# Appendix B1

## Protocol for HepG2 Cells + Receptor + Reporter and/or β-gal plasmids for Use in Steroid Hormone Receptor Assays

## (Provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA)

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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 β- 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% $CO_2$/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% $CO_2$/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)

a. 0.65 ml of phenol red-free MEM **without any additives.**
b. 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.
c. Carefully add appropriate amounts of receptor, promoter, and β-gal plasmids. This may vary depending on the application. A suggestion for amounts is as follows:

**Estrogen Assay Example**

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

a. Mix **very gently** and let sit at RT for at least 5 min.
b. To each well of the 24 well plate containing HepG2 cells, carefully add 25 µl of the TransIT/DNA complex.
c. Place plate in incubator and allow to incubate for 3 hr at 37°C.

Treating cells.

a. 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).

b. After the 3 hr incubation, aspirate the media and add 0.5 ml/well of the chemical diluted in media.

c. Return plate to incubator and incubate for 24 hr. Collect cell lysate for β-gal and luciferase assays.

## Lysis Procedure

**1. MATERIALS:**

a. Phosphate Buffered Saline (1X PBS).
b. Tris base.
c. Trans-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA).
d. Glycerol.
e. Phosphoric Acid.
f. Triton X-100.
g. 1M Dithiothreitol (DTT).
h. Transfected cells plated in 24-well plate.

**2. EQUIPMENT AND SUPPLIES:**

a. 1-200 $\mu$l Pipettor
b. Multi-channel pipettor,1-100 $\mu$l
c. 1-200 $\mu$l pipette tips
d. Pipette aid
e. Vacuum system with hazardous waste flask attached
f. pH meter
g. 5 3/4" Pasteur pipette
h. 500 ml squeeze water bottle
i. 96 well ELISA plate
j. 96 well Plate, white
k. 250 ml Glass beakers
l. 100 and 200 ml Graduated cylinders
m. Stirrer and stir bars

**3. PREPARATION:**

**a. 5X Lysis Solution.**

a. Weigh out 3.03g Tris Base and 0.695g CDTA and place in 250 ml beaker.
b. Dissolve completely in 60 ml of $dH_2O$.
c. Measure 100 ml glycerol in 100 ml graduated cylinder, pour into fresh 250 ml beaker.
d. Rinse 100 ml cylinder with Tris base/CDTA. Add to glycerol in 250 ml beaker. Mix well.
e. pH to 7.8 with phosphoric acid ($H_3PO_4$) if necessary.
f. Add $dH_2O$ to 200 ml.
g. 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 $dH_2O$. Add **30 µl** 1M DTT per **10 ml** 1X lysis solution. Make fresh each time. Make up enough 1X lysis solution to dispense 65µl 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 µl of 1X lysis solution per well.

c. Let sit at room temperature for 20 min, rocking occasionally.

d. Transfer 30 µl of cell lysate to 96 well ELISA plate. This will be used for the β-galactosidase assay.

e. Transfer 20 µl of cell lysate to a 96 well white plate. This will be used for the luciferase assay.

# ß-Galactosidase Assay Using Chlorophenol Red-β-D-galactopyranoside

## 1. MATERIALS AND SOURCES:

a. Chlorophenol red-ß-D-galactopyranoside (CPRG).
b. Disodium phosphate ($Na_2HPO_4$•$7H_20$).
c. Monosodium phosphate ($NaH_2PO_4$•$H_20$).
d. Potassium chloride (KCl).
e. Magnesium sulfate ($MgSO_4$•$7H_2O$).
f. β-Mercaptoethanol (2-ME).

## 2. EQUIPMENT AND SUPPLIES:

a. Spectrophotmetric 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 µl pipettor
h. 1-100 µl pipet tips
i. Pipettor reservoirs
j. 0.2 µ Filter unit
k. 96 well ELISA plate
l. 1 L beaker
m. 50 ml centrifuge tube, polypropylene, sterile

## 3. CPRG BUFFER PREPARATION:

a. Weigh out in 1 L beaker:
b. 16.1 g $Na_2HPO_4$•$7H_20$
c. 5.5 g $NaH_2PO_4$•$H_20$
d. 0.75 g KCl
e. 0.25 g $MgSO_4$•$7H_2O$
f. Dissolve in 800 ml of distilled water with stirring.
g. Adjust pH to 7.8.
h. Transfer to 1000 ml graduated cylinder. Bring up to 1000 ml with distilled water.
i. Filter sterilize. Store at room temperature.

## 4. ASSAY PROCEDURE:

a. Pipet 30 µl of cell lysate into a 96 well plate (usually done in triplicate).

b. **PER WELL OF 96 WELL PLATE**, add 170 µl of CPRG reagent made up as follows: 80 µg CPRG dissolved in 20 µl distilled water, 150 µl of CPRG buffer, and 0.84 µl 2-ME (1/200 dilution).

c. Using multi-channel pipettor, dispense 170 µl of CPRG reagent into each well containing lysate. For plate blank, use 30 µl of lysis solution and add 170 µl 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.**

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