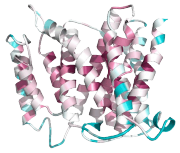
Stable Cell Line Production



INTRODUCTION

There are many different approaches for establishing stable cell lines, depending on the type of expression you're interested in (inducible vs. constitutive) and the construct that you are incorporating. This protocol is specific for the establishment of cell lines that constitutively express GFP-tagged proteins. The end result that you're looking for is a population of cells in which >95% of the cells are expressing your fusion protein at approximately the same level. This allows large-scale biochemical analyses of the fusion protein and monitoring of localization throughout the cell cycle, and greatly simplifies many other microscopy techniques as well.

PROTOCOL

1. Clone your protein of interest into one of Clontech's Living Colors vectors (EGFP, EFYP, ECFP, etc.) These vectors encode kanamycin resistance for selection in bacteria and neomycin (G418) for selection in mammalian cells.
2. Characterize expression of the fusion protein in your cell type of interest using transient transfections (we use HeLa cells for most of our stable cell lines, and QIAGEN's Effectene transfection reagent to give >80% transfection efficiency). You want to be sure that your fusion protein is full-length and not degraded (can probe with GFP antibody on a Western blot; Roche's monoclonal anti-GFP works well at 1:1000). If you have an antibody to the endogenous protein you can also compare the level of expression of the fusion protein to the endogenous protein. Many proteins mislocalize when overexpressed. Include coverslips in the dish that can be fixed to check localization by fluorescence microscopy.
3. To set up for the stable cell line selection, split cells and transfect with your construct. The following day, replace the standard media with media containing G418. Over time this will select for cells that have stably incorporated the GFP plasmid into their genomic DNA. The amount of G418 required to kill cells not expressing the construct will vary from cell line to cell line. We have titrated the amount required for our HeLa cell lines, and this can be a starting point for other cell lines. Initially we use 400 ug/ml G418 in the media when selecting for stable clones. This selection can take anywhere from 1 to 2 weeks. Carefully change the media in the dish every day, taking care not to pipette directly onto the cells. At some point there will be a massive cell death and most of the cells will wash off the bottom of the dish, leaving colonies of stable cells behind.
4. To pick colonies, prepare 24-well dishes with 1 ml media containing G418 in each well. Rinse the dish with PBS and then add warm PBS containing 5% trypsin (1 ml standard trypsin-EDTA plus 19 ml PBS). Colonies can be picked on an inverted light microscope, although an inverted fluorescence microscope allows you to monitor the fluorescence signals in the colonies that you're picking. Using a Gilson pipette with a sterile yellow tip, lower the tip to the surface of the colony of interest and scrape and suck gently until you've pulled it up into the tip. Transfer to a well in the 24-well plate. Repeat with other colonies.
5. In a day or two, when the wells are confluent, rinse with PBS and trypsinize with 100 ul of trypsin-EDTA. Split into one well of a 6-well plate (for passaging) and one well of a 24-well plate that also contains a 13 mm coverslip. These coverslips can then be fixed in a day or two to screen the colonies to decide which should be put down and which are worth keeping (and whether they require further subcloning to reach > 95% homogeneity).
6. All clones that you are interested in keeping should be passaged from the 6-well plate into at least 3 dishes: one for freezing as Passage 0 (in case something goes horribly wrong in the future you can always go back to this one), one for further passaging (if the line is already clonal), and one for subcloning (if required). Seed at clonal density for subcloning (e.g. 10 ul from a 1:5 split of the 6-well dish into a 10 cm dish).
7. When you have your clone or subclone of interest, you can then use a lower amount of G418 for maintenance (200 ug/ml for HeLa cells). Keep track of passage numbers (stability of the clones will vary and some may be thrown out after a few passages). Periodically freeze down samples from early passages so that you can always go back to them.