Lipofectamine 2000 Transfection Protocol with Luciferase Reporter Genes

HEK293 cells are BSL-2; zebrafish ZF4 cells are BSL-1. Perform steps 1) and 4-8 in the tissue culture hood. Starting with step 10, the work can be done at your bench when using BSL-1 cells, but must be done in the tissue culture hood for BSL-2 cells until after lysis is complete.

Day Minus 1:
1) Trypsinize three T75 flasks of ZF4 cells, or 1-2 T75 flasks of HEK293 cells:
   Pour off old medium.
   Pipet 3 ml trypsin/EDTA solution, swirl over cell monolayer, and pour off.
   Pipet 2 ml trypsin/EDTA solution. Let sit on cells until they have detached. Rap bottom of flask and/or swirl to facilitate detachment of cells. HEK293 cells detach quickly; ZF4 cells take over 10 minutes (put in 28°C incubator while waiting). After all cells have detached, per flask, add 10 mL of prewarmed culture medium that contains 10% bovine growth serum (BGS) and 1% Penn/Strep solution. Pipet up and down repeatedly to disaggregate cells (10X pipetting repetitions). If starting with more than one T75 flask, combine cells into one flask and mix.

2) Count density of cells using a hemacytometer. To do this, put the special hemacytometer cover slip over the gridded area. Using a Pasteur pipet, feed a bit of cell soup to the notch between the cover slip and the hemacytometer slide using capillary action (Did you remember to mix the cells before pipetting? The cells settle quickly.). Count the total number of cells located on four 4X4 grids. As a rule, count cells that are “liners” when overlapping the left and top lines, but not the right or bottom lines. Use the middle mag. objective of the inverted microscope, and one 4X4 grid will fill your field of view.

3) To obtain the density of your trypsinized culture in cells/mL, divide your total cell count by 4 (since you counted four 4X4 grids) and multiply by $10^4$ (each 4X4 grid corresponds to $10^{-4}$ mL). Adjust the desired volume of cells to a density of $4 \times 10^5$ cells/mL. Pipet 2.5 mL each per well of 6-well TC dish. Incubate O.N.

Day 1: (volumes for transfection of one well of 6-well dish)
4) Check cells in 6-well plate to make sure OK. Remove medium and replace with prewarmed culture medium containing 10% BGS, but lacking antibiotics. Put back in incubator. You can do this ahead of time (1 to several hours).
5) In 12 x 75mm clear culture tube, mix 125 µL OptiMEM (at room temp.) plus plasmid DNA(s) (Typically, I use 200 ng each reporter plasmid (firefly luciferase reporter for experimental samples and pRLSV as renilla luciferase reporter to use as normalization control) and 5-50 ng each expression plasmid). To increase precision, make master premixes and aliquot premix into individual tubes.
6) Per transfection sample, mix 125 µL OptiMEM (at room temp.) plus 1.5 µL Lipofectamine 2000. Make a master mix containing enough for all samples. Drip L2000 directly into OptiMEM; i.e., do not let it run down inside of tube. Incubate at R.T. for 5 minutes, but no longer.
7) Add 126.5 µL L2000/OptiMEM mix to DNA mixture and vortex gently to mix. Incubate at R.T. for 20 minutes.
8) Dropwise using a P1000, add DNA/L2000/OptiMEM mix to one well of cells in 6-well plate. Swirl gently when finished with one plate and put back in incubator.
9) Incubate cells approximately 48 hours. It is not necessary to replace medium containing L2000.

Day 3: harvesting cells and making extracts
10) Aspirate off medium and discard in autoclave waste bag. Wash monolayer with 2 mL cold PBS, and aspirate to discard. Remove PBS wash completely by using Pipetman to get last microliters.
11) Dilute enough 5X passive lysis buffer from Promega kit with MilliQ water to have enough for all samples.
12) Add 250 µL 1X passive lysis buffer to each well of cells. Put on rotary shaker with quick shaking for 10 minutes at R.T.
13) Remove lysis buffer and cell glop to 1.5 mL Eppi tube. Vortex briefly. Store in ice from this point.
14) Centrifuge top speed in microfuge, 1 minute, R.T.
15) Save supernatant (your extract) to clean Eppi tube. Store at -80°C until ready to assay.

Luciferase assay using Promega Dual Luciferase Assay Kit
1) Thaw enough LAR II substrate/buffer at R.T. for all samples (keep dark in a drawer while thawing). Dispense 100 µL per luminometer tube. DO NOT ADD YOUR EXTRACTS TO THESE TUBES UNTIL READY AT THE LUMINOMETER!!! (Step 5).
2) Thaw Stop & Glo Buffer to R.T. Make up enough Stop & Glo substrate / buffer for all samples (add 1/50 Stop & Glo reagent to desired amount buffer).
3) If frozen already, thaw transfected cell lysates; then keep cold on ice.
4) I use RAW DATA settings on luminometer: measurement delay time = 5.0 sec; measuring time = 10.0 sec
5) Add 20 µL cell lysate to LAR II in luminometer tube at R.T. Mix by pipetting up and down with P20.
6) Put into luminometer to read firefly luciferase activity.
7) Pull out drawer, remove tube, add 100 µL Stop & Glo reagent/buffer to tube. Mix by gently vortexing. Put back into luminometer and close drawer to begin measurement. This will read renilla luciferase to use as normalization control.
8) Include a 20 µL sample of passive lysis buffer as background readings for both firefly and renilla luciferases.