

Phosphodiesterase Assays On FlashPlate[®] Microplates

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Introduction

Multiple forms of phosphodiesterase have been reported in many tissues.⁽¹⁾ Cyclic nucleotide phosphodiesterases catalyze the breakdown of cyclic 3',5'-adenosine monophosphate (cAMP) and/or cyclic 3',5'-guanosine monophosphate (cGMP) to their corresponding 5' nucleotides. The degradation of these cyclic nucleotides, which serve as second messengers in hormonal and photic transduction systems, terminates their formational role which is thought to be mediated by the cyclic nucleotide-dependent protein kinases.⁽²⁾

Over the last several years, many novel agents have been identified that exert selective inhibitory effects on the various molecular forms of phosphodiesterase present within different cells.⁽³⁾ There continues to be a growing interest in the pharmaceutical industry to participate in the rapidly evolving area in the discovery of selective phosphodiesterase inhibitors.

Using FlashPlate platforms, which are white, opaque, 96- and 384-well scintillant coated microplates, new applications have been developed which enable the study of phosphodiesterase activity on both cAMP and cGMP. These applications can be used to measure phosphodiesterase activity, or the inhibition of phosphodiesterase activity, in a fully homogeneous assay. This provides a platform which is very suitable for high throughput screening and provides reproducible and dependable results.

PerkinElmer offers cAMP (SMP001) and cGMP (SMP002) FlashPlate assays, originally developed to quantitate cAMP and cGMP in serum, plasma and urine samples in a 96-well format. To optimize an assay for phosphodiesterase activity detection, it was necessary to alter the assay configuration. Studies were done that involved converting from a 96-well to a 384-well format, optimizing buffer formulations, terminating conditions, and DMSO effects.

The application involves adding a source of phosphodiesterase and [¹²⁵I]cAMP or [¹²⁵I]cGMP to a FlashPlate well which contains an antibody to either cAMP or cGMP. The phosphodiesterase will catalyze the breakdown of the [¹²⁵I]cAMP or [¹²⁵I]cGMP to their corresponding 5' nucleotides. The antibody will not bind to the catalyzed product; therefore, the decrease in the amount of [¹²⁵I]cAMP or [¹²⁵I]cGMP will be detected.

To screen for inhibitors of phosphodiesterase, a source of phosphodiesterase, [¹²⁵I]cAMP or [¹²⁵I]cGMP, and compounds are added to a FlashPlate well as above. If the compound is found to be an inhibitor of phosphodiesterase, the catalyzed breakdown of cAMP or cGMP will not occur; therefore, a decrease in the amount of [¹²⁵I]cAMP or [¹²⁵I]cGMP will not be detected.

Since this assay is homogeneous (i.e., does not require separation of bound from unbound [¹²⁵I]cAMP or [¹²⁵I]cGMP), the capability to process a large number of samples, as would be necessary in high throughput screening of drug candidates, is now possible.

Methods, Results, and Discussion

Materials

cAMP FlashPlate (96-well) and Assay Buffer (NEN Catalog#SMP001)
[¹²⁵I]cAMP (NEN, Cat. #NEX130)
cGMP FlashPlate (96 well) and Assay Buffer (NEN, Cat. #SMP002)
[¹²⁵I]cGMP (NEN, Cat. #NEX131)
Basic FlashPlate HTS, 384-well (NEN, Cat. #SMP400)
Phosphodiesterase I (Sigma, Cat. #P6903)

Experimental Elements

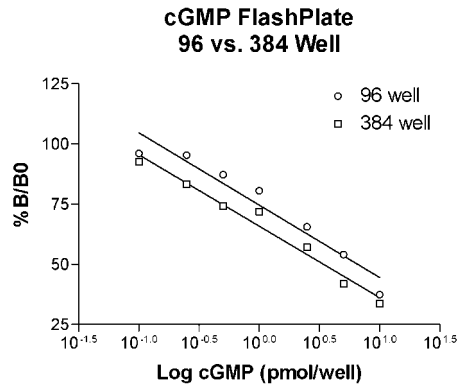
Convert the 96-well assay to a 384-well assay platform
Determine the optimal amount of [¹²⁵I]cAMP or [¹²⁵I]cGMP
Titrate phosphodiesterase to determine sensitivity
Enzyme Termination
DMSO effects
Counting Time

1

96-well to 384-well Conversion

In order to develop a reproducible and dependable assay, studies were done to determine optimal assay conditions to convert from a 96-well to a 384-well format. Most of the studies described here were done using the [¹²⁵I]cGMP assay.

The anti-cGMP antibody is used at a 1:650 titer in the 96-well format and 0.2 ml is added/well. A titration of this antibody was done and the volume/well was decreased to 0.05 ml/well.

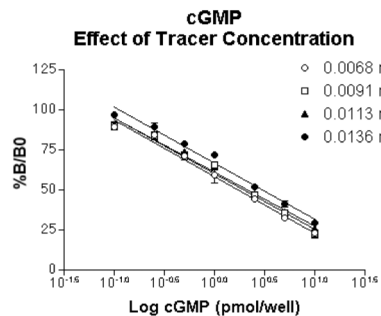
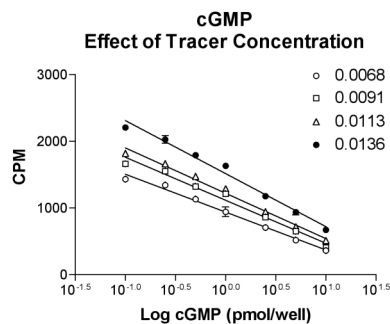


It appears that the amount of antibody needed to get similar results in the 384-well format is 1/4 as compared to the 96-well format. The titer remained the same, but the volume/well decreased 4X.

2

Optimal [¹²⁵I]cGMP Concentration

A study was done to determine the optimal [¹²⁵I]cGMP concentration. Several concentrations of [¹²⁵I]cGMP were added to FlashPlate wells coated with anti-cGMP antibody and allowed to bind for 18 hours at room temperature.



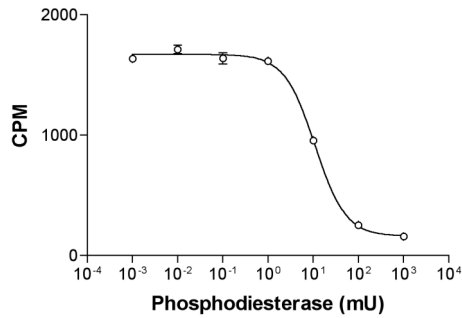
It appears that the tracer concentration used in the original assay may be increased two-fold to obtain approximately 25% more counts/well if desired. When the data is normalized, the curves are approximately the same regardless of tracer concentration. This would be true for both 96-well and 384-well format.

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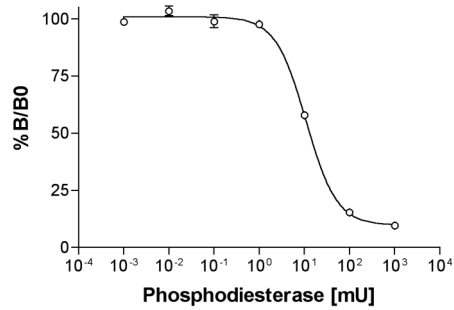
Phosphodiesterase Titration

Phosphodiesterase was titrated to determine the range of the assay.

Phosphodiesterase Assay using $[^{125}\text{I}]\text{cGMP}$ as Substrate



Phosphodiesterase Assay using $[^{125}\text{I}]\text{cGMP}$ as Substrate



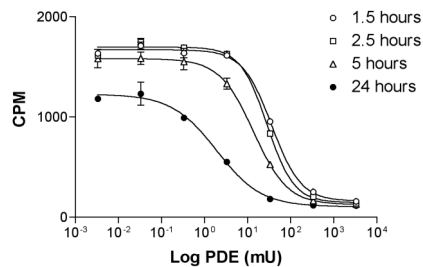
The phosphodiesterase activity that is used is capable of degrading the cyclic nucleotides almost completely.

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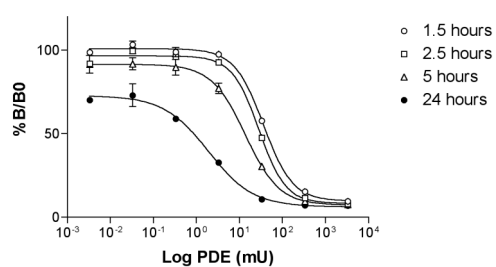
Enzyme Termination

To control for consistency due to possible incubation time differences from plate to plate, a study was done to determine the effect of 1 mM EDTA on stopping phosphodiesterase activity.

Phosphodiesterase Effect of Incubation Time without EDTA Addition

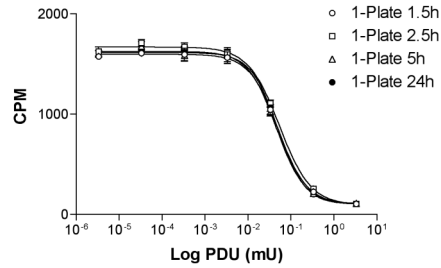


Phosphodiesterase Effect of Incubation Time without EDTA Addition

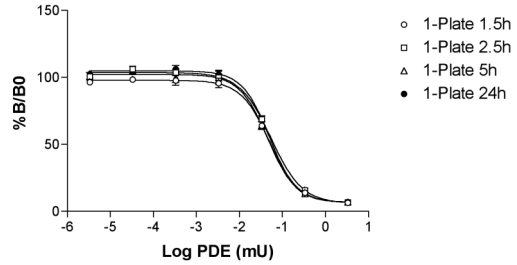


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**Phosphodiesterase
Effect of Incubation Time
with EDTA Addition Post 1 Hour
Reaction Time**



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Effect of Incubation Time
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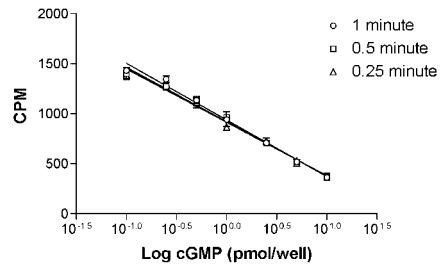
As can be seen in the above graphs, without the addition of EDTA, there are differences between incubation times. When stopping the assay after a 1 hour incubation, results are almost identical.

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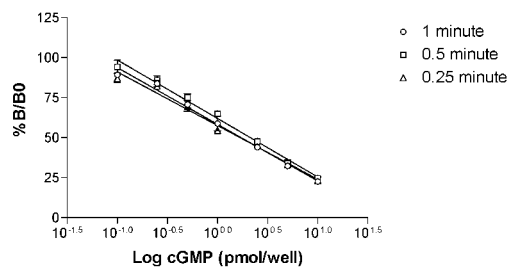
Counting Time

A study was done to determine if the counting time can be reduced from 1 minute/well.

**Phosphodiesterase Assay
Effect of Counting Time**



**Phosphodiesterase Assay
Effect of Counting Time**

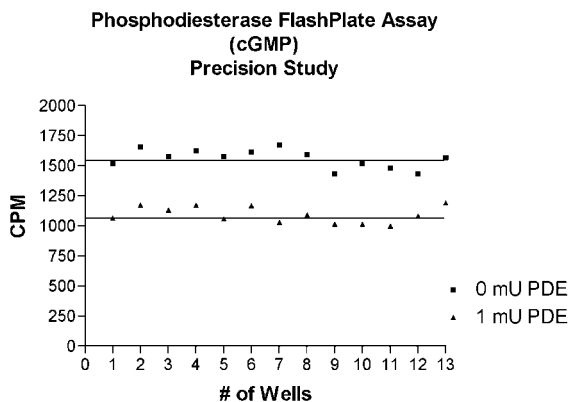


Decreasing the counting time to 0.5 or 0.25 minute does not significantly affect performance.

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Precision

A study was done to determine precision of the assay. Thirteen wells were assayed with 0 and 1 mU phosphodiesterase (PDE)/well.

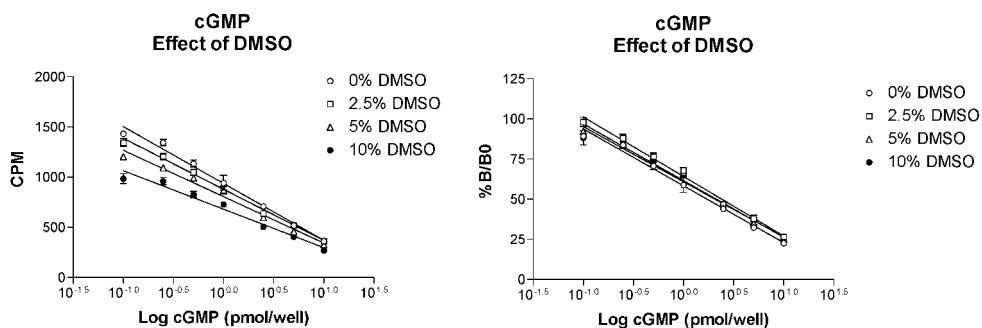


The C.V. obtained for the 0 and 1 mU PDE levels was 5.0% and 6.3%, respectively.

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Effect of DMSO

A study was done to determine the tolerance to DMSO.

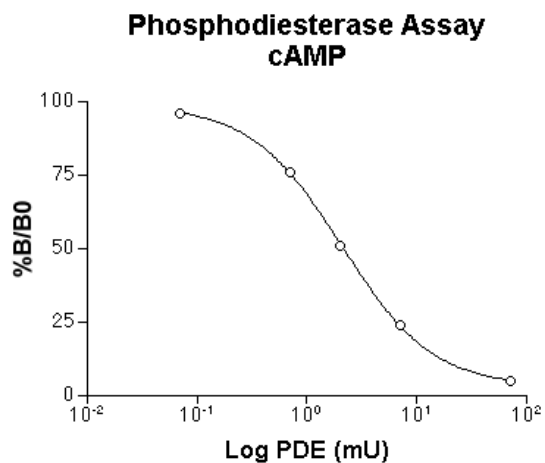


DMSO at concentrations up to 10% v/v does not appear to affect performance of the assay.

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cAMP Phosphodiesterase Assay

A titration of phosphodiesterase was performed using [¹²⁵I]cAMP as the substrate.



Whether using cAMP or cGMP, results are similar for Phosphodiesterase I.

Conclusion

Using FlashPlate technology, homogeneous assays can be performed to measure phosphodiesterase activity on both cAMP and cGMP. This technology eliminates the need for separation, enables kinetic studies, and provides the capability to process a large number of samples in a HTS setting with excellent precision.

References

1. Etrade, M., Grondin, P., Cluzel, J., Bonhomme, B., Doly, M., (1998) *Eur J Pharmacol* 352:2-3, 157-163
2. Kincaid, R., Manganiello, V., (1988) *Methods in Enzymology* 159, 457-459
3. Weishaar, R.E., Cain, M.H., Bristol J.A. (1985) *J Med Chem* 28:5, 537-545

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