MILLIPORE

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Title: **Cell-based Receptor Binding Assays Performed with the MultiScreen® Assay System**

Introduction

Cell-based receptor binding assays are a fundamental part of drug discovery programs and a valuable tool for elucidating the mechanisms of a drug's biological effect. Various filtration techniques have been used for separating the bound labeled ligand from the free labeled ligand in receptor binding assays. The purpose of this technical brief is to introduce the MultiScreen assay system, which has been designed to fulfill the many needs of cell-based receptor binding assays.

The MultiScreen system is a reliable and versatile system for performing a wide variety of ligand binding assays. It is a rapid, convenient, and reproducible analytical tool for sample preparation, incubation, washing, and quantitative collection of both filtrates and filters. Consisting of a 96-well filter bottom plate, a vacuum filtration manifold for washing, and a punching and collection unit, the MultiScreen system delivers high reproducibility with precision and accuracy for a wide range of applications.

The filtration plates contain hydrophilic microporous Durapore® membranes of polyvinylidene fluoride (PVDF), and are available in three pore sizes: 0.22 µm (GV plate), 0.45 µm (HV plate), and 0.65 µm (DV plate). Consistent and durable, the membrane demonstrates extremely low non-specific protein binding (NSB) and broad chemical compatibility.

Recent research continues to demonstrate the high performance of the MultiScreen system in a variety of cell- based receptor binding assays. In this publication, we will present the procedures and results of four studies successfully using the system with cells or receptors from diverse origins, and assorted receptor purification techniques. These studies illustrate the MultiScreen system's non-toxicity, its use for Scatchard analysis, and its reliability in Interleukin 1, Angiotensin II, and C5a receptor binding assays. Furthermore, the system will be demonstrated not only to be comparable or superior to existing methodology, but also able to dramatically increase a laboratory's sample throughput capabilities.

Study No. 1: Cell Viability

Dr. Denis Snider of the National Institutes of Health conducted experiments with 5 1 Cr-labeled mouse splenic B lympho- cytes to determine the retention of viable whole cells using all three MultiScreen filter plates.

Procedure

After labeling cells with 51 Cr, Dr. Snider measured the 51Cr present on the filters with that lost to the filtrate. In this way, he assessed the ability of the MultiScreen system to retain viable cells on the filter. Here is an outline of his procedure:

1. Mouse splenic B lymphocytes were labeled with 51Cr. The quantity of cells per well was approximately 200,000 in a final volume of 100 µL. The cells were in RPMI 1640 tissue culture media containing 5% FCS.
2. The vacuum manifold was applied for 1 minute to suction out filtrate, which was recovered in a 96-well flat bottom plate. All filtrate was removed within 1 – 2 seconds for the DV (0.65 µm) and HV (0.45 µm) plates, while the GV (0.22 µm) plate required a few more seconds. There was no washing.
3. After removing the underdrain, the filters were dried, punched and deposited into tubes by the MultiScreen punch.
4. The cpm in both the filters and filtrate were determined in a gamma counter.

Results and Discussion

Table 1 shows that each of the plate types demonstrated a high retention of viable whole cells that averaged above 95 percent (n=18). Dr. Snider also observed that this rate was consistent at all locations on a plate. These results confirm not only that the Durapore filtration plates are biocompatible, but also that the washing, vacuuming, and other mechanical functions of the MultiScreen system preserve cell viability.

Table 1. Viability as Determined by ^{51}Cr.

	DV Plate (0.65 µm filter)	HV Plate (0.45 µm filter)	GV Plate (0.22 µm filter)
Average % viable	96.37	95.98	96.50
S.D.	±0.77	±1.11	±0.59
%C.V.	0.8	1.1	0.6

Study No. 2: Scatchard Analysis

In their research on Interleukin 1 (IL-1) receptors, Dr. Patricia L. Kilian and a research team at Hoffmann-LaRoche, Inc., used the MultiScreen system for binding assays to collect both filter-bound and free radiolabeled IL-1.

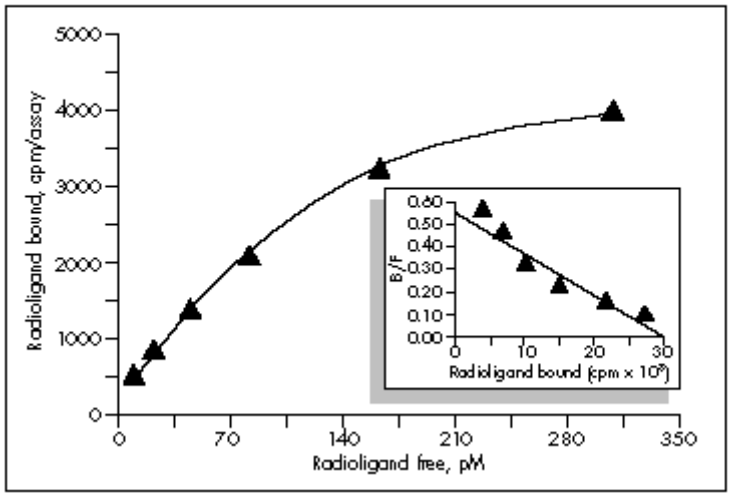
Procedure

The binding characteristics of the 125I-labeled IL-1 to membranes prepared from murine EL-4 thymoma cells were determined.

1. Membranes from EL-4 cells were prepared by homogenization and differential centrifugation, as described by Paganelli et al.
2. Membrane suspension (approximately 30 µg protein/well) was added to MultiScreen-HV plates.
3. Various concentrations of 125I-IL-1 were added. NSB was determined by addition of unlabeled IL-1. The final incubation volume was 150 µL.
4. Plates were incubated for 1 hour at 37 C. The wells were aspirated by use of the vacuum manifold. The filters were washed three times with buffered saline.
5. After the membranes dried, they were punched and collected into 12 x 75 mm tubes. The counts of the 125I filter-collected material (bound) were determined.

Figure 1. Saturation Curve, MultiScreen-HV Plate.

IL-1 Binding to Thymoma Cell Fragments. IL-1 Scatchard Plot (Inset).



Results and Discussion

Binding of the 125I-IL-1 to EL-4 membranes in an experiment using the MultiScreen-HV plate is shown in Figure 1. Binding characteristics (Bmax ,Kd) were similar to that determined with experiments using HATF nitrocellulose filters except that the Bmax value was consistently 20 – 30% lower than that observed with HATF filters. The reproducibility of experiments performed with the MultiScreen-HV filter plates was very good and suitable for competitive inhibition assays.

Horuk has reported that the Durapore membranes contained in the MultiScreen plates are superior to both nitrocellulose or glass fiber filters for IL-1 receptor assays due to the Durapore membranes' extremely low non-specific binding. These results are consistent with other research which demonstrated the extremely low NSB of Durapore membranes for insulin, human chorionic gonadotropin, albumin, IgG and the activated complement anaphylatoxin C3a. Further IL-1 research with the MultiScreen system has demonstrated its versatility with both whole cell and cloned receptor binding assays.

Study No. 3: Angiotensin II Binding to Adrenal Preparations

Dr. Mitchell Steinberg used the MultiScreen system at Lilly Research Labs for investigating the binding of radiolabeled Angiotensin II to adrenal membranes.

Procedure

A. Preparation.

Adrenal membranes were prepared from the capsular portion (glomerulosal layer attached) of rat adrenal glands by differential centrifugation. Homogenized capsules were centrifuged 10 minutes, 1000g, 4 C; supernatant was centrifuged 30 minutes, 30,000 x g, 4 C. The pellet was resuspended in 50 mM Tris and stored in aliquots at -70 C until used.

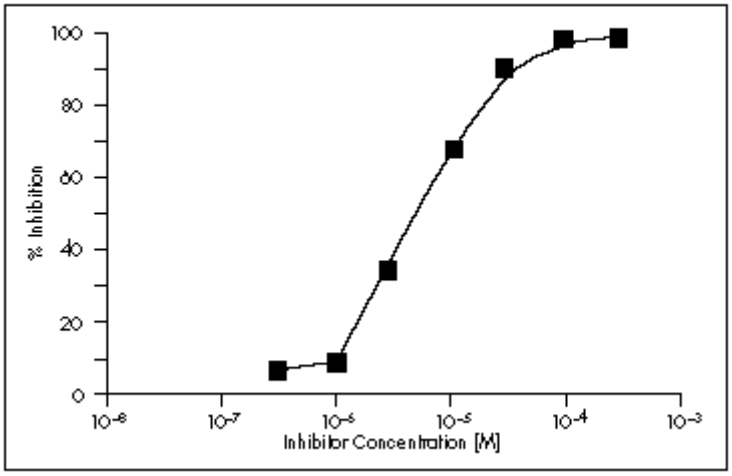
B. Assay procedure.

1. 125 I-Angiotensin II was added to adrenal membranes in MultiScreen-HV (0.45 µm) plates and incubated 90 minutes at room temperature. Each 250 µL incubate consisted of the following (final concentration): 50 mM Tris, 120 mM NaCl, 5 mM MgCl2 , 1 mM dithiothreitol, 0.05% bovine serum albumin, 0.1 nM 125I- Angiotensin II and 8 – 15 µg adrenal membrane protein.
2. Antagonists were added in concentrations from 10 nM to 100 µM. Non-specific binding was measured in the presence of 0.1 µM SAR 1 , Ile8 -Angiotensin II.
3. Binding was terminated by applying vacuum to filter plates.
4. Receptor-ligand complex trapped on filters was washed three times with 300 µL ice-cold wash solution (50 mM Tris, 120 mM NaCl, 5 mM MgCl2 , 1 mM dithiothreitol).
5. Filter discs were dried, punched out and counted in a gamma counter (52% efficiency).

Results and Discussion

Total binding in the absence of inhibitor is 150 fmol Angiotensin II (0.1 nM AII) bound per mg protein. Specific binding represented 96% of total binding. Figure 2 shows the Angiotensin II inhibition curve obtained with a protypical experimental drug on the MultiScreen-HV plates. This rapid screening system is in routine use to determine the binding affinities of various novel chemical compounds.

Figure 2. Inhibition of ^{125}I-Angiotensin II Binding Using an Adrenal Gland Preparation.



Study No. 4: Complement Component C5a Receptor Studies

Dr. Martin S. Springer of Merck, Sharp and Dohme Research Laboratories repeated experiments into identification of the polymorphonuclear leukocyte C5a receptor using the MultiScreen system. He compared the system with earlier harvester techniques using PEI-coated glass-fiber filters, and evaluated both GV (0.22 µm) and HV (0.45 µm) plates.

Procedure

1. The assay buffer (50 mM HEPES, pH 7.5, containing 1 mM CaCl2 , 5mM MgCl2 , 100 µM phenylmethyl-sulfonyl fluoride, 0.5% BSA, and 0.1% bacitracin) was added to wells.

125I-C5a, unlabeled C5a, and PMN membrane receptor fragments (typically 0.5 µg) were added sequentially to a final reaction volume of 200 µL.

2. After incubation for 60 minutes, assay buffer was added, and samples were filtered through the MultiScreen-GV and -HV plates or Whatman GF/C glass-fiber filter treated with 0.33% polyethylenimine (PEI).
3. Buffer was again added to wells for washing filters.
4. Filters were dried and counted.

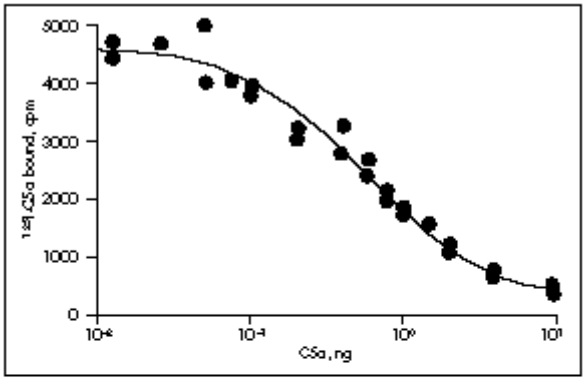
Results and Discussion

Dr. Springer determined that the MultiScreen-HV plate allows the human PMN cell membrane fragments to pass through, resulting in about a 30 percent loss of counts. The MultiScreen-GV plate, however, retained the purified receptor and produced considerably lower CVs and NSBs than the original cell harvester method. In addition, results using MultiScreen-GV plates were similar to those obtained with his manual method generally reserved for important samples.

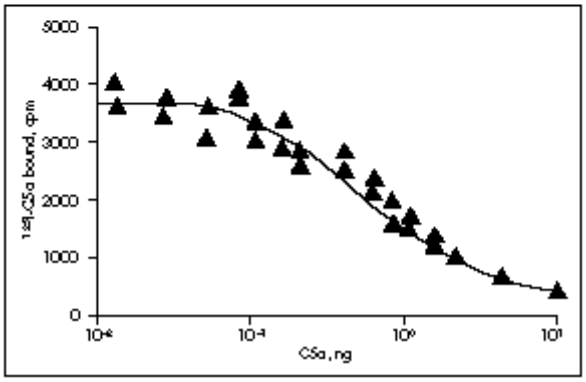
In choosing larger pore sizes in MultiScreen plates, users must weigh the benefits of filtration speed against the loss of additional cell receptor fragments able to pass through the membrane. In two of these studies, IL-1 and Angiotensin II, however, the lower cell retention of the larger pore sizes was not significant. For high loadings or crude cell homogenates, new MultiScreen glass fiber filter plates (available in Type B or C) may be better suited to the assay.

Figure 3. Competitive C5a Binding to Human PMN Membranes.

A. MultiScreen-GV Plate.



B. Cell Harvester with PEI-Treated Glass-Fiber Filters.



Conclusion

Cell-based receptor binding assays are commonly used in the pharmaceutical and biotechnology communities as valuable tools to assess the potential biological activities of novel compounds. Drug discovery and basic research programs require more rapid and reliable procedures to process large numbers of unknown compounds for activity. In addition, a more quantitative evaluation of the unknown agonists or antagonist interactions such as Scatchard analysis is required.

The MultiScreen system can be used with confidence in a wide range of receptor binding assays, from drug screenings to Scatchard analysis, with whole cells from many sources or purified receptor membrane fragments prepared by assorted methodologies. Well suited to processing large numbers of samples from preparation and incubation through filtration and punching, the system offers speed, reproducibility, accuracy, and precision while protecting cell viability. Glass fiber filters are appropriate for crude homogenates, or for high loads; however, Durapore membranes (GV, BV or HV) are required for use with purified receptors.

The MultiScreen system, therefore, meets the need for a reliable, rapid assay and separation system that can be readily adapted into existing cell-based receptor binding assay procedures.

All of the plate types used in this method are also available as opaque plates for direct reading in a microplate scintillation counter.

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