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^2 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.

$MgCl_2$ 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_2). 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 µl
Fugene : 60 µl
DNA (totally) : 15 µg
} 5 µl / well

75 ng DNA per well

FuGene (ul) / DMEM+FuGene (ul) = 0.06
DNA µg / Fugene µl = 0.25
psvAR0 , MMTV-Luc = 1 : 100
AR13 , MMTV-Luc = 2 : 100 (for cytotoxicity determination)

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

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 µl is added to a column in a microtiter plate. 5 µl is added to the each well containing the cells using a 100µl 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 µl compound + 50 µl media is added according to the scheme

	Final conc.:	
Plate 1	**Compound x**	
Row B:	**0.01 nM R1881**	**0.02 nM R1881** x 12 wells + media
Row C:	0.025, 0.05, 0.10	**uM** x 4 wells + **0.02 nM R1881**
Row D:	0.20, 0.39, 0.78	----------" ----------
Row E:	1.56, 3.13; 6.25	----------" ----------
Row F:	12.5, 25, 50	----------" ----------
Row G:	**0.01 nM R1881**	**0.02 nM R1881** x 12 wells + media
Plate 2		
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 + **0.02 nM R1881**
Row F:	10; 50; 100 nM	OHF x 4 wells + **0.02 nM R1881**
Row G:	500; 1000; 5000nM	OHF x 4 wells + **0.02 nM R1881**

Solvents

Hydroxyflutamide and R1881: Stock solutions in freezer no.

Positive antagonism control: Hydroxyflutamide (2 x conc.)

10.000 nM:	5 µl 5 mM + 2.5 ml media
2000 nM:	5 µl 1 mM + 2.5 ml media
1000 nM:	5 µl 500 µM + 2.5 ml media
200 nM:	5 µl 100 µM + 2.5 ml media
100 nM:	5 µl 50 µM + 2.5 ml media
20 nM:	5 µl 10 µM + 2.5 ml media
10 nM:	5 µl 5 µM + 2.5 ml media
2 nM:	5 µl 1 µM + 2.5 ml media

Positive agonism control: R1881 (2 x conc.)

20 nM:	5 µl 10 µM + 2.5 ml media **– (not to be added)**
4.6 nM:	500 ul 20 nM + 1.665 ml media
2 nM:	5 µl 1 µM + 2.5 ml media
0.46 nM	500 ul 2 nM + 1.665ml media
0.2 nM:	5 µl 0.1 µM + 2.5 ml media
0.02 nM:	4**0 µl 0.01 µM + 20 ml media**
0.046 nM	500 ul 0.2 nM + 1.665 ml media
0.002 nM:	5 µl 0.001 µM + 2.5 ml media
0.0046 nM:	500 ul 0.02 nM + 1.665 ml media
0 nM:	5 µl EtOH + 2.5 ml media.

Compound x	**(2 x conc.)**
100 µM:	25 µl 20 mM + 5 ml media
50.0 µM:	1 ml 100 µM + 1 ml media
25.0 µM:	1 ml 50 µM + 1 ml media
12.5 µM:	1 ml 25 µM + 1 ml media
6.25 µM:	1 ml 12.5 µM + 1 ml media
3.13 µM:	1 ml 6.25 µM + 1 ml media
1.56 µM:	1 ml 3.13 µM + 1 ml media
0.78 µM:	1 ml 1.56 µM + 1 ml media
0.39 µM:	1 ml 0.78 µM + 1 ml media
0.20 µM:	1 ml 0.39 µM + 1 ml media
0.10 µM:	1 ml 0.20 µM + 1 ml media
0.05 µM:	1 ml 0.10 µM + 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 $MgCl_2$ (8 µl 1M $MgCl_2$/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 DMEM/F12 + 10 % FBS for a 25cm^2 flask DMEM/F12 + 1% PSF	xx ml xx ml 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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