Abstract

The microplate format has become increasingly popular in biological experimentation due to its small size, easy sample handling, increased throughput, decreased sample preparation, and small sample volume. The need to perform increasing numbers of assays has sparked interest in high throughput, microplate compatible radioisotope counters. Many of these biological experiments are performed on glass fiber filters or synthetic membranes. These media may be used to separate specific components of an assay mixture for counting, or target substances may be immobilized for analysis with radiolabeled or luminescent probes. The diversity of assays requires a number of different filter types and sizes. We have developed a microplate scintillation and luminescence counter and associated accessories with which to carry out many of these procedures in several popular formats. Described in this paper are some experiments that have been conducted to demonstrate the feasibility of using microplate-based instrumentation to carry out several classes of important biological assays.

Introduction

Modern techniques used in biological and pharmaceutical research involve the assay of large numbers of samples. Various biological agents and drug candidates are routinely evaluated by assessing their ability to affect binding to neurotransmitter and cell surface receptors, enzymatic activity, cell growth, and immune function. Test compounds are typically run through a large panel of tests to evaluate their potential as therapeutic agents. Many assays are conducted by collecting bound material on glass fiber or synthetic membranes using a cell harvester or filtration manifold and washing through the unbound tracer. The filter must then be assayed in a liquid scintillation counter (LSC) or gamma counter to assess the action of the test compound. In recent years, the microplate format has become popular for these types of tests. Microplates provide a convenient and efficient format, in which replicates, blanks, and controls can be arrayed in a convenient matrix for pipetting with multichannel pipettors or with automated liquid handling systems (e.g., MultiPROBE™ by Packard Instrument Company, Meriden, Connecticut). Conducting experiments in a microplate reduces sample handling and radioactive waste, since entire assays can often be performed in a single plate. Until very recently, filters, extracts, or supernatants resulting from these assays had to be placed into individual LSC vials or gamma tubes for analysis, resulting in high costs and low sample throughput.

We have developed the TopCount® Microplate Scintillation and Luminescence Counter in response to the needs of investigators conducting many of these assay types. This instrument is a fully automated radioisotope and luminescence microplate counter, designed using the principle of all-reflective optics,¹ which can assay up to 12 samples simultaneously. This design prevents optical crosstalk effects, providing improved accuracy and dynamic range for both screening and quantitative assays.

We have also developed accessories specifically designed for counting samples isolated on filters. Experiments involving labels which may not remain firmly attached to the filter after addition of scintillation fluid, such as ligands
bound to receptors, must be counted on filter disks that are physically isolated from each other to eliminate artifacts caused by optical crosstalk or sample migration. To this end, samples can be collected using a FilterMate™ cell harvester onto UniFilter™ plates (Packard Instrument Company).² UniFilters are specially designed filtration plates containing discrete filter disks and integrated filter support screens. UniFilter plates can be counted directly on TopCount. Alternatively, material collected using a commercially available filtration system manufactured by Brandel®, Inc. (Gaithersburg, Maryland) can be punched from a continuous filter sheet after harvesting into discrete microplate wells for counting.² If large amounts of material are collected on a small surface area, the filter can become clogged, and self-absorption effects can cause low and/or variable counting efficiency. To optimize reproducibility and maximize throughput, we have developed several methods of harvesting and counting on small or large discrete filters or continuous filter sheets, depending on the amount and type of material to be processed.

With a number of biological assays, it is also possible to use a microplate well, rather than a filter, as a solid support to immobilize cells or specific molecules. Examples include coated-well RIA, adherent cell proliferation, and cellular receptor binding. The microplate itself can be treated using a number of commercially available processes to promote cell adhesion and to specifically bind target molecules. After simple washing, the plate can be counted directly in the TopCount, thus eliminating tedious punching and harvesting steps altogether.

We have conducted receptor binding, cell proliferation, and in-plate assays on TopCount with excellent results. This paper describes new equipment used to prepare and count microplate samples, and details the results of our experiments.

Materials and Methods

All reference assays were carried out on a Tri-Carb® 2500TR LSC or Cobra® gamma counter (Packard Instrument Company). To evaluate performance for receptor binding assays, we used the [³H]-benzodiazepine (Cat. No. NED-002) and [¹²⁵I]-endothelin-1 (Cat. No. NED-009) NENQuest™ Drug Discovery Systems (DuPont-NEN Research Products, Boston, Massachusetts). All sample preparation and incubation steps were carried out according to the kit instructions.

To test new accessories, we used a modular FilterMate cell harvester. This harvester can be configured to simultaneously process samples in either 24- or 96-well formats. It is designed to accommodate the UniFilter filtration plates, which contain discrete glass fiber filter disks in individual wells, simulating a discrete filter in an LS vial. The diameters of the disks are 14 mm and 7 mm for 24-well and 96-well plates, respectively. UniFilter plates are available with GF/B™ and GF/C™ (Whatman®) filter materials.

We used as our reference filtration method the Brandel 24-well (4 x 6) cell harvester (Model No. MPR-24) and filter punch/deposit device (Model No. MPDR-24) which deposits each of the 24 filter disks simultaneously into wells of a 24-well microplate. Filters so placed can be counted directly in TopCount or removed for assay in a conventional LSC.

We processed standard competition curves in triplicate using the FilterMate cell harvester and both 24-well and 96-well UniFilter filtration plates, each containing GF/B filter disks. After harvesting, the plates were dried and MicroScint® (Packard Instrument Company) scintillation cocktail was added to each well (125 µl and 25 µl, respectively). Finally, we counted each UniFilter plate on TopCount using the appropriate preset counting conditions. For a control assay, we processed an additional curve using the Brandel equipment. The 24 samples were transferred into a 24-well microplate using the Brandel punch system described above. 125 µl of MicroScint cocktail was added to each well and the plate counted in TopCount. The filters were then removed, solubilized, and assayed for absolute activity (DPM) in the discrete-vial LSC. Total count samples were assayed similarly.

We also investigated the feasibility of counting discrete-membrane microplates directly in the TopCount. Here, we used white MultiScreen™ HV filtration plates (Millipore Corporation, Bedford, Massachusetts, Cat. No. SA2M060E2) and a MultiScreen vacuum manifold.³ These devices are also designed for biological assays such as enzyme activity, cell growth, and receptor binding. We set up duplicate competition curves for the [¹²⁵I]-endothelin kit on two plates. After processing, one plate was attached to a
reflective backing plate. We added 10 µl of MicroScint cocktail to each well and counted the plate in the TopCount. The samples on the remaining control plate were punched into 12 x 75 mm gamma tubes using the MultiScreen punch device and counted in the gamma counter.

For cell proliferation assays, we used mouse spleen cells cultured to 10⁶ cells/ml in a standard 96-well culture plate. After growth, the cells were stimulated with increasing amounts of anti-CD3. Prior to harvesting, the cells were pulsed with 74 KBq (2 µCi) [³H]-thymidine per well. We harvested all samples simultaneously into a UniFilter-96 plate (GF/C), using the FilterMate cell harvester, dried the plate, added MicroScint cocktail (25 µl) to each well, and counted the plate on the TopCount. Finally, we removed each of the filter disks and placed them into small LSC vials. We then solubilized the samples and assayed them for DPM on our conventional LSC.

To demonstrate performance for in-plate assays such as coated-well RIA or cellular receptor binding, we chose the [¹²⁵I]-TSH IRMA assay. We first coated a solvent-resistant microplate (PicoPlate, Packard Instrument Company, Cat No. 6005162), a commercially available white microplate (Microlite™, Dynatech Laboratories, Chantilly, Virginia, Cat. No. 011-010-7411), and white strip wells (Microlite Removawell™, Dynatech) with a universal solid phase consisting of ovalbumin/biotin and streptavidin. We then added diluted anti-TSH/biotin conjugate (Dr. Alain Baret; Laboratoire Trichereau, Nantes, France) to each well. After incubation and washing, we incubated TSH standards and [¹²⁵I] anti-TSH antibody (Amersham® Corporation, Arlington Heights, Illinois) with the first antibody. We then washed the microplates again, and added 300 µl/well of MicroScint cocktail to each well prior to counting on TopCount. Finally, we washed the strips, broke them into discrete wells and counted each well in the gamma counter.

Results and Discussion

Receptor Binding Assays

Receptor binding assays conducted on tissue homogenates, whole cells, or cloned receptors generally require the use of discrete filters, since radioligands bound to the filter tend to elute or migrate in the scintillation fluid, or require solubilization. We investigated several alternatives for counting on discrete filters. To evaluate the performance of TopCount compared to a traditional LSC when using conventional methods, we compared the counting results for the samples processed with the Brandel equipment. We found that TopCount gave an absolute ³H counting efficiency of 54%, with a correlation coefficient of 0.99. This indicates excellent counting linearity, as well as high efficiency due to optimum counting geometry. We also calculated the percent bound, and plotted it against dose for each of the standard points (Figure 1A). The superimposed competition curves also demonstrate equivalent counting performance.

We evaluated the data from each of the UniFilter experiments in a similar fashion. Figure 1B illustrates the competition curves for samples on the UniFilter-96 plate and control samples processed with the Brandel equipment. Again, excellent correlation is observed. Finally, after converting all counting results to DPM, we calculated K_d and B_max for each experiment by conducting a Scatchard analysis. A summary of all results appears in Table 1.

Relative efficiency is based on CPM obtained on a traditional LSC. Literature values (DuPont-NEN data sheet) for the benzodiazepine receptor system are K_d = 1.4 nM and B_max = 0.25 nM. These results provide conclusive evidence that several combinations of harvesting equipment and filtration plates in conjunction with the TopCount instrument can be used to accurately and rapidly process and analyze receptor binding samples isolated on glass fiber filter media.

<table>
<thead>
<tr>
<th></th>
<th>Relative Efficiency</th>
<th>Correlation (R²)</th>
<th>K_d</th>
<th>B_max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brandel Punch/Deposit System</td>
<td>119%</td>
<td>0.99</td>
<td>1.3 nM</td>
<td>0.21 nM</td>
</tr>
<tr>
<td>FilterMate/UniFilter-24</td>
<td>90%</td>
<td>0.96</td>
<td>1.7 nM</td>
<td>0.20 nM</td>
</tr>
<tr>
<td>FilterMate/UniFilter-96</td>
<td>56%</td>
<td>0.98</td>
<td>1.8 nM</td>
<td>0.22 nM</td>
</tr>
</tbody>
</table>

Table 1. Summary of receptor binding experiments.
We also carried out a similar evaluation for the $^{125}$I-endothelin receptor kit using the MultiScreen filtration plate system. The competition curves and Scatchard plots from the TopCount and gamma counter assays are illustrated in Figure 2.

Here again, we observe excellent agreement between the TopCount and control experiments. $K_d$ and $B_{\text{max}}$ values agree closely with published results (data not shown) which further demonstrates the feasibility of counting MultiScreen plates directly in TopCount.

**Cell Proliferation Assays**

The ability to rapidly harvest samples onto discrete or continuous filters of 7 or 14 mm diameter is important. The UniFilter plates used for receptor binding assays are also ideally suited for cell proliferation. We processed the
produce results for cell proliferation studies which are indistinguishable from those produced with traditional equipment.

**In-Plate Assays**

For in-plate assays, the ability to accurately discriminate and quantitate samples in the absence of crosstalk is critical. The use of all-reflective optics permits excellent quantitation with either isotopic or luminescent detection. We evaluated the feasibility of carrying out assays in which the radiolabel is specifically bound to the microplate well. We correlated TopCount counting performance using the TSH samples prepared in the Microlite plate for TopCount and the Microlite strips for the gamma counter (Figure 4A) and found the correlation coefficient to be 0.99, with a relative counting efficiency of close to 100%.

Figure 4B illustrates the dose-response curves for both types of microplates tested in the TopCount. The curves are essentially identical, indicating that a number of different microplate supports can be used to produce excellent assay results. The use of microplate wells as solid supports has become increasingly popular for RIA/IRMA as well as receptor binding applications, where cell surface receptors are characterized by immobilizing cells on the microplate and specifically binding a radioligand to them. These results show that TopCount can accurately count these samples with increased throughput and decreased labor.

![Figure 3.](image)

Cell proliferation assay dose-response curves processed using FilterMate cell harvester/UniFilter filtration plates and counted on Topcount and conventional LSC.

Within experimental error, the results are identical. They demonstrate that the FilterMate/UniFilter harvesting equipment can be used with TopCount to quickly and efficiently produce results for cell proliferation studies which are indistinguishable from those produced with traditional equipment.

![Figure 4A.](image)

TSH IRMA assay. A) Correlation of coated-well samples counted on TopCount and gamma counter. B) Dose-response curves of Microlite and PicoPlate TSH samples counted on TopCount.

![Figure 4B.](image)
Conclusions

We have demonstrated that the TopCount Microplate Scintillation and Luminescence Counter is the preferred alternative to conventional LSC for processing a wide variety of samples produced in the course of modern biological and pharmaceutical research. Accessory supplies and equipment such as UniFilter plates and the FilterMate harvester have been designed specifically to accommodate the varying needs of these samples without compromising assay reliability or requiring unconventional processing schemes. Excellent results have been achieved for receptor binding, cell proliferation, and in-plate assays. Many of these samples are isolated on solid supports such as filters, membranes, or microplate wells. Depending on the specific assay, a number of alternative sample preparation methods for use in TopCount can be employed. Regardless of the method used, the resulting samples can be accurately screened and quantitated directly in the TopCount with improved sample handling, increased net throughput, and decreased costs.

References

1. TopCount Topics #13, Crosstalk, Packard Instrument Company.
2. TopCount Topics #10, Receptor Binding Assays, Packard Instrument Company.
3. TopCount Topics #11, Direct Counting of Millipore® MultiScreen® Filtration Plates, Packard Instrument Company.
4. TopCount Topics #9, Cell Proliferation Assays, Packard Instrument Company.
5. TopCount Topics #7, Solid-phase RIA in Microplates, Packard Instrument Company.

Brandel is a registered trademark of Brandel, Incorporated.
NENQuest is a registered trademark of E.I. DuPont de Nemours & Company.
GF/B and GF/C are registered trademarks of Whatman Specialty Products Division, Whatman Paper, Ltd.
MultiScreen is a registered trademark of Millipore Corporation.
Microlite is a registered trademark of Dynatech Laboratories, Incorporated.
Amersham is a registered trademark of Amersham Corporation.