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the tube by scraping the surface with a pipet tip, and deposit it in a sterile culture tube containing LB-amp. The culture should be incubated overnight at 37°C with agitation before proceeding to step 4.

STEP 4. DNA PREPARATION

OriGene provides a plasmid preparation service for all of our HuSH-29 customers. To receive larger quantities of each plasmid, use catalog number DNA05 to receive 5 ug of each construct for the price of $100 USD each ($400 USD for all 4).

Miniprep method (for producing up to 40 ug plasmid from 5 mL liquid culture)

Isolate DNA from the liquid cultures by using your preferred method. OriGene routinely uses Marligen's High Purity Plasmid Miniprep Kits or similar systems. Follow the manufacturer's protocol for isolation, and elute the DNA in 50 uL of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Determine the concentration of each sample, and store the DNA at -20°C.

Midiprep method (for producing up to 500 ug plasmid from 100 mL liquid culture)

Add 5 mL of liquid culture grown several hours or overnight to a sterile flask containing 95 mL of LB-amp. Incubate overnight at 37°C with agitation. Isolate DNA from the liquid culture using your preferred method. OriGene routinely uses Marligen's High Purity Plasmid MidiPrep Kits or similar systems. Follow the manufacturer's protocol for isolation, and elute the DNA in 4 mL of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Determine the concentration of each sample, and store the DNA at -20°C.

HuSH-29 constructs are ideal for use on hard-to-transfect cells and cells lines, thanks to the option of retroviral infection.

Introduction of gene specific shRNA into mammalian cells via transient transfection

Transient transfection is the simplest method of introducing HuSH-29 constructs into your target cells. OriGene recommends transfecting each HuSH construct separately as well as all constructs together into an easily transfectable cell line (such as HEK293T or NIF3T3 cells) to determine the construct or pairing of constructs that produces the most complete knockdown effect, before proceeding to work with a more retractable cell line or primary culture.

STEP 1. PLATE CELLS

The day before transfection, passage cells into the desired cell container. Plate an amount of cells expected to achieve 50-80% confluency on the following day (see Table I for examples). Grow the cells overnight at 37°C in a 5% CO_2 incubator.

STEP 2. PREPARE TRANSFECTION MIXTURES

Dilute the transfection reagent* into serum-free medium without antibiotics (Invitrogen's OptiMEM solution is a good example). Do not let the transfection reagent come into contact with the side of the tube; instead, pipet the reagent directly into the medium. Gently flick the tube or pipet up and down to mix. Incubate for 5 minutes at room temperature. Follow the manufacturer's recommendations for ratios and volumes of reagent and DNA (see Table II for examples).

**Many commercially available transfection reagents are convenient for transient transfection. Choose a reagent that is compatible with your target cells. OriGene routinely uses lipid based transfection reagents such as FuGENE 6 or Lipofectamine 2000 on HEK293T cells for validation experiments, and the protocol here is based on those manufacturers' recommendations. Be sure to follow the specific protocol for your transfection reagent.*

Dilute the plasmid DNA into serum-free medium without antibiotics. Gently flick the tube or pipet up and down to mix. Combine the tube of reagent/medium with the tube of DNA/medium, and gently mix. Incubate for 15-45 minutes at room temperature.

TABLE I. SEEDING DENSITY OF TARGET CELLS 1 DAY PRIOR TO EXPERIMENT

Vessel type	Seeding density of cells	Volume of media
T-75 flask	10^6 cells	18 mL
T-25 flask	3 x 10^5 cells	16 mL
10 cm dish	7 x 10^5 cells	12 mL
6 well plate	10^5 cells	2 mL/well
12 well plate	5 x 10^4 cells	1 mL/well
24 well plate	2 x 10^4 cells	500 uL/well
96 well plate	4 x 10^3 cells	50 uL/well

TABLE II. VOLUMES RECOMMENDED FOR TRANSFECTION REACTIONS

Vessel type	OptiMEM	Transfection reagent	OptiMEM	shRNA plasmid	cDNA expression plasmid**
T-75 flask	400 uL	24 uL	400 uL	8.0 ug	80 ng
T-25 flask	125 uL	8 uL	125 uL	2.5 ug	25 ng
10 cm dish	300 uL	18 uL	300 uL	6 ug	60 ng
6 well plate	50 uL	3 uL	50 uL	1 ug	10 ng
12 well plate	25 uL	1.5 uL	25 uL	0.4 ug	4 ng
24 well plate	10 uL	0.6 uL	10 uL	0.2 ug	2 ng
96 well plate	2.5 uL	0.2 uL	2.5 uL	0.05 ug	0.5 ng

** The plasmid designed to overexpress the target gene may be included in some wells to directly compare the effects of overexpression with and without shRNA targeting. This is most useful when your target cells do not endogenously express the target gene, or express it at very low levels.

STEP 3. ADD TRANSFECTION MIXTURE TO CELLS

Remove culture vessel from incubator. For many transfection reagents, it is not necessary to change the medium to a serum-free solution prior to transfection, but check the manufacturer's recommendations for details. Slowly add the transfection mixture dropwise to the culture medium. Rock the plate gently to mix the solution into the media, then return the vessel to the incubator. For many transfection reagents, it is not necessary to change the media after transfection, but follow the manufacturer's instructions for your particular transfection reagent. Incubate the cells at 37°C in a 5% CO_2 incubator for 48 hours before harvesting for RNA analysis, or 72 hrs before harvesting for protein analysis.

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HuSH-29 constructs can be used for transient or stable transfection, or retroviral infection, of your target cells.