Transfection and Subsequent Transduction Protocols for Single shRNA Expression Constructs From TRC1, 1.5, or 2 Library Clones

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Day 1 – Seed HEK293FT cells in 6-well plates (one well per shRNA construct)

Trypsinize HEK293FT cells and count. Seed 300,000 ($3x10^5$) cells/well of a 6-well plate in the evening.

Sigma Aldrich's DMEM (D6429), completed with 10% FBS and 5.5mL Antibiotic-Antimycotic is a recommended medium option.

Note: The cells should be ~30% confluent at the time of transfection, so you can adjust the number of cells seeded and the time elapsing between seeding and transfection in order to get cells to approximately 30% confluence.

Day 2 – Transfect HEK293FT cells with shRNA construct & packaging DNA

i) Change the media on the HEK293FT cells two hours prior to transfection,aspirate the media off the HEK293FT cells and replace with 1.5mL of fresh, complete DMEM medium.

ii) Each well to be transfected will require:

- 400µL serum-free DMEM (D6429)
- 2.0µg of Packaging Vector Mix (PVM) [100ng/µL]

Note: The packaging vectors used in this lentiviral system are pΔ8.9 and pCMV-VSV-G. Packaging Vector Mix. (PVM) consists of 3 parts pΔ8.9 to 1 part pCMV-VSV-G. In this protocol, for instance, 2.0µg of PVM contains 1.5µg of pΔ8.9 and 0.5µg of pCMV-VSV-G, typically concentrated at 100ng/uL.

- 2.0µg shRNA construct DNA [100ng/µL]
- 12µg Polyethyleneimine (PEI) [1.0mg/mL]

Prepare one 2mL micro-centrifuge tube for each unique shRNA construct to be transfected. In the microcentrifuge tube, combine 400µL serum-free DMEM (or OPTI-MEM), 2.0µg PVM, and 2.0µg shRNA construct DNA. Mix well.

Then, add 12µg PEI to each tube and mix well by vortexing on the highest speed for 15 sec. Incubate the tubes at room temperature for 15 minutes. After incubation, add the entire contents of a tube to a single well of HEK293FT cells.

Note: When preparing many transfections, it may be more efficient to prepare a master mix of serum-free DMEM and PVM, and then aliquot the mix into individual tubes.

Day 3 – Change the media on the HEK293FT cells

Note: Prior to changing media, users who have transfected positive control constructs (i.e., TurboGFP, RFP, etc.) may take the opportunity to check transfection efficiency under a fluorescence microscope at ~12 hours after transfection. It is advisable to check again at a later time point, as 12 hours is sometimes not soon enough to gauge transfection efficacy by GFP.

12-14 hours after transfection aspirate the media off the HEK293FT cells and replace with 2.0mL of fresh, complete DMEM. THIS IS TIME ZERO for harvesting the virus.

Day 4 – Production of Lentiviral particles

No action is required with respect to the transfected HEK293FT cells on this day. For those users following this protocol immediately with viral transduction, please refer to the below steps for instructions on preparing cells for transduction, which should be done at this time.

Seed recipient cell line for viral transduction:

For HCT116 (neomycin-sensitive) cells, seed 300,000 ($3x10^5$) cells/well of a 6-well plate. Plate in completed medium. HCT116 cells grow well in Sigma Aldrich's McCoy's 5A (M9309), completed with 10% FBS and 5.5mL Antibiotic-Antimycotic. Make sure to seed at least two additional wells to be used as non-transduced controls during puromycin selection.

Day 5 – Harvest viral supernatant off HEK293FT cells

To harvest lentivirus for immediate freezing:

One 5mL syringe and one 0.45µm cellulose acetate syringe filter are required for each well to be harvested.

Draw the media from one well into the syringe, and dispense through the 0.45µm cellulose acetate filter into a sterile 15mL conical. Seal conical and store at -80C.

Note: It is not advised to freeze/thaw cycle viral supernatant multiple times, as each freeze/thaw cycle results in loss of downstream transduction efficacy.

To harvest lentivirus for immediate transduction:

Perform this step one well at a time, so as to minimize the time cells spend without media. Aspirate the media from a well of HCT116 (neomycin-sensitive) cells. One 5mL syringe and one 0.45µm cellulose acetate syringe filter are required for each well to be harvested. Draw the media from one well into the syringe, and dispense through the 0.45µm cellulose acetate filter directly onto the HCT116 cells. Repeat with fresh syringes and filters for each well to be transduced. Add 2µL of 1000x polybrene into each well.

Rock gently to mix.

Day 6 – Change media on transduced cells:

16 hours post-transduction, aspirate the media from each well of transduced cells, taking care to avoid cross contamination.

Add 2mL of completed media to each well (McCoy's 5A for HCT116).

Day 7 – Begin puromycin selection:

Aspirate the media from each well of transduced cells, taking care to avoid cross-contamination. Add 2mL of completed media to each well (McCoy's 5A for HCT116). Add puromycin to each well, including the nontransduced control wells. Puromycin should be added at a pre-calculated, kill curve-determined concentration. HCT116 (neomycin sensitive) cells, for instance, can be selected at puromycin concentrations between 1µg/mL and 10µg/mL. Ideally, the lowest concentration of puromycin that reliably kills non-transduced cells within two days should be used.

Day 8 & Beyond – Continue puromycin selection:

For the duration of puromycin selection, each day:

Examine cells by microscopy. Non-transduced controls should die at a much faster rate than wells containing transduced cells, although all wells will show signs of cell death, as non-transduced cells are selected out of the population. Aspirate the media from each well of transduced cells, taking care to avoid cross-contamination. Add 2mL of completed media to each well (McCoy's 5A for HCT116). Add puromycin to each well, including the non-transduced control wells. Puromycin selection can be discontinued when all non-transduced control cells are dead.