Appendix B2

Protocol for Chimeric ERα-Mediated Reporter Gene Expression in MCF-7 Cells

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TRANSCRIPTIONAL ACTIVATION: CHIMERIC ERα-MEDIATED REPORTER GENE EXPRESSION IN MCF-7 CELLS

Thawing of cells:

1. Remove vial of cells from liquid nitrogen tank and thaw with hands.
2. Add cells to 10ml of prewarmed 10% fetal bovine serum (FBS; Intergen) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) and incubate at 37C.
3. Change media after approximately 24hrs, then pass cells (see below) at confluence, after approximately another 30hrs, to one T-75 flask in 15ml DMEM/10% FBS. At confluence (approximately 15 million cells) split the T-75 to two T-75s, then the two T-75s to five T-75s. Cells can then be maintained by splitting each T-75 to three new T-75s every three days. For assays one confluent T-75 flask can be used for 5 6-well plates (~500,000 cells per well).

Note: cells are used for transfection assays until their responsiveness diminishes noticeably. We have found this to be after approximately 10 passages, but this will vary depending on the initial passage number of the cells.

Passage of cells:

1. Aspirate off media into collection flask containing bleach.
2. Add approximately 2ml prewarmed trypsin to the T-75 flask (1ml to a T-25 flask) and briefly rinse the cells. Aspirate off the trypsin.
3. Add 2ml fresh trypsin (1ml to a T-25 flask) to rinsed cells and incubate at 37C for approximately 5min.
4. Firmly tap the flask enough to dislodge the cells from the inner surface of the flask. Verify under a microscope that the cells free of the surface and floating singly.
5. Add approximately 10ml DMEM/10% FBS (<5ml for a T-25 flask) and transfer the media and cells to a 50ml sterile disposable centrifuge tube.
6. Centrifuge at 500g for 1min.
7. Carefully remove media/trypsin.
8. Resuspend in an appropriate volume of media: 15ml DMEM/10% FBS per T-75 for maintenance, or 12ml DMEM/5% FBS-DCC (see recipe) per 6-well plate for transfection assays. Initially add a small volume (~5ml) and gently breaking up clumps of cells by repeatedly resuspending with a Pasteur pipette before adding the rest of the media and then mixing thoroughly.

Note: for transfection assays, an extra well (single 35mm Petri dish) is also plated as a blank, which is not transfected or dosed.

Transient transfection assay:

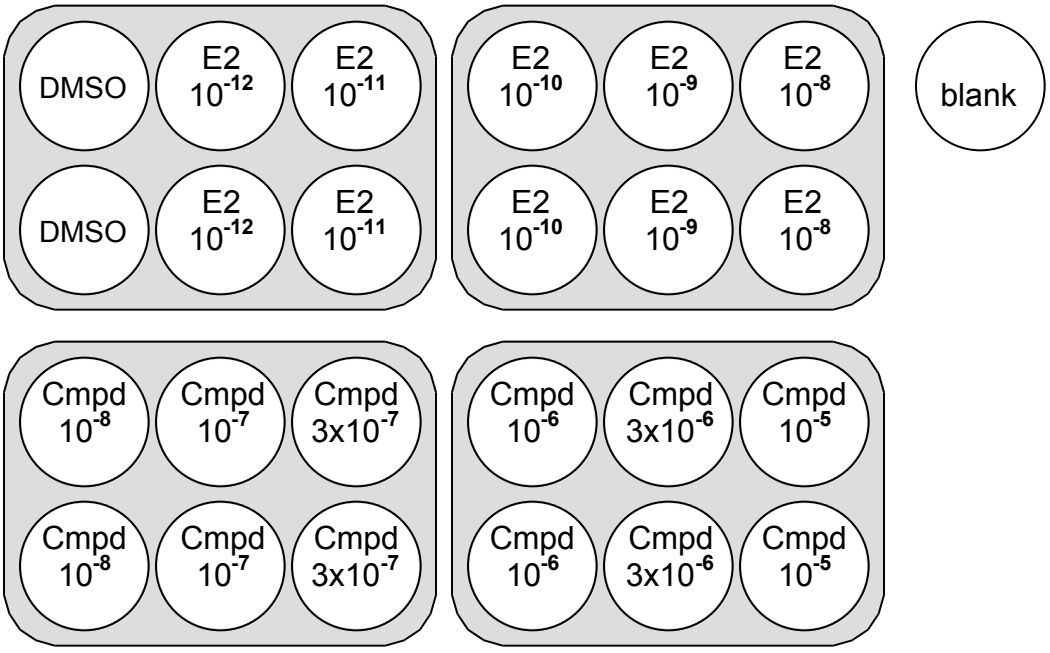
Cells split into 6-well plates in DMEM/5% FBS-DCC are incubated 7hrs at 37C to allow attachment to the plate surface. Transfection is typically performed by the calcium phosphate coprecipitation method outlined in Molecular Cloning (Sambrook, Fritsch, and Maniatis) and summarized below; an alternative effective method follows the Lipofectamine reagent protocol (Gibco).

1. Add appropriate amounts of maxiprepped DNA (0.2µg/well Gal4-ERα def; 1.5µg /well 17m5-G-Luc; 0.15µg /well pCMV-lacZ) to 100ul/well TE (see recipe) in a sterile tube.
2. Add 15µl/well 2M $CaCl_2$ (see recipe) dropwise to the DNA/TE mixture with gentle vortexing.
3. Add the DNA/TE/ $CaCl_2$ solution dropwise to 120µl/well 2xHBS (see recipe) with gentle vortexing.
4. Incubate 20min at room temperature.
5. Add 235µl of the mixture dropwise to each well of each 6-well plate.

6. Incubate at 37C for 16hr.

Dosing of cells:

1. Rinse cells twice with prewarmed sterile phosphate-buffered saline (PBS).
2. Aspirate off PBS and add 2ml DMEM/5% FBS-DCC.
3. Dose cells by adding 2µl of test compound dissolved in appropriate solvent (often dimethyl sulfoxide) at various concentrations in duplicate. A typical scheme is shown below for a weak estrogen of interest as well as 17β-estradiol (E2) (positive control). Concentrations are expressed as final concentration (M) in the 2ml media.
4. Incubate 20hr at 37C.



Harvesting of cells:

1. Aspirate off media.
2. Rinse each well twice with 1ml cold PBS.
3. Place plates on incline and aspirate off all remaining PBS.
4. Add 100µl lysis buffer (see recipe) per well with freshly added protease inhibitor cocktail (to 1x) and dithiothreitol (DTT; for 2mM final concentration add 8µl of 1M DTT per well).
5. Tap plates firmly to distribute lysis buffer.
6. Freeze plates for at least 20min.
7. Thaw plates, tap firmly again, and place on incline until cell debris sinks to the bottom and upper clear lysate can be removed.

Luciferase assay:

1. Add 10µl of cell lysate from each well to duplicate wells of an opaque 96-well plate. Because each treatment is represented by duplicate wells in the 6-well plate, there are now four wells per treatment in the 96-well plate.
2. Add 100µl Luciferase Reaction Buffer with 2mM freshly added DTT (add 0.2µl of 1M DTT per well) and 2mM freshly added ATP (add 2µl of 100mM ATP per well).

3. Set Luminoskan 96-well luminometer to inject 25µl of 0.5x luciferin (Molecular Probes; diluted in Luciferase Reaction Buffer) and read for 10sec.

β-Galactosidase assay:

1. Add 10µl of cell lysate from each well to wells of a transparent 96-well plate.
2. Add 100µl of β-galactosidase reaction buffer with 2.7µl β-mercaptoethanol per well freshly added.
3. Add 25µl 4mg/ml o-nitrophenyl-B-D-galactopyranoside (ONPG; Sigma).
4. Incubate until a pale yellow color appears (0.5-2hrs).
5. Measure absorbance at 420nm.

Calculations:

$$\text{Luciferase activity for each well (units/µl/hr)} = \frac{(\text{luc reading} - \text{blank luc reading})}{(\beta\text{-gal reading} - \text{blank } \beta\text{-gal reading})} \frac{1}{(\mu\text{l of lysate used, i.e. } 10\mu\text{l})} \frac{1}{(\text{hrs before reading } \beta\text{-gal})}$$

$$\text{Fold induction} = \frac{(\text{luc activity in well of interest})}{(\text{luc activity in vehicle well})}$$

Notes:
*calculations are averaged across the four readings for each treatment.
*typical fold induction for highest concentration of E2 relative to DMSO is ~20-fold

RECIPES:

TE:
1mM Tris (0.158g/l)
0.1mM EDTA (0.372g/l)
*dissolve in glass-distilled H_2O
*pH to 8.0
*sterilize with 0.22µ filter
*store at 4C

2x HBS:
280mM NaCl (16g/l)
10mM KCl (0.74g/l)
1.5mM $Na_2HPO_4 \cdot 2H_2O$ (0.27g/l)
12mM dextrose (2g/l)
50mM HEPES (10g/l)
*dissolve in glass-distilled H_2O
*pH to 7.05
*sterilize with 0.22µ filter
*store at 4C; long-term -20C

2M $CaCl_2$
2M $CaCl_2 \cdot 6H_2O$ (367.5g/l)
*dissolve in glass-distilled H_2O
*sterilize with 0.22µ filter
*store at -20C

5x Lysis Buffer:
125mM glycylglycine, pH 7.8 (25ml of 500mM/100ml)
20mM EGTA (4ml of 500mM/100ml)
50% glycerol (50ml of 100% glycerol/100ml)
5% Triton X100 (5ml of 100%/100ml)
0.75mM Spermine (0.75ml of 100mM/100ml)
300mM KCl (9.7ml of 3.1M/100ml)
75mM NaCl (1.5ml of 5M/100ml)

Luciferase Reaction Buffer:
25mM glycylglycine (25ml of 500mM/500ml)
15mM $MgSO_4$ (7.5ml of 1M/500ml)
4mM EGTA (4ml of 500mM/500ml)

β-Galactosidase Reaction Buffer:
60mM Na_2HPO_4 (30ml of 1M/500ml)
40mM NaH_2PO_4 (20ml of 1M/500ml)
10mM KCl (5ml of 1M/500ml)
1mM $MgSO_4$ (0.5ml of 1M/500ml)

100x Protease inhibitor cocktail:

1µg/µl Aprotinin

100µg/µl phenylmethylsulfonyl fluoride (PMSF)
others can be added as described in Molecular Cloning (Sambrook, Fritsch, and Maniatis)

Trypsin:

1. Prepare Solution A as follows:
   NaCl 4.2g
   Tris base 1.6g
   KCl 0.2g
   Na_2HPO_4 0.52g
   1N NaCl 7.5ml
2. Mix and allow to stand at 4C overnight.
3. Adjust pH to 7.4 and adjust volume to 500ml with water.

4. Mix together 450ml Solution A with 0.25g trypsin and 1.25ml of 0.2M EDTA (pH 7.5) and adjust volume to 500ml with water.
5. Sterilize through a 0.22µ filter.

Dextran-Coated Charcoal-treated Fetal Bovine Serum (FBS-DCC):

A. Preparation of DCC – make fresh every time.

1. Suspend 3.12g of activated charcoal (BDH – decolorizing powder activated, 33032 4E) into 50ml of 10mM tris (pH 7.4) in a 50ml disposable centrifuge tube.
2. Centrifuge 10min at 180g. Remove Tris and repeat two more times with fresh Tris.
3. Resuspend pellets in 50ml of 10mM Tris and transfer to a sterile 100ml bottle.
4. SLOWLY, while stirring, add 0.31g dextran T70 (Pharmacia) and continue to stir for 20min.

B. FBS stripping – 500ml.

1. Add 100 IU sulfatase (ICN) to a 500ml bottle of FBS (Intergen).
2. Incubate 2hrs at 37C with magnetic stirring.
3. Add 10ml fresh DCC.
4. Incubate overnight at 4C with magnetic stirring.
5. Centrifuge at 4200g for 10min. Pour supernatant into a fresh bottle.
6. Add 10ml fresh DCC.
7. Incubate with magnetic stirring at 56C for 45min.
8. Cool with magnetic stirring to 4C in ice bath (~30min).
9. Repeat steps 5-8 two more times.
10. Centrifuge a final time at 4200g for 10min. Pour supernatant into a fresh 500ml bottle.
11. Filter the stripped serum into a sterile 500ml bottle using a 0.22µ filter (e.g. Gelman VacuCap90). Do not overload the filter – may need to change filter several times.
12. Aliquot 25ml of filtered serum into 50ml sterile tubes and store at -20C.