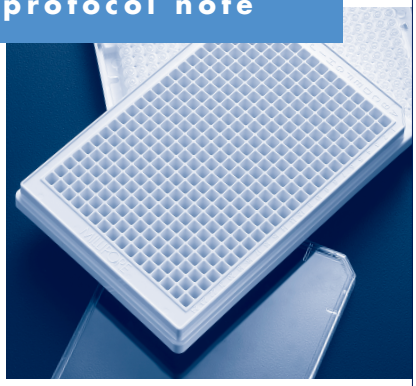
protocol note



MultiScreen®$_{HTS}$ 384-well Filter Plate with Glass Fiber Filter

High throughput method for in-plate receptor-ligand binding assays and radioisotope counting

Introduction

Heterogeneous filter binding assays have been used for the purpose of characterizing receptor-ligand binding. To date, most assays have been performed on 96-well platforms. The new MultiScreen$_{HTS}$ 384-well filter binding format increases the density of samples that can be screened in a single plate, making it possible to significantly reduce the amount of reagents for each assay. MultiScreen$_{HTS}$ filter plates are also designed for binding reactions and analyses to be performed directly in the plate.

General Considerations

This protocol note provides an overview of the basic receptor-ligand binding reaction which can be performed in Multiscreen$_{HTS}$ 384-well filter plates and provides guidance in scaling this assay from the 96-well format. Individual binding assay parameters (such as receptor concentration, ligand concentration, buffer components, etc.) can differ greatly from one system to another and will necessarily need to be optimized for each system.

Reactions

Displacement binding is a method by which a constant amount of radiolabeled ligand is competitively displaced by a non-labeled competitor ligand. This is done with a titration of the non-labeled ligand to estimate the relative binding affinity for the receptor (IC50, Ki) or is used to screen large libraries of compounds for their ability to displace the radioligand.

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Materials

1. MultiScreen$_{HTS}$ 384-well Filter Plate with glass fiber filter (MZFB N0W or MZFC N0W)
2. MultiScreen$_{HTS}$ Vacuum Manifold (MSVM HTS 00)
3. Robotic liquid handling system for automated assays (strongly preferred) or multichannel pipette for manual assays
4. Liquid scintillation counting equipped with plate reader for 384-well plates (i.e. MicroBeta Trilux® Plate Reader)
5. Liquid scintillant cocktail (i.e. Packard Supermix™ or PerkinElmer Optifluor™)
6. Oven capable of maintaining 25 °C to 50 °C
7. Assay reagents:
   - **a)** Reaction buffer (defined by user) including buffer (i.e. Tris), salts (i.e. NaCl), essential co-factors
   - **b)** Receptor preparation. May be in the form of purified recombinant receptor, partially purified cellular membranes or tissue homogenates
   - **c)** Labeled ligand. ^{3}H or ^{125}I labeled with high specific activity (typically 50Ci/mmol or higher).
   - **d)** Unlabeled ligand and/or tests compounds for performing displacement binding assays

Displacement Binding

For each well in the MultiScreen$_{HTS}$ 384-well filter plate, 100 µL total; reaction volume:

10 µL	Radioligand dilution at approx. Kd concentration
10 µL	Compound(s) being tested at single concentration or serial dilutions
10 µL	Diluted receptor preparation
70 µL	Incubation buffer
=100 µL	total well

Note: the suggested volume is 50 – 100 µL for binding reactions in the 384-well format. When converting from 100 – 200 µL assays performed in 96-well format to 384-well format, keep in mind that adding the same mass of receptor in a smaller volume will result in increasing its effective concentration and can lead to radio-ligand depletion (i.e. binding affinity appears lower than actual since a higher concentration of ligand is required). This is of particular concern if the final receptor concentration is significant compared to the binding affinities of the radioligands or the compounds being measured. Therefore, the amount of receptor should be reduced according to volume to prevent ligand depletion as long as good radiometric signal is maintained. These parameters should be determined by the user.

Protocol

General Binding Protocol for In-Plate Receptor-Ligand Binding*:

1. If required, add 100 µL 0.5% PEI to each well of the MultiScreen$_{HTS}$ 384 (Cat # MZFBN0W or MZFCN0W) plates to be tested and allow to incubate at 4 °C, 2 – 24 hours. (Do not allow PEI solution to dry. Use a lid to prevent evaporation.)
2. Wash treated wells 2 times with cold (4 – 8 °C) incubation buffer.
3. As required, thaw or reconstitute receptor preparation and radiolabeled ligand on ice. Prepare or acquire stock solutions of compounds to be screened.
   - **a)** Dilute radiolabeled ligand in incubation buffer.
   - **b)** Dilute compounds to be screened in incubation buffer.
   - **c)** As required, dilute receptor preparation in incubation buffer.
4. In each well, add compounds first then radiolabeled ligand, mix then add diluted receptor preparation (final volume of reaction should be 50 – 100 µL).
5. Incubate 1 – 2 hours at room temperature.
6. Vacuum filter reaction volume using the vacuum manifold (8 – 15" Hg).
7. Wash each test well 5 – 10 times by adding and then vacuum filtering 100 µL of incubation buffer.
8. After the last wash, apply vacuum until the glass fiber filter is almost dry.
9. Allow plates to dry at 50 °C for at least 1 hour. Alternatively, dry plates overnight at 37 °C.
10. When using tape, place clear sealing tape over bottom of plate.
11. Add 5 – 10 µL of scintillation cocktail (e.g., Optifluor) to each well.
12. Place clear sealing tape over top of plate†.
13. Let the plate stand for at least 2 hours (best signal may be obtained with overnight for incubation).
14. Assay plates for radioactivity on a beta scintillation counter (e.g., MicroBeta TriLux Plate Reader [coincidence mode recommended] or equivalent: see Radioisotope Counting).

* If out-of-plate incubation is desired, transfer incubations from separate solid-bottom plate to prewashed filter plate wells (steps 1 and 2). Quickly continue from step 6.

† If using a MicroBeta Trilux 32 plate stacker, use every other slot for a total of 16 plates. If 32 plates are desired, tape bottom of cassette instead of bottom of filter plate.

Radioisotope Counting

Radioactivity (scintillation counting) can be measured directly in the Multiscreen$_{HTS}$ 384-well filter plate. The design allows the plate to be analyzed from the top (top counting) or from both sides simultaneously (coincidence counting). For best signal to noise ratios, it is recommended to count in the coincidence mode. Low energy isotopes such as ^{14}C and ^{3}H require the use of scintillation fluid. For best results, it is recommended to use 5 – 10 µL of scintillation cocktail per well when counting a 384-well filter plate. For optimal results and consistency, the scintillation cocktail should be allowed to mix with the sample for at least an hour before counting. In some instances, longer incubations (up to 24 hours) may result in even higher counting efficiencies. The type and amount of scintillation cocktail, and how much time should elapse prior to counting to produce the best and most consistent results, should be determined by the user.

Typical counting efficiencies for ^{3}H labeled compounds are listed in Table 1.

$$\text{Counting Efficiency} = \frac{\text{CPM measured in counting instrument}}{\text{Disintegration per minute (DPM)}}$$

Counting efficiency values for 96-well filter plates are typically greater than for 384-well plates. This is especially true when the underdrain of the 96-well filter plate is removed. The design of the MultiScreen$_{HTS}$ 384-well filter plate allows for direct coincidence counting through the underdrain without any need to remove it.

It is important when converting from CPM to moles of bound ligand per mass of protein in the assay (i.e. fmol/mg) to use the appropriate counting efficiency value before fitting data. The same amount of bound radioligand in the 96-well filter plate will give more CPM than an equivalent amount in the 384-well filter plate. However, the determined specific bound amount (i.e. fmol/mg) will be the same if the appropriate counting efficiency value is used.

The signal to noise, however, is typically the same or better in the 384-well format depending on counting mode, scintillant, etc. (Figure 1).

Important Note: Different assays have different counting criteria and levels of signal. For the most accurate results, counting efficiency should be determined by the operator by adding a known DPM of isotope and counting.

Coincidence Counting Efficiencies

^{3}H Millipore Plate Type	Cat#	# Wells	Packard Optifluor % ^{3}H Efficiency at 50K DPM	Wallac Supermix % ^{3}H Efficiency at 50K DPM
MultiScreen$_{HTS}$ 384 FB	(MZFB N0W)	384	8.7	13.7
MultiScreen$_{HTS}$ FB	(MSFB N0B)	96	29.4/51.5*	38.9/67.1*
MultiScreen$_{HTS}$ 384 FC	(MZFC N0W)	384	7.1	6.7
MultiScreen$_{HTS}$ FC	(MSFC N0B)	96	36.6/60.3*	45.8/72.5*
^{125}I Plate Type	**Cat#**	**# Wells**	**Packard Optifluor % ^{125}I Efficiency at 50K DPM**	**Wallac Supermix % ^{125}I Efficiency at 50K DPM**
MultiScreen$_{HTS}$ 384 FB	(MZFB N0W)	384	20.4	22.0
MultiScreen$_{HTS}$ FB	(MSFB N0B)	96	58.1	80.0
MultiScreen$_{HTS}$ 384 FC	(MZFC N0W)	384	18.4	18.7
MultiScreen$_{HTS}$ FC	(MSFC N0B)	96	63.9	82.2

Table 1. *Typical radiometric counting efficiencies values for 50,000 DPM ^{3}H and ^{125}I labeled compounds as determined in the Wallac Microbeta Trilux Counting Instrument with 10 µL (384-well) or 50 µL (96-well) Packard Optifluor or Wallac Supermix (scintillation cocktail). Values presented are the average of 20 efficiency determinations.*

* ^{3}H values for 96-well filter plate are listed with underdrain on/with underdrain removed.

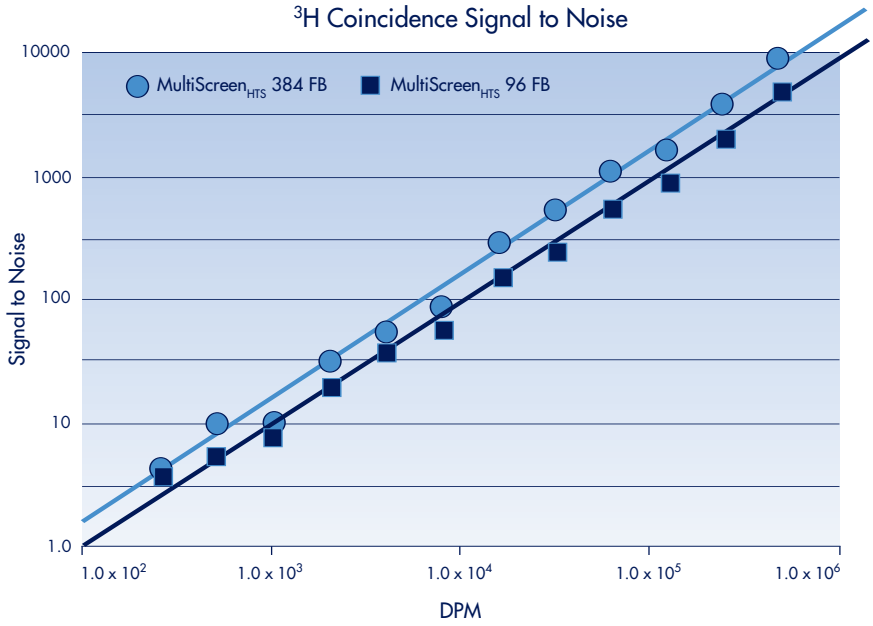

Figure 1. *The design of the MultiScreen$_{HTS}$ 384-well filter plate allows for radioactivity detection equivalent to the 96-well MultiScreen$_{HTS}$ filter plate. Signal to noise was determined for the MultiScreen$_{HTS}$ 384-well and the MultiScreen$_{HTS}$ 96-well filter plates with FB glass fiber with ^{3}H labeled ligand. Counting was performed in the Wallac MicroBeta TriLux Liquid scintillation counter in default coincidence counting mode with Optifluor (PerkinElmer) liquid scintillant cocktail. CPM were determined as a function of initial DPM loading. The CPM values were divided by the average CPM of the remaining wells that did not contain radioactive material.*

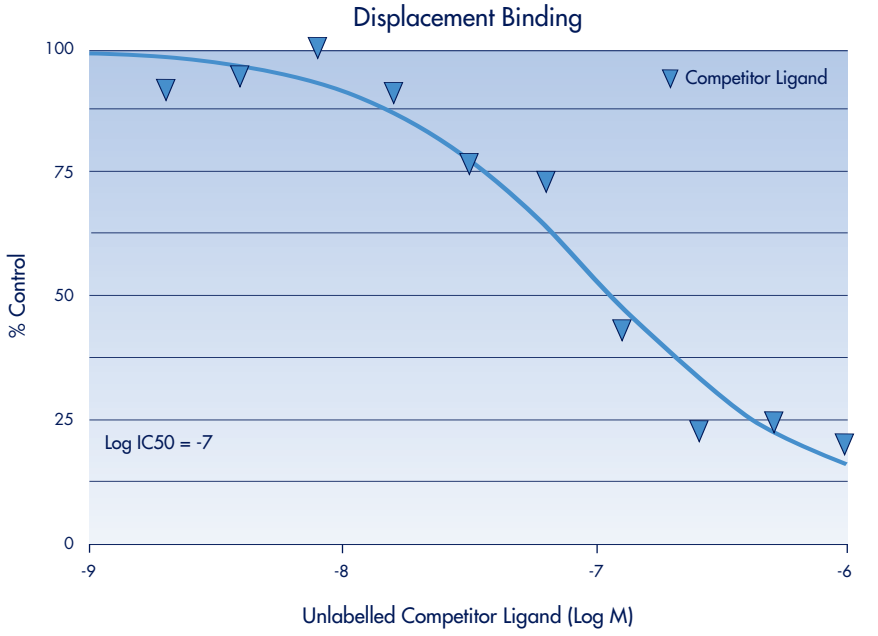

Figure 2. *Radiolabeled ligand-binding inhibition is determined with a constant radiolabeled ligand concentration and serial dilutions of unlabelled competitor ligand. Data are normalized to the specific binding of radiolabeled ligand observed in the absence of competitor (% Control).*

Data Analysis

Displacement Binding

Radioligand-binding inhibition is determined with a constant radioligand concentration usually at or around the Kd value for the receptor. Serial dilutions of an unlabelled competitor ligand were normalized to a control binding experiment without unlabelled ligand (% Control) (see Figure 2). Relative affinity values (IC50) are determined by fitting binding inhibition values by non-linear regression using Prizm® data software (www.graphpad.com). For screening of compounds, a single concentration is used as a first screen to identify those compounds which prevent radioligand binding, followed up by the quantitative displacement binding described above to determine IC50 values.

Summary

- The use of filter plates for in-plate receptor-ligand binding assays eliminates the need for a separate incubation plate thereby decreasing the number of manipulations and reducing radioactive solid waste.
- Optimizing 384-well plates for coincidence counting gives highly quantitative data that can be collected at very low levels of radioactivity. The data remains linear over several orders of magnitude of signal.
- Receptor-ligand binding assays in 384-well MultiScreen$_{HTS}$ filter plates demonstrate the same robustness and reliability as 96-well assays but use half the reagents in half the assay volume.

Related Literature

AN086EN00: MultiScreen$_{HTS}$ 384-well Receptor-ligand Binding Application Note

PF1150EN00: MultiScreen$_{HTS}$ Glass Fiber Filter Plates Data Sheet

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