PROFLUOR™ PKA ASSAY: EXCELLENT Z´-FACTOR VALUES MEAN RELIABLE RESULTS

Promega introduces the ProFluor™ PKA Assay for measuring protein kinase A activity using purified kinase in a multiwell plate format. The simple protocol and the stability of the PKA R110 Substrate signal provides an assay ideally suited for high-throughput screening applications.

Introduction

Protein kinases and their signaling cascades regulate essentially every cellular activity—from proliferation to migration to metabolism and death. In addition, kinases are implicated in a variety of diseases. Many viral oncogenes, such as v-*src* and v-*abl*, are protein kinases, suggesting a role in cancers. Kinases also play roles in the inflammatory response and are implicated in other diseases such as Alzheimer's disease. Because of their fundamental role in cellular activity, kinases are promising targets for drug therapies. Additionally they are important targets when assessing cytotoxicity of therapeutic agents. The ProFluor™ PKA Assay[a,b] is a robust protein kinase A (PKA) assay designed for use in a multiwell format that is easily amenable to high-throughput screening.

Assay Principle

The ProFluor™ PKA Assay measures PKA activity using purified kinase in a multiwell plate format and involves "add, mix and read" steps only (Table 1). The user performs a standard kinase reaction with the provided bisamide rhodamine 110 peptide substrate (PKA R110 Substrate). The provided substrate is nonfluorescent (Figure 1; reference 1). After the kinase reaction is complete, the user adds a Termination Buffer containing a Protease Reagent. This simultaneously stops the reaction and removes amino acids specifically from the nonphosphorylated PKA R110 Substrate, producing highly fluorescent Rhodamine 110. Phosphorylated substrate is resistant to protease digestion and remains non-fluorescent (Figure 1). Thus, fluorescence is inversely correlated with kinase activity (Figure 2).

The ProFluor™ Assay produces Z´-factor values greater than 0.8 in either 96- or 384-well plate formats.

Table 1. General Assay Format for 96-Well Plates and 384-Well Plates.

Step	Description	96-Well	384-Well
1.	Add diluted kinase and PKA R110 Substrate in 1X Reaction Buffer.	25µl	5µl
2.	Add diluted ATP in 1X Reaction Buffer.	25µl	5µl
3.	Mix plate 15 seconds and incubate 20 minutes at room temperature.		
4.	Add diluted Protease Reagent in 1X Termination Buffer.	25µl	5µl
5.	Mix plate and incubate for 30 minutes at room temperature.		
6.	Add diluted Stabilizer Reagent in 1X Termination Buffer.	25µl	5µl
7.	Mix plate and read at 485/530nm.		

Robust, User-Friendly Assay

We used the statistical calculation for Z´-factor to assess assay reliability. Z´-factor, the "screening window coefficient," compares the assay dynamic range to data variation (2). Generally, assays that have Z´-factor values greater than 0.5 are considered good assays; a Z´-factor of 1.0 would indicate a perfect assay. The ProFluor™ Assay produces Z´-factor values greater than 0.8 in either 96- or 384-well plate formats (Figure 3).

A good kinase assay should also provide IC_{50} values comparable with previously published data. In inhibitor titrations for PKA Peptide Inhibitor (PKI; Cat.# V5681) and staurosporine, the assay produced IC_{50} values of 4.2nM and 25.7nM, respectively, comparable to the published values of 3nm for PKI (3) and 18nM for staurosporine (4).

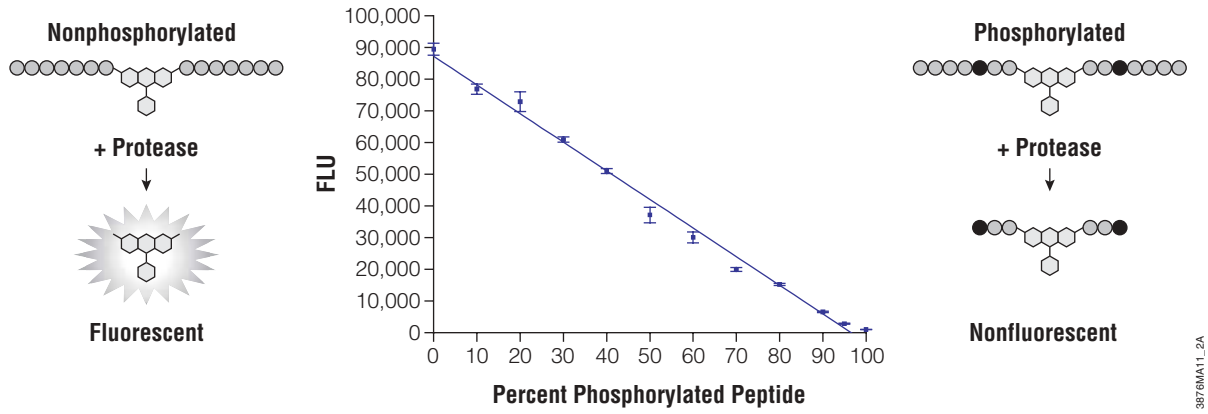

Figure 1. Schematic and graph demonstrating that the presence of a phosphorylated amino acid (black circles) blocks the removal of amino acids by the protease. The graph shows the average FLU (n = 6) obtained after a 30-minute Protease Reagent digestion using mixtures of nonphosphorylated PKA R110 Substrate and phosphorylated PKA R110 Substrate. The total peptide concentration was 5µM in 50µl of Reaction Buffer A to which 25µl of Protease Reagent diluted in Termination Buffer A was added. (FLU = Fluorescence Light Unit, excitation wavelength 485nm, emission wavelength 530nm; $r^2 = 0.992$) As the concentration of the phosphopeptide increases in the reaction, FLU decrease.

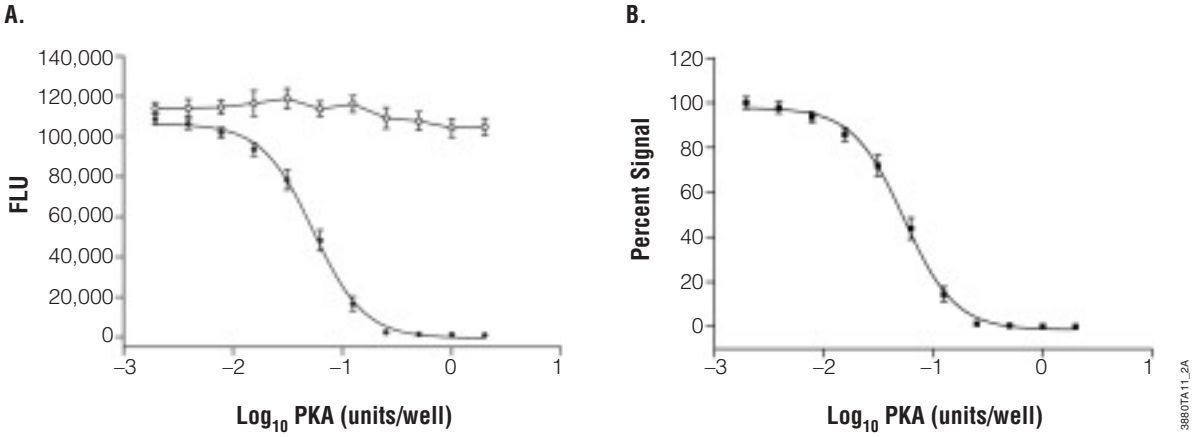

Figure 2. Amount of kinase activity is inversely correlated with fluorescent output. Results of an assay that was performed according to the kinase titration protocol described in Technical Bulletin #TB315. The assay was performed in a solid black, flat-bottom, 96-well plate with ATP (solid circles) and without ATP (open circles) **(Panel A)**. Data points are the average of 4 determinations, and error bars are ± S.D. Curve fitting was performed using GraphPad Prism® 3.0 sigmoidal dose-response (variable slope) software. The R^2 value is 0.99; EC_{50} is 0.05 units/well PKA (Cat.# V5161). Normalizing the data allows for a quick determination of the amount of PKA required to provide the percent conversion desired **(Panel B)**.

High-throughput screening applications often require the ability to process plates in large batches. The stable fluorescent signal of the PKA R110 Substrate in the ProFluor™ Assay allows such batch-mode processing. Figure 4 shows the averages ± S.D. of fluorescence values for an assay performed in a solid black, flat-bottom 96-well plate. The fluorescence measurements were made at time zero (after adding Stabilizer and mixing) and four hours later. The average of five such determinations indicates that the signal showed less than ten percent change in fluorescence intensity over four hours.

Summary

The ProFluor™ PKA Assay is a robust assay suitable for high-throughput screening applications. The assay consistently yields excellent Z´-factor values, produces IC_{50} data comparable to published data, and allows the flexibility of batch-mode processing.

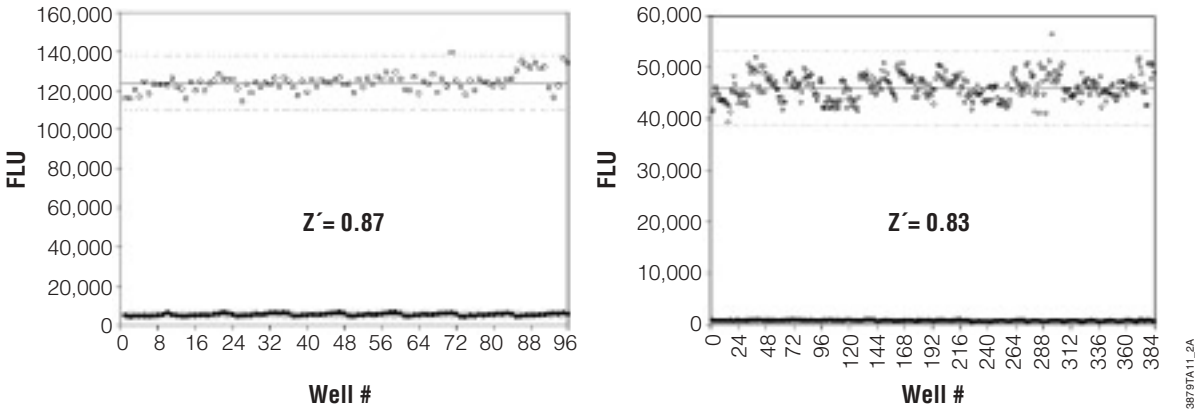

Figure 3. Z´-factor values obtained in both 96- and 384-well plates. Results of Z´-factor analysis are shown for 96-well plates **(Panel A)** and 384-well plates **(Panel B)**. The assay was performed manually according to the protocol described in Technical Bulletin #TB315 in solid black, flat-bottom plates with ATP (solid circles) and without ATP (open circles). Solid lines indicate the mean, and the dotted lines indicate ± 3 S.D. Amount of PKA (Cat.# V5161) used was 0.125 units/well in 96-well plates and 0.05 units/well in 384-well plates.

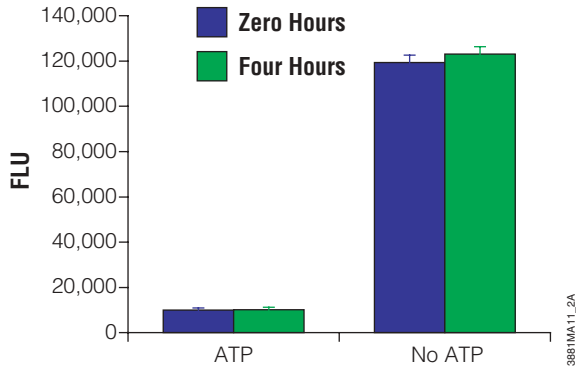

Figure 4. Stable fluorescence signal allows for high-throughput batch processing. The bar chart shows averages ± S.D. of FLU values (n = 48) collected from an assay performed in a solid black, flat-bottom 96-well plate using 0.25 units/well PKA (Cat.# V5161) as described in Technical Bulletin #TB315, with and without ATP, at time 0 and 4 hours later. The average of 5 such determinations on each plate indicated that the signal increased less than 10% in four hours.

References

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2. Zhang, J.H. *et al.* (1999) *J. Biomol. Screening* **4**, 67–73.
3. Walsh, D.A. and Glass, D.B. (1991) *Meth. Enzymol.* **201**, 304–16.
4. Hidaka, H., Watanabe, M. and Kobayashi, K. (1991) *Meth. Enzymol.* **201**, 328–39.

Protocol

ProFluor™ PKA Assay Technical Bulletin #TB315, Promega Corporation

www.promega.com/tbs/tb315/tb315.html

Ordering Information

Product	Size	Cat.#
ProFluor™ PKA Assay[a,b]	4 plate	V1240
	8 plate	V1241
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500u	V5161
cAMP-Dependent Protein Kinase Peptide Inhibitor	1mg	V5681

[a]Patent Pending.
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