

Protocol for cloning MEF lines by limited dilution

Medium used throughout (in our lab at least) is DMEM (we get from Invitrogen but shouldn't matter) with 10% fetal bovine serum (FBS; we get from Atlanta Biologicals but shouldn't matter). Note that we grow all our cells in the absence of antibiotics to avoid introduction of mycoplasma. We screen for mycoplasma regularly by staining fixed cells for DNA (DAPI/Hoechst) and looking for extranuclear stain.

This has been optimized for MEFs and will vary a bit with different cell types.

1. Thaw cells (1 mL) by taking out of liquid nitrogen (or -80°C freezer), putting at 37°C water bath to thaw quickly and minimize time spent in DMSO. Add thawed cells to 9 mL DMEM with 10% FBS and put all into 10 cm culture dish. Let recover at least overnight and grow to near confluence (density not critical but check that they look "happy" and not overgrown/clumping).
2. Add 100 μ l of DMEM+FBS to each well of two 96-well plates. Put plates in incubator to warm medium. This and later cell addition to 96-well plates is best performed using a 12-channel pipette and a individually wrapped, sterile, disposable trough to take up solution. Single channel pipette obviously also works just more tedious and prone to contamination.
3. Resuspend cells and count with hemocytometer.
4. Dilute cells into 10 mL of medium to 30 and 50 cells/mL (or 3 and 5 cells/100 μ L). Use to plate your 96-well plates with 100 μ l/well. This will yield two plates, one with 3 cells per well and the other at 5 cells per well.
5. Return to incubator at 37°C with 5% CO₂. Note that cells survive poorly at very low densities so 3-5 cells/well is optimized for enough survival to yield individual/"pure" clones without too many mixed cell populations. If you plate at 1 cell/well, expect about half as many clones to grow up.
6. Two days after plating, start monitoring each well. Any well with 2 or more colonies should be marked and ignored in the future. Those with a single clone growing marked for later expansion.
7. When a well approaches confluence, take up and use all to plate into one well of a 6-well plate. Within one month of plating, any clone that is going to grow is up and moved to 6 well plates. To re-plate cells from a 96-well dish to a 6-well dish, wash cells quickly with 100 μ L trypsin (e.g., Gibco 0.05% trypsin-EDTA), add a fresh aliquot of 100 μ L trypsin, let sit at room temperature for a few minutes before adding 200 μ L DMEM to resuspend cells (with trituration) and transfer into 6-well dish containing 2mL DMEM. Note that this cloning procedure using 2 96-well plates typically yields ~30-40 clones, which is well more than needed to screen for indels. It is not a bad idea to screen colonies arising at different times after plating in case doubling time is a phenotype of your KO lines.
8. When cells in wells of 6-well plates are near confluence, take up cells with 1 mL trypsin/EDTA, collect and add 2 mL medium, then use this 3 mL cell suspension to transfer each clone into one 10 cm plate (1.5 mL; for further expansion and freezing/storage) and 2 wells (0.75 mL each; for DNA sequencing from genomic DNA) of a 6 well plate.
9. When near confluence, take cells from 10 cm plate, suspend with trypsin into a total of 10 mL DMEM/FBS, pellet cells and resuspend into 1 mL DMEM/FBS. Aliquot 0.45 mL into each of two cryovials, add 50 μ l DMSO (final = 10% DMSO) and freeze. Collect cells from 6 well plates, pellet and freeze pellets for analyses. We use one of these two tubes for purification of genomic DNA for PCR and DNA sequencing. The other can be used as backup or for T7 endonuclease assay.

10. We prefer to screen for indels by DNA sequencing, after PCR amplification of the region around the targeted exon. If screening by immunoblot or other techniques, feel free to modify how you freeze down cell pellets.
11. It is a good idea to record dates of cloning and to keep track of passage number. This may prove to be important if you have cell cycle defects or other phenotypes that provide a strong selective pressure which can cause your phenotype(s) to migrate over time.
12. Also note, particularly if you don't do this much, that every clone is different and may grow at a different rate than parental, or be smaller in size, or different in appearance. This may be the result of random cloning differences or a phenotype of your KO. This is another reason why it is important to have several different KO lines for comparison.
13. You now have pure clones and ideally at least two independent clones from each of at least two different guide RNAs, to help ensure against off-target effects of CRISPR. Four clones yielding the same phenotype, even though they may vary in severity is strong evidence of a phenotype. That said, it is very important that you plan "rescue experiments" in which you put back or re-express the deleted gene product. If expression of the gene/protein deleted reverses or partially reverses your phenotype you have again demonstrated that the phenotype is not an off-target effect and you are ready to publish your findings. We have used both plasmids and lentiviruses to express the deleted protein. MEFs don't transfect well, typically not better than 20-40% of cells transfect. While we can get >90% of cells expressing exogenously after lentivirus transduction.