TopCount Topics

TCA-010

# Two Methods of Harvesting Receptor Binding Assays

## Abstract

Ligand-receptor binding studies are widely used for receptor characterization and in high throughput drug screening, but are hampered by inefficient sample preparation and counting. The TopCount Microplate Scintillation Counter, based on a novel radioisotope counting method, significantly improves counting throughput by using multiple detectors, and facilitates automation of sample handling by using standard microplate formats. This paper describes the application of TopCount to ligand-receptor binding studies and compares the performance of TopCount to that of traditional liquid scintillation counting (LSC).

## Introduction

Receptor binding studies are used to characterize receptors and to evaluate potential pharmaceutical agents by assessing their ability to interfere with the specific binding of a radiolabeled ligand to its receptor. After attaining equilibrium with receptors, excess free ligand must be separated rapidly from the bound receptor- ligand complex. Separation has been traditionally carried out on a cell harvester using glass fiber filter media. Filters containing the receptor-ligand complex are typically punched out into vials for counting in a discrete sample LSC or a gamma counter. After counting, it is necessary to dispose of individual vials and, in the case of LSC, large volumes of scintillation cocktail. The expensive and time consuming nature of these operations has limited the use of ligand-receptor binding studies in high throughput pharmaceutical screening studies.

The development of the TopCount Microplate Scintillation Counter makes it possible to significantly expand the use of receptor binding studies. In conjunction with multiple sample harvesters, TopCount increases sample throughput and decreases the amount of labor and waste. Sample processing equipment and filter plates have been designed to fully isolate discrete sample positions, thus preventing physical migration of the labeled ligands in the scintillation fluid. Crosstalk due to ligand migration or optical effects is insignificant.

In this paper, a number of studies are described which demonstrate the use of TopCount for receptor binding assays. TopCount provides results equivalent to those obtained with traditional LSC methods, with reduced handling and disposal costs and without the optical crosstalk or ligand migration problems associated with scintillation counting from continuous filter mats.

## Methods

### Cell Harvesters.

Two filtration manifolds were used with TopCount for filtration onto glass fiber mats. One harvester, manufactured by Brandel (model MPR-24), can filter samples from a variety of tube or well formats, 24 at a time, onto a continuous filter mat (*e.g.*, GF/B or GF/C). The diameter of the filter disk is 14 mm. A punch and deposit device (Brandel model MPDR-24) was used to transfer individual filter disks to separate wells of a Packard 24-well PicoPlate. Ligand migration in the scintillation fluid, which could cause loss of signal and crosstalk between samples, is prevented by the physical isolation of the filter disks.

Filter disks can be punched into deep or shallow well PicoPlates with the Brandel punch. Dry filters can be counted in the shallow well plates by adding up to 150 µl of organic cocktail (MicroScint-O) to the wells. Alternatively, moist filters may be counted without drying by using an

PAN0022 8/92 Printed in U.S.A.

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| | Relative Efficiency | Correlation ($R^2$) | $K_d$ | $B_{max}$ |
|---|---|---|---|---|
| Brandel punch/ deposit | 119% | 0.99 | 1.32 | 0.21 |
| MicroMate & UniFilter-24 | 90% | 0.96 | 1.68 | 0.20 |
| MicroMate & UniFilter-96 | 56% | 0.98 | 1.81 | 0.22 |

**Table 1.**

Correlation of results of benzodiazepine competition assays performed with various harvesting/counting methods. Relative efficiency was determined from the linear regression of the count rate for the method indicated versus the count rate for the filters counted in conventional LSC vials. The correlation coefficient of the regression is indicated. $K_d$ and $B_{max}$ were determined by Scatchard analysis. When the filters were counted in LSC vials, $K_d$ ranged from 1.24 to 1.74, and $B_{max}$ ranged from 0.17 to 0.22.

| | NSB/$B_0$ (ratio) 5 mg | 25 mg |
|---|---|---|
| MicroScint-O, 1 ml TopCount | 1046/2772 (38%) | 236/451 (52%) |
| MicroScint-20, 1 ml TopCount | 1171/3313 (35%) | 152/406 (37%) |
| Ultima Gold, 3 ml LSC | 1235/3589 (34%) | 315/760 (41%) |

**Table 2.**

Counts obtained withTopCount and conventional LSC for maximum binding ($B_0$) and non-specific binding (NSB) samples in a $^3$H receptor binding assay with large tissue masses as indicated. After harvesting on a Brandel system, filters (GF/B) were extracted for 24 hours in the scintillation cocktails indicated and counted on TopCount or conventional LSC.

emulsifying cocktail, MicroScint-20 or -40, although the counting efficiency may be lower than with MicroScint-O. Larger volumes of scintillation cocktail (1.0 ml) may be added to filters in deep well plates, and the plates can be sealed and incubated with shaking to solubilize ligands in either organic or emulsifying cocktails.

The other filtration manifold, a MicroMate 196 Cell Harvester (Packard Instrument Company), simultaneously harvests either 24 or 96 samples in standard microplate format into 24- or 96-well UniFilter plates. These filter plates contain discrete glass filter disks, physically isolated from each other to prevent optical crosstalk and physical migration of the radioligands. Since the filtration and counting are done in the same plate, the handling and transferring of individual filter disks is completely eliminated. The diameters of the filter disks are 14 mm and 7 mm, respectively. UniFilter plates are currently available with GF/B and GF/C glass fiber filters (Whatman). UniFilter plates may be counted by drying the filters, adding MicroScint-O (as little as 20 µl in 96-well plates and 125 µl in 24-well plates) to the wells and sealing with TopSeal-S. Alternatively, MicroScint-20 or -40 may be used.

**Assays.**

Some studies were done with the [$^3$H] benzodiazepine NENQuest Drug Discovery System (NEN-DuPont, #NED-002). Samples prepared from this kit contain about 1 mg of receptor homogenate per tube. The assays were performed according to the protocols supplied with the kit. The binding of the [$^3$H] flunitrazepam agonist was inhibited by unlabeled flunitrazepam. Receptors were harvested onto GF/B glass fiber filters, and the filters were dried and counted on TopCount with MicroScint-O. In some cases, the filters were solubilized before conventional LSC counting, and DPM results were calculated. Then, absolute TopCount efficiencies could be determined by correlation to the conventional LSC results. TopCount CPM were normalized to DPM values to prepare Scatchard plots of the results. Results of these assays with various harvesting and counting methods are summarized in Table 1.

Other experiments were done with $^3$H-labeled ligands and receptor homogenate preparations containing up to 25 mg of tissue per tube. The receptors were harvested onto GF/B or GF/C filters and counted with MicroScint-O or MicroScint-20. The TopCount results were correlated to conventional LSC CPM results (Table 2).

**Brandel Harvester and PicoPlates.**

To evaluate the uniformity of the harvesting, punching, and counting operation using the Brandel equipment, 24 replicates of the maximum binding standard of the [$^3$H] benzodiazepine kit were prepared and harvested onto a GF/B filter mat, which was punched into a 24-well shallow PicoPlate. MicroScint-O (150 µl) was added to each well, and the plate sealed and counted in TopCount. Individual filter disks were then removed, solubilized, and counted in a traditional LSC. With TopCount we observed an average absolute counting efficiency of 57%, with a CV of 4.5%.

A benzodiazepine competition curve was produced with TopCount using similar procedures,

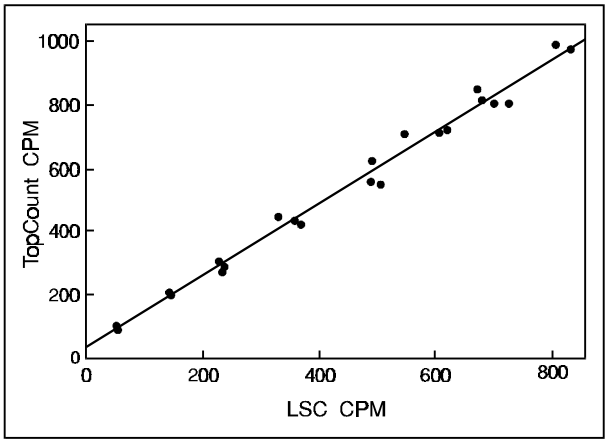


**Figure 1.**
Correlation of CPM obtained with TopCount and conventional LSC in [$^3$H] benzodiazepine receptor competition experiments performed with a Brandel harvester. The slope of the linear regression line is 1.19, and $R^2$ = 0.99.

and a parallel set of samples was prepared for LSC. Figure 1 shows the correlation of the raw counting results from the two methods. The correlation was excellent (Table 1), and the slope is slightly greater than one, indicating a slightly greater efficiency for TopCount. This efficiency is higher than that obtained in conventional LSC due to optimized sample counting geometry. Figure 2 depicts the competition curves prepared from the raw data. The curves are superimposed, confirming the excellent correlation between TopCount and the conventional counter.

Another experiment done using the Brandel harvester and punch/deposit system involved the assay of a tritiated ligand-receptor system containing approximately 700 µg of protein per sample. Figure 3 shows the correlation between

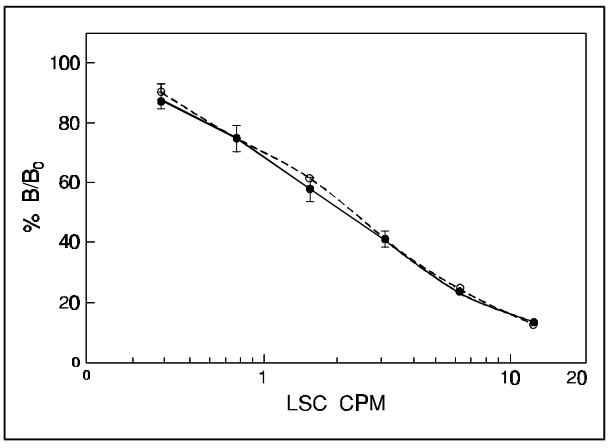


**Figure 2.**
Competition curves obtained with TopCount and conventional LSC for [$^3$H] benzodiazepine receptor assays performed with a Brandel harvester.

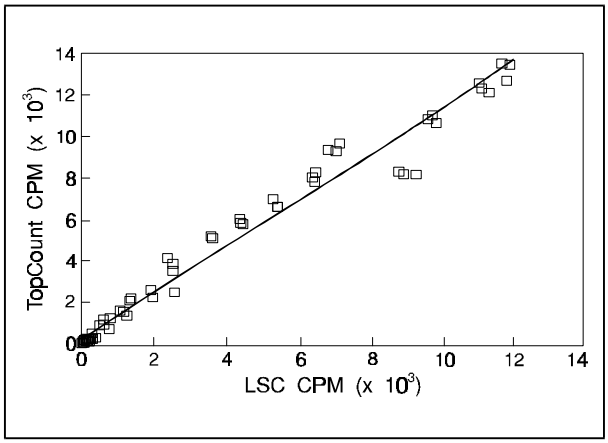


**Figure 3.**
Correlation of CPM results from a receptor binding experiment with TopCount and a parallel experiment with conventional LSC. The receptor preparation contained 700 µg of protein per sample. The filters (GF/B) were dried and counted with an organic cocktail (MicroScint-O) in TopCount, but were solubilized for conventional LSC counting. The slope of the linear regression line is 1.12, and $R^2$ = 0.97.

the TopCount and a conventional LSC. An experiment with a larger protein mass (8 mg) is shown in Figure 4. In this experiment the same filters were first counted in a shallow well plate with MicroScint-O on TopCount and then by conventional LSC. Excellent counting efficiencies and correlations were observed in both experiments.

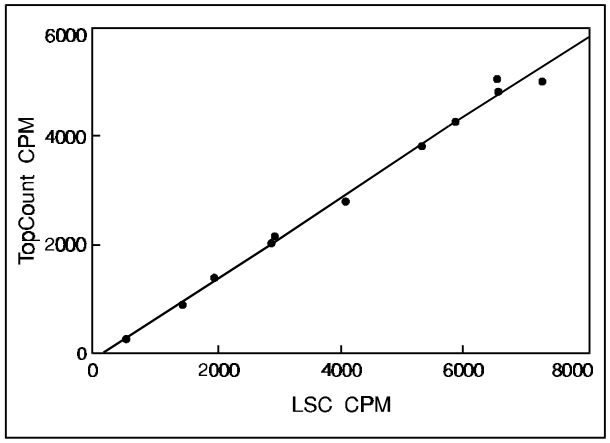


**Figure 4.**
Correlation of CPM results obtained by counting the same filters (GF/C) from a receptor binding experiment first in TopCount and then in conventional LSC using organic cocktails. The receptor preparation contained 8 mg of protein per sample. The slope of the linear regression line is 0.74, and $R^2$ = 0.99.

To study the effect of tissue mass on counting efficiency and the potential for ligand solubilization in the microplate format, maximum binding samples containing either 5 or 25 mg of tissue were harvested with a Brandel harvester and punched into deep well PicoPlates, and 1 ml of either MicroScint-O or MicroScint-20 was added to the wells. Parallel samples were counted by conventional LSC in 6 ml plastic vials with 3 ml of an emulsifier cocktail, Packard Ultima Gold. All the samples were allowed to stand for 24 hours to extract the ligand into the scintillation fluid. The results (Table 2) show that filters can be incubated in scintillation fluid to extract ligands and obtain good counting efficiencies and ratios of NSB/$B_o$ as good as those obtained with conventional LSC, even with an extremely high tissue load (25 mg).

**MicroMate Harvester and UniFilter Plates.** The Packard MicroMate harvester and UniFilter plates were tested by harvesting and counting samples from the NENQuest benzodiazepine kit. A competition experiment was performed on a UniFilter-24 plate, and after counting on TopCount the filter disks were removed from the UniFilter plate, solubilized and counted by conventional LSC. Competition curves similar to those in Figure 2 were obtained. There was a good correlation between the two instruments and a relative efficiency of 90% (Table 1). The $K_d$ and $B_{max}$ determined by Scatchard analysis are listed in Table 1.

UniFilter-96 plates were tested by performing parallel competition experiments with a UniFilter plate counted in TopCount, and with the Brandel harvester and a filter mat counted by conventional LSC. The two experiments correlated well, and the relative efficiency of the MicroMate/TopCount system was 56% (Table 1). Scatchard plots for the two experiments are shown in Figure 5, and the $K_d$ and $B_{max}$ values are listed in Table 1. Another parallel experiment (data not shown) was performed to determine whether the lower count rate observed with the 96 plate was due to lower recovery during harvesting or to lower counting efficiency. Samples were harvested onto a continuous sheet of filter with a 96-well MicroMate harvester, and the filter disks were counted by conventional LSC. The count rate in this experiment was 72% of that in the parallel experiment with the Brandel system, indicating that the lower count rate observed with the 96-well plate was partly due to lower recovery of the receptors on the smaller filter area.

## Conclusions

Harvesting equipment and consumables are available for the TopCount Microplate Scintillation Counter which facilitate high throughput filter binding procedures, such as receptor binding assays. This system has been designed to provide good counting efficiency and uncompromised performance. Whether harvesting with the Brandel harvester and punching into microplates for counting or harvesting and counting in UniFilter plates, the filter disks are physically isolated for scintillation counting to prevent ligand migration between samples in the scintillation cocktail, and optical crosstalk is insignificant. Samples may be counted with a minimum of scintillation fluid, or incubated and counted in larger volumes of cocktail to solubilize ligands.

The UniFilter plate is designed to minimize sample handling. The one piece microplate and filter design allows samples to be harvested and counted in the same microplate. All 96 or 24 samples are handled as a single unit. In addition to the time savings and ease-of-use, there is no danger of sample mix up because the microplate format is maintained throughput the harvesting and counting process.

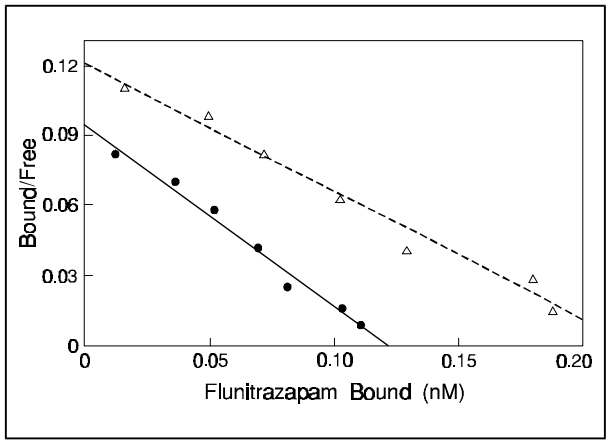


**Figure 5.**
Scatchard analysis of flunitrazepam binding experiments performed with a MicroMate Cell Harvester, UniFilter-96 plate, and TopCount, and with a Brandel harvester and a conventional LSC. The CPM results from both instruments were divided by the absolute efficiencies (Table 1) to calculate the results.