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#### **COMPETITION ASSAY**

# Membrane preparation

Frozen tissue or washed cells are homogenized in 20 volumes of cold lysis buffer (50mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, protease inhibitor cocktail). After a low speed spin (1,000 x g for 3 mintes) to remove large tissue chunks (tissue homogenates), the homogenate is centrifuged at 20,000 x g for 10 minutes at 4 °C to pellet the membranes. The pellet is resuspended in fresh buffer and centrifuged at the same speed a second time. The pellet is then resuspended into buffer (15 ml) containing 10 % sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay. On the day of the assay the membrane preparation is thawed and the pellet resuspended in final assay binding buffer (50 mM Tris, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, pH 7.4).

### Incubation and filtration

The filtration binding assay is carried out in 96-well plates in a final volume of 250  $\mu$ L per well. To each well is added 150  $\mu$ L membranes (3 - 20  $\mu$ g protein for cells or 50 - 120  $\mu$ g protein for tissue), 50  $\mu$ L of the competing test compound and 50  $\mu$ L of radioligand solution in buffer. The plate is incubated at 30 °C for 60 minutes with gentle agitation. The incubation is stopped by vacuum filtration onto 0.3 % PEI presoaked GF/C filters using a 96-well FilterMate<sup>TM</sup> harvester followed by four washes with ice-cold wash buffer. Filters are then dried for 30 minutes at 50 °C. The filter is sealed in polyethylene, scintillation cocktail (Betaplate Scint; PerkinElmer) added and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

#### Data analysis

For each drug concentration, non-specific binding is subtracted from total binding to give specific binding. Data is fitted using the non-linear curve fitting routines in Prism® (Graphpad Software Inc). For competition assays, Ki values are calculated from  $IC_{50}$  values using the formula  $K_i = IC_{50} / (1 + ([S]/K_d))$  where [S] is the radiotracer concentration used in the assay and  $K_d$  is the dissociation constant of the radiotracer.



#### SATURATION ASSAY

# Membrane preparation

Frozen tissue or washed cells are homogenized in 20 volumes of cold lysis buffer (50mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, protease inhibitor cocktail). After a low speed spin (1,000 x g for 3 minutes) to remove large tissue chunks (tissue homogenates), the homogenate is centrifuged at 20,000 x g for 10 minutes at 4 °C to pellet the membranes. The pellet is resuspended in fresh buffer and centrifuged at the same speed a second time. The pellet is then resuspended into buffer (15 ml) containing 10 % sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay. On the day of the assay the membrane preparation is thawed and the pellet resuspended in final assay binding buffer.

#### Incubation and filtration

The filtration binding assay is carried out in 96-well plates in a final volume of 250  $\mu$ L per well. To each well is added 150  $\mu$ L membranes (3 - 20  $\mu$ g protein for cells; 50 - 120  $\mu$ g protein for tissue), 50  $\mu$ L of the unlabeled compound (non-specifics) or buffer and 50  $\mu$ L of radioligand solution in binding buffer. The radioligand is added at up to 8 different concentrations (e.g. 0.2 - 20 nM). The plate is incubated at 30 °C for 60 minutes with gentle agitation. The incubation is stopped by vacuum filtration onto 0.3 % PEI presoaked GF/C filters using a 96-well FilterMate<sup>TM</sup> harvester followed by four washes with ice-cold wash buffer. Filters are then dried for 30 minutes at 50 °C. The filter is sealed in polyethylene, scintillation cocktail (Betaplate Scint; PerkinElmer) added and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

## Data analysis

For each radioligand concentration, non-specific binding is subtracted from total binding to give specific binding. Bound CPM values are converted to fmoles per mg protein. Data is fitted using the saturation analysis non-linear curve fitting routines in Prism® (Graphpad Software Inc). The K<sub>d</sub> (in nM) and B<sub>max</sub> (fmol/mg or sites/cell) are derived from the saturation curve.



#### **KINETIC ASSAY**

### Membrane preparation

Washed cells are homogenized in 10 ml of cold lysis buffer (50mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, protease inhibitor cocktail). The homogenate is centrifuged at 20,000 x g for 10 minutes at 4 °C to pellet the membranes. The pellet is resuspended in fresh buffer and centrifuged at the same speed a second time. The pellet is then resuspended into buffer (5 ml) containing 10 % sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay. On the day of the assay the membrane preparation is thawed and the pellet resuspended in final assay binding buffer (50 mM Tris, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, pH 7.4).

#### Incubation and filtration

<u>Dissociation assays:</u> To each well is added 150 μL of receptor preparation and 50 μL of radioligand solution in buffer. The plate is incubated at 30 °C for 90 minutes (or until equilibrium binding) with gentle rotation. Dissociation is then initiated by addition of a saturating concentration (e.g.  $10 \mu M$ ) of the unlabeled ligand at various time points prior to termination of the assay. The incubation is stopped by vacuum filtration onto 0.3 % PEI presoaked GF/C filters using a 96-well FilterMate<sup>TM</sup> harvester followed by four washes with icecold wash buffer. Filters are then dried for 30 minutes at 50 °C. The filter is sealed in polyethylene, scintillation cocktail (Betaplate Scint; PerkinElmer) added and the radioactivity counted in a Wallac<sup>®</sup> TriLux 1450 MicroBeta counter.

Association assays: The radioligand binding assay is carried out in a final volume of 250  $\mu$ L per well. To each well is added 200  $\mu$ L of receptor preparation followed by 50  $\mu$ L of radioligand solution in buffer at various time points prior to the termination of the assay. The incubation is stopped by vacuum filtration onto 0.3 % PEI presoaked GF/C filters using a 96-well FilterMate<sup>TM</sup> harvester followed by four washes with ice-cold wash buffer. Filters are then dried for 30 minutes at 50 °C. The filter is sealed in polyethylene, scintillation cocktail (Betaplate Scint; PerkinElmer) added and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

### Data analysis

For each time point, non-specific binding is subtracted from total binding to give specific binding. Data is fitted using the non-linear curve fitting routines in Prism® (Graphpad Software



Inc). Dissociation data is analyzed to give  $k_{off}$  and  $t_{1/2}$ . For association curves, a two radioligand concentrations model is used to obtain  $k_{on}$ . For the association analysis,  $k_{off}$  is constrained to the value obtained from the dissociation data.

#### LIVE CELL RADIOLIGAND BINDING ASSAY

### Incubation and washing (adherent cells)

24 - 48 hours prior to the assay, cells are seeded into 24 well plates and grown to near confluence. On the day of the assay, the cells are gently washed with two changes of PBS. The cells are then incubated with PBS (or DMEM) containing the radiolabeled ligand and competing compounds for 60 minutes (or until equilibrium) at 35 °C. The incubation is stopped by gentle washing of the cell layer with ice-cold PBS (three changes). To each well is added NaOH (0.1 M, 100 uL) and the plates incubated overnight. The solution is then transferred to a 24 well counting plate, neutralized and scintillation cocktail added. Radioactivity is counted in a Wallac® TriLux 1450 MicroBeta counter.

## Incubation and filtration (non-adherent cells)

At room temperature, 150  $\mu$ L of cells (50,000 - 150,000 cells) is added to each well, followed by 50  $\mu$ L of "cold" unlabeled antibody or protein solution (for non-specifics) or buffer and 50  $\mu$ L of [\$^{125}I\$]-labeled antibody or protein. The [\$^{125}I\$]-labeled protein is added at up to 8 different concentrations (e.g. 0.13 – 16 nM). The plate is incubated for 120 minutes with gentle agitation on an orbital shaker. The incubation is stopped by mild vacuum filtration onto GF/C filtermats (presoaked in buffer with BSA) using a 96-well FilterMate harvester. Washing is accomplished using an initial wash with ice-cold assay buffer followed by several washes with ice cold PBS. Filters are then dried under a stream of warm air. Scintillation cocktail (Betaplate Scint; PerkinElmer) is added to the filters and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

### Data analysis

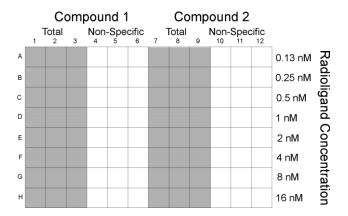
Bound CPM values are converted to fmoles bound [ $^{125}$ I]protein or [ $^{3}$ H]ligand per assay well and plotted against radioligand concentration (nM). Data is fitted using the saturation analysis non-linear curve fitting routines in Prism® (Graphpad Software Inc) to derive the K<sub>d</sub> (in nM) and receptor density ( $B_{max}$ ; fmol/mg or sites/cell) values.



## **PLATE LAYOUTS**

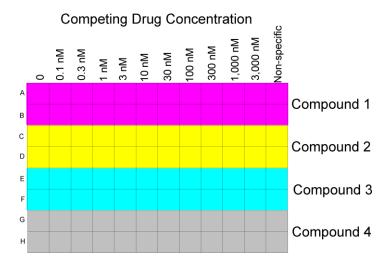
# Competition assay

Typical plate layout for 4 competing drugs with  $K_i$  values in the range of 3 - 30 nM. Duplicate replicates.



# Saturation assay

Typical plate layout for 2 radiolabeled compounds with  $K_d$  values in the range of 0.5 - 2 nM. Triplicate replicates.





# Kinetic assay

Typical plate layout for a dissociation assay for a radiolabeled compound with  $t_{1/2}$  in the range of 15 - 30 min. Four conditions with duplicate replicates at each.

Α		E	В С		С	D				
								120	_	
								90	Diss	
								60	OC.	
								50	Dissociation duration (min)	
								40	n d	
								30	ura	
								20	tior	
								10	π T	
								5	j.	
								0		
								Non-	Spec.	
								Blanks		

Typical plate layout for an association assay. Two radioligand concentrations each with total and non-specifics and duplicate replicates.

Low radioligand concentration					gh ra				
Total		Non-Specific		Total		Non-sp	oecific		
								40	
								30	As
								20	SOC
								10	iati
								7	on o
								5	dura
								4	atio
								3	n (r
								2	Association duration (min)
								1	_
								0.5	
								Blan	ıks