CELL CULTURE – GENERAL PROTOCOL

Media
225 ml DMEM (with 1.5 g/L sodium bicarbonate)
25 ml calf serum
2.5 ml Pen/strep
2.5 ml Glutamax
2.5 ml NEAA
2.5 ml sodium pyruvate
Filter

From frozen culture
1. Dilute vial of cells 1:10 in DMEM (with 10% calf serum)
2. Spin 5' @ setting 3 (Barnes lab)
3. Aspirate off media
4. Resuspend cells in 12 ml media
5. Plate on 100 mm cell culture dish

Splitting cells (1:4)
1. Aspirate off media
2. Rinse with 6 ml 1x filter-sterilized PBS and aspirate off PBS
3. Add 1 ml of trypsin (TripleE; Invitrogen) per 100 mm plate
4. Incubate at 37C for 3-5'
5. Inactivate trypsin with 9 ml complete media (i.e., w/serum) per 1 ml trypsin
6. Pipet up and down
7. Transfer to 15 ml conical bottom tube
8. Pipet up and down 15-20x in conical bottom tube
9. Spin down 2' at ~2000g
10. Aspirate off media – DON'T ASPIRATE THE PELLET!
11. Add 10 ml media to 4 new plates
12. Resuspend cells in 8 ml
13. Pipet 2 ml per plate (for a total volume of 12 ml)

Freezing cells
1. Aspirate off media
2. Rinse with 6 ml 1x filter-sterilized PBS
3. Aspirate off PBS
4. Add 1 ml trypsin
5. Incubate at 37C for 3-5'
6. Inactivate trypsin with 9 ml of complete serum and transfer to a 15 ml conical bottom tube
7. Pipet up and down 15-20x in conical bottom tube
8. Spin down 2' at ~2000g
9. Aspirate off media
10. Add 1 ml serum/10%DMSO per 100 mm plate that was spun down
11. Pipet up and down and transfer to cryovials
12. Place vials in Styrofoam box in -80C
13. Transfer to liquid nitrogen tank after 24h at -80C

Transfection Protocol (12 well plate):

1. Day before transfection: plate cells at 0.4–2.0 x 10^5 cells/well (40-80% confluent)
    a. **Make sure you have filtered medium that is free of proteins, antibiotics and serum for complex formation with Superfect reagent.**

2. On the day of transfection:
    a. Dilute supercoiled DNA in TE buffer to 75 ul volume with cell growth medium containing no serum, proteins or antibiotics.
    b. Spin down.
    c. Add appropriate volume of Superfect reagent [7.5 ul] (Qiagen).
    d. Incubate samples for 5-10 minutes at RT to allow complex formation.
    e. While complex formation takes place, aspirate medium from dish and wash cells once with PBS.
    f. Add appropriate volume of cell growth medium (containing serum and antibiotics – 400 ul) to the reaction tube containing transfection complexes. Mix by pipetting up and down 2x and transfer total volume to the cells.
    g. Incubate cells with the transfection complexes for 2-3 hours under normal growth conditions
    h. Remove medium by aspiration.
    i. Wash cells 3-4x with PBS.
    j. Add fresh complete cell growth medium.

Culture format	DNA:Superfect ratio	DNA (ug)	Final vol. of DNA in SF med.	Vol. Superfect reagent (ul)	Vol. of Serum-containing med (ul)
6-well	1:2	2	100	4	600
	1:5	2	100	10	600
	1:10	2	100	20	600
12-well	1:2	1.5	75	3	400
	1:5	1.5	75	7.5	400
	1:10	1.5	75	15	400

Use 12-well plate @ 1:5 ratio of DNA:Superfect to start and 1:1, 1:10 and 1:50 ratios of control:experimental constructs (control construct is phrl-CMV luciferin [from Promega and given to us by Daniel in Boyer's lab):

1. pGL3-CMV (3 replicates each DMSO or TCDD)
2. wtFoxQ1b(-7.5)_Luc (3 replicates each DMSO or TCDD)
3. FoxQ1b(-7.5)Δ1-7ahre_Luc (3 replicates each DMSO or TCDD)
4. 2dluc (from T. Gasiewicz @ U. Rochester) (3 replicates each DMSO or TCDD) – this construct has 2 consensus AhREs driving luciferase expression

Too many samples to start?

- alternatively #1-4 (3 replicates each DMSO or TCDD) at 1:10 ratios of control:experimental constructs.
- Total: 24 wells.

Plate reader set-up (for me)

1. KC4
2. System/reader/dispenser
    a. Volume: 2 ml (70% EtOH in 50 ml conical tube); Rate: 300
    b. Volume: 2 ml (H20); Rate 300
    c. Prime air
    d. Substrate (protected from light), prime #1 with firefly luciferase substrate (Volume: 1100) (make sure yellow is dispensed), prime #2 with Renilla luciferase substrate
    e. Notes:
        i. Need 5 ml for 4, 24-well plates
        ii. Lyse in 100 ul (can use 50 ul)
            1. Rock 15' (or freeze O/N)
            2. Transfer 20 ul to 96 well plate
            3. (cleaner version – transfer to tube, vortex, spin down at ma for 5' and take 20 ul of supernatant)
    f. Protocol:
        i. Open dual luciferase
        ii. Settings/layout/type sample
        iii. Options/export/Microsoft excel
3. Clean
    a. Purge reagent (1100 ul)
    b. 70% EtOH
    c. H20
    d. air