TopCount Topics

TCA-029

High Efficiency Count Mode for SPA and Cytostar-T™ Assays

Abstract

The TopCount™ family of microplate scintillation and luminescence counters has been used successfully for many years in the high throughput screening of compounds for biological activity. Many of these assays are performed using SPA technology. A related new technology, Cytostar-T scintillating microplates, promises high throughput screening in cell-based assays. The ongoing drive toward higher throughput, lower sample volumes, and decreased costs has resulted in the need to maximize the signals obtained from individual samples. To meet these needs, Packard has developed a novel approach to increase count rates of SPA and Cytostar-T assays in the TopCount. This approach, High Efficiency Count Mode, maximizes the signal obtained from these as well as other radiometric assays in both 96-well and the new 384-well format. This application note describes High Efficiency Count Mode and presents results which demonstrate that counting efficiencies for ^{3}H-labeled SPA samples can be increased by a factor of two to five depending on the level of quenching in the sample. Counting reproducibility remains excellent as a result of the temperature-controlled detectors.

Introduction

The TopCount Microplate Scintillation and Luminescence Counter detects photons in microplate wells produced by the interaction of radioactive labels with solid or liquid scintillators, as well as those produced by chemiluminescent reactions. The use of patented single photomultiplier tube (PMT), time-resolved techniques permits the accurate quantitation of emissions with a single PMT per well. These techniques allow the discrimination of true events from background noise by analyzing the differences in afterpulse characteristics of the scintillator as compared to background, which is composed mostly of PMT noise. In turn, this permits the use of opaque microplates, which are preferred for their elimination of optical cross-talk effects.

SPA and Cytostar-T technologies (both available from Amersham International plc) produce photons through the specific interaction of radiolabels that are bound to a scintillating plastic solid support, such as a microsphere or microplate well, which contain organic scintillants in the plastic matrix. SPA generally produces fewer photons than traditional homogeneous liquid scintillation counting, which results in a lower counting efficiency. As a result, counting times must be increased to achieve a comparable statistical counting accuracy, which is undesirable because it decreases the overall assay throughput.

To achieve the higher throughputs required for many screening assays, Packard has developed a new method of counting these samples on the TopCount. This technique is called High Efficiency Count Mode (HECM). The existing counting modes have also been revised to reflect these new developments. A brief summary of each counting mode follows.

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Normal Count Mode (NCM)

This mode optimizes both counting efficiency and signal-to-noise ratio for assays with low non-specific signal.

- Detected events must have two or more photons to be accepted as a true decay.
- Single-photon events are rejected as background.
- NCM is the recommended mode for most liquid scintillation methods using MicroScint™ or FlexiScint™, and proximity assays using PVT-SPA beads, Cytostar-T, or FlashPlates®.

High Efficiency Count Mode (HECM)

This counting mode maximizes counting efficiency for assays that can tolerate relatively high non-specific signals.

- Certain single-photon and all multiple photon events are accepted as true decays.
- Low-energy, single-photon events are rejected as background by an adjustable lower level discriminator.
- HECM can be used to enhance the response for low signal PVT-SPA, Cytostar-T, and FlashPlate assays.
- It is also used in Cerenkov counting applications.

High Sensitivity Count Mode (HSCM)

This mode maximizes signal-to-noise ratio for measuring high energy radionuclides and those assays which use long lifetime scintillators.

- Events must have at least three photons to be accepted as true decays.
- All single- and dual-photon events are rejected to reduce backgrounds to very low levels.
- HSCM is used primarily for LumaPlate™ and SPA applications using yttrium silicate beads.

The reader is referred to TopCount Topics #3 (TCA-003) "Theory of TopCount Operation" for a complete description of how the pulse discrimination modes function.[1]

High Efficiency Count Mode uses energy discrimination to reject unwanted background while retaining high counting efficiency. Background counts result mostly from PMT noise, and are typically of very low energy. The TopCount's isothermal counting chamber ensures that PMT noise is both low and consistent. In contrast, true counts appear as higher energy signals in the multichannel analyzer (MCA). Figure 1, below, illustrates this relationship.

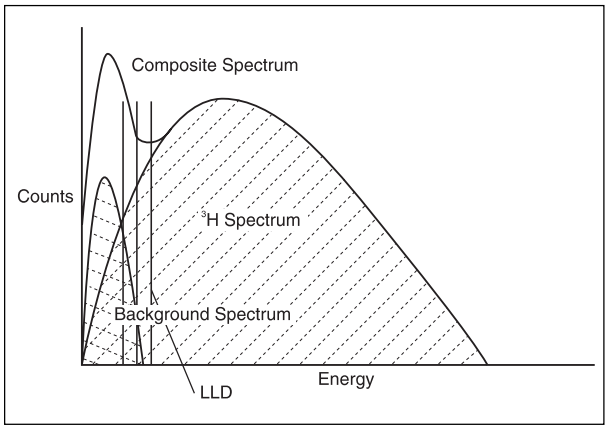

Figure 1.
Relationship of isotope and background energy spectra in HECM.

The successful use of HECM requires that the user determine the optimum counting region based on required counting efficiency and allowable background. The TopCount is capable of counting in two energy regions simultaneously. These regions are selected by defining appropriate Lower and Upper Level energy Discriminators (LLD and ULD, respectively). Energy signals occurring above the LLD but below the ULD will be processed, while those occurring below the LLD and above the ULD will be rejected.

The optimum region is defined by setting the LLD in such a way as to minimize background while retaining adequate counting efficiency. Figure 1 illustrates that as the LLD is increased, more of the background spectrum is rejected. At the same time, some valid events from the ^{3}H spectrum are also rejected. As the LLD is increased further, background will be significantly reduced. However, efficiency, particularly for ^{3}H, will also be affected. Because of the slopes of both of the spectra, small changes to the LL channel settings may have significant effects on both background and efficiency.

Excellent counting efficiency and robust assay performance can be maintained by setting the LLD appropriately using the procedure prescribed by Packard. It is possible to further optimize HECM for specific assay requirements. A judgement must be made regarding whether to further increase counting efficiency while allowing higher back-

grounds, or whether to decrease background at the expense of some efficiency. The choice depends on user preference, assay characteristics, required sensitivity, signal/noise (S/N) ratio, etc. If, after optimizing the system as recommended, further background reduction is warranted, background or blank subtraction may be employed. In general, background subtraction is needed only for extremely low count rate assays.

HECM has been characterized for several applications, including SPA and Cytostar-T. Where appropriate, the use of HECM in 384-well microplates has also been investigated. The following describes the results of these investigations.

Materials and Methods

^{3}H-SPA

All experiments were performed with PVT-SPA beads labeled with ^{3}H from the SPA color quench kit (#TRKQ.7080, Amersham). Where appropriate, the beads and other assay reagents were diluted in 50 mM Tris-HCL buffer (pH 7.2) containing 15% (w/v) glycerol to maintain the beads in suspension[2] (*i.e.*, the beads neither settle nor float to the surface).

To evaluate the effect of the LLD setting on counting efficiency and background, a series of samples were prepared in white 96-well and 384-well microplates (OptiPlate™-96, #6005190, OptiPlate-384, #6005214, Packard) along with a parallel set of background samples. This microplate was counted several times in HECM, each time varying the LLD by a small amount. The experiment was repeated, this time using the "Background Subtract" feature.

To evaluate the ability of the TopCount to correct for color quench, a set of quenched SPA standards were prepared according to the procedure outlined in the instructions provided with the SPA color quench kit.[3] Eleven levels of quench were made by serially diluting a stock solution of tartrazine yellow dye to give a range of quench levels. Aliquots of individual tartrazine dilutions were combined with aliquots of diluted stock SPA beads and buffer in the recommended ratios to give a total volume of 200 µL in the 96-well microplate (50 µL in the 384-well microplate).

Quench curves were established on the TopCount in both HECM and NCM using recommended procedures.[3,4] Unquenched SPA samples were counted in each mode to determine the reference CPM level for that mode. A quench correction curve was then constructed. This curve corrects color quenched CPM's to the previously determined reference (unquenched) CPM. A moderately quenched sample selected from the original twelve was counted repeatedly against the quench curve to determine the repeatability of the quench correction. This sample, which was selected to give approximately 30% relative efficiency, was cycled 20 times in each mode to determine reproducibility of both counting and quench correction. Automated cycle counting involves repeated load-unload cycles, which tests not only counting reproducibility, but also positioning reliability. Count times were programmed so that a minimum of approximately 3000 gross counts were collected. Raw CPM, tSIS, and quench-corrected CPM (QC-CPM) were collected.

A comparison was made between suspended and settled SPA beads. As above, two samples of beads were prepared in 50 mM Tris-HCL. One set contained 15% glycerol, while the other did not. The samples which did not contain glycerol were allowed to settle in the instrument overnight prior to counting in HECM, and were resettled prior to counting in NCM. Counting protocols were as above.

^{125}I-SPA

A standard curve was prepared using the [^{125}I]-cAMP SPA kit (#RPA.556, Amersham) to demonstrate the use of HECM in an actual assay. Triplicate samples of each standard were prepared in an OptiPlate-96 according to the directions included with the assay kit.[5] All liquid transfers were done using the MultiPROBE® 104 Robotic Liquid Handling System (Packard) equipped with fixed, washable tips. Diluted cAMP standards, [^{125}I]-cAMP, antiserum, PVT-SPA beads, and assay buffer were dispensed into the appropriate wells to a total volume of 200 µL. After overnight incubation and settling, the microplate was counted in HECM. Each sample was counted for three minutes after a five minute pre-count delay. The microplate was recounted in NCM using the Nuclide Library settings for ^{125}I-PVT-SPA.

Cytostar-T

Cytostar-T is a new assay technology that consists of clear-bottom 96-well microplates in which the bottom surface contains scintillants. It is intended for cell-based assays where uptake or binding can be directly measured by the production of specific

signals when a radiolabel is in close proximity to the bottom.[6] Cytostar-T microplates were obtained from Amersham (#RPNQ.0160). Although the microplates are not supplied with white backing tapes, Packard supplies and recommends the use of these self-adhesive white stickers (#6005199, Packard) under the clear bottoms to optimize reflectivity and enhance counting efficiency in the TopCount. The best performance will be achieved with the tapes affixed to the microplate. An alternative for those wishing to periodically examine the cell cultures is to loosely place the backing tape on the TopCount's microplate carriage prior to counting.

Samples were prepared by spotting and drying 25 µL aliquots of diluted radiochemicals ([^{3}H]-thymidine, [^{125}I]-thyroxine, [^{14}C]-thymidine, in 50 mM PBS buffer) into selected wells. Absolute activities were checked and verified on a liquid scintillation or gamma counter. All samples were prepared in triplicate.

The microplates were counted in HECM using optimized regions on the TopCount for five minutes after a five minute pre-count delay to dark adapt the microplate. The microplates were recounted in NCM using standard instrument settings. In order to determine the effect of the backing tape, the microplate was counted in both modes with no tape, tape placed on the microplate carriage, and tape attached to the underside of the microplate.

Results

^{3}H-SPA

Figure 2 summarizes the CPM and backgrounds obtained for the 384-well microplate in HECM when the LLD is increased. Note that at very low LLD settings, background can be quite high. As the LLD is increased, background decreases. This decrease is quite rapid at critical settings which represent the background spectrum endpoint. Note also that high counting efficiency is maintained as the LLD is increased, although there are slight losses with each higher LLD setting. The use of background subtraction further reduces background, but has very little effect on the sample CPM. The choice of whether to use background subtraction is dictated by assay requirements. Based on these results, a LLD setting of Channel 4.5 was chosen for the 384-well microplate. In a parallel experiment, the optimum LLD for the 96-well microplate was found to be at Channel 5.5.

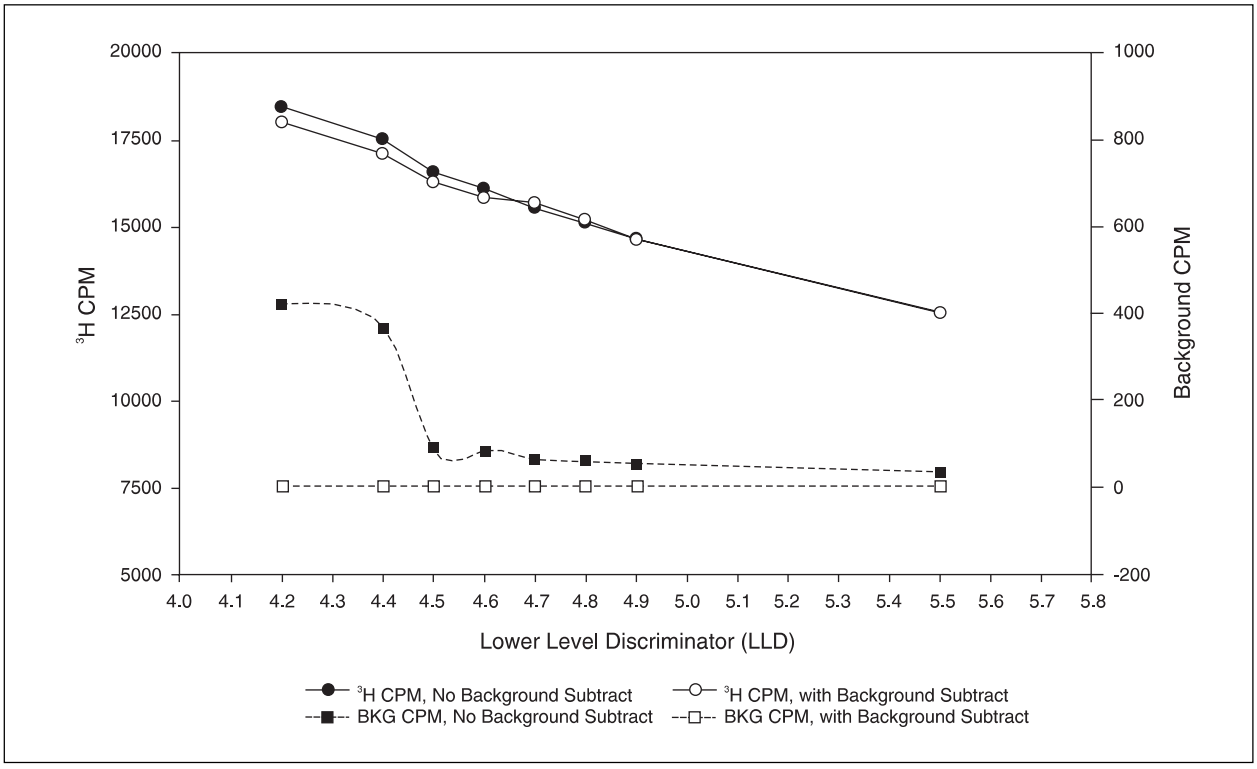

Figure 2.
Effect of LLD setting in HECM on ^{3}H and background CPM, 384-well microplate.

Quench Level	Tartrazine Conc. (mg/mL)	Normal Count Mode			High Efficiency Count Mode			% Increase in CPM
		^{3}H CPM	tSIS	% Relative Efficiency	^{3}H CPM	tSIS	% Relative Efficiency	
1	0.000	2841	19.30	99.7	5269	18.30	101.7	85
2	0.002	2347	17.75	82.4	4650	17.09	89.8	98
3	0.004	1979	16.74	69.4	4168	16.45	80.5	111
4	0.008	1567	15.39	55.0	3662	16.30	70.7	134
5	0.010	1339	14.56	47.0	2967	15.30	57.3	122
6	0.016	997	13.34	35.0	2465	14.06	47.6	147
7	0.020	873	13.07	30.6	2099	13.90	42.6	141
8	0.031	576	12.20	20.2	1488	13.54	40.5	158
9	0.063	328	11.32	11.5	1294	13.29	32.7	295
10	0.080	229	10.81	8.1	1072	12.89	28.7	368
11	0.125	160	10.42	5.6	904	11.51	17.5	465

Table 1.
^{3}H-SPA quench curve counting data in HECM and NCM, 96-well microplate.

Using the results of this experiment, SPA quench correction curves were established in both optimized HECM and NCM. The data obtained for the curves are shown in Table 1, while Figure 3 illustrates the relationships among CPM, counting efficiency, and quench.

These results demonstrate that HECM can produce a dramatic improvement in ^{3}H counting efficiency and quench resistance. In some cases, signals increase by over 400%. It is important to note that the relationship of efficiency to the quench parameter tSIS remains the same. This indicates that HECM will enhance efficiency across a wide range of quench levels. It also suggests that quench correction will be accurate and effective in HECM.

The sample used to illustrate quench correction repeatability has a quench level that is consistent with typical SPA assays.[2] The data obtained from 20 repeat counts of this sample are listed in Table 2.

Table 2 demonstrates the accuracy and precision of quench correction in HECM. Since SPA samples are corrected to an unquenched reference CPM (quench corrected CPM, or QC-CPM) obtained in each mode,

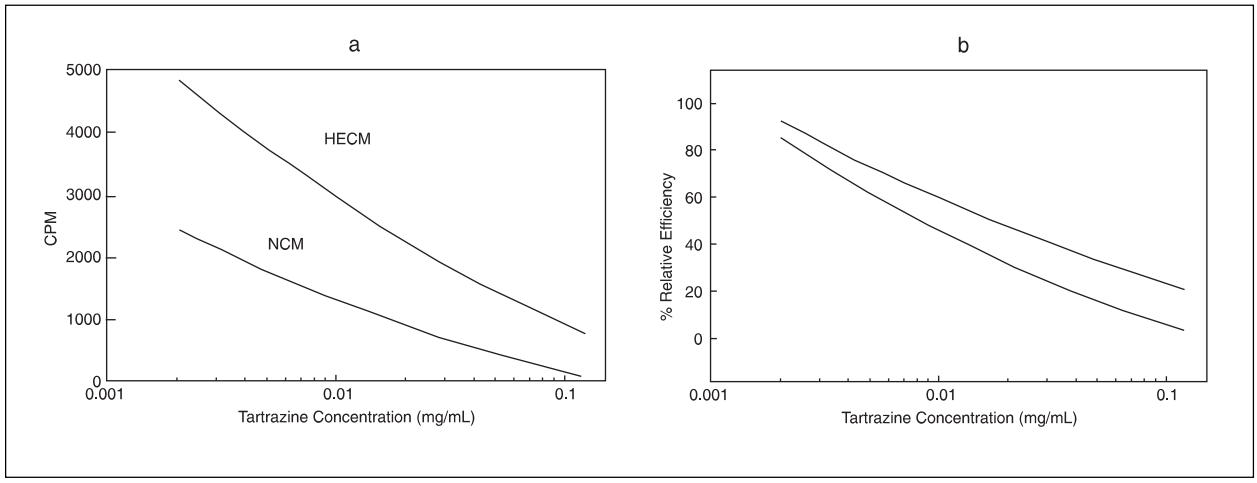

Figure 3 (a and b).
3a shows the relationship between ^{3}H CPM and quenching agent (tartrazine) concentration for both HECM and NCM. 3b illustrates the relationship between relative efficiency and tSIS for HECM and NCM. Results obtained from a 96-well microplate. The improvement for relative efficiency increases with increasing quench.

	Normal Efficiency Count Mode (NCM) Reference CPM = 2849				High Efficiency Count Mode (HECM) Reference CPM = 5179				
Cycle Number	^{3}H CPM	tSIS	QC-CPM	% Diff. from Ref CPM	^{3}H CPM	tSIS	QC-CPM	% Diff. from Ref CPM	% Increase in CPM
1	948	12.97	3260	14.4	2255	13.87	5740	10.8	138
2	936	13.13	3072	7.8	2147	14.26	4840	-6.5	129
3	903	13.20	2907	2.0	2096	14.18	4980	-3.8	132
4	916	13.27	2941	3.2	2097	14.14	5137	-0.8	129
5	904	13.42	2678	-6.0	2096	14.11	5217	0.7	132
6	905	13.19	2943	3.3	2062	14.11	4863	-6.1	128
7	915	13.51	2586	-9.2	2080	14.07	5216	0.7	127
8	925	13.45	2701	-5.2	2065	14.06	5224	0.9	123
9	925	13.35	2715	-4.7	2054	14.18	4971	-4.0	122
10	914	13.26	2822	-1.0	2072	14.23	4851	-6.3	127
11	929	13.29	2877	1.0	2055	14.10	5066	-2.2	121
12	903	13.52	2558	-10.2	2052	13.90	5482	5.8	127
13	929	13.32	2877	1.0	2043	14.08	5120	-1.1	120
14	909	13.42	2635	-7.5	2055	14.05	5205	0.5	126
15	914	13.24	2871	0.8	2033	14.01	5131	-0.9	123
16	918	13.55	2657	-6.7	2045	14.17	4908	-5.2	123
17	907	13.33	2757	-3.2	2045	13.99	5311	2.6	126
18	907	13.30	2789	-2.1	2078	14.24	4694	-9.4	129
19	927	13.31	2800	-1.7	2050	13.98	5274	1.8	121
20	923	13.43	2732	-4.1	2063	14.06	5168	-0.2	124
Average	918	13.32	2809	-1.4	2077	14.09	5120	-1.1	126
Std Dev	12	0.14	165		48	0.10	235		4
% CV	1.3	1.0	5.9		2.3	0.7	4.6		3.5

Table 2.

Accuracy and precision of ^{3}H-SPA quench correction in HECM and NCM. The reference CPM was determined separately in each mode by counting unquenched samples.

the QC-CPM values are different for HECM and NCM. By comparing the QC-CPM obtained for each repeat with the reference CPM, one observes that each individual calculated value is generally within the ± 10% window established by Amersham for accurate quench correction of SPA samples, and on average, the QC-CPM values accurately reflect the reference values within less than 2%. In the above example, HECM more than doubled the raw CPM, and the repeat statistics are comparable for both counting modes. These results confirm that HECM produces high quality quench correction in addition to significantly higher signals.

Bead Dispersion	^{3}H CPM NCM	^{3}H CPM HECM	% Increase
Suspended	18176	22617	24.4
Settled	21061	29203	38.7

Table 3.

Comparison of ^{3}H CPM of suspended and settled SPA beads.

The results of the comparison of suspended and settled ^{3}H-SPA beads are illustrated in Table 3. It is clear from these results that HECM can produce equivalent improvements in CPM when used with settled bead assays. The magnitude of the increase in CPM of course depends on the specific type of assay being performed.

^{125}I-SPA

Figure 4 illustrates the performance of the [^{125}I]-cAMP SPA kit. Figure 4a shows the increase in raw CPM obtained when the assay was counted in HECM. Across all of the samples, the increase averaged 34%. Figure 4b shows the standard curves, which are equivalent. In HECM, the ratio of B_o/NSB was 15.25, while in NCM, it was 12.84. This, together with the higher signal obtained in HECM, demonstrates that assay sensitivity and performance may be as good or better in HECM.

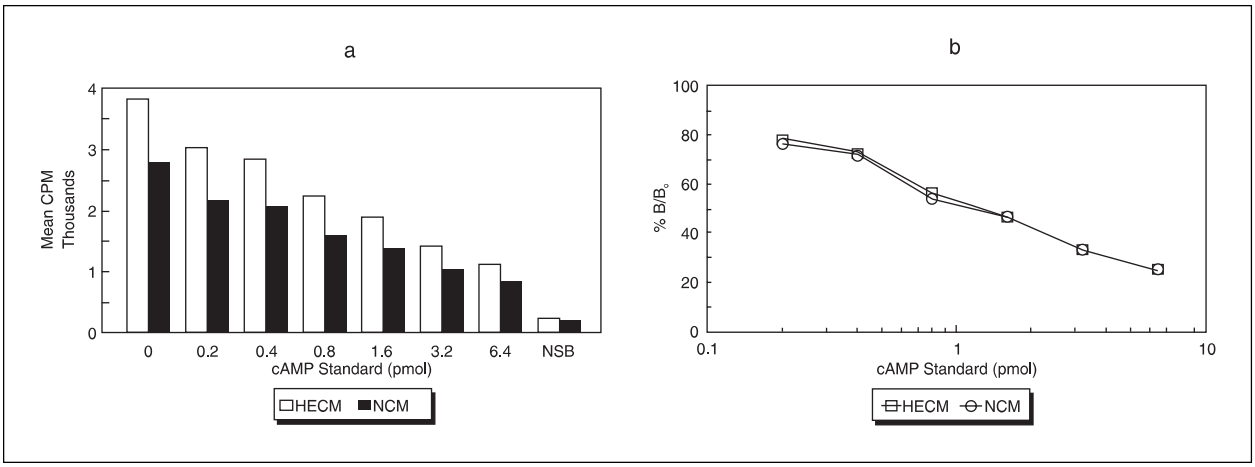

Figure 4 (a and b).

[^{125}I]-cAMP SPA standard curve in HECM and NCM. 4a illustrates CPM levels, 4b illustrates standard curves.

Cytostar-T

Table 4 summarizes the results obtained for the Cytostar-T experiments. Efficiency increases significantly when the microplates are counted in HECM. Moreover, the use of backing tape further increases the signal. Another important observation is that the degree of signal increase is greatest with low energy nuclides such as ^{3}H and ^{125}I. This indicates that HECM will produce the largest signal increase with these low energy nuclides. HECM permits the use of low energy nuclides in Cytostar-T assays which ordinarily would have prohibitively low counting efficiency.

It is also important to note that these results were obtained by spotting the labeled compound directly onto the Cytostar-T microplate. Absolute counting efficiencies will differ in actual cell-based assays, where the cell line and label location affect the degree to which the label interacts with the microplate. Nonetheless, HECM will still enhance the signal over that obtained in NCM. For researchers who find that attaching the backing tape is not an option due to the necessity to periodically check the status of the cells, placing the tape loosely on the microplate carrier provides a viable alternative.

Backing Tape	Isotope	Normal Count Mode (CPM)	High Efficiency Count Mode (CPM)	% Increase in CPM
Attached	^{3}H	3346	7264	117
	^{125}I	3130	4547	45
	^{14}C	30830	37729	22
Loose	^{3}H	1833	4978	172
	^{125}I	1878	3196	70
	^{14}C	23708	32235	36
None	^{3}H	1099	3178	189
	^{125}I	1396	2534	82
	^{14}C	16362	23053	41

Table 4.

Cytostar-T counting results in HECM and NCM. White backing tape supplied by Packard (#6005199). LLD = 5.0. HECM increases counting efficiency by a factor of two for low energy isotopes. In addition, the use of reflective backing tape doubles efficiency again.

Conclusions

High Efficiency Count Mode (HECM) can significantly increase the CPM obtained from many low signal assays. It is particularly useful for low energy SPA, Cytostar-T, and FlashPlate applications. The magnitude of the increase depends on the assay and sample characteristics, as well as the LLD selected for the assay. In high throughput screening applications, this increase in signal will allow shorter count times and, as a result, higher counting throughput. Coupled with the development of the TopCount HTS for counting 384-well microplates, this represents a significant advance in the processing of microplate-based samples. By virtue of using temperature controlled detectors, counting reproducibility remains excellent with twelve simultaneously counting detectors. TopCount offers the maximum throughput possible for counting both 96-well and 384-well microplates.

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6. Cytostar-T kit, instructions, Amersham International plc.

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