Troubleshooting Forum

Molecular Biology Techniques Q&A

Clone Selection in Weakly Adherent Cells

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "Cell Culture" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

How can I select for stable GFP expression in weakly adherent cells?
(Thread 31171)

Q I want to generate Lewis lung carcinoma (LLC) cells that stably express GFP. The plasmid I am working with confers neomycin resistance, so it is selectable with G418. The first thing I plan to do is generate a G418 kill curve. I have done some reading on how to set this up, but I haven't found any reference stating what number of cells I should use. I want to span 12 concentrations of G418 between 100 and 1200 $\mu\text{g/mL}$ and perform this in duplicate on a 24-well plate. Does anyone know what concentration of cells will work best for this?

My other concern is that LLC is a mixed population of cells. Most grow in suspension, but some are adherent, so I can't rely on loss of adhesion to indicate viability. Can I monitor viability while replacing the G418 and media by taking an aliquot and counting the viable and dead cells with trypan blue? Alternatively, is there a more efficient way to accomplish this?

Once I discover the appropriate amount of G418 to use, I plan to transfect these cells using Lipofectamine 2000. I haven't decided how to isolate the GFP-positive G418 resistant clones, but I heard that flow cytometry and methylcellulose in the media make picking clones with a pipet much easier.

A I hate to count cells, so I use the MTS assay for determining the percentage of live cells. When the cells are 10% confluent on a 48-well plate, I treat them with G418 for 3–7 days and then run the MTS assay. In general, 500 $\mu\text{g/mL}$ G418 is a concentration that works well for most cell types I have used. If you are going to count the cells using trypan blue, a cell density of ~50% confluency may give better results.

Flow cytometry may be the only way to isolate nonadherent cells expressing GFP.

Q I'm relatively new to cell culture, so I have some concerns about this cell line. Although I see both adherent and nonadherent cells, can I assume that I have a collection of genetically identical cells? Are the differences in how they grow and in their cell morphologies just due to these cells being weakly adherent? I need to be certain that the clones resulting from transfecting these cells will be more or less identical to the original cells. In other words, if I isolate a GFP-positive clone that is not adherent, will some of its progeny be adherent—like the original population of cells—when I culture it?

A According to the ATCC, cells in this line are loosely adherent and floating. If you select for only adherent cells, I don't know if some will begin to float later. After transfection, you can try selecting adherent and floating cells separately. For adherent cells, the selection will be easier since the dead cells will be removed with the change of medium. For floating cells, you will have to use flow cytometry to separate the GFP-positive cells from the dead ones. About 2 weeks after selection of your clones, you should know if some of the adherent cells begin to float, and vice versa.

Q I thawed the cells in the ATCC vial and grew them in T-25 culture flasks for 3 days, then I split them into T-75 flasks so I could freeze some vials. Before splitting, I did a viability count to monitor the health of the cells. To split the cells, I collected them by pipetting

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media onto the sides of the flask and tapping gently to lift the adherent cells. Trypsinization was not necessary. Then I spun the cells at 1200 RPM for 5 min at 4°C and discarded the supernatant. I resuspended the pellet in fresh warm DMEM and took out an aliquot for viability counting using trypan blue.

After the split, I found ~50% dead cells in each of my four cultures. I don't think 50% viable cells are ideal for storing in freezing vials. I am afraid that I collected dead cells as well as living cells when spinning down the cultures. Since my cells are mostly growing in suspension, how can I collect and discard the dead cells? Is it possible to retain them in the supernatant?

A Your centrifuge force is too high and may actually cause cell death. You can spin down your cells at 100× g for 5 min. With continuous culture, the dead cells will be diluted out. It is possible to separate dead cells from live cells using centrifugation. One method is to layer 18 mL cell suspension onto 12 mL Ficoll-paque mixture in a 50-mL Corning centrifuge tube. Centrifuge these tubes at 400× g for 15 min. The live cells will collect at the interface and the dead ones will form a pellet at the bottom of the tube.

Q If I want to feed my cells while they are plated on a 6-well plate, is it okay to spin the plate for 5 min at 800 RPM to pellet the cells, then carefully replace the media and gently resuspend the cells in the well itself?

A I do not know if it is possible to spin the plate; it will be difficult to pellet cells on such a big surface area. It is better if you transfer the cells to 2-mL microfuge tubes and spin at 100× g for 5 min.

Q You said that you plate the cells and grow them to 10% confluence before treating with G418. Do the cells continue to grow after the addition of G418? Do you start with so few cells because you expect to see 100% confluent cells after 3–7 days?

A Yes, cells do grow continuously if the G418 is not at a lethal concentration. By plating cells at low density, you can easily see the difference between the lethal and nonlethal concentrations.

Most cells are slow to die even at 500 µg/mL G418. I usually start the selection at the

lethal concentration instead of starting at low concentrations and increasing the dose gradually.

Q In that case, do you need to split the cells at some point during the course of culturing to collect data for the kill curve? Or do you just let them grow until they are confluent, and at that end point count the dead and living cells?

A You will need to split the cells 1:5 before selection and split again when they are confluent. For adherent cells, the dead cells will be removed when the medium is changed. Only G418-resistant cells will continue to grow. With your mixed culture, it will be much more difficult to separate the living floating cells from the dead ones.

A Another solution to the problem of a mixed culture including floating and adherent cells is to use plates coated with

lysine, ornithine, laminin, collagen, or fibronectin. One or more of those coating molecules should facilitate most of your cells in adhering to the plate. This will make G418 selection easier to observe and the isolation of transfected clones much more straightforward. Poorly attached cells tend to move around on the plate even when it isn't repeatedly disturbed, which can result in mixed colonies and require several repeated sub-cloning steps to resolve.

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BioTechniques 51:163-165 (September 2011)
doi 10.2144/000113732

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