

# TopCount *Topics*

TCA-015

## Quench and Quench Correction

### Abstract

The TopCount® Microplate Scintillation and Luminescence Counter is capable of analyzing radiolabeled microplate samples in a wide variety of formats using liquid and solid scintillation counting techniques. Quench is a common interference in scintillation counting which reduces the sample's apparent activity. In most applications, the level of quench is constant; hence, it need not be considered when comparing the results of a set of samples. However, some experiments produce samples having different amounts of quench. The apparent activities of these samples must be corrected to yield accurate final results. This typically requires a quench indicating parameter and a set of quenched standards. The TopCount incorporates several proven methods of correcting for quench. This paper describes these methods and details several practical protocols for creating quenched standards for use in the TopCount. The paper also describes and presents results of experiments conducted to demonstrate the performance of the TopCount in a variety of quench correction modes.

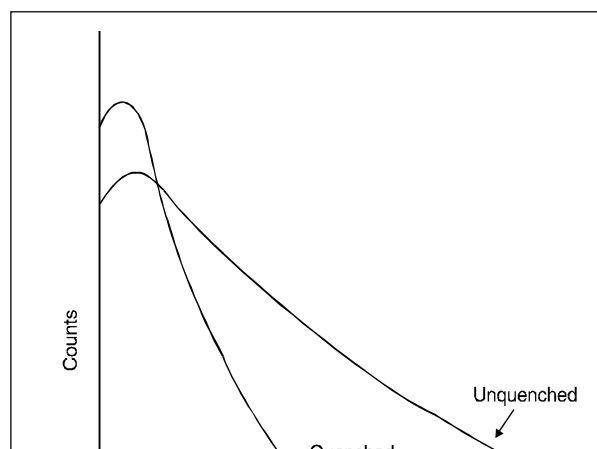
### Introduction

#### **Quench and Quench Correction**

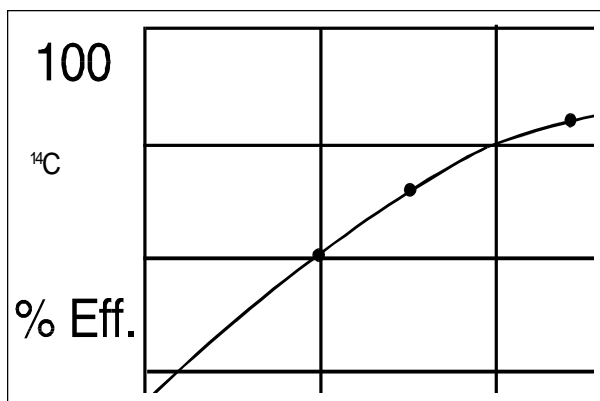
Quench is a common phenomenon in liquid scintillation counting, in which specific components found in the sample interfere with the production and/or transmission of light, thereby reducing counting efficiency. This loss of efficiency is related to both the concentration and the strength of the quenching compound in the sample. There are two primary types of quench. *Chemical quenching* occurs when the compound interferes with the scintillation process, causing non-radiative dissipation of energy. This reduces the apparent energy of the decay event and the number of photons produced, resulting in a loss of counting efficiency. *Color quenching* is an optical phenomenon whereby photons produced by

the scintillation cocktail are absorbed in the colored sample prior to reaching the photomultiplier tube (PMT). Although the mechanisms are different, each type of quench causes changes in the radionuclide spectrum and loss of count rate (Counts Per Minute or CPM, Figure 1).

A series of unknown samples containing similar amounts of quench can be counted and the results analyzed directly using the samples' CPM values. However, a series of samples containing variable amounts of chemical and/or color quenching agents will count at markedly different efficiencies, and will produce CPM results which are dependent on the quench level. To accurately compare the results of all samples, the individual samples' quench levels must be determined and the samples must be corrected to absolute activity (disintegrations per minute or DPM). Traditionally, this is done by counting a series of known activity, progressively quenched standard samples, measuring both their quench level and counting efficiency, and using this information to produce a quench correction curve (Figure 2). The counting efficiency of the unknown sample is then



**Figure 1.**  
Examples of unquenched and quenched spectra.



**Figure 2.**  
Example of a TopCount quench correction curve.

automatically determined by interpolation from the curve, and the sample's DPM is automatically calculated by dividing the CPM by the efficiency.<sup>1</sup>

To assess quench levels, Packard has developed two unique quench indicating parameters based on multichannel analysis of the radionuclide spectrum. Both of these parameters function by quantifying the changes in spectral shape caused by quenching. The first quench parameter is based on the sample spectrum, and involves the calculation of the mean pulse height (transformed Spectral Index of the Sample - tSIS).<sup>2</sup> This parameter correlates changes in counting efficiency with changes in the shape of the sample spectrum. With particularly severe quench, loss of CPM and efficiency is accompanied not by a change in spectral shape, but by a reduction in spectrum height. When this point is reached, it is no longer possible to accurately correct for quench using any sample-based quench parameter. The second parameter is based on an external gamma source (the external standard). Traditional external standard parameters are derived from the shape of the external standard spectrum. A unique approach introduced by Packard involves a reverse sum transformation of the external standard spectrum (transformed Spectral Index of the External standard - tSIE),<sup>3</sup> which eliminates artifacts caused by varying sample volumes, vial materials, and low activity samples, and results in greater dynamic range.<sup>2</sup>

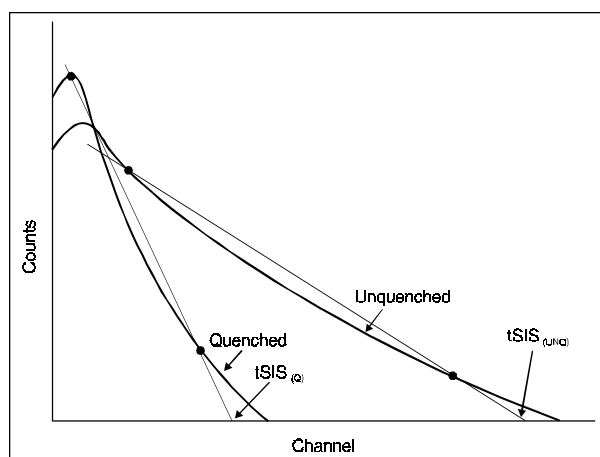
A third approach to quench correction, not involving a quench parameter, is applicable in specific situations when all of the unknown samples are expected to be at a constant quench level. Here, it is possible to measure efficiency directly by preparing a sample at the expected quench level and adding to it a known amount of the radionuclide (internal standardization). Once the counting efficiency has been determined for that sample, it is applied to all unknown samples.

### Quench Correction on TopCount

The TopCount Microplate Scintillation and Luminescence Counter is a versatile instrument capable of measuring radiolabeled and luminescent microplate samples from a wide variety of applications. Radiolabeled samples may be prepared in a number of formats including traditional liquid scintillation counting using MicroScint™ cocktails and solid scintillation counting<sup>4</sup> using LumaPlates™. Liquid counting is affected by both chemical and color quenching, whereas solid scintillation counting using the LumaPlate eliminates chemical quench and will only exhibit color or absorption quenching. TopCount incorporates both sample and external standard quench parameters.

With TopCount, Packard introduces a new sample quench parameter based on proven methods in traditional LSC: 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 sample's 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. This process is illustrated in Figure 3. 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, also shown in Figure 3. 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.

TopCount can also be equipped with an external standard source used to determine tSIE in 24-well microplates. The tSIE quench parameter is derived in the same way as the tSIS. It allows extremely accurate measurement of both single and dual label DPM values over a wide range of quench levels and isotope ratios, and has the additional advantage of being independent of radionuclide and activity level.



**Figure 3.**

Calculation of the tSIS quench indicating parameter.

In this paper, protocols for creating quench correction curves for liquid and solid scintillation counting are described. All methods of quench correction are employed in a series of experiments designed to characterize the quench correction performance of TopCount. Results are presented for these experiments for both single and dual label counting with chemical and color quench.

## Experimental

### Protocols for Quench Correction

For assays requiring correction of variably quenched samples, a series of quenched standards must be made and assayed in the TopCount to produce a quench correction curve which can be stored for further use. The following procedures are recommended for making the standards:

#### I. Liquid Scintillation Counting:

- A. Calculate the total volume of MicroScint cocktail needed, accounting for the number of standards, number of replicates, sample volume and well size, adding at least 50% to the total; *e.g.*, eight standards in triplicate at 250  $\mu\text{L}$  each plus 50% equals 9 mL total volume. It is recommended that the quenched standards be made using the MicroScint cocktail being used in the actual assay.
- B. Make a bulk radiolabeled cocktail solution by adding to the cocktail a sufficient amount of the radionuclide stock solution so that each sample will contain approximately 100,000 DPM; *e.g.*, 100,000 DPM per 250  $\mu\text{L}$  for 9 mL equals  $3.6 \times 10^6$  DPM or 1.62  $\mu\text{Ci}$ . Mix thoroughly.

- C. Aliquot equal volumes of the solution into eight liquid scintillation vials, labeled "Standard 1" through "Standard 8." This is the minimum number of standards recommended. A maximum of 20 is possible. Verify activity in each vial by counting in a traditional LSC, collecting at least 160,000 counts (0.5%  $2\sigma$  value), and discarding any vials that do not fall within 2% of the average.

- D. To produce a quench curve, the eight standards must be progressively quenched with increasing amounts of a suitable quenching compound. Common chemical quench agents include nitromethane, carbon tetrachloride, and methyl salicylate. Color quench curves can be made using a variety of aqueous or organic dyes such as titan yellow, sudan red, or commercially available food dyes. To make a *chemical quench curve*, follow the procedures listed in Section E1. To make a *color quench curve*, follow the procedures listed in Section E2.

#### E. Quenching the standards:

1. Add the volumes of nitromethane listed in Table 1 to each of the eight vials. If another chemical quench agent is used, double the listed volumes. Mix thoroughly.
2. a) Make a stock solution of the color quench compound. Yellow food dye concentrate is used directly in this example, or alternatively, titan yellow can be used at 5 mg/mL, or sudan red at 1 mg/mL.
  - b) Dispense 1 mL of the appropriate MicroScint cocktail into eight LS vials. Make eight stock quenching solutions by adding to each vial the amount listed in Table 2. Mix each thoroughly.
  - c) To each "standard" vial, add 20  $\mu\text{L}$  per mL of the appropriate color quenching solution. Mix thoroughly.

- F. Aliquot solutions of the quenched standards into the desired microplate wells. The accuracy of this operation should be better than  $\pm 2\%$ . It is recommended that the microplate used for the quenched standards be the same type of microplate used for the actual assay. Because the location of each standard will later be defined using the "Plate Mapping" feature in the TopCount, the standards can be placed anywhere on the microplate. A maximum of ten

Standard Vial Number	$\mu\text{L}$ Nitromethane/mL of Cocktail
1	0.0
2	0.3
3	0.6
4	1.3
5	2.4
6	3.6
7	4.8
8	6.3

**Table 1.**

Chemical quench volumes.

replicates is allowed. Heat seal the microplate and mix thoroughly.

- G. Assay an identical aliquot of the unquenched “Standard 1” solution for DPM in a standard LSC to determine the DPM of the standard set. The accuracy of this measurement will directly affect the accuracy of the quench curve. It is recommended that this measurement be done in triplicate and at least 160,000 total counts be collected.
- H. Set up and count the standards, following the instrument operation manual, entering the DPM value obtained in G and defining the plate map in accordance with the layout of the standards. If using tSIS as the quench parameter, it is recommended that the samples be counted long enough to collect at least 10,000 total gross counts to ensure maximum accuracy.

## II. Solid scintillation counting:

Follow the instructions above for creating a color quench curve, with the following changes:

Quenching Solution Number	$\mu\text{L}$ Yellow Food Dye Concentrate/mL of Cocktail
1	0
2	0.5
3	1.1
4	2.3
5	4.5
6	9.0
7	16.0
8	25.0

**Table 2.**

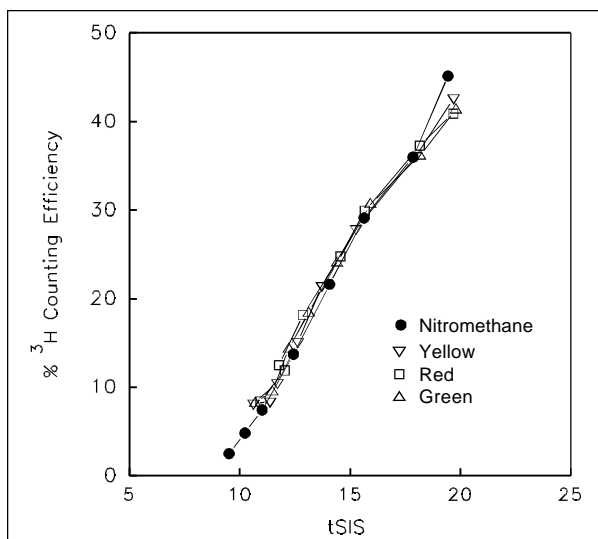
Color quench volumes.

- A. It is highly recommended that the bulk radiolabeled solution be made in the buffer or media being used in the assay. If this is not practical, water may be used. Calculate total volume required as in IA. This time, assume 50  $\mu\text{L}$  per well for the 96-well plate and 200  $\mu\text{L}$  for the 24-well plate.
- B. The radionuclide solution must be non-volatile, preferably a protein or amino acid such as thymidine. Do not use  $^3\text{H}_2\text{O}$  or  $\text{Na}^{125}\text{I}$ .
- C. It is recommended that a non-volatile aqueous or organic dye be used as the quenching agent. Make the stock quenching solutions as in Table 2 using assay buffer or water rather than MicroScint cocktail as the diluent.
- D. Pipet 50  $\mu\text{L}$  (96-well) or 200  $\mu\text{L}$  (24-well) of each labeled solution into LumaPlate wells.
- E. Thoroughly dry (at 50 °C or less) and heat seal the LumaPlate.

### Liquid Scintillation Counting Performance

To evaluate single label performance for liquid scintillation counting, a series of chemical and color quenched standards were prepared in solvent-resistant PicoPlates™. For maximum efficiency with highly quenched samples, polystyrene OptiPlates™ are recommended. [ $^3\text{H}$ ]-thymidine was used as the label in MicroScint-20. Nitromethane and red, yellow and green food dyes were used as the quenching agents. Standards were made according to Tables 1 and 2 and counted in the TopCount to determine tSIS (96-well plate), tSIE (24-well plate), and counting efficiency. Figure 4 illustrates the quench curves for the PicoPlate-96 using tSIS, and Figure 5 depicts the curves for the PicoPlate-24 using tSIE.

Note that in both cases, the quench curves for the chemical quenched samples and all of the color quenched samples are superimposed, even at  $^3\text{H}$  efficiencies below 10%. This indicates that tSIS and tSIE are independent of quench type and, for most experimental situations, a single chemical or color quench curve can be used to correct all samples. However, for severely quenched samples, it is advisable to set up a quench curve using a quench agent which is similar to the unknown samples being processed using the curve. The nitromethane and yellow quench curves were used to interpolate DPM values for some of the standards run as unknowns. Figure 6 illustrates DPM recovery for the 96-well plate using a chemical quench curve, while Figure 7 illustrates DPM recovery for the 24-well plate using



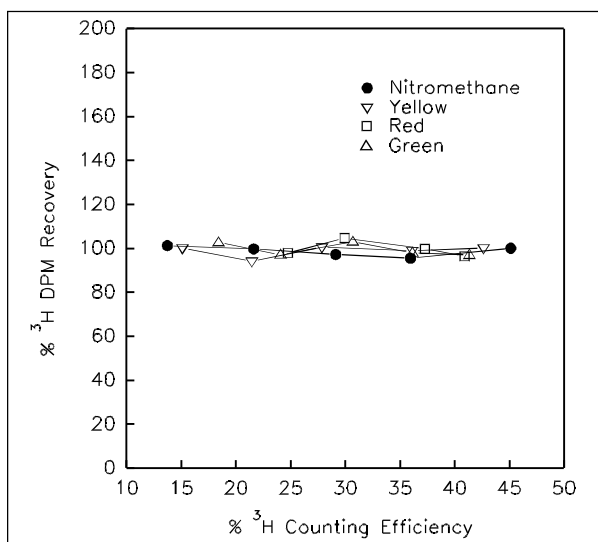
**Figure 4.**

TopCount<sup>3</sup>H quench correction curves, PicoPlate-96, tSIS.

a color quench curve.

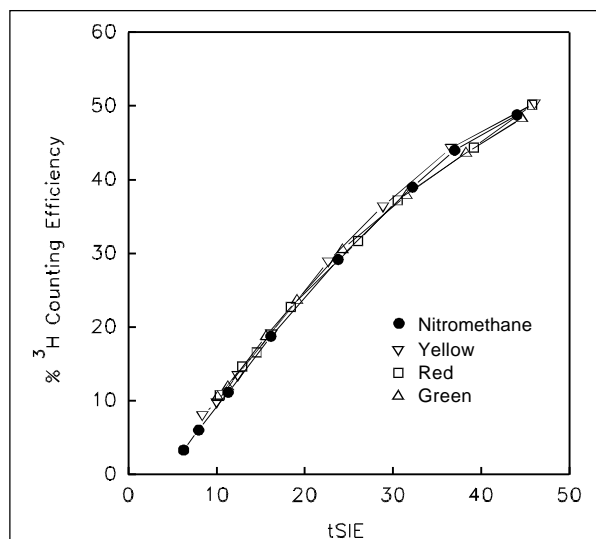
DPM recovery is linear to within  $\pm 5\%$  even at <sup>3</sup>H efficiencies of less than 10%, regardless of what type of quench is used for the standards and/or samples. This indicates that tSIS and tSIE are excellent quench correction parameters for counting LSC samples in microplates. These graphs also indicate that tSIE has a wider dynamic range than tSIS, thus permitting accurate quench correction for more highly quenched samples.

Dual label liquid scintillation counting is also possible on TopCount. When assaying dual label samples that have varying degrees of quench, it is recommended that tSIE be used. Because tSIE is



**Figure 6.**

<sup>3</sup>H DPM recovery using tSIS chemical quench curve (nitromethane), PicoPlate-96.

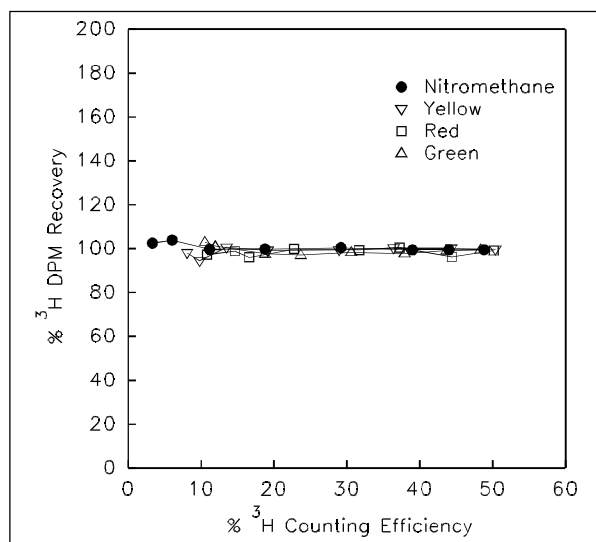


**Figure 5.**

TopCount<sup>3</sup>H quench correction curves, PicoPlate-24, tSIE.

independent of the sample spectrum, it is not sensitive to changes in quench or isotopic ratios. A series of experiments were performed by first creating dual label quench curves based on tSIE in the 24-well format using <sup>3</sup>H and <sup>14</sup>C chemically quenched standards made following the earlier recommendations. Three additional series of quenched samples were prepared. These contained both <sup>3</sup>H and <sup>14</sup>C in the amounts listed in Table 3.

After creating the dual label quench curves, both the standards themselves and the dual label samples were assayed for DPM using the curves. Preset <sup>3</sup>H/<sup>14</sup>C counting regions were selected from the nuclide library, although custom regions can be used by setting the intermediate discriminator to the



**Figure 7.**

<sup>3</sup>H DPM recovery using tSIE color quench curve (yellow), PicoPlate-24.

endpoint of the lower energy nuclide. Figures 8 through 10 illustrate DPM recovery as a function of quench.

Excellent DPM recovery is again achieved over widely varying quench levels and isotope ratios. Even when the higher energy isotope is five times more abundant, TopCount is able to accurately separate the isotopes at heavy quench levels using tSIE. Separation and recovery of each nuclide is accurate to  $\pm 10\%$  even at  $^3\text{H}$  efficiencies of 10%. This is further demonstrated by linear recovery when assaying the single label samples against the dual label quench curves (data not shown).

A final experiment was performed, this time to demonstrate the ability to correct for quench in filter samples. The UniFilter™ plate, which consists of 96 or 24 discrete filter disks arranged in the microplate format, was developed for researchers conducting filtration assays on receptor binding of crude natural products and synthetic compounds.<sup>5</sup> Many of these compounds leave a colored residue on the filter, even after harvesting and extensive washing. By setting up a color quench correction curve on a UniFilter plate, it is possible to correct for varying color in the filtered samples. The procedures outlined above were generally followed, but the total volume per well was reduced to 35  $\mu\text{L}$  for the UniFilter-96. Total volumes for the UniFilter-24 plate should not exceed 200  $\mu\text{L}$ . Figure 11 illustrates the quench curve obtained in this experiment.

As observed earlier, this curve can be used to correct samples containing a variety of colors.

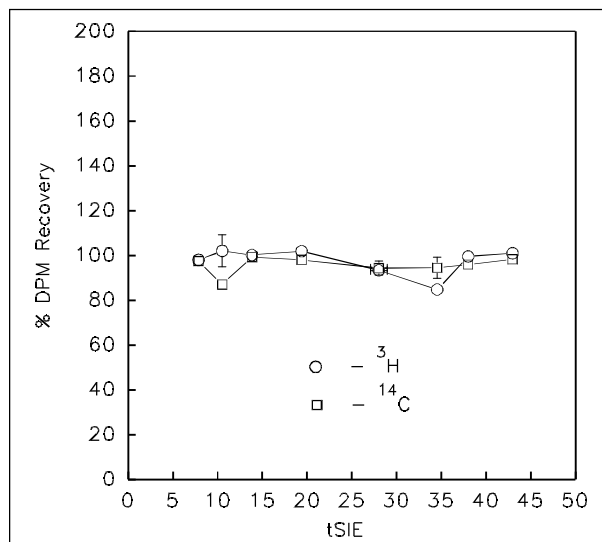
Set	DPM Ratio (H-3:C-14)	H-3 DPM	C-14 DPM
1	5:1	44517	9785
2	1:1	44517	48521
3	1:5	10342	48521

**Table 3.**  
Dual label DPM samples.

### Solid Scintillation Counting Performance

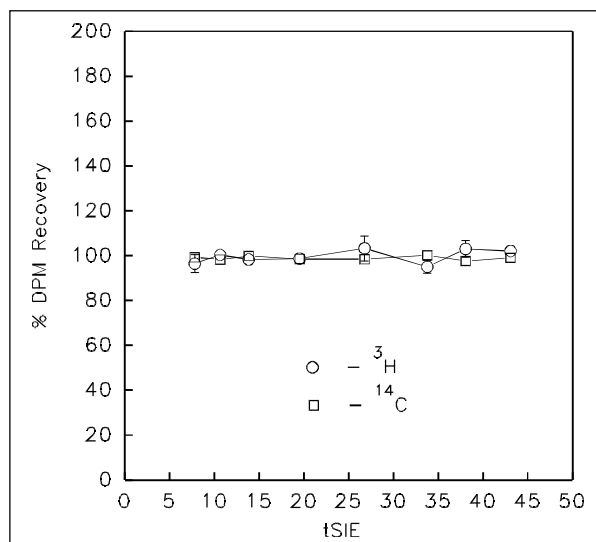
An experiment was conducted using a LumaPlate in a simulation of a  $^{51}\text{Cr}$ -release cytotoxicity assay.<sup>6</sup> Samples produced by this assay are typically colored due to pH indicator dyes present in the cell culture medium. To demonstrate quench correction for this assay, a color quench set was produced using the recommended procedures and used to create a quench correction curve. Here, rather than determining absolute DPM on a LSC, the unquenched standard CPM value was used as the DPM, eliminating the need to independently measure DPM. This strategy produces final results which are corrected to the unquenched CPM value, and can be used in cases where it is impractical or unnecessary to measure the absolute DPM, such as in percent bound calculations. Figure 12 illustrates the quench curve produced by this method.

Note the smooth curve and that the unquenched efficiency is 100%. This demonstrates that  $^{51}\text{Cr}$ -release assays on LumaPlates can be color quench corrected using tSIS to a reference CPM over a range of typically encountered quench levels.



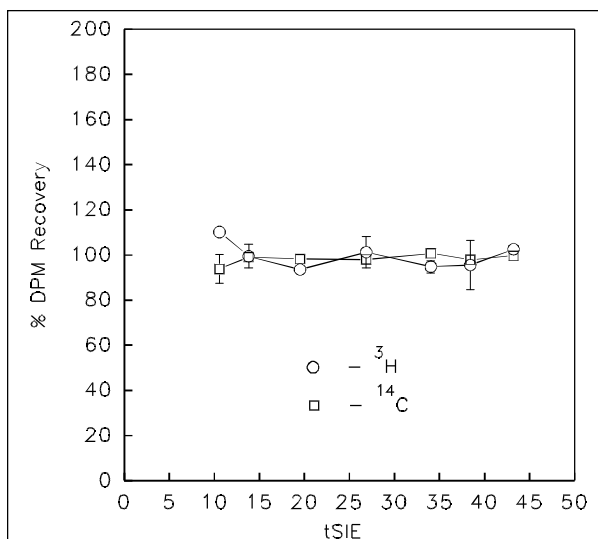
**Figure 8.**

Dual label DPM recovery in a PicoPlate,  $^3\text{H}:^{14}\text{C}$  Ratio = 5:1.



**Figure 9.**

Dual label DPM recovery in a PicoPlate,  $^3\text{H}:^{14}\text{C}$  ratio = 1:1.



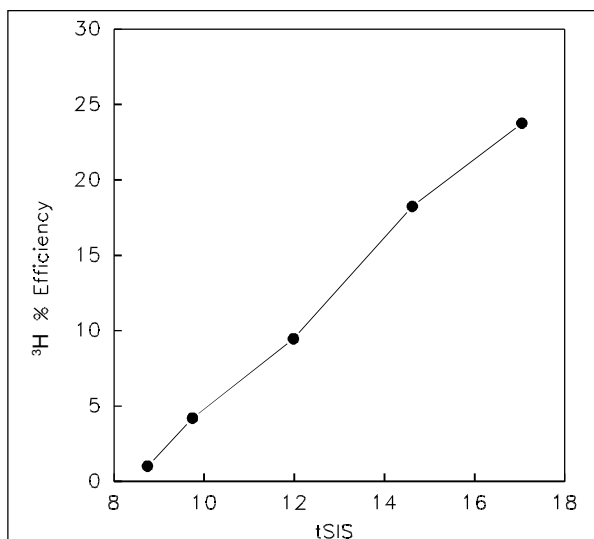
**Figure 10.**

Dual label DPM recovery in a PicoPlate, <sup>3</sup>H:<sup>14</sup>C ratio = 1:5.

Dual label quench correction on LumaPlates over a range of quench levels is also possible using tSIE. To demonstrate this, a series of color quenched standards were prepared for both <sup>3</sup>H and <sup>14</sup>C using the general procedures documented above. In addition, a set of dual label samples covering the same quench range were made at an activity ratio of 1:1. After setting up the quench curves on TopCount, both the single and dual label samples were assayed for DPM. Figures 13 and 14 summarize DPM recovery for this experiment.

Again, linear DPM recovery is observed for both <sup>3</sup>H and <sup>14</sup>C over a range of quench levels. Also, as shown previously, recovery remains linear regardless of the actual color of the samples.

Finally, constant quench dual label performance was evaluated for solid scintillation counting, although it is applicable to liquid counting as well. This situation occurs in many assays in which individual samples have very similar chemistry and, therefore, essentially identical quench levels. A series of stock solutions were prepared which contained [<sup>3</sup>H]- and [<sup>14</sup>C]-thymidine at ratios varying between 10:1 and 1:10, as well as single label controls. The solutions contained equivalent total amounts of thymidine, so that the samples would be at a constant quench level. 50 µL aliquots of each solution were pipetted into wells on a LumaPlate-96, as well as into LS vials for DPM determination on a traditional LSC. After drying and sealing, the LumaPlate was counted in the TopCount. The two counting regions, A and B, were determined by setting the intermediate discriminator to the endpoint of the lower energy nuclide, thus excluding spill-up of the lower energy nuclide into the higher energy region.

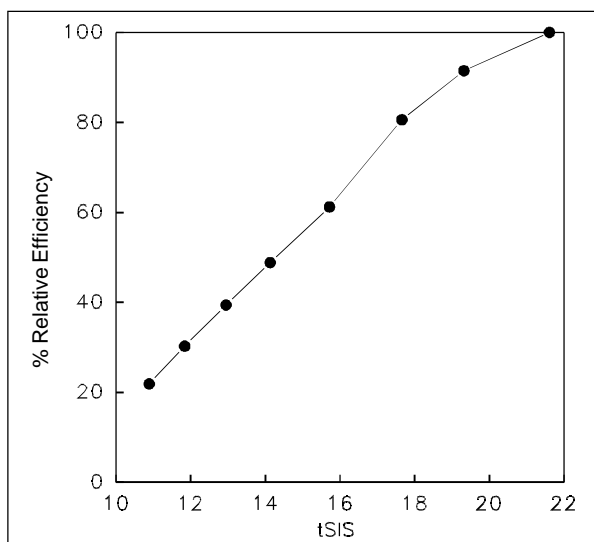


**Figure 11.**

<sup>3</sup>H color quench correction curve (yellow) on UniFilter-96 filtration plate.

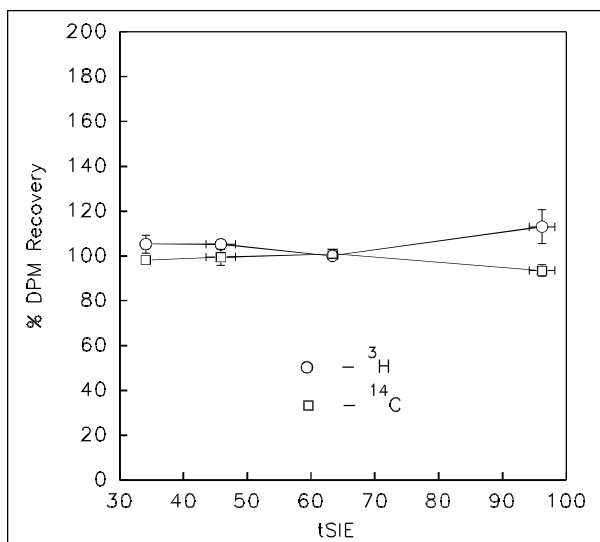
After counting, the single label samples were used to determine the efficiency of each nuclide in each counting region. These efficiencies were used along with CPM<sub>A</sub> and CPM<sub>B</sub> to calculate the DPM's of the individual radionuclides present in the samples. A complete description of these calculations can be found in the Packard publication "*Liquid Scintillation Analysis, Science and Technology*."<sup>2</sup> Figure 15 illustrates the DPM recovery performance for this assay.

Linear recovery for both <sup>3</sup>H and <sup>14</sup>C is observed over a wide range of activity ratios. This demonstrates that accurate dual label quench correction can be achieved easily when the level of quench is expected to be constant over the entire sample set. Further-



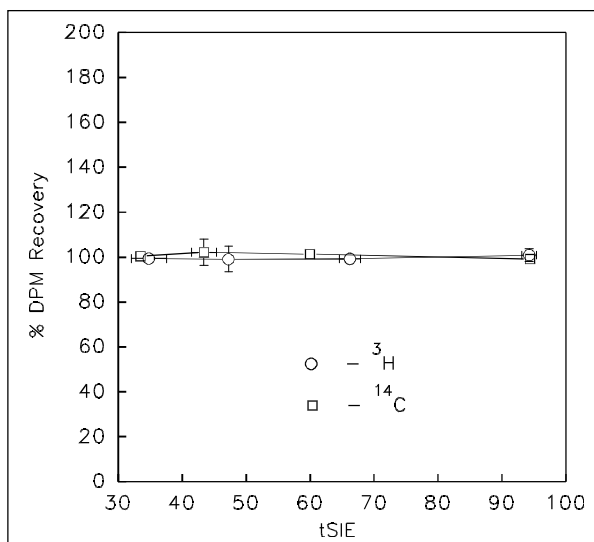
**Figure 12.**

<sup>51</sup>Cr color quench curve (yellow) on a LumaPlate-96.



**Figure 13.**

Dual label DPM recovery in a LumaPlate, <sup>3</sup>H:<sup>14</sup>C ratio = 1:1.



**Figure 14.**

Dual label DPM recovery in a LumaPlate, <sup>3</sup>H, <sup>14</sup>C single label standards.

more, processing of results using the above referenced equations can easily be automated using TopCount's Tandem Processing software and a simple spreadsheet macro.<sup>7</sup>

## Conclusions

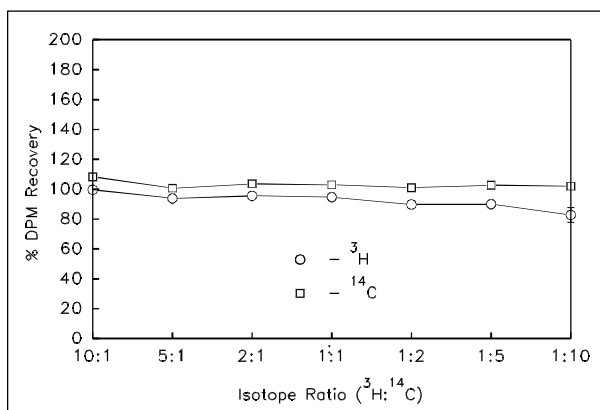
The ability to accurately correct for quench is a prominent feature of the TopCount Microplate Scintillation and Luminescence Counter. Two quench indicating parameters, tSIS and tSIE, allow quench correction for a wide variety of conditions in both liquid and solid scintillation counting applications. tSIS is shown to be an excellent general purpose parameter which is independent of chemical or color quench. tSIE can be used for both single and dual label assays for the most accurate, widest dynamic range quench correction, and is also independent of quench type. Both parameters reduce or eliminate dependence on sample activity level, and back-

ground count rate. As a result, they allow accurate DPM calculations over a wide dynamic range.

Depending on the assay and the quench correction requirements, several options are available for quench correction on TopCount. Examples of these options are described, and practical experimental protocols which allow the investigator to correct for quench are detailed. Results based on those protocols are presented which demonstrate excellent performance over a wide range of conditions.

## References

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3. Jones, D.K., Tomisek, J.D., Park, E., and Young, H.M. 1986. *Reverse sum quench measurement using a liquid scintillation counter*. U.S. Patent Number 4,633,088.
4. TopCount Topics #2, *Solid Scintillation Counting*, Packard Instrument Company.
5. TopCount Topics #12, *Biological Applications of Microplate Scintillation Counting*, Packard Instrument Company.
6. TopCount Topics #8, *Counting <sup>51</sup>Cr Released in Cytotoxicity Assays*, Packard Instrument Company.
7. TopCount Topics #6, *TopCount Tandem Processing Using Automated Spreadsheets*, Packard Instrument Company.



**Figure 15.**

Constant quench dual label DPM recovery on a LumaPlate-96.