

## **Appendix B3**

### **Protocol for HepG2 Cells + Receptor + Reporter and/or $\beta$ -gal plasmids for Use in Steroid Hormone Receptor Assays**

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## TransIT Transfection Method of HepG2 Cells for Use in Steroid Hormone Receptor Assays

### 1. MATERIALS AND SOURCES:

- a. TransIT-LT1 Transfection Reagent, supplier: Mirus Corporation, CAT. #: MIR 2300.
- b. 1X Phosphate Buffered Saline Solution.
- c. Plasmid DNA's of choice: i.e., receptor, reporter, and/or -gal plasmids.
- d. Phenol red-free Minimum Essential Medium (MEM).
- e. Complete phenol red-free Minimum Essential Medium (MEM), with stripped (or charcoal/dextran treated) fetal bovine serum.
- f. 0.02% EDTA.
- g. Trypsin, 2.5%.
- h. Dimethyl sulfoxide.
- i. 1M Sodium pyruvate.
- j. L-glutamine (100X).

### 2. EQUIPMENT AND SUPPLIES:

- a. Incubator with 5% CO<sub>2</sub>/air, 37°C
- b. Vortexer
- c. 10 µl, 100 µl, 200 µl, and 1000 µl Eppendorf pipettor or equivalent
- d. pipet tips
- e. 1, 2, 5, 10, 25, and 50 ml pipets
- f. 500 ml screw cap glass bottles, sterile
- g. 24 well tissue culture plates
- h. 15 and 50 ml centrifuge tubes, sterile, polypropylene
- i. 17x100, polypropylene snap-cap tubes, sterile, round bottom
- j. 1.5 ml siliconized polypropylene screw-cap vials

### 3. PREPARATION:

- a. 0.12% Trypsin/0.02% EDTA.

In 500 ml sterile screw cap glass bottle, sterilely transfer 190 ml 0.02% EDTA. Add 10 ml of 2.5% trypsin. Store at 4°C.

- b. Complete phenol red-free MEM.

To 500 ml of phenol red-free MEM, add 0.5 ml 1M sodium pyruvate solution, 10.0 ml glutamine, and 50 ml resin-stripped (or charcoal dextran treated) fetal bovine serum. Store 4°C.

### c. Chemicals.

Dissolve chosen chemical to make a 0.1M stock solution using appropriate vehicle. Make serial dilutions in 1.5 ml polypropylene screw-cap vials to yield a standard curve of concentrations varying from  $10^{-5}$  M to  $10^{-11}$  M (may be changed as necessary).

## 4. PROCEDURE:

### Plating Cells.

- a. Aspirate medium from 150 mm plate of 75-80% confluent HepG2 cells and rinse with 10 ml of 0.02% EDTA.
- b. Place 10 ml of 0.12% trypsin/0.02% EDTA on plate.
- c. Place in incubator until cells begin to detach (~5 min).
- d. After cells have detached, pipette vigorously to remove the cells and transfer to 50 ml polypropylene centrifuge tube containing complete phenol red-free MEM.
- e. Rinse plate with complete phenol red-free MEM and add to tube.
- f. Centrifuge at 1000 RPM for 5 min at 4°C.
- g. Carefully aspirate supernatant and resuspend the pellet in phenol red-free complete MEM.
- h. Take cell count. Plate cells in 24-well tissue culture dishes at  $10^5$  cells/0.5 ml complete phenol red-free MEM. Swirl the plate gently to spread cells evenly in wells.
- i. Place cells in 37°C incubator with 5% CO<sub>2</sub>/air for 18 hours.

### Transfecting Cells.

In a 17x100 ml round bottom, polypropylene, snap cap tube, add the following reagents: (For transfection of a 24-well tissue culture plate)

1. 0.65 ml of phenol red-free MEM without any additives.
2. Appropriate amount of TransIT LT1 reagent. For every µg of DNA plasmid, add 2 µl of TransIT LT1 reagent. (11 µl of TransIT LT-1 reagent is needed for the suggested amounts of plasmid listed in 3. Below.) Mix very gently and let sit at RT for at least 5 min.
3. Carefully add appropriate amounts of receptor, promoter, and -gal plasmids. This may vary depending on the application. A suggestion for amounts is as follows:

Androgen Assay

Receptor Plasmid: 7 ng/well  
pCMV Plasmid ( -gal): 30 ng/well  
Promoter Plasmid: 200 ng/well

4. Mix very gently and let sit at RT for at least 5 min.
5. To each well of the 24 well plate containing HepG2 cells, carefully add 25  $\mu$ l of the TransIT/DNA complex.
6. Place plate in incubator and allow to incubate for 3 hr at 37°C.

Treating cells.

1. Dilute chosen chemicals 1:1000 in complete phenol red-free MEM, to create final concentrations ranging from  $10^{-5}$  to  $10^{-11}$ M (this may vary as necessary).
2. After the 3 hr incubation, aspirate the media and add 0.5 ml/well of the chemical diluted in media.
3. Return plate to incubator and incubate for 24 hr. Collect cell lysate for -gal and luciferase assays.

## Lysis Procedure

### 1. MATERIALS:

- D. Phosphate Buffered Saline (1X PBS).
- E. Tris base.
- F. Trans-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA).
- G. Glycerol.
- H. Phosphoric Acid.
- I. Triton X-100.
- J. 1M Dithiothreitol (DTT).
- K. Transfected cells plated in 24-well plate.

### 2. EQUIPMENT AND SUPPLIES:

- k. 1-200  $\mu$ l Pipettor
- l. Multi-channel pipettor, 1-100  $\mu$ l
- m. 1-200  $\mu$ l pipette tips
- n. Pipette aid
- o. Vacuum system with hazardous waste flask attached
- p. pH meter
- q. 5 3/4" Pasteur pipette
- r. 500 ml squeeze water bottle
- s. 96 well ELISA plate
- t. 96 well Plate, white
- u. 250 ml Glass beakers
- v. 100 and 200 ml Graduated cylinders
- w. Stirrer and stir bars

### 3. PREPARATION:

- a. **5X Lysis Solution.**
  - d. Weigh out 3.03g Tris Base and 0.695g CDTA and place in 250 ml beaker.
  - e. Dissolve completely in 60 ml of dH<sub>2</sub>O.
  - f. Measure 100 ml glycerol in 100 ml graduated cylinder, pour into fresh 250 ml beaker.
  - g. Rinse 100 ml cylinder with Tris base/CDTA. Add to glycerol in 250 ml beaker. Mix well.
  - h. pH to 7.8 with phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) if necessary.
  - i. Add dH<sub>2</sub>O to 200 ml.

- j. Add 5 ml of 100% Triton X-100 (solution will look cloudy/milky). Store room temperature.
  - b. **1X Lysis Solution.**  
In 50 ml centrifuge tube, dilute 5X Lysis Solution to 1X by diluting 1 ml 5X lysis solution into 4 ml dH<sub>2</sub>O. Add 30  $\mu$ l 1M DTT per 10 ml 1X lysis solution. Make fresh each time. Make up enough 1X lysis solution to dispense 65 $\mu$ l per well.
4. **PROCEDURE:**
- a. Aspirate media from wells and rinse with 0.5ml of PBS per well.
  - b. Aspirate PBS from wells and with multi-channel pipettor; dispense 65  $\mu$ l of 1X lysis solution per well.
  - c. Let sit at room temperature for 20 min, rocking occasionally.
  - d. Transfer 30  $\mu$ l of cell lysate to 96 well ELISA plate. This will be used for the  $\beta$ -galactosidase assay.
  - e. Transfer 20  $\mu$ l of cell lysate to a 96 well white plate. This will be used for the luciferase assay.

## **$\beta$ -Galactosidase Assay Using Chlorophenol Red- $\beta$ -D-galactopyranoside**

### **1. MATERIALS AND SOURCES:**

- a. Chlorophenol red- $\beta$ -D-galactopyranoside (CPRG).
- b. Disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ).
- c. Monosodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ).
- d. Potassium chloride (KCl).
- e. Magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ).
- f. -Mercaptoethanol (2-ME).

### **2. EQUIPMENT AND SUPPLIES:**

- A. Spectrophotometric microplate reader, with a 575 nm filter and kinetics capability
- B. Multi-channel pipettor
- C. Graduated cylinder, 1000 ml
- D. Balance
- E. Stir plate
- F. Magnetic stir bar
- G. 1-100  $\mu\text{l}$  pipettor
- H. 1-100  $\mu\text{l}$  pipet tips
- I. Pipettor reservoirs
- J. 0.2  $\mu$  Filter unit
- K. 96 well ELISA plate
- L. 1 L beaker
- M. 50 ml centrifuge tube, polypropylene, sterile

### **3. CPRG BUFFER PREPARATION:**

- j. Weigh out in 1 L beaker:

16.1 g	$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
5.5 g	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
0.75 g	KCl
0.25 g	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- k. Dissolve in 800 ml of distilled water with stirring.
- l. Adjust pH to 7.8.
- m. Transfer to 1000 ml graduated cylinder. Bring up to 1000 ml with distilled water.
- n. Filter sterilize. Store at room temperature.

**4. ASSAY PROCEDURE:**

- a. Pipet 30  $\mu$ l of cell lysate into a 96 well plate (usually done in triplicate).
- b. PER WELL OF 96 WELL PLATE , add 170  $\mu$ l of CPRG reagent made up as follows: 80  $\mu$ g CPRG dissolved in 20  $\mu$ l distilled water, 150  $\mu$ l of CPRG buffer, and 0.84  $\mu$ l 2-ME (1/200 dilution).
- c. Using multi-channel pipettor, dispense 170  $\mu$ l of CPRG reagent into each well containing lysate. For plate blank, use 30  $\mu$ l of lysis solution and add 170  $\mu$ l of CPRG reagent.

Set spectrophotometer microplate reader to kinetic endpoint and read the plate at 575 nm at 1 min intervals for 30 min to obtain Vmax. Samples will change from yellow to dark red as reaction occurs.