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## Creating a stable cell line expressing gene specific shRNA via transfection

Stable integration of a HuSH-29 construct into a cell line allows you to study the effects of knockdown over a longer time course than transient transfection studies would allow. Stable cell lines can be clonally produced, assuring that every cell in the population contains the HuSH-29 plasmid.

### STEP 1. TRANSFECTION

Transfect the cells using the protocol above for transient transfection. After transfection, do not change the medium until the next day.

### STEP 2. SELECTION

The day after transfection, change the medium to fresh growth medium containing 0.5-1 ug/mL. Continue to grow and passage the cells as necessary, maintaining selection pressure by keeping 0.5-1 ug/mL puromycin in the growth medium. After 1-2 weeks, a large number of the cells will be killed by the puromycin, indicating that they did not take up or have lost the HuSH-29 plasmid with the puromycin resistance cassette. The cells that remain growing in the puromycin-containing medium have retained the expression plasmid, which stably integrates into the genome of the targeted cells.

### STEP 3. CLONAL SELECTION

Select clonal populations of cells by transferring a well-isolated single clump of cells (the clonal ancestor and cells divided from it) into a well of a 24 well plate; repeat to select 5-10 clonal populations. Continue growing these cells in selection medium for 1-2 additional passages. At this time, each well contains a clonal population of stably transfected cells, which can be maintained in normal growth medium without the selection pressure of puromycin. These populations can be used for experiments or stored under liquid nitrogen in growth medium with 10% DMSO and 20% FBS for future use. You can verify the integration of the shRNA plasmid by isolating total RNA from the cells and performing RT-PCR to amplify the hairpin insert.

## Infection of gene specific shRNA into cells via retrovirus

This protocol has been optimized to produce infectious, replication-incompetent retroviral particles that can efficiently transfer genes into a variety of mammalian cell types *in vitro* or *in vivo*. Retroviral infection allows for the delivery of shRNA plasmids to most dividing mammalian cell types, including many difficult-to-transfect cells.

### STEP 1. PREPARE THE PACKAGING CELLS

The day before transfection, plate the retroviral packaging cells* of appropriate tropism at recommended dilutions. Distribute cells evenly about the plate, and incubate at 37ºC in 5% $CO_2$. Since subconfluent cells are best suited for transfection and potentially generate the highest viral titer, plan to grow the cells to approximately 60-70% confluency. Plating approximate 3 x $10^6$ cells per 10 cm cell culture dish should achieve this level of confluence by the following day.

**Choose a packaging cell line whose species specificity is compatible with your target cell line. OriGene routinely uses PT67 (Clontech) or Phoenix (Orbigen) cells for this purpose.*

### STEP 2A. TRANSIENTLY TRANSFECT THE PACKAGING CELLS

Transfect the shRNA plasmids** into the packaging cells by following the procedure above for transient transfection. Incubate for 2-3 days at 37ºC in 5% $CO_2$, and proceed directly to step 3.

***You may wish to do a parallel transfection with a marker plasmid (such as one that expresses EGFP) as a positive control for transfection efficiency.*

### STEP 2B. STABLY TRANSFECT THE PACKAGING CELLS (SKIP IF YOU PERFORMED STEP 2A)

Transfect the shRNA plasmids individually (and separately, a plasmid without a puromycin resistance cassette) into the packaging cells by following the procedure above for transient transfection. One day after transfection, add 0.5-1 ug/mL puromycin to the medium for positive selection. Incubate the cells for 3-5 days at 37ºC in 5% $CO_2$. Confirm that all the negative control cells (those transfected with a puromycin sensitive plasmid) are dead before proceeding.