High Density Receptor Ligand Binding Assays in the MultiScreen®HTS 384-well Glass Fiber Filter Plate

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Abstract

Radiometric filter binding assays are acknowledged for their ability to identify and confirm lead compounds in drug discovery. They can be limited in their utility due to throughput, cost, and amenability to automation. Data obtained using a new 384 well filter plate demonstrate that automated quantitative and screening assays can be developed that are equivalent to assays run in a 96-well format.

Using the Muscarinic M1 G-protein Coupled Receptor as a model system, we achieved accurate and reproducible determinations of binding affinity (Kd) and IC50 results for known ligands on the 384-well plate device. As a result of being able to incubate the reaction mixture in the filter plate and configure the assay using half the reaction volume, significant reductions in reagent costs and radioactive waste were achieved. Use of the fully-automated 384 filter plate makes it possible to develop higher throughput, lower cost radiometric receptor binding assays.

Introduction

Receptors are biological macromolecules located in the cell membrane, the cytoplasm or the cell nucleus that are capable of highly specific binding to chemical ligands. Upon binding of a receptor by a ligand, signal transduction events occur that regulate various biological processes required for proper growth and function of the cell.

Many disease states can be treated by regulating the activity of receptors and/or their downstream effector molecules. For instance, uncontrolled cellular proliferation during cancer can be reduced by blocking the activity of growth factor receptors. In addition to blocking the activity of receptors, many diseases can be treated by replacing the activity of an endogenous ligand with a more potent chemical entity, either when the production of the ligand is limited or changes in the receptor alter its specificity for the natural ligand. Thus, a major focus in drug discovery is the indentification of those drugs which bind specifically to cellular receptors to reduce (antagonists) or augment (agonists) a biological process.

A significant portion of the drug discovery process involves the systematic screening of large compound libraries for specific binding to various cellular receptors. For decades, heterogeneous filter binding assays have been used for this purpose, particularly under more challenging conditions such as when the receptor is prepared from unpurified tissue homogenates or cell membrane fragments. To date, most radioreceptor filter binding screening has been performed on 96-well platforms. However, a bottleneck has been created which has made it necessary to develop higher throughput platforms that make it possible to perform adequate library screening in a timely and cost effective manner. The 384-well filter binding format presented here better addresses these needs by increasing the number of samples that can be screened in a single plate, reducing the amount of reagents for each assay, and by performing and quantifying the binding reactions directly in the plate.

Methods

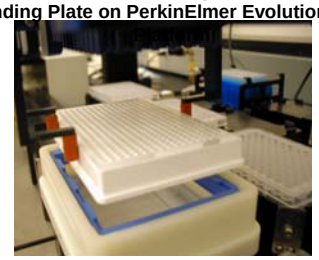
MultiScreen (Millipore, FB glass fiber cat# MAFB N0B) and MultiScreen$_{HTS}$ 384 (Millipore, FB glass fiber) filter plates were pretreated with 0.1% polyethyleneimine (PEI) and washed with appropriate assay buffer.

Saturation Binding: Unless otherwise stated, all binding experiments were performed by mixing the reagents in the filter plate and incubation to equilibrium was performed directly in the plate as well. 8.75 µg (96 well) or 4.38 µg (384 well) of a human Muscarinic M1 receptor expressing transgenic CHO cell membrane fragment bound receptor preparation (PerkinElmer) was incubated [200 ul (96 well), 100 ul (384 well)] with serial dilutions of radiolabeled ligand [(^{3}H-scopolamine (PerkinElmer NEN)]. After 1 hour incubation in the filter plate, plates were washed 10 times with binding buffer then vacuum. The plates were dried completely before the addition of Opti-Fluor® Scintillation cocktail (PerkinElmer cat# 6013199) Scintillation counting and analysis was performed on a Wallac Microbeta® TriLux scintillation counter in default coincidence counting mode. Non-specific binding was determined in a separate experiment with an excess of unlabeled competitor ligand [pirenzipine (Sigma)]. Specific binding was calculated as non-specific activity subtracted from total activity. Binding constants (Kd) were determined by fitting specific binding by free ligand concentration by non-linear regression and Scatchard analysis (shown) using Prizm data software (www.Graphpad.com).

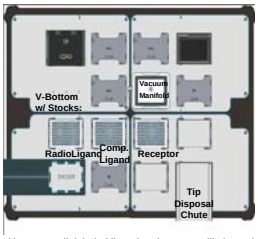
Displacement Binding: Receptor amounts used were the same as for above. Radioligand binding inhibition was determined with a constant radioligand concentration (0.6 nM ^{3}H-scopolamine) and serial dilutions of unlabelled competitor ligand (pirenzipine) as compared to a control binding experiment without unlabelled ligand (% Control). Relative affinity values (IC50) were determined by fitting binding inhibition values by non-linear regression using Prizm data software.

For out-of-plate assays, reagents were mixed as above but incubating in V-Bottom 96 well plates before being transferred to PEI treated 384 well filter plates. Subsequent washes and analyses were performed the same as above.

View of MultiScreen$_{HTS}$ 384 well Filter Binding Plate on PerkinElmer Evolution™ P^3



Automated Assays with MultiScreen®$_{HTS}$ on PerkinElmer Evolution™ P^3 Platform



Radioligand, competitive non-radiolabeled ligand and receptor dilution, mixing, and filter plate washing were all performed on the PerkinElmer Evolution™ P^3 platform. The programs allows the user to input number of plates to run, number of filter plate washes, and whether the receptor-ligand binding reaction is performed directly in a filter plate or in a V-Bottom incubation plate followed by transfer to a filter plate.

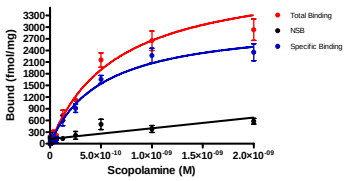
Binding Parameter Determination on 96 and 384 Well formats

Binding Parameter	96 well	384 Well	Literature *
Bmax (fmol/mg)	3288 +/- 129	3285 +/- 164	1100 to 8900
^{3}H Scopolamine Log Kd (M)	-9.1 +/- 0.2	-9.3 +/- 0.3	-9.3
Pirenzipine Log IC50 (M)	-6.9 +/- 0.1	-7.0 +/- 0.2	-6.5 +/- 0.1

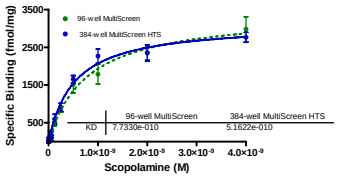
* Haga K and Haga T, "Purification of the Muscarinic Acetyl Choline Receptor from Porcine Brain" J. Biol. Chem., 260 (1985): 7927-7935

G-Protein Coupled Receptor (GPCR) Binding on MultiScreen® Plate with PEI Treated FB Glass Fiber

384 Muscarinic M1 Binding

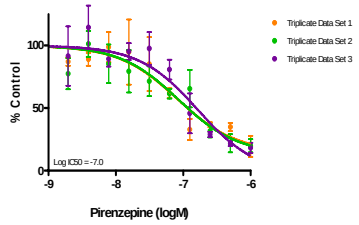


Human Muscarinic M1 Receptor Binding: 96 and 384 well FB Filter Plates



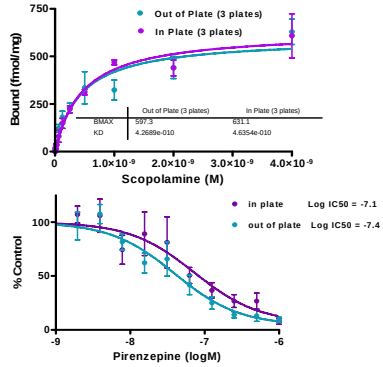
Reproducibility of Binding Parameter Determination On Multiscreen HTS Plate

384 Displacement Binding: Pirenzepine Displacement of Scopolamine Binding to Human Muscarinic M1 Receptor



Radioligand binding inhibition was determined with a constant radiolabeled scopolamine concentration (0.6nM) and serial dilutions of unlabelled pirenzipine as compared to a control binding experiment without unlabelled pirenzipine (% Control). Relative affinity values (IC50) were determined by fitting displacement binding inhibition values by non-linear regression using Prizm data software.

Equivalence of In-Plate and Out of Plate Muscarinic M1 Receptor-Ligand Incubation



Pirenzepine Displacement of Scopolamine

Summary

•Receptor-ligand binding assays can be performed in a 384 well filter plate format with the same robustness and reproducibility as 96 well filter plate formats.

• Receptor ligand binding assays were performed with a G-protein Coupled Receptor (GPCR), the human Muscarinic M1 receptor. Accurate and reproducible determinations of receptor specific activity (Bmax), binding affinity (Kd) and IC50 values of competitor ligands were measured. Values obtained in 96 well and 384 well FB glass fiber plates were in agreement with each other and literature values.

•By optimizing the 384 well filter plate for coincidence counting, highly quantitative data can be collected at very low levels of radioactivity over a large range.

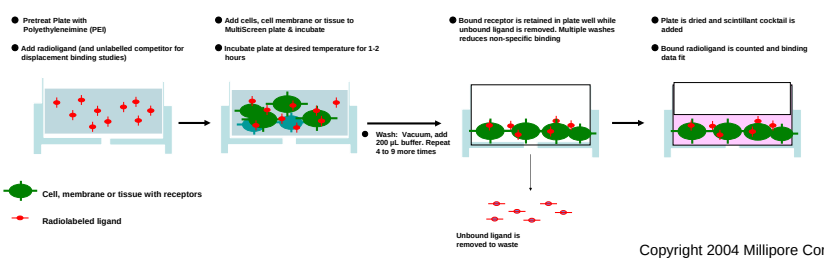
•Receptor ligand binding in the 384 well filter plate was performed with half the reagents in half the assay volume as the 96 well format.

•Cost savings through the use of less reagents can be achieved without sacrificing sensitivity.

•Receptor-ligand binding assays can be performed directly in the filter plate ("in-plate") decreasing the number of manipulations, and reducing radioactive solid waste by eliminating the need for a separate incubation plate.

•The of 384 well filter plate can be used to screen large libraries of compounds in a high density platform.

Schematic of In-Plate Receptor/Ligand Binding in MultiScreen Glass Fiber Filter Binding Plates



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