

Appendix B2

Protocol for the COS Cell Binding Assay

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COS CELL BINDING ASSAY

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1. Day 1- Monday

Plate 400,000 COS-1 cells/well of 6 well plate in 3 ml 10% bovine calf serum, DMEM-H/20 mM Hepes, glutamine, pen/strep (use stock of 2 M Hepes, pH 7.2, sterile filter)

(200,000 cells/12 well plate with 2 ml media for Scatchard analysis)

2. Day 2, prepare DNA

0.95 ml 1.08x TBS/well

2 µg AR DNA for 6 well comp binding (0.1-3 µg AR DNA for 12 well, 3 µg for GAL/VP vectors)

0.11 ml DEAE-dextran

(250 mg/50 ml, autoclaved water, sterile filter made fresh)

Aspirate media, add 1 ml DNA solution, incubate 30 min at 37°C, aspirate media

Add 3 ml/6 well of chloroquine-media (2 ml/12 well)

Prepare 5 mg/ml chloroquine in dH₂O fresh, sterile filter, add 1 ml of 5 mg/ml chloroquine to 100 ml 10% BCS/DMEM-H, 20 mM Hepes media

Incubate 3 h at 37°C, aspirate media

Glycerol shock 4 min at RT with 1 ml/6 well (or 12 well) of 15% glycerol in 10% BCS/DMEM-H

Aspirate, wash carefully 1X with 3 ml 1xTBS/6 well (2 ml/12 well)

Add 3 ml 10% BCS DMEM-H, incubate overnight in incubator

3. Day 3, leave in 10% serum containing media until next day

4. Day 4, aspirate (don't wash), set up tubes for binding assay:

Use 600 µl/6 well of 5 nM [³H]R1881 labeling solution in serum free/phenol red free with or without 100 fold excess unlabeled R1881 for nonspecific binding control (400 µl/12 well)

For calculations, prepare 0.625 ml/well for all h and h+c wells in serum-free, phenol red-free media

To make h + c, # h+c wells x 0.625 ml, take this volume from 5 nM hot solution, add cold R1881 so final is 100 fold higher (500 nM) unlabeled R1881 with 5 nM [³H]R1881

Incubate 2 hr at 37°C (for Scatchard in 12 well, after 2 h labeling, take 100 µl for free counts)

For ligand dissociation experiment:

Add 10,000 fold excess of cold R1881 (50 μ M final) in 0.1 ml serum free media (350 μ M, 7X stock)

Amount to prepare: 100 μ l x total # wells + 0.5 ml extra

Spread plates out in incubator, start timer, incubate at 37°C for times indicated

Remove at indicated time, aspirate using radioactive flask; wash carefully 1X with 3 ml PBS

Aspirate to dry, harvest in 500 μ l 1X sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 6.8) for 6 or 12 well, add 4 ml scintillation fluid and count

	<u>500 ml</u>	<u>final conc</u>	<u>4 liters</u>
2X TBS:	8.18 g NaCl	280 mM NaCl	65.44 gr NaCl
	0.23 g KCl	6 mM KCl	1.84 g KCl
	0.147 g CaCl ₂ -2H ₂ O	2 mM CaCl ₂	1.18 g CaCl ₂ -2H ₂ O
	0.1 g MgCl ₂ -6H ₂ O	1 mM MgCl ₂	0.8 g MgCl ₂ -6H ₂ O
	0.128 g NaH ₂ PO ₄ -H ₂ O	1.8 mM NaH ₂ PO ₄	1.02 NaH ₂ PO ₄ -H ₂ O
	3.03 g Tris	50 mM Tris pH 7.4	24.24 g Tris
pH to 7.4			

	<u>500 ml</u>	<u>4 liters</u>	<u>final</u>	<u>MW</u>
1.08X TBS:	4.42 g NaCl	35.34 g NaCl	51.2 mM NaCl	58.44
	0.121 g KCl	1.0 g KCl	3.24 mM KCl	74.56
	0.08 g CaCl ₂ -2H ₂ O	0.64 g CaCl ₂ -2H ₂ O	1.08 mM CaCl ₂	147.02
	0.055 g MgCl ₂ -6H ₂ O	0.439 g MgCl ₂ -6H ₂ O	0.54 mM MgCl ₂	203.3
	0.067 g NaH ₂ PO ₄ -H ₂ O	0.54 g NaH ₂ PO ₄ -H ₂ O	0.972 mM NaH ₂ PO ₄	137.99
	1.636 g Tris	13.09 g Tris	27 mM Tris pH 7.4	121.14
pH to 7.4				
or 270 ml 2XTBS + 230 ml H ₂ O = 1.08xTBS				