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Transient Transfections in 12 well plates

1. Day before transfection (for CV1, HepG2 cells) trypsinize a ~90% confluent T75. (Wash flask with 10ml PBS followed by addition of 1ml of trypsin/EDTA. Incubate 5 minutes at 37˚C or until cells are falling off plate.)

2. Add 9mls of media to flask and resuspend well. If you are doing a transfection in which you will be using ligand where **charcoal stripped serum** is necessary, split cells and plate in charcoal stripped serum and use this stripped serum from here on out.

3. In a 15ml conical tube, dilute 1ml of cell suspension into 10ml media (11ml total).
    a. Thoroughly mix cells by pipeting up and down
    b. 1ml of this cell suspension is enough cells for 1 12-well plate
    c. Dilute 1 ml in 11ml media (12 mls total) per 12 well plate
    d. Plate 1ml of 1:12 dilution per well (use 5 or 10 ml pipet to plate)

4. Following day, check confluency of cells. Cells should be roughly 60-70% confluent before transfecting. You can wait an extra day if the cells are too sparse.

5. Aspirate media and **add fresh media** approx 3 hours before transfection, ie: addition of precipitate to wells. *Do not have to change Pasteur pipet between wells when aspirating.*

6. Dilute reporter and expression constructs to 0.5 or 1ug/ul (or less for renilla) for easier pipeting.

7. Mix DNA and transfection reagents.
    Mix as follows for **each triplicate**:
        Reporter: 12ug
        PGC-1: 1.5ug
        PPAR: 1.5ug
        RXR: 0.375ug
        Renilla: 600ng

    a. **Each well should have the same amount of DNA. Empty expression vector should be used in place of expression constructs in control wells.** Total volume is120µl DNA + H_2O per triplicate
    b. **Master mixes should be used as much as possible to cut down on splay**. For instance, if you can mix up all the reporter and renilla and water first, then aliquot, then add in the different expression vectors, this would be ideal!!!
    c. Per triplicate add 120µl Buffer A (incubate 10') + 240µl Buffer B (incubate 15') **ADDITION OF BUFFER A AND B SHOULD BE SLOW AND SLOWLY MIXED 5-7 TIMES WITH PIPET**
    d. Add 155µl (instead of 160 to make up for pipetting error) of this mixture per well dropwise slowly as not to disturb the cells.

8. After addition of DNA to the wells, allow cells to incubate ~14-18 hrs.

9. Following 14-18 hour incubation, wash cells ~3x with PBS (~1ml per well).
    a. Following each wash, assess if all the precipitate has been removed in order to decide if more washes are necessary.

10. Refresh media. If adding ligand, ligand should be added to the media before adding fresh media to the wells. In most cases, charcoal stripped serum should be used when adding ligand. In this case, charcoal stripped serum should be used when initially plating the cells.

11. Luciferase assay should be performed 48 hours post wash.

12. Aspirate media and wash cells once with PBS. Aspirate.

13. **Add 100ul PBS** to each well and freeze the plate at -80 for several hours. Thaw the plate and scrap well with cell lifter. Pipet cells up and down several times to make homogenous cell suspension.

14. **Transfer 20µl of cell** suspension to a non-sterile 96w plate.

15. **Add 20µl of Stop-Glo reagent**. (Dilute substrate 1:100 in dilution buffer; substrate is @ -20°C; dilution buffer is at 4°C).
    a. NEVER leave Stop-Glo reagent UNCAPPED OR AT ROOM TEMP. It is highly volatile and will evaporate. Aliquot amount of stop-glo diluent you will need then bring to freezer and uncap substrate tube only long enough to remove desired amount (this is very important or we will run out of substrate long before diluent).

    b. **Incubate 10'. Read** on plate reader using the "Lumin 5sec TCL" parameter file. (May read for at least 1 hr). Note that the "Lumin 5sec TCL" parameter file is a "direct" mode file. This is equivalent to HIGH SENSITIVITY! If sensitivity is too high, you can use the "Lumin filtered" parameter file (filter mode). As an aside, the filter mode further reduces cross-talk between wells.

16. See "Chameleon Plate reader" Protocol for more detail on using the instrument

ITEM	Catalog number	Vendor
Dual-Glo™ Luciferase Assay System	PRE2980 (10x100ml)	Fisher
White stickers for bottom of 96w-plate	6005199	Perkin Elmer
12 well tissue culture plates	08-772-29	Fisher
96 well white plates, sterile, clear bottom	07-200-566	Fisher (Costar #3610)
96 well solid white plate, nonsterile	07-200-589	Fisher (Costar #3912)

17. When graphing results, graph the Raw luciferase values, the raw renilla values and the normalized values and place on same page in order to help you make conclusions on whether or not the experiment worked.

Transfection Buffer A	
Final Conc.	Stock
0.5 M $CaCl_2$	7.35 g (m.w. 147)
0.1 M HEPES	2.38 g (m.w. 238)
CRITICAL pH to 7.09 w/ 1M NaOH	
Q.S. to 100 mls and filter sterilize	
USE Tissue Culture grade H_2O!	
Wait 24 hrs b/f use; store at 4°C; warm to RT b/f use	

Transfection Buffer B	
Final Conc.	Stock
0.28 M NaCl	3.28 g (m.w. 58)
0.05 M HEPES	2.28 g (m.w. 238)
0.75 M NaH_2PO_4	1.5 ml (0.1 M stock)
0.75 M Na_2HPO_4	1.5 ml (0.1 M stock)
CRITICAL pH to 7.09 w/ 1M NaOH	
Q.S. w/ to 200 mls and filter sterilize	
USE Tissue Culture grade H_2O!	
Wait 24 hrs b/f use; store at 4°C; warm to RT b/f use	

Phosphate buffer stocks are in the door of the TC fridge
0.1 M NaH_2PO_4 1.20g/100ml (sterile filter)
0.1 M Na_2HPO_4 1.42g/100ml (sterile filter)

ITEM	Catalog number	Vendor
SV40 Renilla (phRL-SV40)	PRE6261	Fisher
CMV Renilla (phRL-CMV)	PRE6271	Fisher
96 well white plates, sterile, clear bottom	07-200-566	Fisher (Costar #3610)

Notes:

1. For differentiating cells, you may need to wait 72 hours before reading the plate post transfection. This will be dependent on tube formation, although all of your experiments should be done the same way.
2. When differentiating L6 and C2C12 cells, differentiation media should be added after the precipitate is washed off the cells. We use 2%-hourse serum DMEM (4.5 g/L glucose) for myotube differentiation.
3. **When differentiating C2C12 to myotubes**, you will **need to gelatin coat the plates** first by covering the surface (approx 1ml for 12 well plate, 100ul for 96 well plate), incubating 5 minutes, removing gelatin, waiting 5 additional minutes prior to plating. The Cat# for the gelatin coating is ES-006-B from Specialty Media.

Oleate addition:

When adding oleate as the ligand, use the Oleate/BSA purchased from SIGMA (Oleic Acid, O-3008, 5ml). For each different lot, call SIGMA to verify the concentration of Oleate and BSA. Each LOT will be different. For example, you will need to dilute the Oleate into your media at approx a 1:14 dilution for 250µM final concentration. Also, don't forget to add BSA (essentially FA free, SIGMA, A6003) to the control wells. For the BSA, make a 100mg/ml stock (depending on lot) in water, sterile filter, and store at 4ºC.

For example:
If protein concentration is 109mg/ml and the oleic acid is 2:1 albumin
MW for BSA=66430g/mol

109/66430=.00164 or 1.6mM
0.00164mol * 2= 0.0328mol/L or 3.28mM
For 100uM
Dilute1:31.79