

TopCount *Topics*

TCA-014

The Measurement of Luminescence .

Signal linearity and detection limit of various chemiluminescent and bioluminescent chemistries.

Abstract

TopCount microplate scintillation counter may be used as an automated microplate luminometer with all glow-type or enhanced flash-type luminescent chemistries. The data shown here documents TopCount's performance with various luminescent chemistries. The signal linearity, detection limit, and reproducibility of TopCount are as good or better than those of a dedicated microplate luminometer. Advantages of TopCount over other luminometers include, a plate stacker, bar code system, thermo-regulation, an on-board IBM®-PC compatible 386 computer, and the option of isotopic counting.

Introduction

TopCount is a microplate scintillation counter, the hallmark of which is its versatility. TopCount may be used for the high throughput screening of isotopic samples in the 24- or 96-well microplate format, or luminescent assays in the 96-well format. TopCount's fast digital circuitry enables luminescent measurements via Single Photon Counting (SPC).

TopCount offers major advantages over other luminometers on the market. First, TopCount is the only luminometer with a plate stacker. Up to 32 plates may be loaded, and TopCount's bar code system allows one to run multiple protocols within a stacker load. Second, TopCount provides a counting environment which is thermoregulated. Samples may be counted at a constant temperature, 15° to 35°C, regardless of ambient temperature fluctuations.

With the introduction of luminescence counting, TopCount expands its application versatility. Presented here are data demonstrating TopCount's util-

ity as a luminometer using a variety of popular glow and enhanced flash luminescent chemistries. TopCount is ideal for those labs wishing to convert from isotopic to non-isotopic assays, since both methods may be used on the same instrument. Applications include immunoassays, DNA probe assays, cell growth assays, and reporter gene assays.

Luminescent chemistries have come a long way from their early days as a trade show curiosity. The new glow chemistries equal or exceed the sensitivity of isotopic labels. The major advantages of luminescent over isotopic labels are safety, easy handling, and significantly lower disposal costs. With respect to colorimetric detection, luminescence is more sensitive, has a larger dynamic range, and does not require the addition of a stopping reagent.

The following six luminescent chemistries were examined on TopCount using serial enzyme dilutions to demonstrate linear range and detection limit:

1. Lumi-Phos™ 530 (dioxetane) with alkaline phosphatase, Lumigen® (Detroit, Michigan).
2. Enhanced Chemiluminescence (ECL; luminol) with horseradish peroxidase, Amersham® (Arlington Heights, Illinois).
3. Lumi-Gal™ 530 (dioxetane) with b-galactosidase, Lumigen.
4. Luciferase Assay System (bioluminescence; firefly), Promega® (Madison, Wisconsin).

5. Enliten™ (bioluminescence; firefly) ATP Assay System, Promega.
6. XO(isoluminol) with xanthine oxidase, A. Baret (Nantes, France).¹

Chemistries 1, 2, 3, and 6 are classified as glow-type luminescent chemistries, whereas 4 and 5 are classified as enhanced flash-type chemistries. Flash-type chemistries such as chemiluminescent acridinium esters can not be measured on TopCount since they require a reagent injection system.

Methods

All assays were done using Dynatech® (Chantilly, Virginia) MicroFLUOR® black 96-well microplates. Black plates were chosen over white plates due to their low background and the fact that there is no requirement for a count delay. The plates were counted on TopCount using the SPC mode at 24°C with a count time of 1.2 seconds per well. For instrument comparison, the plates were also counted on a commercially available microplate luminometer (ML) at room temperature. TopCount data is presented as counts per second (CPS), and the ML data are presented as the percentage of maximum intensity. The substrate blank was determined for all assays using 5 ml of diluent plus the appropriate substrate. This background value was subtracted from all dilutions. The detection limit is defined as the enzyme (or ATP) concentration corresponding to a value greater than the mean of the substrate blank plus two standard deviations.

LumiPhos 530: 5 ml of serially diluted (1:10 in PBS/0.2% BSA) avidin-alkaline phosphatase (Sigma®, St. Louis, Missouri) was added per well in triplicate. 50 ml of Lumiphos 530 was added, the plate was incubated for one hour at 37°C, and then counted.

ECL: 5 ml of serially diluted (1:10 in PBS/0.2% BSA) horseradish peroxidase (Sigma) was added per well in triplicate. 50 ml of ECL substrate (1:1 ratio of kit reagents #1 and #2) was added, the plate was incubated for 30 minutes at ambient temperature, and then counted.

Lumi-Gal 530: 5 ml of serially diluted (1:10 in PBS/0.2% BSA) b-galactosidase (Sigma) was added per well in triplicate. 50 ml of Lumi-Gal 530 was added, the plate was incubated for one hour at 37°C, and then

counted.

Luciferase Assay: 5 ml of serially diluted (1:10 in PBS/0.2% BSA) luciferase (Sigma) was added per well in triplicate. 50 ml of luciferin substrate was added, the plates were shaken for five seconds, and then counted immediately.

Enliten: 4 ml of serially diluted (1:10 in ATP-free water) ATP was added per well in triplicate. 50 ml of Luciferin/Luciferase Reagent was added, the plates were shaken for five seconds, and then counted immediately.

Baret XO: 5 ml of serially diluted (1:10 in PBS/0.1% BSA) xanthine oxidase (XO; Biozyme®, San Diego, California) was added per well in triplicate. 100 ml of signal reagent¹ was added, the plates were shaken for two minutes, and then counted after a 20 minute incubation at room temperature.

Stacker Test: 100 ml of the Baret signal reagent containing 10^{-15} moles of xanthine oxidase was pipetted manually into 24 wells (every other row and column) of a 96-well black MicroFLUOR plate. This single plate was loaded into the TopCount stacker and then counted for four cycles. Each cycle consisted of plate movement from the front stacker (plates to be counted)

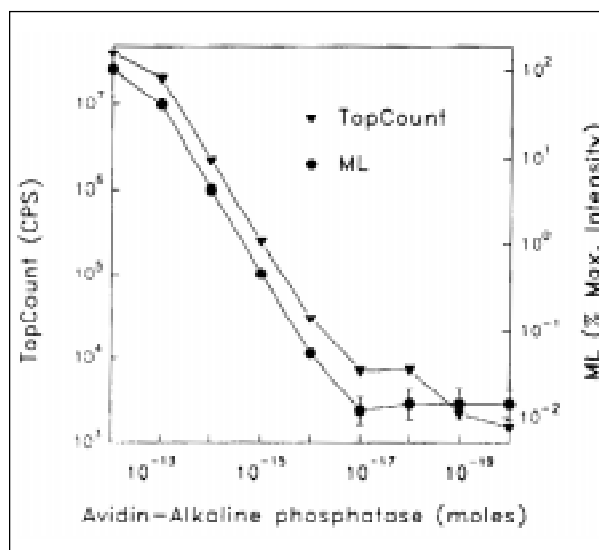


Figure 1.

Instrument response comparison. The dynamic range and detection limit of avidin-labeled alkaline phosphatase was measured using the glow-type chemiluminescent substrate, LumiPhos 530. Serial enzyme dilutions (1:10) and substrate were added to a black microplate and counted on TopCount and another microplate luminometer, ML.

into the counting chamber, out to the rear stacker (plates counted), and then reverse-stacked into the front stacker. The purpose of this test was to demonstrate the consistent positioning of microplates within the counting chamber. Data are presented for each well as a percentage of the mean for each well.

Results

Avidin-labeled alkaline phosphatase was measured using LumiPhos 530 (Figure 1). Both TopCount and the ML produced a linear range of three decades, 10^{-13} to 10^{-16} moles of alkaline phosphatase. The detection limit on TopCount was 0.1 attomoles, whereas the detection limit on the ML was 10.0 attomoles.

Data using the Amersham Enhanced Chemiluminescent (ECL) substrate are presented in Figure 2. The linear range for both instruments was only two decades, 10^{-13} to 10^{-15} moles of horseradish peroxidase. However, the detection limit was better on TopCount, 0.01 attomoles, than on the ML, 0.1 attomoles.

The Lumi-Gal 530 and Luciferase Assay System substrates are designed to measure b-galactosidase and luciferase, respectively. The genes for these enzymes have been sequenced and have been used in reporter gene assays.^{2,3} The data shown in Figures 3

and 4 were generated using dilutions of purified enzyme, rather than cell lysates. The linear range for b-galactosidase was similar on both instruments, 10^3 to 10^0 mU/ml. However, TopCount's detection limit was better, 10^{-4} versus 10^{-1} . The linear range for luciferase detection was similar on both instruments, 10^{-14} to 10^{-17} moles, although TopCount had a detection limit which was one decade more sensitive, 0.01 attomoles.

The detection limit for ATP is determined largely by the sensitivity of the kit. The Enliten ATP Assay System is a bioluminescence detection kit which is designed to measure down to 0.1 femtomoles of ATP. As shown in Figure 5, TopCount achieved this detection limit, 0.1 femtomoles, and had a linear range of 10^{-12} to 10^{-15} moles. The ML was less sensitive with a linear range of 10^{-12} to 10^{-14} , and a detection limit of 10.0 femtomoles.

The dynamic range for xanthine oxidase detection using the Baret chemistry was 10^{-13} to 10^{-17} moles of XO on TopCount and 10^{-13} to 10^{-16} on the ML, Figure 6. The detection limit for TopCount was 10.0 attomoles of XO, whereas the detection limit on the ML was 0.1 femtomoles. The Baret XO chemistry is ideally suited for high volume assays due to its long-term signal

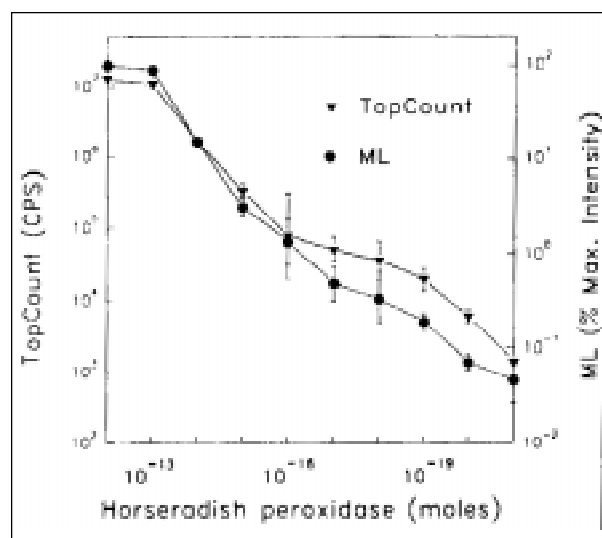


Figure 2.

Instrument response comparison. The dynamic range and detection limit of horseradish peroxidase was measured using the glow-type chemiluminescent substrate, ECL. Serial enzyme dilutions (1:10) and substrate were added to a black microplate and counted on TopCount and ML.

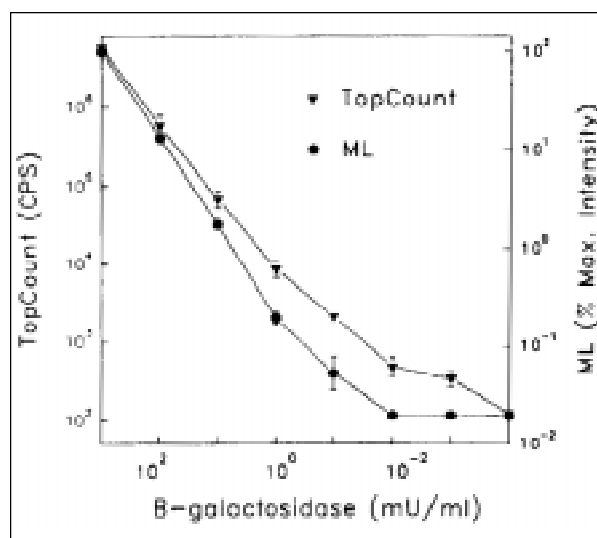


Figure 3.

Instrument response comparison. The dynamic range and detection limit of b-galactosidase was measured using the glow-type chemiluminescent substrate, Lumi-Gal 530. Serial enzyme dilutions (1:10) and substrate were added to a black microplate and counted on TopCount and ML.

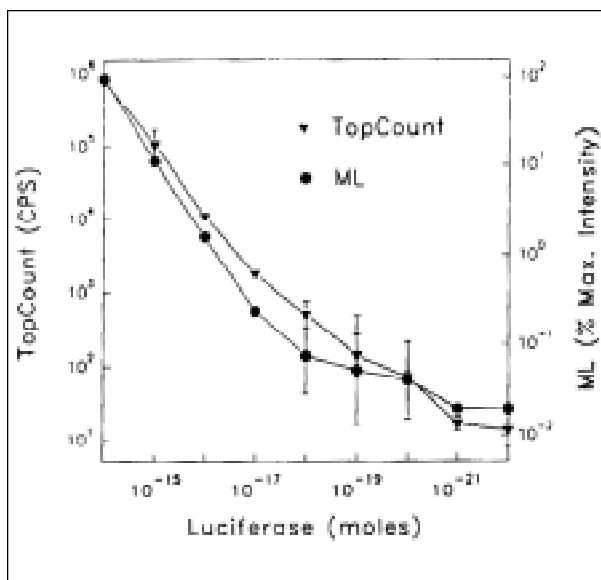


Figure 4.

Instrument response comparison. The dynamic range and detection limit of luciferase was measured using the enhanced flash-type chemiluminescent substrate from the Luciferase Assay System kit. Serial enzyme dilutions (1:10) and substrate were added to a black microplate and counted on TopCount and ML.

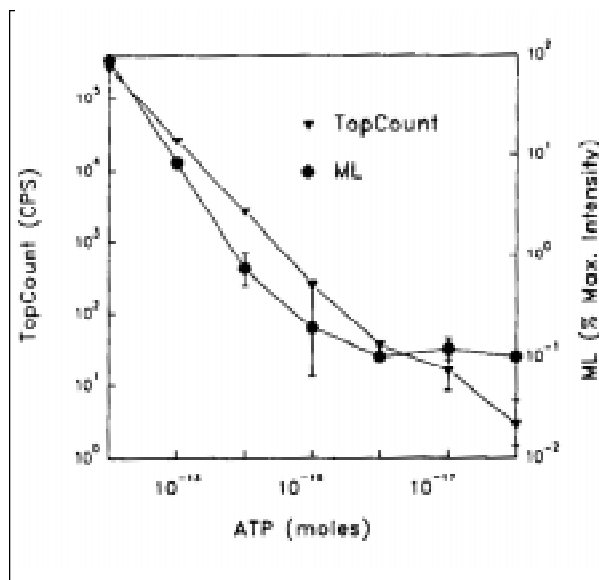


Figure 5.

Instrument response comparison. The dynamic range and detection limit of ATP was measured using the enhanced flash-type chemiluminescent substrate, Enliten. Serial dilutions (1:10) of ATP and substrate were added to a black microplate and counted on TopCount and ML.

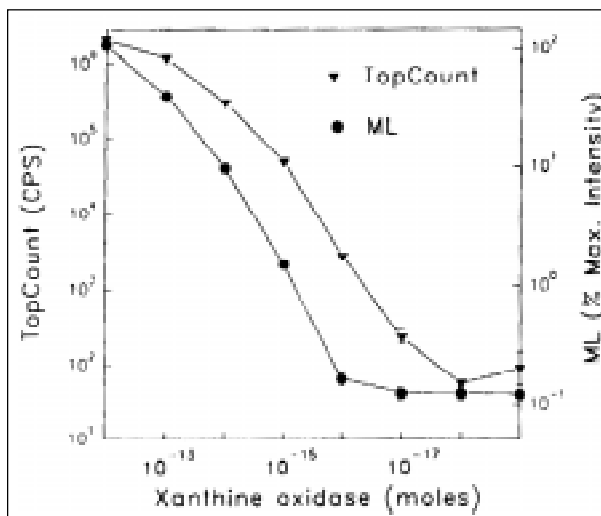


Figure 6.

Instrument response comparison. The dynamic range and detection limit of xanthine oxidase was measured using the Baret glow-type chemiluminescent substrate. Serial enzyme dilutions (1:10) and substrate were added to a black microplate and counted on TopCount and ML.

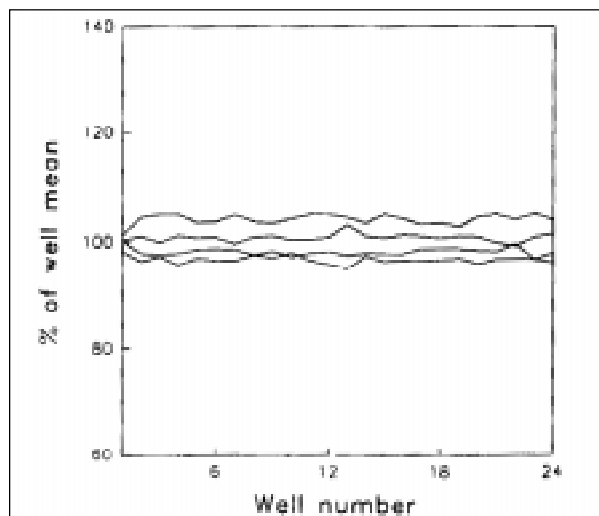


Figure 7.

TopCount stacker test. A single black microplate containing an identical volume of luminescent probe pipetted manually into 24 wells (every other row and column) was counted on TopCount using the plate stacker. The plate was counted for four cycles with a 45 minute delay prior to each cycle.

stability, allowing full utilization of TopCount's stacker capacity.¹

The results from the stacker test are presented in Figure 7. The four curves correspond to the four cycles. Down shifting of the curves was due to decreasing photon emission over time; this is normal for luminescent chemistries. The count delay between cycles was 45 minutes.

Conclusion

All of the chemiluminescent and bioluminescent chemistries tested performed well on TopCount. Data were as good or better than that obtained on a dedicated microplate luminometer. TopCount's stackers enable one to automate the counting of multiple plates, and the bar code system allows the counting of multiple protocols. However, when using bioluminescent probes (e.g., Luciferin/Luciferase), the so-called enhanced flash chemistries which have a relatively short light output, single plate counting may be required. Flash chemistries, such as chemiluminescent acridinium esters, require reagent injection in front of the photomultiplier and cannot be measured with TopCount.

Since TopCount's counting chamber is thermoregulated, the effects of changing ambient temperature will not affect counter response. The temperature can be set (15°-35°C) to an optimal temperature for a specific luminescent chemistry to obtain consistent counting results.

Although both white and black opaque microplates may be used for luminescence counting on TopCount, under most circumstances black plates are preferred due to their lower background. For example, an identical volume of a luminescent substrate counted in both black and white plates produced backgrounds of 63 and 613 CPS, respectively. Optical crosstalk on both black and white plates is less than 0.05%, but is the lowest with black plates. In addition, black plates, unlike white plates, will not phosphoresce, thereby obviating the need for dark adaptation prior to measurement. The dynamic range of TopCount in the SPC mode is approximately six orders of magnitude. This is based on PMT photon saturation above 3×10^7 CPS and an instrument background of approximately 30 CPS.

References

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3. Beale, EG, et. al. A rapid and simple chemiluminescent assay for *Escherichia coli* - galactosidase. *BioTechniques.* 12 (3) 320-324, 1992.

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