MONIKA'S CLONAL CELL LINE PROTOCOL

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Day 1 Plating Cells

1. Plate cells in a six well dish such that they are at 60-80% confluent for the actual transfection the next day (I can't remember exactly what this is but I suspect it is around $3x10^5$ cells/well in a six well dish). Keep in mind this is plenty of cells, as one doesn't need to use large dishes or lots of DNA for making stables; in fact you are shooting for colonies so it is better not to get a super-transfection to ensure clonality (that is the optimistic way of looking at poor transfections).

Day 2 Transfecting Cells

I like to change the media on the recipient cells first thing in the morning before a transfection, pray to the transfection gods and hope for the best. You are welcome to use my ritual if you are uncomfortable with making one up yourself but I strongly recommend sticking with one ritual for each technique.

3. ***Make DNA-liposomes.***
   I set these up using OptiMEM (OMEM) from InVitrogen (cat #11058-021) (PBS probably works as well). Warm 50mls to 37°C and warm about 10mls to room temperature for setting up the liposomes. I use 2-2.5ul Lipofectamine 2000 (InVitrogen, cat #11668019) for each 1ug of plasmid DNA. For a 6 well dish use 2ug of DNA for a 100mm dish anywhere from 10-20μg works well. Set these up in a total volume of 200μl and 1000μl respectively.

   NEVER use polypropylene tubes, ALWAYS use polystyrene tubes
   (I use Falcon 2058, Fisher cat# 14-959-1a).

   a) For each transfected well aliquot a total of 2μg of DNA in a total volume of 100μl of OMEM in Falcon 2058 tubes. Leave at room temp for 5 minutes.
   b) Make a "master" mixture of OMEM and Lipo2000 in a Falcon 2058 tube with 5μl of Lipo2000 for each 95μl of OMEM. Let stand at room temp or 5 minutes.
   c) Add 100μl of the Lipo/OMEM mixture to the DNA/OMEM mixture *dropwise*. Let stand at room temp for 10-20 minutes. During this time rinse your cells off once with warm OptiMEM (GIBCO) (or PBS), add 2ml OptiMEM and place them at 37°C.

4. When you are ready to transfect you DNA, remove the media from the cells, add 800μl of warm OMEM to the DNA/lipo mixture and add dropwise to the cells. Incubate at 37°C for 1-2 hours. Call this t=0. Agitate the plate gently every 30minutes to distribute the liquid over the monolayer (if paranoid). Note: 3T3 cells can take this stuff really well but Rat1 cells appear to have a problem with Lipofectamine (I am not sure about Lipofectamine2000 however).

5. 3T3 cells - Add 2ml of media (again I use OMEM as I care nothing about finances) with 20% FCS (no antibiotics) and incubate overnight.
   Rat1 cells – Remove DNA/lipo mix immediately after 2hours. Rinse the cells twice with 10%FBS in media without antibiotics. Then add back full media with antibiotics and incubate overnight.

Day 3 Fresh media.

6. The next day (about 19 hours later) split each well into two 100mm dishes. I would do a 1/100 and 1/5 in each dish respectively.

Day 4 Selection

7. At around 42-72 hours after t=0 add G418 to a final concentration of 400μg/ml (if using puromycin use 3-4μg/ml). For Neo selection you can add it on day 3, you can add puro around 72 hours after the DNA is added.

   For neo selection cells should be proliferating. It doesn't really matter for puro selection, as it will wipe out everything. I like to select the cells in Neo or Puro for the amount of time it takes to kill every single cell on a mock transfected plate twice (i.e. after all the cells are dead add drug to another naive plate and wait for it to die as well)

8. At some point around 8-14days after the transfection has started one might want to think about picking clones. Ideally, this should be done with cloning rings (which can be purchased ready to go). Alternatively one can pick clones with a pipetman, which takes a bit of practice and doesn't yield as many cells. In these two cases I like to pick them into a 12 or 6 well dish and maintain them under selection. A third method involves serial dilutions into 96 well dishes immediately after selection. One can also use FACS sorting into 96 well dishes dropping a single cell in each. To really ensure that you have a clone from a single cell you can go through two rounds of cloning. (In reality most are not willing to do this and will clone them once).

Key points

a) Cells are best transfected at fairly high density but still subconfluent (i.e. 80%).
b) Cells should be split the day after transfection to allow for a couple rounds of DNA synthesis.
c) Always carry three extra wells (plates): one without any treatment, one mock transfection (no DNA just liposomes) and one positive control with and empty vector. For some cells (like Rat1 fibroblasts), the treatment with liposome alone can cause death. The positive control will serve as a useful control to let you know if your gene has toxic effects.
d) If cloning via rings or picking the cell colonies need to be well spaced. This is why a 1/100 dilution works well. If you cannot get clearly individual cells then clone as best you can and put them directly into 100mm dishes, here they should grow as individual colonies.