

## **Appendix B2**

### **Protocol for CHO Cells + hAR + Luciferase Assay**

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**Protocol for CHO Cells + hAR + Luciferase Reporter Gene Assay**

**Day 1** A suspension of CHO cells (ATCC batch no. ) is made in DMEM/F12 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St.Louis,MO) and with 10% DCC serum (BioWhitaker). A 75cm<sup>2</sup> cell culture flask with almost confluent CHO cells is washed with 5 ml PBS and is trypsinated with 3 ml 0.05 % trypsin containing 0.02 M EDTA at 37 °C. Cells are diluted and cell number is determined (approx.  $120 * 10^4$  cells/ml). Calculate how much cell suspension, that is necessary and dilute the cell suspension with media so that every well is added 7000 cells (70,000 cells/ml). Cells are seeded in white microtiter plates from Packard with 100 µl / well.

**Passage of cells**

**Day 2** 9a.m.: Cells are approx. 90 % confluent. Cells are transfected with cDNA and FuGene 6 (Roche) according to the table. Cells are incubated for 5 hours (14 a.m.) Turn the plates on paper towel and add media +/- hormones and chemicals (p.3). Cells are incubated for 20 hours (11a.m)

**Day 3** Remove media by turning the plates on paper towel.

MgCl<sub>2</sub> is added to lysis buffer. All wells are added 20µl lysis buffer and incubated for 15 min on a shaker. Prepare luciferin solution containing (2 ml luciferin/ATP is added 2 ml lysis buffer with Mg Cl<sub>2</sub>). Protect from light. Measure luciferase activity on the BioOrbit Galaxy Luminometer directly in the plates by injection of 40 µl luciferin solution per well. The chemiluminiscense generated from each well is measured over a 1 sec interval after an incubation time of 2 sec.

Transfection scheme

The expression vector pSVAR0, AR13 and the MMTV-LUC reporter plasmid were both provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam.

Optimum AR reporter gene assay conditions:

For 200 wells:

DMEM/F12	: 940 $\mu$ l	} 5 $\mu$ l / well
Fugene	: 60 $\mu$ l	
DNA (totally)	: 15 $\mu$ g	

75 ng DNA per well

FuGene ( $\mu$ l) / DMEM+FuGene ( $\mu$ l) = 0.06

DNA  $\mu$ g / Fugene  $\mu$ l = 0.25

psvAR0 , MMTV-Luc = 1 : 100

AR13 , MMTV-Luc = 2 : 100 (for cytotoxicity determination)

	$\mu$ g DNA	Plate 1 - 6	Plate x (cytotoxicity)
DMEM/F12 without serum		3290 $\mu$ l	600 $\mu$ l
FuGene		210 $\mu$ l	38.3 $\mu$ l
psvAR0 0.1 $\mu$ g/ $\mu$ l (batch no. )	0.53 $\mu$ g	5.3 $\mu$ l	AR13(0.082 $\mu$ g/ $\mu$ l) 3.11 $\mu$ l
MMTV-Luc 2.252 $\mu$ g/ $\mu$ l (batch no. )	51.97 $\mu$ g	23.1 $\mu$ l	6.087 $\mu$ l

DMEM/F12 without serum is added to a 15 ml plastic vial. FuGene is added without touching the walls of the vial. Gently mix and incubate for 5 min at room temperature. cDNA is added to another 15 ml vial. The diluted FuGene solution is added drop-wise to the cDNA. Gently mixing. The solution incubates for 15 min at room temperature. 8\* 250  $\mu$ l is added to a column in a microtiter plate. 5  $\mu$ l is added to the each well containing the cells using a 100 $\mu$ l 8-channel pipette. Be sure that the cDNA is distributed well (DNA should lie as grain of sand in the media instantly or the day after).

## Overview of plates

Don't use row A and H

**50  $\mu$ l compound + 50  $\mu$ l media** is added according to the scheme

	<b>Final conc.:</b>	
<b>Plate 1</b>	<b>Compound x</b>	
Row B:	<b>0.01 nM R1881</b>	<b>0.02 nM R1881</b> x 12 wells + media
Row C:	0.025, 0.05, 0.10	<b>uM</b> x 4 wells + <b>0.02 nM R1881</b>
Row D:	0.20, 0.39, 0.78	-----”-----
Row E:	1.56, 3.13; 6.25	-----”-----
Row F:	12.5, 25, 50	-----”-----
Row G:	<b>0.01 nM R1881</b>	<b>0.02 nM R1881</b> x 12 wells + media
<b>Plate 2</b>		
Row B:	0; 0.001; 0.0023 nM	R1881 x 4 wells + media
Row C:	0.01; 0.023; 0.1 nM	R1881 x 4 wells + media
Row D:	0.23; 1.0; 2.3 nM	R1881 x 4 wells + media
Row E:	0; 1; 5 nM	OHF x 4 wells + <b>0.02 nM R1881</b>
Row F:	10; 50; 100 nM	OHF x 4 wells + <b>0.02 nM R1881</b>
Row G:	500; 1000; 5000nM	OHF x 4 wells + <b>0.02 nM R1881</b>

Solvents

Hydroxyflutamide and R1881: Stock solutions in freezer no.

**Positive antagonism control: Hydroxyflutamide (2 x conc.)**

10.000 nM:	5 $\mu$ l 5 mM + 2.5 ml media
2000 nM:	5 $\mu$ l 1 mM + 2.5 ml media
1000 nM:	5 $\mu$ l 500 $\mu$ M + 2.5 ml media
200 nM:	5 $\mu$ l 100 $\mu$ M + 2.5 ml media
100 nM:	5 $\mu$ l 50 $\mu$ M + 2.5 ml media
20 nM:	5 $\mu$ l 10 $\mu$ M + 2.5 ml media
10 nM:	5 $\mu$ l 5 $\mu$ M + 2.5 ml media
2 nM:	5 $\mu$ l 1 $\mu$ M + 2.5 ml media

**Positive agonism control: R1881 (2 x conc.)**

20 nM:	5 $\mu$ l 10 $\mu$ M + 2.5 ml media – (not to be added)
4.6 nM:	500 $\mu$ l 20 nM + 1.665 ml media
2 nM:	5 $\mu$ l 1 $\mu$ M + 2.5 ml media
0.46 nM	500 $\mu$ l 2 nM + 1.665ml media
0.2 nM:	5 $\mu$ l 0.1 $\mu$ M + 2.5 ml media
0.02 nM:	<b>40 <math>\mu</math>l 0.01 <math>\mu</math>M + 20 ml media</b>
0.046 nM	500 $\mu$ l 0.2 nM + 1.665 ml media
0.002 nM:	5 $\mu$ l 0.001 $\mu$ M + 2.5 ml media
0.0046 nM:	500 $\mu$ l 0.02 nM + 1.665 ml media
0 nM:	5 $\mu$ l EtOH + 2.5 ml media.

**Compound x (2 x conc.)**

100 $\mu$ M:	25 $\mu$ l 20 mM + 5 ml media
50.0 $\mu$ M:	1 ml 100 $\mu$ M + 1 ml media
25.0 $\mu$ M:	1 ml 50 $\mu$ M + 1 ml media
12.5 $\mu$ M:	1 ml 25 $\mu$ M + 1 ml media
6.25 $\mu$ M:	1 ml 12.5 $\mu$ M + 1 ml media
3.13 $\mu$ M:	1 ml 6.25 $\mu$ M + 1 ml media
1.56 $\mu$ M:	1 ml 3.13 $\mu$ M + 1 ml media
0.78 $\mu$ M:	1 ml 1.56 $\mu$ M + 1 ml media
0.39 $\mu$ M:	1 ml 0.78 $\mu$ M + 1 ml media
0.20 $\mu$ M:	1 ml 0.39 $\mu$ M + 1 ml media
0.10 $\mu$ M:	1 ml 0.20 $\mu$ M + 1 ml media
0.05 $\mu$ M:	1 ml 0.10 $\mu$ M + 1 ml media

Media, buffers, compounds etc.

Lysis buffer: 25 mM Trisphosphate pH 7.8 (adjusted with phosphoric acid)  
 15 % glycerol  
 1 % Triton X-100  
 1 mM DTT  
 Stored in sterile 50 ml vials at -20°C  
 Before use: add 8 mM MgCl<sub>2</sub> (8 µl 1M MgCl<sub>2</sub>/ml buffer)

Requirement: x \* 10 ml lysis buffer + x \* 4.5 ml luciferin/ATP  
 (Freezer no. )

Requirement of media: DMEM/F12 + 10 % DCC x ml + x ml for counting xx ml  
 DMEM/F12 + 10 % FBS for a 25cm<sup>2</sup> flask xx ml  
 DMEM/F12 + 1% PSF xx ml

Compounds: Comp.1 Mw. xx g/mol  
 Supplier: Lot no.: Purity:  
 Stocksolution of 20 mM (x mg to x ml EtOH)  
 Date: Person:  
 Remarks:

Ethanol: Merck pro analysis UN 1170, K 27773283-020

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