Data Sheet
Radioligand Binding Assay
Protocols

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info@giffbio.com
www.giffordbioscience.com

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COMPETITION ASSAY

Membrane Preparation

Frozen tissue or washed cells are homogenized in 20 volumes of cold lysis buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail). After a low speed spin (100 x g for 3 minutes) to remove large tissue chunks (tissue homogenates), the homogenate is centrifuged at 17,000 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 for a second time, again at 4 °C. 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 $MgCl_2$, 0.1 mM EDTA, pH 7.4).

Incubation in Radiotracer

The filtration binding assay is carried out in 96-well plates in a final volume of 250 µL per well. To each well is added 150 µL membranes (3 - 20 µg protein for cells or 50 - 120 µg protein for tissue), 50 µL of the competing test compound and 50 µL 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™ 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.

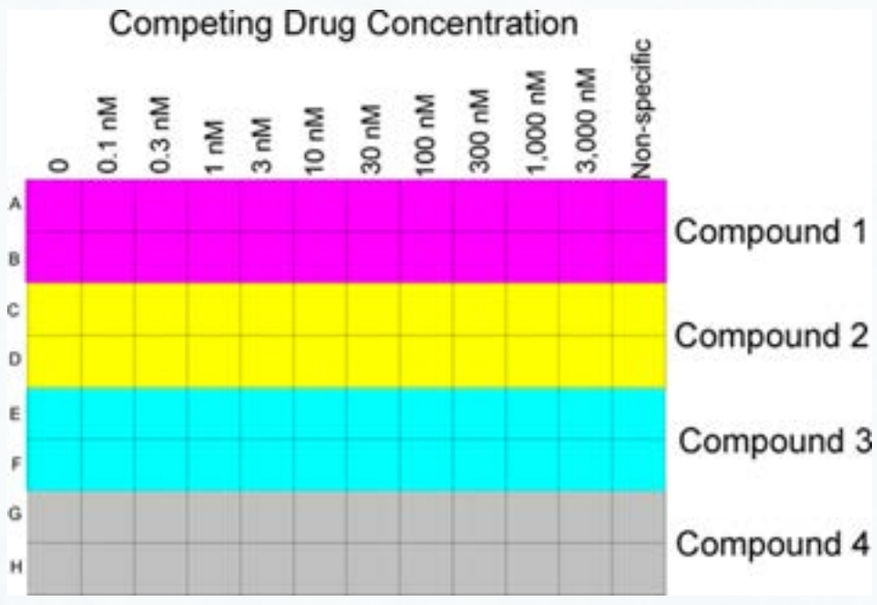

Figure 1: Typical plate layout for 4 competing drugs with Ki values in the range of 3 - 30 nM. Duplicate replicates.

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, K_i 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.

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SATURATION ASSAY

Membrane Preparation

Frozen tissue or washed cells are homogenized in 20 volumes of cold lysis buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail). After a low speed spin (100 x g for 3 minutes) to remove large tissue chunks (tissue homogenates), the homogenate is centrifuged at 17,000 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 for a second time, again at 4 °C. 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 µL per well. To each well is added 150 µL membranes (3 - 20 µg protein for cells; 50 - 120 µg protein for tissue), 50 µL of the unlabeled compound (non-specifics) or buffer and 50 µL 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™ 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.

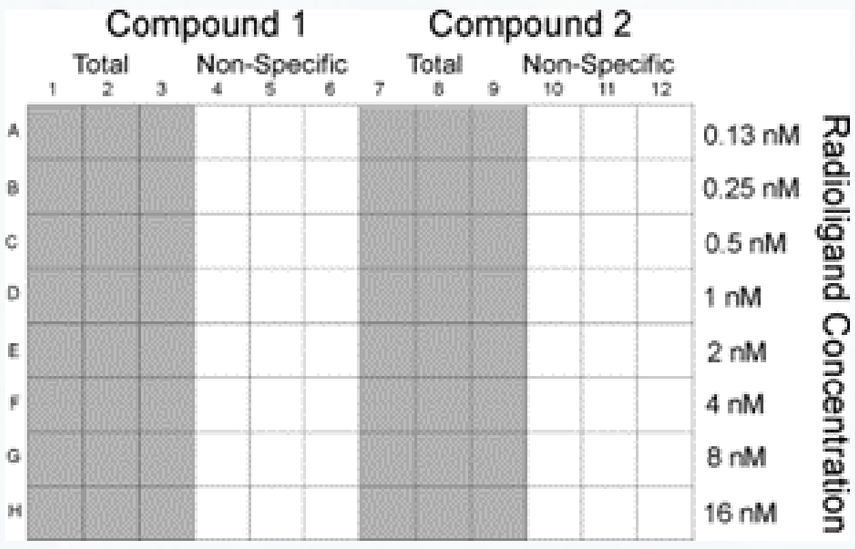

Figure 2: Typical plate layout for 2 radiolabeled compounds with Kd values in the range of 0.5 - 2 nM. Triplicate replicates.

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_d (in nM) and B_{max} (fmol/mg or sites/cell) are derived from the saturation curve.

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KINETIC ASSAY

Membrane preparation

Washed cells are homogenized in 10 ml of cold lysis buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail). The homogenate is centrifuged at 17,000 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 for a second time, again at 4 °C. 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 $MgCl_2$, 0.1 mM EDTA, pH 7.4).

Incubation and filtration

Dissociation assays: 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 µM) 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™ 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.

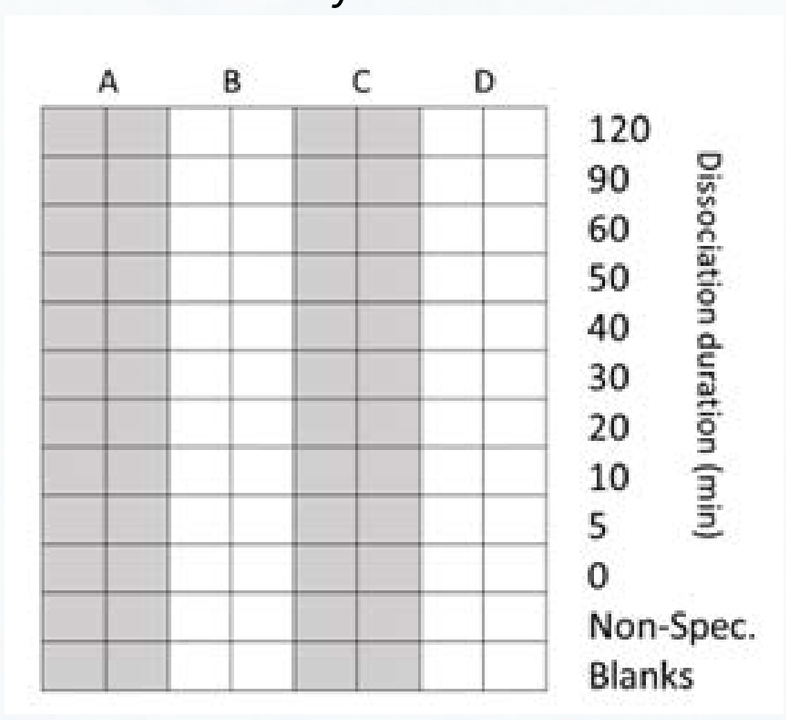

Figure 3: Typical plate layout for a dissociation assay for a radiolabeled compound with t1/2 in the range of 15 - 30 min. Four conditions with duplicate replicates.

Association assays: The radioligand binding assay is carried out in a final volume of 250 µL per well. To each well is added 200 µL of receptor preparation followed by 50 µL 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™ harvester followed by four washes with ice-cold wash buffer.

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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.

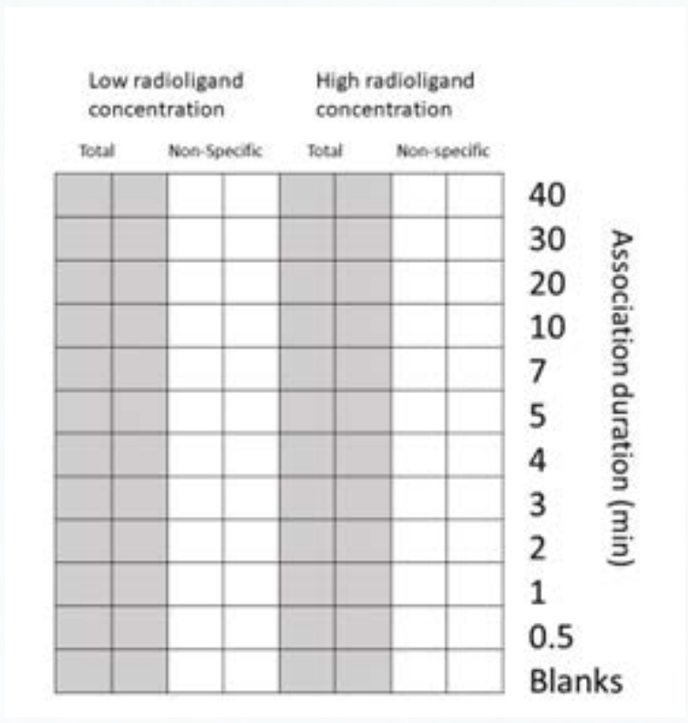

Figure 4: Typical plate layout for an association assay. Two radioligand concentrations each with total and non-specifics and duplicate replicates.

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.

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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 µL of cells (50,000 - 150,000 cells) is added to each well, followed by 50 µL of “cold” unlabeled antibody or protein solution (for non-specifics) or buffer and 50 µL 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_d (in nM) and receptor density (B_{max}; fmol/mg or sites/cell) values.

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