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Aspiration and Color Quench Correction on FlashPlate®

Abstract

The accuracy of photonic-based assay methods may be adversely affected by the use of colored compounds. Colored medium acts as a filter, "quenching" or attenuating the light signal and potentially leading to false estimates of binding activity.

A specific advantage of NEN FlashPlate microplate technology is the ability to perform color quench correction or to eliminate the effect of color quenching by means of an aspiration step. The following experiments demonstrate the effective use of aspiration, and the application of color quench correction, when aspiration is undesirable.

Background

Color quenching occurs in assays when colored compounds are introduced into the microtitre plate wells. It is an optical phenomenon whereby photons produced by the scintillation cocktail are absorbed in the colored sample prior to reaching the photomultiplier tube (PMT). This type of quench causes changes in the radionuclide spectrum and loss of count rate. When comparing results of unknown wells against a control, color quenching may lead to false estimates of activity. Therefore, correction parameters must be applied to yield accurate final results. The experiments discussed in this file were read on the TopCount® Microplate Scintillation and Luminescence Counter from Packard Instruments.

The TopCount unit utilizes a new sample quench parameter, called the transformed Spectral Index of the Sample (tSIS). This parameter is automatically determined for each sample and is calculated by applying the reverse sum transformation to the sample spectrum. The tSIS is calculated by first determining the samples spectral endpoint. The reverse sum calculation is then applied by summing the counts in individual multichannel analyzer (MCA) channels starting at the endpoint and proceeding from right to left along the spectrum, generating a transformed spectrum. A line through two points along the transformed spectrum is calculated, and its intersection with the channel axis determines the tSIS value. In the presence of chemical or color quenching, the sample spectrum shifts to lower apparent energies, as fewer photons are produced, or those that are produced are absorbed in the colored sample. The tSIS value shifts accordingly with the level of quench in the sample. This shift is used to produce a quench correction curve. TSIS permits greater dynamic range with fewer artifacts caused by low activity samples and background cpm contributions because background counts are most often found in the lowest energy channels. It is also independent of the absolute color of a colored compound, since spectral distortions caused by differences in absolute color do not affect the spectrum endpoint.

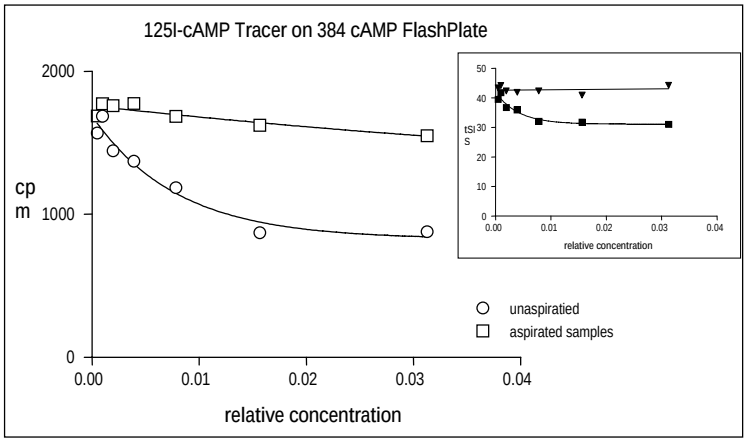
Aspiration

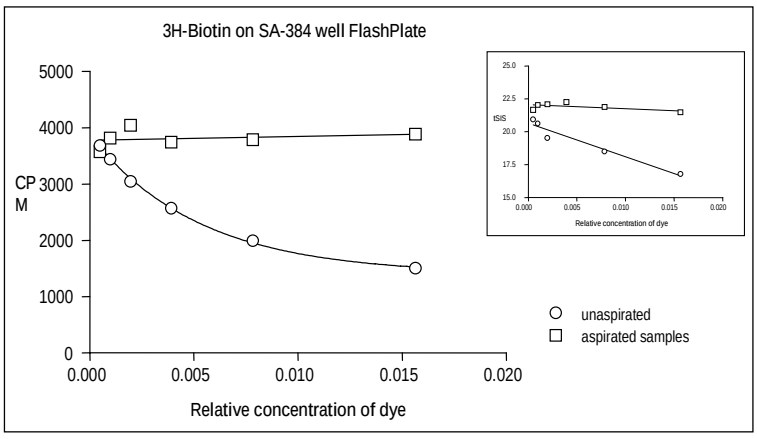
A significant advantage of the FlashPlate microplate platform over other homogeneous assay technologies is the ability to eliminate color quench artifacts by simply aspirating the colored medium. The bound radioactivity will remain on the plate while the colored solutions causing the quench are removed.

Color quench elimination was tested with [^{3}H]-labeled samples using a 384-well streptavidin FlashPlate and [^{3}H]biotin. Color quench elimination was also demonstrating using [^{125}I]-labeled samples using a cAMP 384-well FlashPlate and [^{125}I]cAMP tracer.

A constant amount of the appropriate tracer and serial dilutions of a red dye were added to the plates and allowed to bind under standard assay conditions. The plates were then read in a Packard TopCount HTS counter, determining both cpm and tSIS for each well. TSIS is inversely proportional to quench. The plates were aspirated and read again

Results





Both [^{3}H]- and [^{125}I]-labeled samples show increasing quench in the presence of the colored dye solution. After aspiration the cpm and tSIS curves are approximately horizontal with no correlation to

the concentration of dye that had originally been present. The quench problem had been eliminated by the simple step of aspirating the liquid in the wells and reading the plate.

Quench Correction

Although aspiration can be done successfully on FlashPlates, there are a few assay situations when aspiration is undesirable. Examples include: kinetic assays, in which the reaction is monitored in real time, binding assays, where aspiration of the medium would disrupt equilibrium, if equilibrium has not yet been reached, and in a HTS setting where totally homogeneous assays are desired.

In such situations, it is often possible to correct for color quenching by establishing a quench correction curve, and correcting unknown samples to a reference quench level. The following experiment demonstrates the feasibility of correcting a FlashPlate-based assay for color quenching.

Procedure

A series of 48 samples, each containing a [^{125}I]-labeled ligand and no competitor, was prepared in a FlashPlate, producing 48 replicates of a "zero standard." The binding reaction was allowed to proceed overnight, then the unbound radioligand was removed through aspiration. Using standard instrument settings for ^{125}I FlashPlate, all samples were counted on a Packard TopCount, to establish a reference unquenched cpm value, and to find any outliers.

Next, a series of ten yellow dye dilutions of graduated levels of intensity was prepared. 200 µL of each dye dilution was added in quadruplicate to the appropriate wells, producing a series of ten "quenched standards." The FlashPlate containing the “quenched standards” was counted again and the relative efficiency was determined for each dye dilution (relative efficiency = cpm with dye divided by initial cpm). The quench curve calculated from these results was entered into the TopCount using the protocol number that had been assigned for the initial counts. This quench curve can be used repeatedly for any samples having the same isotope, sample conditions and count conditions. The protocol setting on the TopCount must be set up for the “dpm” mode to correct for quenched samples. The corrected “dpm” results that are produced from a quench curve as described here represent cpm values as if the sample was not quenched (see Figure 1 and Table 1).

Finally, a set of samples (subset of the standards), known to have relative efficiency greater than 15%, was selected for counting as unknowns. These samples were assayed against the quench curve to determine the accuracy of quench correction. The samples were counted for one minute each, to simulate actual assay conditions (see Table 2 and Figure 2).

Results

Table 1 and Figure 1 illustrate the quench correction curve obtained by counting progressively quenched FlashPlate samples. The smooth, monotonic form and shallow slope of the curve suggest that accurate quench correction can be achieved over a wide range of quench levels.

Figure 1

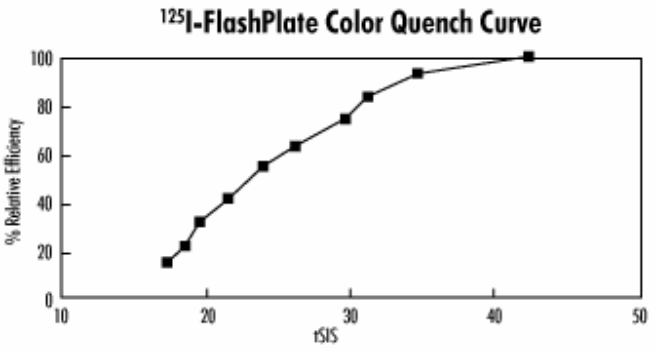


Table 1

Sample	%Eff.	tSIS
1	99.6	42.3
2	93.1	34.7
3	83.5	31.2
4	74.6	29.6
5	63.6	26.1
6	55.2	23.9
7	42.1	21.5
8	32.4	19.5
9	22.2	18.5
10	15.3	17.3

FlashPlate Quench Curve Data

Table 2 summarizes the results of counting progressively quenched samples against the quench curve. Note that cpm and efficiency decrease as a function of increasing quench, while dpm remains constant. The results reflect typical statistical variation obtained with count times of one minute. Variation can be reduced by improving count statistics, obtained by increasing either the count time or the level of sample activity.

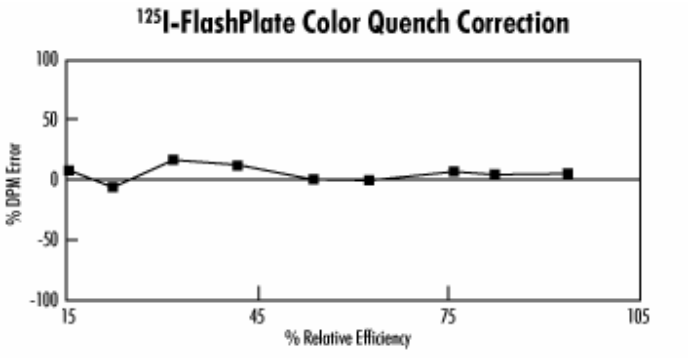
Table 2

Sample	Average cpm	Average "dpm"	% Eff.	% dpm Error
1	1998.3	2200.5	93.7	3.2
2	1754.3	2205.8	82.3	3.5
3	1616.0	2252.3	75.8	5.6
4	1333.3	2100.3	62.5	-1.5
5	1142.5	2140.8	53.6	0.4
6	890.0	2392.0	41.7	12.2
7	677.5	2493.0	31.8	16.9
8	471.8	2022.5	22.1	-5.1
9	333.3	2308.5	15.6	8.3

Correction of progressively quenched FlashPlate samples to reference "unquenched" cpm. Reference cpm = 2132

Percent dpm Error was calculated for each sample by dividing the reference cpm value (2,132 cpm) into the dpm reported by the TopCount, and plotting the result against counting efficiency. As shown in Figure 2, samples that are color quenched to varying degrees can be accurately corrected to an unquenched reference level.

Figure 2



Recommended Protocol

For applications requiring color correction for samples, a series of quenched standards must be generated and run in the TopCount to produce a color quench correction curve. This data is then stored in the TopCount for use in correcting color quench in future assays using the same conditions.

Procedure

1. Set up the FlashPlate with a constant amount of tracer bound per well. It is important for the validity of the data that the wells have a consistent amount of radioisotope bound per well. Set up at least triplicate points.
2. Aspirate and wash the plate.
3. Read the plate on the TopCount.
4. Prepare the series of dye solutions.
5. Add the series of dye solutions to the wells.
6. Read the plate on the TopCount.
7. Construct a table of efficiencies vs tSIS from the TopCount data. This information must now be entered into the protocol used for counting the application. This is done from a menu for Edit Quench Curve from the Directory menu.
8. The output must include dpm. The experiment as described above will not give true dpm, only relative cpm corrected to the absence of color quench. The output is changed the same way as described above for adding tSIS to the output results. A true dpm correction can be applied if the initial cpm are corrected for counting efficiency.

Notes:

a. The volume for the quench curve generated should be the same volume as the application.
b. The buffer used for the quench curve should be the same or closely similar to that of the application.
c. The quench levels in the quench curve should cover a full range of quench; i.e. 100% to 0% quench.
d. The range of radioactivity per well in the quench curve should encompass the entire range of the application. This range should, at a minimum, cover 1,000-10,000 cpm.
e. Specific quench curves must be generated for different isotopes.

f. Quench is independent of the absolute color of the compound, since spectral distortions caused by different colors do not affect the spectrum endpoint. However, yellow is the preferred color for use in the generation of the quench curve. Yellow food dye or titan yellow can be used.

Conclusion

An advantage of FlashPlate is the ability to eliminate the need color quench correction with the conduct of an aspiration step. In applications where aspiration is undesirable, however, quench correction is readily accomplished. Color quench correction should be considered in FlashPlate-based assays where samples are either colored or contain high levels of protein, and where media cannot be aspirated prior to counting. Using standard plate counting methods, an accurate quench correction curve can be readily generated for FlashPlate.