Cloning cells with the agarose method - Lindberg Lab

This is a method to pick 48 individual colonies from a plate of antibiotic-selected cells in 15 minutes without the use of those tedious cloning rings. It is based on bacterial plate agarose overlay methods. We have been using this method for over 20 years in the lab with excellent success, making over 300 cell lines.

Prepare ahead of time:

2% agarose in water. Make 100 ml in a tissue culture glass bottle (ie has not seen detergent) by repetitive microwaving (with loose top!) of 2 g molecular biology grade agarose and 100 ml Millipore water; and repeated swirling after heating (gloves needed!) until dissolved. Keeps on the shelf. (Sterilized by boiling.) *Re-microwave for each use.*

2x DMEM Make 50 ml of 2x DMEM by diluting 10x DMEM (Sigma D2429) 5x with water. So, 10 ml of 10x DMEM, 2.5 ml of 7.5% bicarbonate, 0.5 ml 1 M HEPES stock and 0.5 ml of 200 mM glutamine stock; bring to 50 ml with Millipore water and pass through a sterile filter. (Can add Pen-Strep, depending on your sterile technique). Keeps in the refrigerator 6 months.

10 cm dishes with 50-100 clones each (or fewer). Clones must be <u>well-separated</u> but each clone should have at least 50 cells. Hold plate up to the light and carefully mark 50 or so clones with a sharpie on the bottom of the plate.

Truncated 200 ul pipet tips, sterile (USA Scientific 1011-8410**) a 200 ul pipetman, a 48-well plate**
In a pinch you can cut a few mm off regular tips and then autoclave. But it isn't worth the time.

Growth medium (with extra serum, ie 20%). Again, you may want to add Pen-Strep, though at some point you should eliminate this for proper cell culture technique. Some cell lines may require conditioned medium or extra serum when first cloned.

Clone Picking Method:

Briefly warm 3 ml of 2x DMEM in a 37 degrees bath in a sterile 50 ml tube. (should be at room temp at procedure start).

Microwave the 2% agarose (opened slightly) until homogenous and melted and put in hood hot. If boiling, it will take a while for it to cool to the correct temperature (about 50 C); that's ok you have stuff to do.

Organize the other reagents and put them in the hood.

Put 100 ul of growth medium in each well of a 48-well plate.

Circle your colonies using a dark sharpie out of the hood. It is easy to do if you hold the plate up to the light out. (Don't tilt it!)

Then going back to the hood, aspirate off the medium - you must remove ALL of the medium. Cover the plate again (leaving it open dries out the cells) and work quickly from now on.

Add 3 ml of hot melted agarose to the 3 ml of 2x DMEM, close the tube, swirl, and test the temperature on your cheek. Should be warm but NOT hot (think baby bottle**)**; clumps mean it is too cold (start over again). Quickly pour this solution right onto the middle of the plate. It will spread out and harden within minutes. (If using different size plates, adjust volumes. You want only a thin layer of agarose on the cells so oxygen can diffuse thru).

Punch out colonies with your pipetman, using a new tip for each and working with two hands (one to stabilize). Put each clone into separate well, shaking cells off of the agarose plug by repeated up-and-down pipetting, as per the YouTube video ("Cloning Cells with the Agarose Method", by ilindb). Bubbles tell you that particular well has already received a clone. Be careful not to release the pipetman sharply, as your agarose plug will go up into the shaft.

The most critical parameter is getting the temperature right. You can practice out of the hood with a thermometer if you like. If too hot, cells do not survive. If too cold, the liquid will not cover plate and colonies will dry out.

Sterile technique tips: do not work over your plates. Keep the recipient plate covered; remove cover only when ready to add a cell plug.

Add more enriched growth medium to each well (halfway up) and place in incubator for one week or longer, depending on growth rate of your cells. Some cell lines need conditioned medium to grow well (filter first to get rid of cells).

Note: not all clones will survive, though most will (90% of HEK and CHO, 70% of AtT-20).

Functional Testing

When the first dozen or so wells are 70% confluent, perform a functional assay. First score the wells from 1 (least confluent) to 4 (most confluent) by writing on the well. You need to do this now, because in 2-3 days all of the wells will be confluent.

For example, if your protein is secreted, you can add 100 ul of Optimem overnight and remove this medium the next day for an ELISA, Western blot, or enzyme assay (a spacing-adjustable pipetman really helps to make the transition from 48 to 96 well plates: I use a VIAFLO 300 ul- it is pricey, but saves a LOT of operator time if you use 96-well plates for ELISAs). As soon as possible, trypsinize and split the positive wells- even if you have no data yet. If your original wells become overconfluent before you have results, the cells may die.

I usually assay clones in two sets of 12-20, as they become confluent, and I number the colonies in the order that they arise.

If your protein is not secreted, you will need replicate plates. When you set up the replicate plate from trypsinizing your mother plate, try to normalize the wells by differential cell addition. You can lyse these cells in stop solution for Western blotting; or make RNA for PCR analysis of expression; or otherwise select clones with the attributes you want.

Correcting for number of cells you originally had, as per score above (or Ponceau stain or some other normalization method), select 3-4 clones- best, good, good, and light expression). For overexpression, usually you will want the most expression per the least cell number. But always keep a range and especially lightly expressing clone- you never know when the effect of expression level itself will need to be tested!

Again, after selection, trypsinize wells containing the best clones and put in T-25s (with enriched growth medium). **RE-FEED the 48-well plate!** This is your backup!

Freeze your proven clones before doing ANYTHING else. Make 3 ampoules from each.

I suggest both an independent secondary functional test and a backup freeze of the most popular clone of at least 6 amps.

Total time from cell transfection to freezing amps- about 4-5 weeks, depending on cell growth rate.

NOTES

If you foolishly have not used a 10-cm plate or a peelaway flask during your selection to get clones but instead a regular T25, you can punch multiple holes around the top of the flask with a soldering iron and use a (sterile) forceps to pull off the top of the flask- it will be smoky, but the cells do not seem to care. Proceed with the agarose.

We most often use Western blots of cells/ and or medium to determine protein overexpression. For knockout, if the protein is inabundant, you may need to analyze mRNA with a PCR-based assay. Choose primers that flank an intron so DNA contamination is not an issue. We use the Zymo RNA kit followed by the iScript kit for reverse transcription.