

Phospholipid FlashPlate[®] PLUS – A Novel Platform To Measure Enzyme Activity On Lipid-Associated Substrates Without Extraction

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Introduction

Many intracellular and intercellular processes are membrane-mediated. A wide variety of research has been done on the reconstitution of biological membranes as a method to study these processes. A variety of artificial membrane systems and various other methods have been used for studying functional molecules that are incorporated in or bound to biological membranes.

Using the FlashPlate technology – white, opaque, 96- and 384-well scintillant coated microplates – a new method has been developed which enables the study of these functional molecules in an easier, more convenient platform. The Phospholipid FlashPlate uses a novel technology (patent pending) which enables the attachment of lipid substrates to the FlashPlate surface, providing a biologically natural environment for presentation of the substrate. This provides a platform which is more suitable for high throughput screening by eliminating the need for time-consuming extraction steps and provides more reproducible and dependable results.

Introduction (continued)

The substrate of interest is incorporated by hydrophobic interactions into a hydrophobic layer, such as an artificial lipid layer, on a FlashPlate surface. If the substrate is an amphipathic molecule labeled on the hydrophilic fragment, when hydrolyzed by enzyme activity, the labeled hydrophilic fragment will migrate into the aqueous phase, thereby no longer being associated with the FlashPlate surface. Since the scintillant is not activated by radioactive decay when the label is not in close proximity of the surface, the radioactive product can no longer be detected and the counts decrease during the reaction. This platform may also be used for unlabeled substrates when they become radioactively labeled via an enzymatic reaction (lipid kinase assays). In this case, the surface-associated radioactivity increases during the reaction.

This method of performing lipid-associated enzyme assays eliminates the need for extraction steps that can be laborious and time consuming, and can cause environmental and health risks. The capability to process a large number of samples, necessary in high throughput screening of drug candidates, is now possible.

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Methods, Results, and Discussion

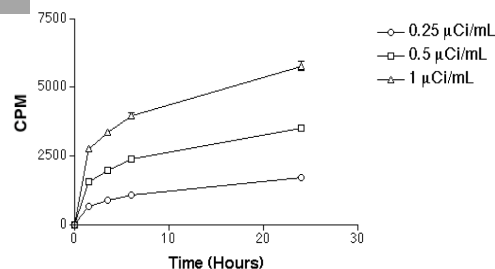
Sphingomyelinase Assay Utilizing [³H]Sphingomyelin as Substrate

In order to develop a reproducible and dependable assay, studies were done to determine the optimal coating and hydrolyzing conditions of the substrate.

Studies were done to determine the optimal “coating” concentration and time for incorporating [³H]sphingomyelin (PerkinElmer Life Sciences) onto the Phospholipid FlashPlate. Concentrations of 0.25, 0.5 and 1 μCi/ml in a Tris-based buffer were added at 0.2 ml volume/well at room temperature and the FlashPlates were read on a Packard TopCount® at intervals.

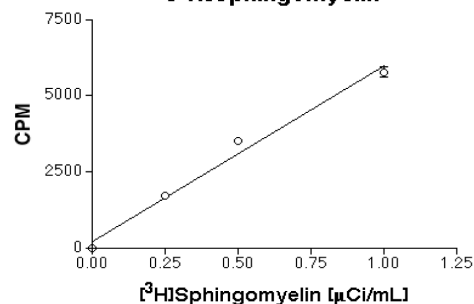
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Phospholipid FlashPlate Effect of Coating Concentration and Coating Time of [³H]Sphingomyelin



The maximum counts/well were obtained after overnight incubation. The counts/well obtained increase linearly with increased concentration of [³H]sphingomyelin up to 1 μCi/ml. This shows the consistency and the enormous capacity that the Phospholipid FlashPlate has for substrate coating.

Phospholipid FlashPlate Effect of Coating Concentration of [³H]Sphingomyelin

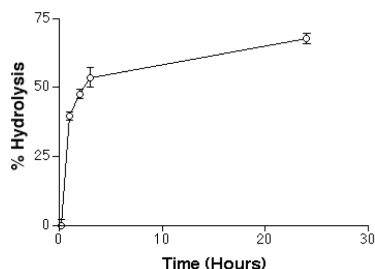


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Next, a study was done to determine the amount of time needed to hydrolyze the maximum amount of substrate (0.2 μ Ci/well). Adding 0.2 units/ml of sphingomyelinase

(Sigma #S7651) diluted in PBS containing 1 mg/ml CaCl_2 and 1 mg/ml MgCl_2 (BioWhittaker), the Phospholipid FlashPlate was incubated at room temperature and read at several time points over a period of 24 hours.

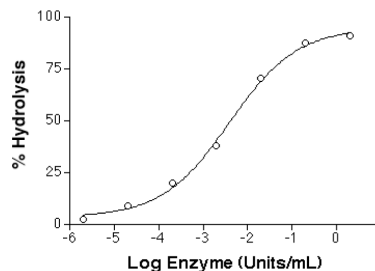
Phospholipid FlashPlate Sphingomyelinase Assay



Most of the hydrolysis of the substrate occurred in the first 3 hours of incubation.

A titration of the enzyme was then done to determine the maximum possible hydrolysis of the substrate over a 24 hour period.

Phospholipid FlashPlate Sphingomyelinase Assay

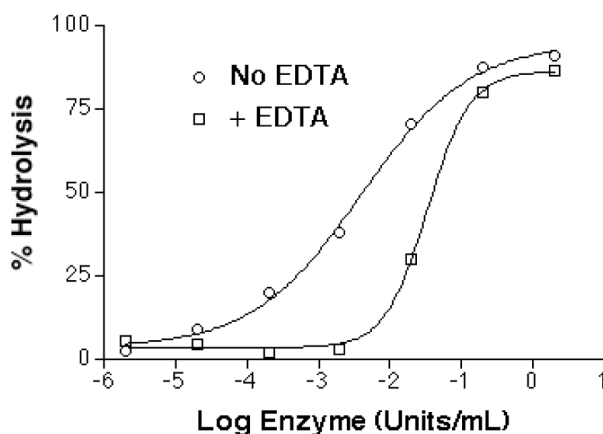


When 2 units/ml of sphingomyelinase were added to the FlashPlate well coated with [^3H]sphingomyelin, approximately 100% of the substrate was hydrolyzed. This shows that the substrate was presented to the enzyme in a way that complete hydrolysis would be accomplished.

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Since sphingomyelinase is magnesium dependent, inhibition by EDTA (5 mM), a magnesium chelator, was also investigated.

Phospholipid FlashPlate Sphingomyelinase Assay



After adding EDTA to the buffer, the EC_{50} changed about 10-fold, from 0.0039 to 0.0336 units/ml. This shows that with the decreased concentration of magnesium, the enzyme is not hydrolyzing the substrate as efficiently.

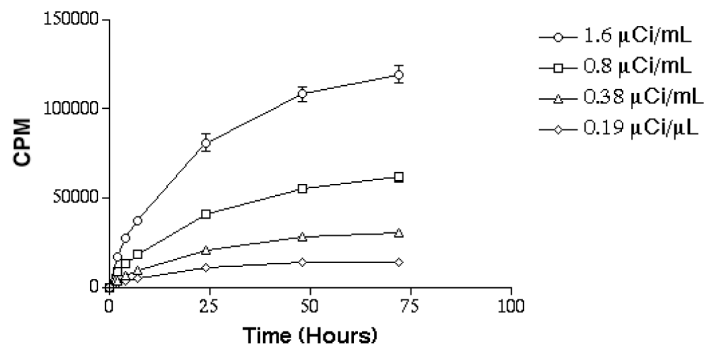
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Phospholipase C Assay Utilizing [³H]PIP₂ as Substrate

In order to validate this platform, another substrate/enzyme system was evaluated. Studies were done to determine optimal conditions for coating and hydrolyzing [³H]PIP₂.

Studies were done to determine the optimum conditions for “coating” [³H]PIP₂ (NEN custom preparation) onto the Phospholipid FlashPlate. Concentrations of 1.6, 0.8, 0.38, and 0.19 μCi/ml in a Tris-based buffer were added at 0.2 ml volume/well and incubated overnight at room temperature, and the FlashPlates were read on a Packard TopCount at intervals. Results are shown below.

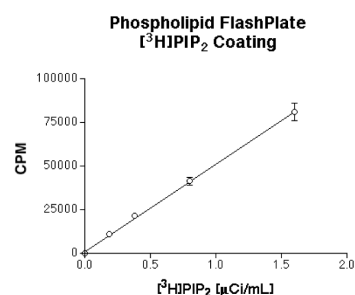
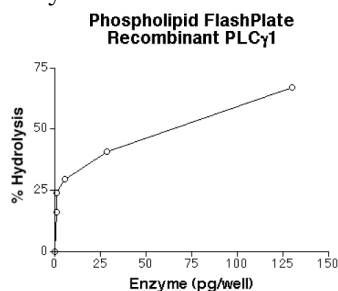
**Phospholipid FlashPlate
[³H]PIP₂ Coating**



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As can be seen from the graphs, substrate incorporation increases with time up to 72 hours. The cpm/well increases linearly with increased concentration of [³H]PIP₂ up to 1.6 μCi/ml. As demonstrated with the [³H]Sphingomyelin, that data shows the consistency and the enormous capacity that the Phospholipid FlashPlate has for substrate coating.

Varying amounts of enzyme were added which were then all allowed to hydrolyze substrate for 24 hours.

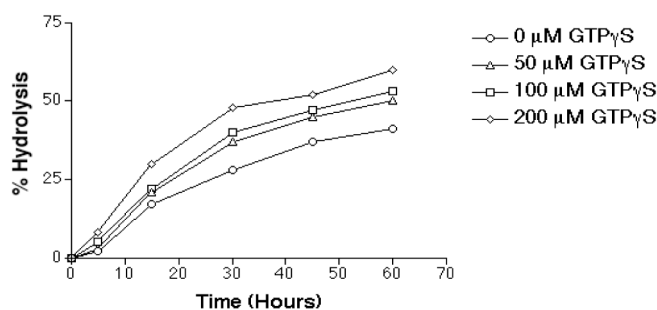


The amount of hydrolysis of the substrate increases in 24 hours with the amount of enzyme added up to 125 pg/well.

Stimulation of phospholipase C in HL60 cytosol by GTPγS was investigated. This was done to determine if the enzyme can be activated and measured using this technology. Varying concentrations of GTPγS (ABS, Inc.) were added to the well in the presence of HL60 and the enzyme activity was measured over time.

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Phospholipid FlashPlate GTP γ S Stimulation of HL60 Cytosol



GTP γ S increased the amount of hydrolysis of the substrate by HL60 cytosol. This shows that this platform may be used to measure enzyme activation through GTP γ S stimulation.

An important feature needed for high throughput screening is the reproducibility of results. A study was done to determine the intra- and inter-plate precision of both substrate coating and the substrate hydrolysis precision. Ninety-six (96) wells/plate were tested to determine the intra-plate and inter-plate %CV for the substrate coating and twelve (12) wells/plate were tested for the corresponding determinations for the substrate hydrolysis. The counts obtained for each well within each plate were used to determine intra-plate precision and the % binding of [3 H]PIP $_2$ was used to determine the inter-plate precision.

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Phospholipid FlashPlate Lot #	Intra-plate % CV	% Binding
1	5.8	20.3
2	4.5	19.8
3	3.9	20.3
4	5.4	21.1
5	3.4	19.5
6	3.3	18.9
7	3.3	19.5
8	3.5	18.8
9	3.8	18.6
10	3.6	17.7
1	3.9	20.6
2	3.5	20.7
3	4.8	20.7
4	3.8	20.9
5	3.7	19.8
1	3.6	18.9
2	3.8	18.1
3	3.9	18.1
4	3.6	18.8
5	3.0	19.3
6	5.4	17.5
7	5.9	18.2
8	5.4	17.1
9	4.8	16.8
10	5.7	17.5
11	5.7	18.7
12	4.9	16.8
13	5.8	17.1
14	3.8	16.8
15	5.0	17.0
16	3.0	15.8
17	3.2	14.7
18	2.8	16.1
19	2.9	15.7
Mean (N = 34)	4.2	18.4
SD		1.7
Inter-plate % CV		9.2

Phospholipid FlashPlate Lot#	Intra-plate % CV	% Hydrolysis
1	8.4	80.9
2	4.2	81.3
3	4.4	77.5
4	7.2	75.3
5	6.1	79.5
1	6.2	82.3
2	7.0	81.8
3	6.4	81.9
4	5.8	82.4
5	6.1	82.0
1	11.3	80.6
2	11.2	79.7
3	8.8	82.7
4	5.9	81.2
5	11.1	81.2
6	5.9	81.0
7	11.2	87.0
8	8.6	83.4
9	8.7	84.3
10	7.6	82.3
11	9.1	85.0
Mean (N = 21)	7.7	81.6
SD		2.5
Inter-plate % CV		3.1

As shown above, precision characteristics of the Phospholipid FlashPlate substrate coating and substrate hydrolysis are easily less than 10% CV overall, very suitable for high throughput screening. In order to verify that the release of surface-bound counts was in fact due to hydrolysis of the [3 H]PIP $_2$, individual wells were aspirated and extracted with two volumes of chloroform-methanol under acidic conditions (CHCl $_3$:MeOH:HCl, 2:1:0.01). The relative distribution of radioactivity in the organic and aqueous phase represents labeled PIP $_2$ and IP $_3$, respectively.

Enzyme Source	%[³ H]IP ₃ in Aqueous Phase	%[³ H]PIP ₂ in Organic Phase
HL60 cytosol (n=10)	89 +/- 7%	11 +/- 1%
Recombinant PLC (n=3)	97 +/- 0.5%	3 +/- 0.3%

As shown, release of radioactivity from the Phospholipid FlashPlate was due predominantly to the hydrolysis of the bound [³H]PIP₂, with both crude PLC preparations from HL60 cytosolic extracts and recombinant PLC (a generous gift of Drs. Smita Ghosh (Brandeis University), John Lowenstein (Brandeis University) and Sue Goo Rhee (NIH)). Control wells showed little or no release of radioactivity (data not shown). Under both conditions, the radioactivity was distributed mostly in the aqueous phase (~90% and above) indicative of the cleaved product, [³H]IP₂. This result shows that labeled IP₃ was being specifically released from the Phospholipid FlashPlate, and that surface-bound labeled PIP was in fact a viable substrate for both crude enzymatic PLC preparations of HL60 cytosolic extracts, as well as the purified recombinant PLC.

Conclusion

This novel Phospholipid FlashPlate platform enables the attachment of lipid enzyme substrates that may be used in signal decreasing (hydrolysis of surface-bound substrate) assays or signal increasing assays (addition of label, such as with lipid kinase, to surface-bound substrate) by a patent pending process.

The data presented demonstrates the use of the Phospholipid FlashPlate to immobilize lipid substrates to format assays which are suitable for HTS. This technology eliminates the need for extraction and other labor intensive steps, enables kinetic studies, and provides the capability to process a large number of samples in an HTS setting with excellent specificity and precision.

There is a patent pending on the Phospholipid FlashPlate PLUS. FlashPlate is a registered trademark of Packard Instrument Company licensed exclusively to PerkinElmer Life Sciences, Inc. The FlashPlate product is protected under US Patent 5,496,502 and foreign equivalents, to all of which PerkinElmer Life Sciences, Inc. holds an exclusive, worldwide license. TopCount is a registered trademark of Packard Instrument Company.



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