

Demonstration of Assay Miniaturization Using Phospholipid FlashPlate[®]

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Abstract

FlashPlate® is a white, opaque, 96- or 384-well scintillant coated microplate. The Phospholipid FlashPlate uses a novel technology (US Patent 5,972,595) which enables the attachment of lipid substrates to the FlashPlate surface through a hydrophobic interaction. This allows for the quantitative and qualitative determination of the activity of various enzymes that act on membrane-bound substrates, without the need for organic extraction. The Phospholipid FlashPlate platform is more suitable for high throughput screening (HTS) by eliminating the need for time-consuming extraction steps, separation and washing.

Assay miniaturization is essential for meeting the cost and throughput goals of screening laboratories. The demands are generally met by performing assays which offer higher density, lower volume formats. 384-well microtiter plates are a convenient platform to increase efficiency and decrease cost of the assays.

This application demonstrates the successful miniaturization of the Phospholipid FlashPlate using different enzyme targets.

Introduction

FlashPlate methods are an ideal detection format for HTS of phospholipids because they are amenable to homogeneous, miniaturized formats. This report focuses on using 384-well format to improve HTS assays of phosphoinositide-specific phospholipase C (PLC) and sphingomyelinase (SMase).

Previously, we have demonstrated the usefulness of FlashPlate technique for 96-well enzyme assays⁽¹⁾. Now we are expanding the 96-well assay format to 384-well format that allows sensitive and accurate measurement of two target enzymes, PLC and SMase. PLCs are a group of enzymes involved in the signal transduction pathway of many plasma membrane receptor mediated events⁽²⁾. These enzymes are specific hydrolases for the phosphoinositides, and hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP₂) to two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. SMase is an enzyme that is involved in apoptosis and the signal transduction of many cytokines and stress-related factors⁽³⁾. SMase hydrolyzes sphingomyelin (SM) to ceramide and phosphorylcholine.

When [³H]PIP₂ and [³H]SM immobilized on the surface of the FlashPlate platform are hydrolyzed by the PLC and SMase, respectively, the light generated by the close proximity of the scintillant can be measured by a Microplate Scintillation Counter. The validation of these solid phase substrate assays is demonstrated by comparing enzyme kinetics.

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Experimental Procedures

Materials

- 96-well Phospholipid FlashPlate (NEN, Cat. # SMP108)
- 384-well Phospholipid FlashPlate (NEN, custom order)
- Substrate Coating buffer (NEN, Cat. # SMP900)
- [³H]PIP₂ (20 Ci/mmol, NEN, custom order)
- [³H]Sphingomyelin (83.5 Ci/mmol, NEN, Cat. # NET1134)
- Purified recombinant phospholipase C was a gift of Drs. Smita Ghosh and John Lowenstein of Brandeis University.
- Sphingomyelinase from *Bacillus cereus* (Sigma Chemicals, Cat. # S7651).

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Methods

PLC Assay

- Immobilization of PIP₂ on FlashPlate: PIP₂ was diluted in substrate coating buffer and 50 µl pipetted into each well of Phospholipid FlashPlate. The plate was incubated overnight at room temperature. After the incubation step, wells were aspirated prior to use in the PLC assay.
- PLC Assay: The assay solution contained 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 0.1 mM CaCl₂, 0.1 mM EGTA, 0.2 mM spermine, 0.02% deoxycholate (DOC), 0.75-6.0 µg/ml PLC, final volume 50 µl/well. Plates were covered and the reaction mixture was incubated at room temperature for 1-24 hours. The bound radioactivity was determined by counting the FlashPlate.

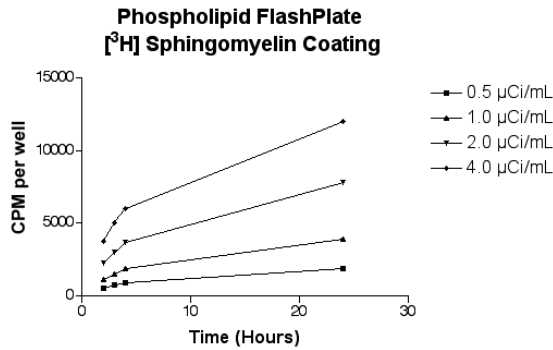
Sphingomyelinase Assay

- Immobilization of sphingomyelin on FlashPlate: [³H]SM was diluted in substrate coating buffer and 50 µl pipetted into each well of Phospholipid FlashPlate. The plate was incubated and aspirated as described above.
- Sphingomyelinase Assay: The assay solution contained 67 mM PBS (pH 7.4), 1 mM CaCl₂, 5 mM MgCl₂, 0.2-1.6 units/ml SMase, final volume 50 µl/well. Plates were incubated at room temperature for 1-24 hours. The bound radioactivity was determined by counting the FlashPlate.

3

Sphingomyelinase Assay Utilizing $[^3\text{H}]$ Sphingomyelin as Substrate

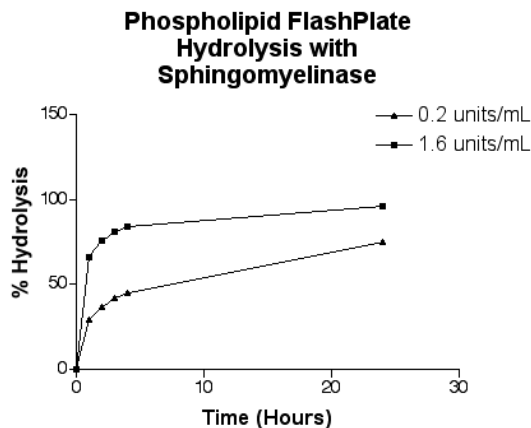
In order to develop a reproducible and dependable assay, studies were done to determine the optimal coating and hydrolyzing conditions of the substrate. Studies were done to determine the optimal "coating" concentration and time for incorporating $[^3\text{H}]$ sphingomyelin onto Phospholipid FlashPlates. Concentrations of 0.5, 1.0, 2.0 and 4.0 $\mu\text{Ci}/\text{ml}$ in a Tris-based buffer were added at 0.05 ml/well at room temperature and the FlashPlates were read on a Packard TopCount[®]NXT[™] HTS at 1-24 hours.



The maximum counts/well were obtained after overnight incubation. The counts/well obtained increase proportionally with increased time. This shows the consistency and the high capacity (0.32 pmol SM bound/well) that the Phospholipid FlashPlate has for substrate coating.

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Next, a study was done to determine the amount of time needed to hydrolyze the maximum amount of substrate (0.025-0.2 μCi coated/well). After adding 0.2-1.6 unit/ml of sphingomyelinase diluted in PBS containing 0.2 mg/ml CaCl_2 and 1 mg/ml MgCl_2 , the Phospholipid FlashPlate was incubated at room temperature and read at several timepoints over a period of 24 hours.

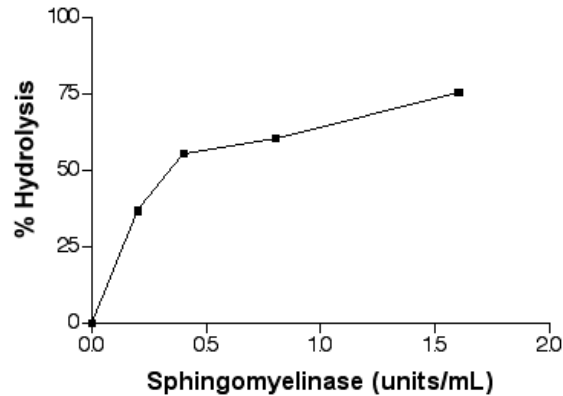


Most of the hydrolysis (45-84%) of the substrate occurred in the first 4 hours of incubation at these enzyme concentrations. When 1.6 units/ml of sphingomyelinase was added to FlashPlate wells coated with 4 $\mu\text{Ci}/\text{ml}$ $[^3\text{H}]$ sphingomyelin, approximately 96% of the substrate was hydrolyzed between 4 to 24 hours. This shows that the substrate was presented to the enzyme in a way that nearly complete hydrolysis would be accomplished.

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A titration of the enzyme was performed to determine the maximum possible hydrolysis of the substrate over a two-hour period.

Phospholipid FlashPlate Sphingomyelinase Assay

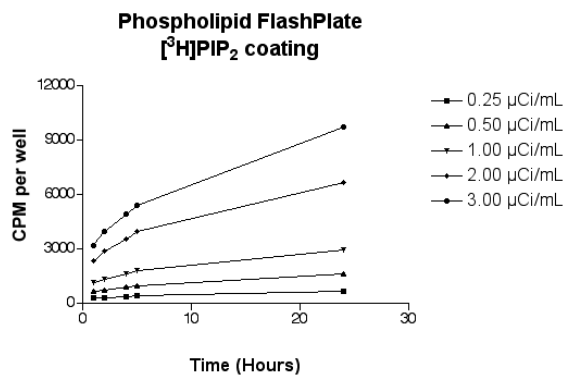


Hydrolysis of wells coated with 4 $\mu\text{Ci/ml}$ [^3H]sphingomyelin increased with increasing concentrations of sphingomyelinase. Greater than 50% hydrolysis of substrate was achieved with enzyme concentrations ranging from 0.4-1.6 units/ml.

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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 onto the Phospholipid FlashPlate. Concentrations of 0.25, 0.5, 1.0, 2.0 and 3.0 $\mu\text{Ci/ml}$ in a Tris-based buffer were added at 0.05 ml/well and incubated 1-24 hours at room temperature, and the FlashPlates were read on a TopCount at 0.5-24 hours. Results are shown below.

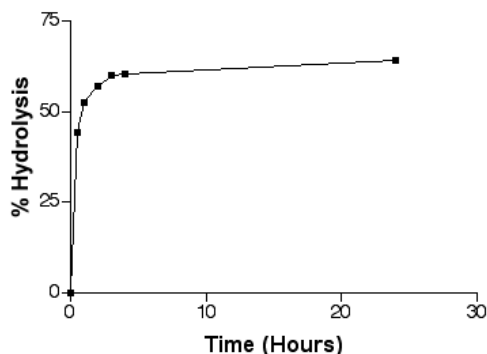


Substrate incorporation increases with time up to 24 hours. The cpm/well increases proportionally with increased concentration of [^3H]PIP₂ up to 3.0 $\mu\text{Ci/ml}$. As was shown with the [^3H]sphingomyelin, the Phospholipid FlashPlate has the consistency and the high capacity (1.35 pmol PIP₂ bound/well) for substrate coating.

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Varying amounts of enzyme (37.5-300 pg/well) were added and then all were allowed to hydrolyze substrate for 1-24 hours.

**Phospholipid FlashPlate
Hydrolysis of [³H]PIP₂ with PLC**



The amount of hydrolysis of the substrate did not vary in 24 hours with the amount of enzyme (150 pg/well) added due to the high enzymatic activity of recombinant PLC. The hydrolysis with 150 pg/well was selected as a representative sample for illustrating enzyme kinetics in the above graph. Similar to results obtained with SMase, most of the hydrolysis (59%) of the substrate occurred in the first 4 hours of incubation with the PLC enzyme.

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In order to evaluate the effect of detergent concentration on PLC activity, two different concentrations of deoxycholate (DOC) were used in PLC assays.

	96-Well Plate		384-Well Plate	
	Mean (n=8)	CV(%)	Mean (n=16)	CV(%)
Bound Counts Buffer Only (CPM)				
0.005% DOC Wells	10258	6.1	9548	8.6
0.02% DOC Wells	9696	7.3	9107	9.6
Counts After Enzyme Hydrolysis (CPM)				
Using 0.005% DOC	4663	5.5	2764	9.6
Using 0.02% DOC	3125	5.2	2100	5.9
% Hydrolysis				
Using 0.005% DOC	55.0	5.5	71.0	9.6
Using 0.02% DOC	68.0	5.2	77.0	5.9

As shown above, purified recombinant PLC demonstrated enhanced hydrolytic activity on addition of the higher concentration of DOC (0.02%) when the 96-well Phospholipid FlashPlate was used. However, the enhancing effect of DOC observed with the 96-well plate (approximately 24%) was less pronounced when the 384-well FlashPlates were used (approximately 8%).

Conclusion

This novel Phospholipid FlashPlate platform enables the attachment of lipid substrates that may be used in signal decreasing assays (hydrolysis of surface-bound substrate).

This application demonstrates the successful miniaturization of the Phospholipid FlashPlate using two enzymes, PLC and SMase.

The adaptation of 96-well to 384-well Phospholipid FlashPlates for the homogeneous phospholipase C and sphingomyelinase assays has been demonstrated. The experimental results indicate that 384-well assay gave a comparable rate of [³H]PIP₂ hydrolysis of 71-77% to 96-well assay with coefficient of variation values of less than 10%.

The data presented demonstrates the use of the Phospholipid FlashPlate to immobilize lipid substrates to format assays that 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 a HTS setting with excellent specificity and precision.

References

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2. Rhee, S. G., Choi, K.D. (1992) *J. Biol. Chem.* 267, 12393-12396.
3. Hannun, Y. J. (1994) *J. Biol. Chem.* 269, 3125-3128.

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