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Applications Note

Title: Use of MultiScreen®$_{HTS}$ 384 Filter Plates on the Wallac MicroBeta®Trilux Plate Reader to Achieve Optimal Radiometric Assay Results

Date: January 2005

Lit. No: AN1018EN00

Abstract

Assays performed in 384-well filter plates provide for higher throughput and reagent savings. Reducing the amount of reagents (e.g., ^{33}P-labeled ATP, Kinase, substrate, etc.) necessarily results in a decrease in maximum achievable signal – so that a loss in counting efficiency can seriously limit an assay's utility. Another consequence of performing assays in a 384-well plate is that each well shares a wall with up to four neighboring wells – creating a significant potential, especially in the case of high energy isotopes like ^{32}P and ^{33}P, of radiometric cross talk (i.e., a beta particle emitted in one well being detected in an adjacent well). By virtue of its ability to detect radiation scintillation in a coincidence mode, the Wallac MicroBeta Trilux plate reader achieves significantly higher counting efficiency and signal to noise ratios than TopCount instruments. In addition, the Trilux plate reader has on-board software that can automatically detect and correct for cross talk. The MultiScreen$_{HTS}$ 384-well filter plate was developed and optimized specifically for high throughput radiometric assays and is designed to be compatible with coincidence counting. Together, the Trilux plate reader and the MultiScreen$_{HTS}$ 384-well filter plate can be used to obtain excellent data quality with higher throughput and lower reagent costs. This applications note details the development of a model kinase screening assay, provides instructions on performing the analysis, and presents representative data highlighting the benefits of coincidence counting and cross talk correction.

Background

The use of molecules that contain high energy radiolabeled compounds is very common in a variety of biological assays. One such assay is based on the kinase-catalyzed transfer of the gamma phosphate from ATP to a peptide substrate. The use of radiolabeled ATP is also a common feature of many nucleic acid based assays.

The high energy beta and gamma radiation emitted from phosphate isotopes offers a challenge for accurately counting signal in a high density 384-well filter plate format. This is especially true when a heterogeneous inhibition profile results in low activity wells being adjacent to high activity ones. Thus, it is essential to maximize the efficiency

of counting for low signals while minimizing the radioactive crosstalk contributed by high signal wells.

The MultiScreen$_{HTS}$ 384-well filter plate is designed to maximize signal by utilizing an optically clear underdrain which enables the plate to be read from above and below the well simultaneously (coincidence counting mode). Additionally, the highly pigmented resin used in the construction of the filter plate is designed for maximum opaqueness between wells. This optical barrier, in combination with Microbeta Trilux crosstalk correction software, effectively eliminates counting artifacts as a consequence of signal crosstalk.

Methods

Liquid Scintillation Counting and Analysis of Reaction Product

10ul (384 well plate) Packard Optiphase™ Supermix (PerkinElmer cat# 1200-439) was added to each test well and allowed to incubate for at least 2 hours before counting. Plates were inserted into 348 well plate cassettes (PerkinElmer cat# 1450-130) before being counted in a Wallac MicroBeta Trilux using coincidence counting mode. Crosstalk correction and normalization protocols were performed as recommended by the manufacturer (PerkinElmer lit. #14501016) and according to Millipore technical note TN1099EN00.

Protein Kinase A Phosphorylation Reactions

Phosphorylation assays were run directly in the phosphocellulose (PH) filter plate (Millipore cat# MZPHN050). The plates were pre-treated with 100ul 1M Tris-HCl pH 7.4. Two reaction mixtures were assembled in 96-well V-bottom plates: buffer A contained cAMP (80nM), Kemptide (Sigma-Aldrich cat. # K1127 - conc. as indicated) and PKC/CaMK inhibitory cocktail (Upstate Inc. cat. #20-119) in 1X ADB Buffer (4mM MOPS, pH 7.2, 5mM β-glycerol phosphate, 1mM EGTA, 0.2mM sodium orthovanadate, 0.2mM DTT); buffer B contained Mg/ATP (6mM/40uM) with ^{33}P-γATP (0.1uCi/reaction), inhibiting peptide (Upstate cat. #20-120) or drug (concentration as indicated) or buffer and Protein Kinase A (Upstate Inc. cat.# 14-114) (amount as indicated) in 1X ADB buffer. The pre-wet filter plates were vacuum evacuated (Millipore vacuum manifold cat# MSVMHTS00) at 8” Hg without being allowed to dry. 15ul of reaction buffer A was added to each test well. To start the reaction, 15ul of reaction buffer B was added to each test well in a timed fashion. Reactions were incubated for 15 minutes. The reactions were stopped by the addition of 50ul 200mM Phosphoric acid. The plates were washed 5 times under vacuum using a vacuum manifold at 8” Hg vacuum level with 100ul phosphoric acid per wash. Scintillation counting was performed as described above.

Conclusions

Counting Efficiency and Signal to Background are Enhanced by the Ability to Perform Coincidence Counting Directly in the Filter Plate

Radioactivity is typically measured indirectly by using a scintillation cocktail to generate light which is then amplified and recorded by photomultiplier tubes (PMTs). Coincidence counting utilizes two PMTs, one located above and the other below the filter plate. This configuration allows for the delineation of background from true isotope counts. This method greatly increases the signal to background ratio.
Due to the optically clear underdrain of the MultiScreen$_{HTS}$ 384-well filter plate, it is possible to perform coincidence counting directly in the filter plate using the PerkinElmer Trilux plate counter. The highly opaque and polished walls of the wells help to direct more light to the upper and lower photomultiplier tubes while preventing light from scattering between the wells.

Perkin Elmer Trilux Plate Reader Mode	Isotope	% Efficiency @ approx. 100K DPM	Isotope ratio Signal to Noise
Coincidence Counting	^{33}P	35.6	29370:1
Top PMT Only	^{33}P	49.5	270:1
Bottom PMT Only	^{33}P	19.3	266:1
Coincidence Counting	^{32}P	98.4 / 37.7 *	98396:1
Top PMT Only	^{32}P	143.2 / 31.2 *	486:1
Bottom PMT Only	^{32}P	107.3 / 30.1 *	181:1

Table 1. Shows radiometric counting efficiencies and signal-to-background values for approximately 100,000 DPM ^{33}P and ^{32}P labeled compounds as determined in the Wallac Microbeta Trilux Counting Instrument with 10µL Wallac Supermix (scintillation cocktail) in 384-well MultiScreen-HTS (MZPH) filter plates.

* ^{32}P values are listed with crosstalk correction off / with crosstalk correction on.

Crosstalk Correction Feature of PerkinElmer Trilux Eliminates Counting Artifacts due to Radiometric Crosstalk

The opaque pigment of the resin used in the construction of the MultiScreen$_{HTS}$ 384-well filter plate prevents scintillant-generated light in one well from reaching an adjacent well. However, the resin is not capable of preventing high energy beta and gamma radiation from penetrating the walls. The ability to perform crosstalk correction in coincidence counting mode on the Trilux plate reader accounts for this source of crosstalk.

Figure 1 shows the difference between empty wells and adjacent wells containing containing approximately 100,000 DPM of ^{33}P either with or without using the crosstalk correction feature. Using the crosstalk correction option, the counts of the adjacent radioactive wells are essentially eliminated as compared to the same wells without the crosstalk correction being applied. Similar results were obtained using crosstalk

correction with ^{32}P (data not shown). For further details on performing crosstalk correction and standardization see Millipore technical note TN1099EN00 (ref. 1).

	33487	498	36247	534	39576	
	455	113	445	102	526	
384 well PH	36748	398	36902	477	40973	
	425	101	400	134	626	Without Crosstalk Correction (CPM)
	36060	514	37752	539	38149	
	498	117	535	126	553	
	39109	418	37650	450	34369	
	471	84	401	85	485	
	38060	394	33851	414	33402	
	233	51	209	55	256	
	45790	0	43555	0	53951	
	0	0	0	0	0	
	52159	0	50676	0	51170	
	0	0	0	0	0	After Crosstalk Correction (CCPM)
	45514	0	53062	0	54045	
	0	0	2	0	0	
	57041	0	53031	0	54261	
	1	0	0	0	0	
	54208	0	53361	0	60181	
	2	2	0	1	0	

Figure 1. Crosstalk correction protocol of the PerkinElmer Trilux eliminates crosstalk. Approximately 100,000 DPM of ^{33}P-ATP was placed in each test well. CPM – counts per minute, CCPM – corrected counts per minute

Combining High Signal-to-background Capability and Crosstalk Correction Enables High Quality Screening in the MultiScreen$_{HTS}$ 384 filter plate

High throughput screening requires a distinct separation between high and low activity. Because samples with high and low activity are often in adjacent wells, it is crucial that a filter plate counting protocol provide adequate separation between samples. Utilizing the MultiScreen$_{HTS}$ 384-well filter plate's abilities to achieve high signal-to-background values and correct for radioactive crosstalk in coincidence counting mode allows for screening to be performed with high energy phosphate isotopes in a high density 384-well plate format.

Figure 2 demonstrates the effect of these capabilities on the screening quality (Z') of a kinase assay. Inhibited and uninhibited Protein Kinase A assays are performed adjacent to each other in a checkerboard pattern. Z' values are increased from 0.2 (unacceptable) to 0.5 (good assay) when crosstalk correction is used in coincidence counting mode. Thus, by the advantage of increased signal-to-background by counting in coincidence

mode and performing crosstalk correction, a significant increase in assay quality can be achieved.

cpm with crosstalk correction

72	3662	40	5294	71	4073
4571	78	5784	142	4895	96
72	4126	86	4068	85	3188
4631	87	4742	192	5207	80
98	3055	162	4330	87	4217
4258	98	4231	89	4782	96

	Z'
With Crosstalk Correction	**0.5**
Without Crosstalk Correction	**0.2**

Figure 2. Effect of crosstalk correction on assay performance. Phosphorylation of kemptide by Protein Kinase A reactions were carried out in the MultiScreen$_{HTS}$ 384-well PH filter plate in a checkerboard pattern. Blue wells indicate reactions that were inhibited with 50nM of the tyrosine kinase inhibitor staurosporin. Z' values were determined by the formula $Z' = 1-((3\sigma_u+3\sigma_i)/(\mu_u-\mu_i))$ (ref. 2) where σ = standard deviation, μ = mean, u and i = uninhibited and inhibited, respectively.

References

1.) Millipore Technical Note TN1099EN00 "Normalization, Standardization and CrossTalk Correction Protocols for Radioactivity Counting of MultiScreen$_{HTS}$ Filter Plates in the Wallac MicroBeta® Trilux"

2.) Zhang, J., Chung, T., and Oldenburg, K., "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays" J. Biomolecular Screening 4:67-73, 1999

Millipore lit. no. AN1018EN00.