

## UPTAKE INHIBITION ASSAY

### Cells (non-adherent)

The uptake assay is carried out in 96-well plates in a final volume of 250  $\mu\text{L}$  per well at 37  $^{\circ}\text{C}$ . 150  $\mu\text{L}$  of cells (50,000 – 150,000 cells) is added to each well followed by 50  $\mu\text{L}$  of the competing test compound or inhibitor for defining non-specific uptake. After 30 min uptake is initiated by adding 50  $\mu\text{L}$  of radiolabeled substrate in buffer. The plate is incubated for a predetermined interval with gentle agitation. 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 cold assay buffer followed by several washes with 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.

### Cells (adherent)

Cells are seeded into 24 (or 96) well plates and incubated in full growth medium until near confluence is obtained. On the day of the assay the medium is aspirated off replaced with 0.15 ml fresh assay buffer (HBSS-HEPES, pH 7.4). To each well is added 50  $\mu\text{L}$  of the competing test compound or inhibitor and the plate incubated for 30 min. Uptake is initiated by adding 50  $\mu\text{L}$  of radiolabeled substrate in buffer. The plate is incubated for a predetermined interval with gentle agitation. The incubation is stopped by washing the cells with three changes of cold PBS. The cells are then lysed with Solvable® (50  $\mu\text{l}$ ). After lysis, scintillation cocktail is added to each well and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

### Synaptosomes

*Tissue preparation:* The brain is rapidly dissected from a rat, cut into pieces and placed into 20 volumes of ice-cold 0.32 M sucrose solution. The tissue is then gently homogenized in a glass homogenizer using several up-and-down strokes of the pestle. After a low speed spin (1,000 x g for 5 min) to remove large tissue chunks, the homogenate is centrifuged at 14,000 x g for 15 minutes at 4 $^{\circ}\text{C}$  to obtain a crude synaptosomal pellet. The synaptosomal pellet is resuspended in Krebs buffer saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. For monoamine uptake, the buffer additionally contains ascorbate and a MAO inhibitor (pargyline). A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay.

*Incubation and filtration:* The uptake assay is carried out in 96-well plates in a final volume of 250  $\mu\text{L}$  per well at 37  $^{\circ}\text{C}$ . To each well is added 180  $\mu\text{L}$  synaptosomes (30 - 80  $\mu\text{g}$  protein) and 50  $\mu\text{L}$  of the competing test compound or inhibitor for defining non-specific uptake. After 30 min, uptake is initiated by adding 20  $\mu\text{L}$  of  $^3\text{H}$ -labeled transporter substrate in incubation buffer. The plate is incubated for a predetermined interval with gentle agitation. The incubation is stopped by gentle vacuum filtration onto GF/C filters using a 96-well FilterMate™ harvester followed by three washes with ice-cold wash buffer. Filters are then dried for 30 minutes at 50 $^{\circ}\text{C}$ . 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 (determined in the presence of a saturation concentration of a transport inhibitor) is subtracted from total binding to give specific binding. Data is fit using the non-linear curve fitting routines in Prism® (Graphpad Software Inc) to obtain the  $\text{IC}_{50}$  value for each inhibitor.

### Plate layouts

Typical plate layout for testing 4 inhibitors with  $\text{IC}_{50}$  values in the range of 5 - 20 nM. Duplicate replicates.

