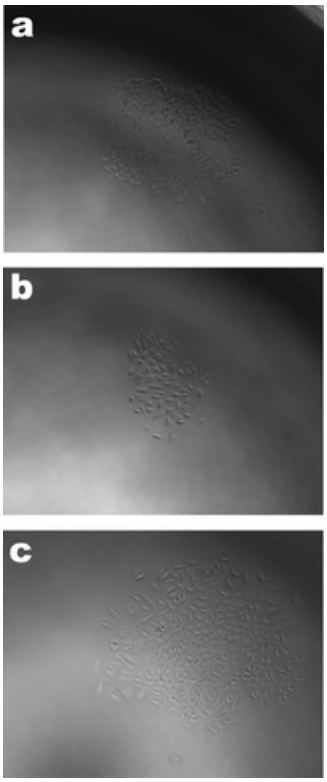

Figure 1: Generation of monoclonal cell lines from expansion of individual A549 cells stably expressing Cas9. A549 cells were transduced with lentiCas9-Blast and then selected with 1 µg/mL blasticidin for 9 days. Single cells were then plated in individual wells of a 96-well plate and left undisturbed for 13 days. (a, b, c) Colonies formed by expansion of single cells for 13 days. 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/generating-stable-cell-lines/

GUIDELINES

Workflow Timeline

Day 0: Seed and Transduce Cells

Day 2-3 (am): Remove media, replace with fresh media containing selection reagent

Day 3-14: Change media as needed

Day 14-18: Expand and harvest stable cell lines

MATERIALS TEXT

Reagents

- DMEM high glucose
- L-alanyl-L-glutamine (or alternative stable glutamine)
- Heat-inactivated FBS
- Polybrene
- PBS pH 7.4 without calcium or magnesium (cations can affect the attachment of adherent cells)
- Microcentrifuge tubes
- 6-well dishes
- Pipettes
- Pipette tips
- Titered lentivirus containing your sequence of interest

Equipment

- Biosafety cabinet
- Pipetman
- Pipettors
- Incubator

Reagent Preparation

DMEM Complete: 10% v/v FBS and 4 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** .

Pro-Tip
Different brands and lots of FBS can promote or inhibit transfection. Test a variety of brands and lots of FBS to find one suitable with your protocols. FBS can be purchased already head inactivated or it can be inactivated in the lab by heating to **56 °C** for **00:30:00** .

SAFETY WARNINGS

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

BEFORE STARTING

Determine the optimal dose of selective reagent for your target cell line. To do this, treat target cells with a range of doses of antibiotic and determine the lowest dose that kills all of the cells.

Additional Considerations Before You Start

- The health of the the target cell line is critical for obtaining accurate results.

- Check the cells for mycoplasma regularly
- Do not over or under-grow your cells.
- Thaw a new vial of cells after 20-30 passages.

- Do not add penicillin/streptomycin to the media.
- Infection efficiency will vary between cell lines.
- It is not recommended that lentiviral supernatants be subjected to multiple freeze-thaw cycles.

Transduction

1 Prepare a batch of DMEM complete + 10 µg/mL polybrene by diluting **20 µl** of 10 mg/mL polybrene into **20 ml** media.

2 Rapidly thaw the lentiviral aliquot at **37 °C** .

3 Prepare a range of dilutions of the lentivirus in DMEM complete + 10 µg/mL polybrene.

Note, this is just a sample of possible dilutions. You may want to try higher/lower dilutions depending on your downstream applications. If you've titered your virus beforehand, you can narrow this range according to the results of your titration.

Dilution	Volume of Lentivirus (µL)	Volume of DMEM complete + 10 µg/mL polybrene (µL)
0	0	500
1:5	300	200
1:10	150	350
1:50	30	470
1:100	15	485
1:500	3	497

Mix dilutions well

4 Add **0.5 ml** of a single viral dilution to each well.

Each well gets one dilution, so a 6-well plate will hold 5 dilutions plus one 'no virus' control well.

5 Perform a "reverse transduction" by seeding 50,000 cells into each well of the 6-well dish. These cells will be added to the wells that already contain 0.5 mL of virus solutions at various dilution.

Make sure to use the polybrene-containing media to make the cell solution in this step.

To seed the cells as described in 5.1 and 5.2.

5.1 Prepare a batch of cells as follows: Dilute 350,000 cells into a total volume of **7 ml** of DMEM complete + 10 µg/mL polybrene. Mix well by pipetting or inverting the tube.

5.2 Aliquot **1 ml** of cell suspension (i.e., 50,000 cells) into each well of the 6-well dish. This brings the total volume in each well up to **1.5 ml** . Since all the media in these wells was made with DMEM complete + 10 µg/mL polybrene, the final concentration of polybrene in each well should be 10 µg/mL.

Pro-Tip
Transducing too many cells relative to the number of virus particles reduces the transduction efficiency, resulting in massive cell death upon antibiotic selection. The number of cells transduced should be enough that they can grow out in a reasonable amount of time, but not so many that they vastly outnumber the virus particles.

6 Incubate the cells with the virus for **48:00:00** - **72:00:00** .

Selection

7 Gently aspirate the media from the cells. Add **1.5 ml** of DMEM complete containing the appropriate antibiotic.

This is the beginning of the selection process, which will begin the selection of a stable cell pool.

8 Observe the dish every day to ensure that the cells in the untransduced well (0 µL lentivirus, above) are dying. Perform regular fluid changes the monitor the growth of the cells.

Pro-Tips

- Depending on the efficiency of your transduction, you will see different degrees of cell death upon antibiotic selection. It is important to monitor these cells regularly and replace the cell media. Cell death by some cells in the culture may adversely affect the surviving cells in the culture, so it is important to do regular fluid changes and maintain optimal growth conditions for the surviving cells. Even in the absence of cell death, the cell media should be changed every 2-3 days to maintain the dose of antibiotic, which may not be stable at **37 °C** .
- To achieve a stable cell pool, the antibiotic selection should last at least as long as it takes the control (untransduced) cells to completely die. After that, the cells may undergo additional seleciton while the population expands. At this time, some researchers reduce the concentration of the antibiotic in culture, or remove the antibiotic entirely. If the antibiotic is reduced or removed from the culture, check the cells regularly to confirm transgene expression.

9 As polyclonal populations of resistant cells start coming through and the individual wells become confluent, expand into larger vessels. A confluent well of a 6-well dish can be expanded into a 10 cm dish. A confluent 10 cm dish can be expanded into two 75 cm^2 flasks.

Pro-Tip
This selection method results in a polyclonal cell population, meaning that the transgene has integrated in different locations in the various cells in the culture. This is because lentiviral integration is random. Given the MOI of the lentivirus used, cells may also exhibit varying numbers of integration events. In other words, if an MOI > 1 was used, some cells may have 1 copy of the transgene, while others have >1 copy of the transgene in their genome. This can result in varying expression levels of the transgene from different cells in the population.

10 Once the polyclonal populations are growing well and have been sufficiently expanded, prepare cell stocks and/or harvest to test for protein expression.

Typically cells transduced with lower dilutions of the virus will have higher levels of expression. Consider expanding populations transduced with a variety of dilutions and pick the population that has the most desirable level of expression.

11 Over time, transgene expression in a polyclonal population may drop. This is because cells that express high levels of the transgene may have reduced growth rates, especially if that transgene is toxic. Eventually, the rapidly growing low-level transgene expressors may take over the culture. To overcome this, consider generating monoclonal lines from the early polyclonal populations.