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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 37°C in 5% CO<sub>2</sub>. 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<sup>6</sup> 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 37°C in 5% CO<sub>2</sub>, 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 37°C in 5% CO<sub>2</sub>. Confirm that all the negative control cells (those transfected with a puromycin sensitive plasmid) are dead before proceeding.

### STEP 3. HARVEST VIRAL SUPERNATANT

When cells are 80-90% confluent, change the medium in the culture vessel to fresh medium normally used on your target cells (i.e., DMEM for HEK293 cells). Twelve hours later, collect the medium as the viral supernatant into 15 ml tubes and centrifuge at 2000 x g for 5 minutes or pass through a 0.45 µm filter to remove cell debris. The clarified supernatant is the viral stock, and can be aliquotted and frozen at -80°C for future use, or used immediately to infect target cells.

### STEP 4. INFECT TARGET CELLS

Add the viral stock with 4 µg/mL polybrene<sup>#</sup> (catalog number H9268, Sigma, St. Louis MO) as growth medium directly onto your target cells that have reached approximately 50% confluency. Incubate at 37°C in 5% CO<sub>2</sub>. 24 hours post-infection, replace the medium with fresh growth medium containing 0.5-1 µg/mL puromycin. Passage as needed, and maintain selection pressure for 1-2 weeks. Most uninfected cells should be killed by the puromycin within 1 week.

*# The efficiency of retroviral infection is enhanced significantly by including polybrene during the infection. This small, positively charged molecule binds to cell surfaces and allows the viral glycoproteins to bind more efficiently to their receptors by reducing the repulsion between sialic acid-containing molecules.*

### STEP 5. CLONAL SELECTION OF STABLY INFECTED CELLS (OPTIONAL)

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 the 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.

You can download the application guide for HuSH-29 at <http://www.origene.com/assets/Documents/AppGuideHuSH29.pdf>.

## TrueClone Citings

### REFERENCES:

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This column focuses on significant scientific discoveries by OriGene's valued customers through their use of OriGene's TrueClone full-length cDNAs. We recognize that such publications are the ultimate value statements coming from the research community.

### STIM1 meets Orail: The Store-operated Calcium Release Mystery: Solved?

It is widely known that calcium functions as an important second messenger in cell signaling, regulating cell metabolism, growth and division, and gene expression. The calcium required for these processes is usually released in a bi-phasic manner. The limited influx from channels within intracellular stores (store-operated channels, or SOCs) is followed by an increase from unknown plasma membrane channels (calcium release activated channels, or CRACs) that are regulated in some fashion by the store-operated channels.

RNAi-based screens revealed STIM1 as one regulator of store-operated calcium. The protein product of the STIM1 gene has an EF-hand domain, which acts as a calcium sensor within the lumen of the endoplasmic reticulum (ER). At low calcium concentrations, STIM1 is relocated into puncta at the cell periphery and signals to the CRAC channels. Recent studies discussed here further explain the interactions between the intracellular stores, STIM1 and the plasma membrane-bound channels.

Localization studies revealed that STIM1 does not enter the plasma membrane, and so must affect the calcium response through interactions inside the cell (Wu et al. 2006). Furthermore, the overexpression of STIM1 alone was insufficient to

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