HuSH-29 Protocols

Plasmid DNA amplification in E. coli

Transforming your HuSH constructs into competent cells allows you to create an eternal stock from which you can produce endless quantities of DNA for your transfection experiments. This simple protocol requires only 30 minutes of hands-on time to generate a glycerol stock and another 30 minutes to purify enough DNA for a transfection experiment.

STEP 1. RESUSPENSION OF LYOPHILIZED SHRNA CONSTRUCTS

Add 100 uL of sterile water into each of the tubes containing shRNA expression plasmids. Vortex the tubes gently or pipet up and down to resuspend the lyophilized DNA. This resuspension produces a DNA solution with an approximate concentration of ~2 ng/uL, which should be stored at –20°C.

STEP 2. TRANSFORMATION

Both electroporation and heat shock are appropriate methods of transformation for amplifying plasmid DNA; use the cells* normally employed in your lab for routine transformations. Example protocols are given below for transformations using chemically competent cells and electrocompetent cells. Be sure to follow the specific recommendations of your competent cell manufacturer.

*Most commercially available competent cells are appropriate for this purpose. Confirm the efficiency of your batch of cells by performing a parallel transformation with the supercoiled control DNA provided with the cells. OriGene recommends using cells with an efficiency of at least 10^6 CFU/ug DNA.

Transformation with chemically competent cells

Briefly thaw on ice a tube of competent cells. Aliquot into prechilled microfuge tubes the appropriate volume of cells for individual transformations (e.g., 10 uL of cells for 1-5 ng supercoiled DNA). Add 1-5 uL of each expression plasmid to an aliquot of competent cells, stir gently with the pipet tip, and incubate on ice for 30 minutes. Perform the heat shock by incubating the mixture of DNA and cells at 42°C for exactly 30 seconds, then removing the cells to ice immediately. Add 250 uL of recovery medium (such as SOC) to the cells, and incubate at 37°C for 1 hour with agitation. Plate several dilutions of the transformation mixture onto separate LB-amp agar plates (try 1%, 10%, and 50% of the transformation reaction on separate plates, each diluted up to 100 uL in SOC). Incubate the plates overnight at 37°C. Store any remaining transformation solution at 4°C in case further dilutions and replating are necessary. The following day, inoculate 2-3 single bacterial colonies from each transformation into individual sterile culture tubes containing 5 mL of liquid medium with 100 ug/mL ampicillin (LB-amp). Incubate overnight at 37°C with agitation.

Transformation with electrocompetent cells

Briefly thaw on ice a tube of competent cells. Aliquot into prechilled microfuge tubes the appropriate volume of cells for individual transformations (e.g., 10 uL of cells for 1-5 ng supercoiled DNA). Add 1-5 uL of each expression plasmid to an aliquot of competent cells, stir gently with the pipet tip, and transfer the mixture to a prechilled electroporation cuvette. Incubate cuvette on ice for 30 minutes. Perform the electroporation with settings optimized for your electroporator, and note the Tau value returned. (This value represents the time required for the current of electricity to cross the membranes of your competent cells, and is a good indication of the efficiency of the transformation process.) Add 250 uL of recovery medium (such as SOC) to the cells, and incubate at 37°C for 1 hour with agitation. Plate several dilutions of the transformation mixture onto separate LB-amp agar plates (try 1%, 10%, and 50% of the transformation reaction on separate plates, each diluted up to 100 uL in SOC). Incubate the plates overnight at 37°C. Store any remaining transformation solution at 4°C in case further dilutions and replating are necessary. The following day, inoculate 2-3 single bacterial colonies from each transformation into individual sterile culture tubes each containing 5 mL of liquid medium with 100 ug/mL ampicillin (LB-amp). Incubate overnight at 37°C with agitation.

STEP 3. CREATING A GLYCEROL STOCK

Remove 425 uL of each overnight liquid culture into a fresh microfuge tube. Add 75 uL sterile glycerol, and gently resuspend. Glycerol is quite viscous, so it's best to use a large bore pipet tip (you may even need to widen your pipet tips by cutting off the end with a sharp blade) or a transfer pipet. When the solution is homogenous, snap freeze the tube in liquid nitrogen or a dry-ice/ethanol bath. Store the glycerol stock at -80°C. If stored properly, this stock can be used for the next several years to inoculate a fresh liquid culture in order to amplify more DNA. Simply remove a small portion of the frozen glycerol stock (thawing the tube is not required) from

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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.