MONITOR PURIFIED PHOSPHATASE ACTIVITY WITH A HOMOGENEOUS, NON-RADIOACTIVE HIGH-THROUGHPUT FLUOROGENIC ASSAY

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We have developed a homogeneous, non-radioactive rapid assay system for monitoring the activity of purified serine/threonine protein phosphatases (S/T-PPases), the ProFluor™ Ser/Thr PPase Assay (Cat.# V1260, V1261). The robust assay performance is manifested by its high Z´-factor values and its excellent dynamic range. The protocol can be completed in as little as two hours, requires only two reagent additions, and the signal is stable for hours. This assay is scalable from single tubes to 384-well plates and is adaptable to automated platforms. We have validated this assay with known selective inhibitors of protein phosphatases.

Introduction

The completion of genome sequencing projects for humans and other organisms, the design and generation of millions of compounds by combinatorial chemistry, and the availability of a large number of natural compounds have heightened the demand for high-throughput enzyme assays. Protein kinases and phosphatases are implicated in a wide variety of cellular functions, and thus they are valid targets for drug discovery. To facilitate the drug discovery process, sensitive, homogeneous, fast and cost-effective assays are needed to screen for inhibitors of these enzymes.

Classical protein phosphatase assays rely on quantitating radioactivity liberated from radiolabeled phosphorylated substrates. These radioactive assays pose safety concerns and require expensive waste disposal. They also require frequent and time-consuming substrate labeling.

Alternative non-radioactive methods have been developed that quantitate liberated phosphate through the formation of a colored complex with malachite green and acidified phosphomolybdate (1). However, such assays are not very sensitive, requiring large amounts of enzymes and high peptide concentrations. Additionally, the presence of inorganic phosphates in samples, solutions or glassware significantly increase the background, affecting the sensitivity and accuracy of these assays. Other assays have been developed that rely on hydrolysis of compounds such as *p*-nitrophenyl phosphate, but the use of such substrates may not reflect dephosphorylation of a true phosphopeptide substrate as shown in recent studies (2).

A sensitive fluorogenic substrate, 3,6-diphosphofluorescein, was recently described for protein tyrosine phosphatases (3), and high-throughput screening assays have been developed based on this substrate. However, this substrate can be acted upon by other enzymes in addition to phosphatases, such as esterases, and hydrolases; therefore this fluorogenic substrate cannot serve solely as a substrate for phosphatases. Pure enzyme preparations have to be used.

Other methodologies, such as fluorescence polarization-based assays, rely on the availability of high-affinity, high-specificity antibodies against the fluorescently labeled phosphopeptides

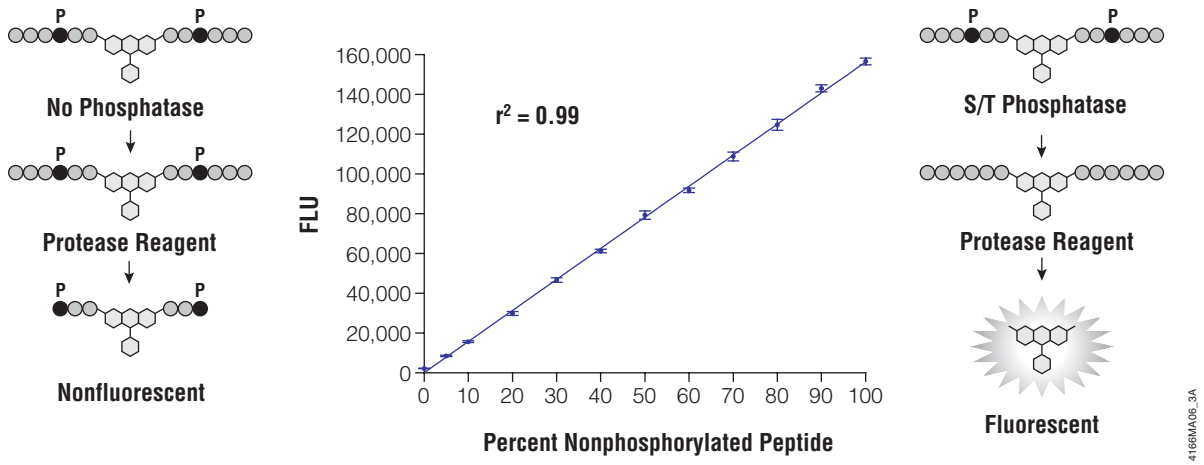

Figure 1. Effect of phosphopeptide content on fluorescence intensity. The graph shows the average FLU (n = 8) obtained after a 90-minute Protease Reagent digestion using mixtures of nonphosphorylated S/T PPase R110 Substrate and phosphorylated S/T PPase R110 Substrate as indicated to mimic a phosphatase titration. The total peptide concentration was 5µM in 50µl of Reaction Buffer B to which 25µl of Protease Reagent diluted in Termination Buffer B was added. (FLU = Fluorescence Light Units, excitation wavelength 485nm, emission wavelength 530nm; $r^2 = 0.999$). Dark circles in the schematics represent phosphorylated amino acids.

(4). These assays require generation of high-affinity antibodies, and they can be affected by interference from fluorescent compounds. Fluorescence resonance energy transfer (FRET)-based assays have limited dynamic range and are affected by interference from other fluorescent compounds as well.

We have developed the ProFluor™ Ser/Thr Phosphatase Assay System[a,b] to overcome the safety issues associated with radioactive assays while maintaining sensitivity and specificity. This rapid, homogeneous assay can be completed in less than 2 hours, has an excellent dynamic range, and is easily adapted to automated platforms for high-throughput applications.

ProFluor™ PPase Substrate Chemistry

Rhodamine 110 (R110) is a highly fluorescent molecule, but when covalently linked via its amino groups in a bisamide form, both its visible absorption and fluorescence are suppressed. Upon enzymatic cleavage of the attached peptides, the nonfluorescent bisamide substrate is converted to the highly fluorescent free R110.

The fluorescence intensity of free R110 is constant from pH 3.0–9.0 (5). Rhodamine 110 exhibits spectral properties similar to fluorescein, with peak excitation and emission wavelengths of 496nm and 520nm, respectively. Free R110 has intense visible absorption (ε496nm ~80,000cm^{-1}M^{-1} in pH 6.0 solution).

ProFluor™ Ser/Thr PPase Assay Principle

We synthesized a bisamide R110-linked phosphopeptide that serves as a substrate for S/T-PPases. Phosphorylation of the peptide renders it resistant to cleavage by the Protease Reagent that is included with the assay system with minimal change in fluorescence. However, when the phosphoryl moiety is removed by a phosphatase, the peptide is cleaved, releasing the highly fluorescent, free R110 molecule (Figure 1). The measured fluorescence correlates with phosphatase activity.

High Sensitivity with the ProFluor™ Ser/Thr PPase Assay

We tested the ability of several phosphatases to dephosphorylate this substrate and used peptidase cleavage and fluorescence output to monitor enzyme activity. The S/T PPase phosphopeptide substrate served as an excellent substrate for all of the enzymes tested within this class (PP1, PP2A, PP2B, and PP2C). As shown in Figure 2, the fluorescence output increased with increasing concentrations of both PP1 and PP2A, and the amounts of enzyme required to dephosphorylate 50% (EC_{50}) of the phosphopeptide by each enzyme was quite low (EC_{50} = 6.98mU for PP1 and 3.28mU for PP2A). As low as 1mU enzyme could be detected using this system (Figure 2).

Excellent Z´-Factor Values and Flexibility

The ProFluor™ Ser/Thr PPase Assay is versatile and is performed at room temperature. The homogeneous format of the assay is easily adapted to single-tube or multiwell formats. The reaction volume required in 384-well plates is one fifth of that required for 96-well plates, offering tremendous savings on the cost of reagent and requiring much less enzyme.

We consistently obtain Z´-factor values greater than 0.8 with PP1 in 384-well plates (Figure 3), indicating a highly predictive, high-quality assay. We obtain similar values with PP2A, PP2B and PP2C. Z´-factor values are statistical indicators of the dynamic range and variability of assay results. Assays that produce Z´-factor values of 0.5 or greater are considered well suited for high-throughput screening (6).

Accurate IC_{50} Values and Stable Signal

We also tested the effect of known selective protein phosphatase inhibitors on their cognate enzymes using the ProFluor™ Ser/Thr PPase Assay. Figure 4 shows that the IC_{50} value (concentration of compound needed to inhibit phosphatase activity by 50%) determined for okadaic acid using PP2A was 1.6nM, while that for PP1 Inhibitor-2 did not inhibit PP2A (not shown). However, PP1 Inhibitor-2 potently inhibited PP1 (IC_{50} = 6.4nM), while okadaic acid was considerably less

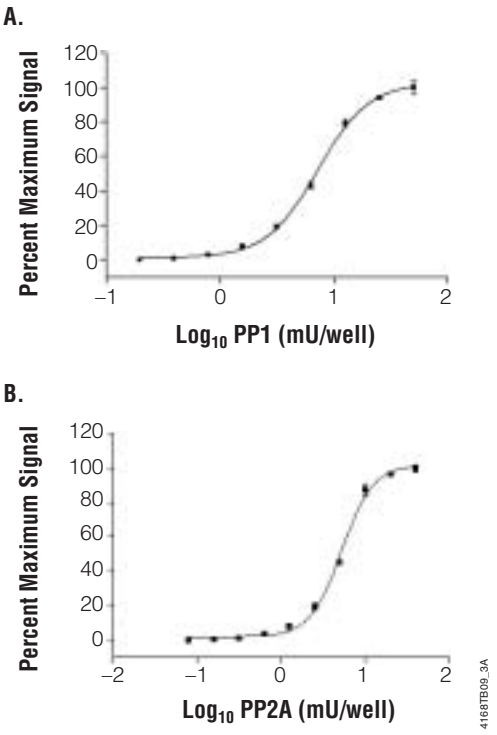

Figure 2. Phosphatase activity is directly correlated with R110 fluorescent output. Results of titration curves performed according to the protocol provided in Technical Bulletin #TB324 using solid black, flat-bottom 96-well plates. **Panel A.** PP1 titration. **Panel B.** PP2A titration. Curve fitting performed using GraphPad Prism® 3.0 software.

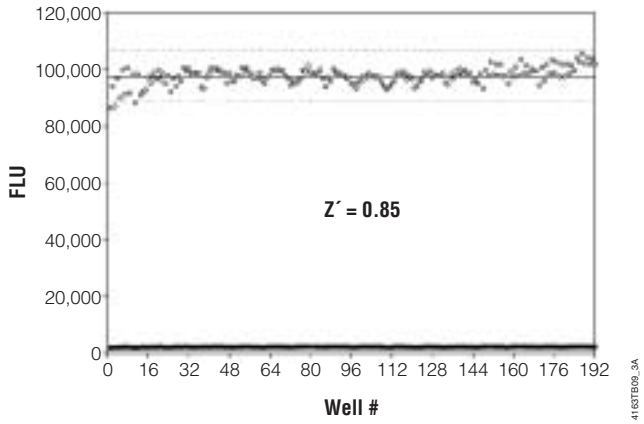

Figure 3. Z´-factor values obtained in 384-well plates. The assay was performed manually according to the protocol provided in Technical Bulletin #TB324 using solid black, flat-bottom plates with phosphatase (open circles) and without phosphatase (solid circles). Solid lines indicate the mean and the dotted lines indicate ± S.D. 6.25 milliunits/well PP1 (Calbiochem Cat.# 539493) was used. Z´-factor was 0.85.

potent (not shown). These IC_{50} values are similar to published values for these inhibitors (7). In these experiments, the signal remained stable at room temperature for at least four hours after the reactions were terminated (less than 10% change). This stability enables researchers to perform batch plate processing in which signal generated from plates can be read at a later time.

Specific Results with Low False-Positive Rate

Assay developers strive to limit the number of false-positive hits. There are four possible outcomes from the combined effect of compounds on phophatase and protease using the ProFluor™ Ser/Thr Phosphatase Assay (Table 1). Compounds that inhibit the phosphatase but not the protease will result in low fluorescence, indicating a positive hit. Compounds that inhibit both the phosphatase and protease will also result in low R110 fluorescence, but such compounds will not be desirable because they lack specificity. Compounds that do not inhibit the phosphatase or protease will show no change in R110 fluorescence relative to control. Compounds that do not inhibit the phosphatase but do inhibit the protease are problematic because these compounds will be picked up as false positives.

To address the issue of false positives resulting from protease inhibition by test compounds, we included another peptide, alanine-alanine-phenylalanine linked to 7-amino-4-methyl-coumarin (AAF-AMC), as a control substrate for protease activity. Since this peptide contains no phosphorylation site, its cleavage is independent of phosphatase activity. The cleavage of this peptide by the protease used in the assay releases free AMC that can be quantified by excitation at 360nm and emission at 460nm.

Table 1. Possible Outcomes of the ProFluor™ PPase Assay.

Test Compound Inhibits:	AMC Fluorescence 460nm	R110 Fluorescence 527nm
Phosphatase Only	⇧	⇩
Protease Only	⇩	⇩
Phosphatase and Protease	⇩	⇩
Neither Enzyme	⇧	⇧

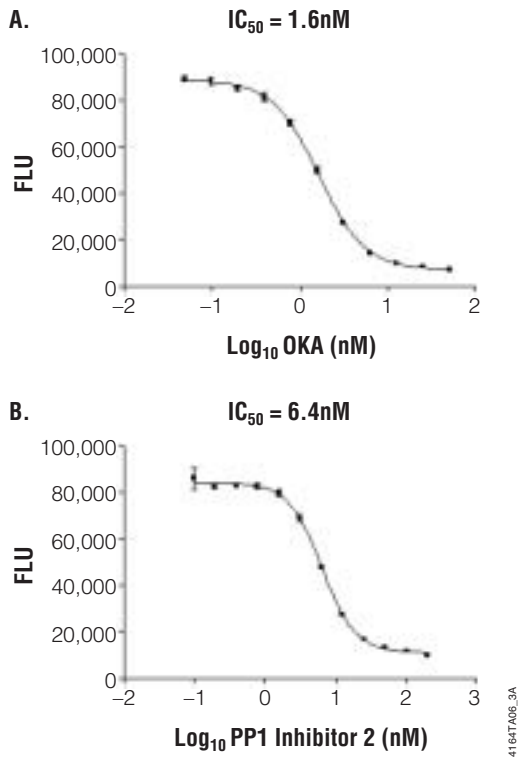

Figure 4. Accurate IC_{50} values. Inhibitor titrations for PP2A (Upstate Biochemicals Cat.# 14-111) using okadaic acid (LC Laboratories, Cat.# O-5857, Panel A) and PP1 (Calbiochem, Cat.# 539493) using PP1 Inhibitor 2 (Panel B). The assay was performed as described in Technical Bulletin #TB324 in solid black, flat-bottom 96-well plates using 10 milliunits/well PP2A or 35 milliunits/well PP1 and the indicated amount of inhibitor. Data points are the average of 4 determinations, and error bars are ± S.D. IC_{50} results were similar to published literature IC_{50} values of 0.6nM for okadaic acid for PP2A and 3.0nM for PP1 Inhibitor 2 for PP1. Curve fitting was performed using GraphPad Prism® 3.0 sigmoidal dose-response (variable slope) software.

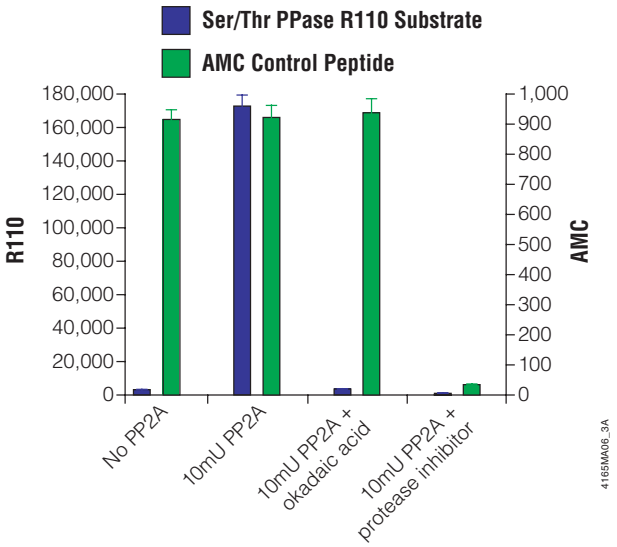

Figure 5. Distinguishing between true hits and false positives. Dark bars indicate fluorescence using excitation at 485nm and emission at 530nm (S/T PPase R110 Substrate), and gray bars indicate fluorescence using excitation at 355nm and emission at 460nm (Control AMC Substrate). The assay was performed as described in Technical Bulletin #TB324 in solid black, flat-bottom 96-well plates. Data points are the average of 8 determinations, and error bars are ± S.D. A compound that only inhibits the phosphatase will produce a decrease in R110 fluorescence but not AMC fluorescence, while a protease inhibitor will decrease both signals.

This protease control substrate allows users to differentiate phosphatase from protease effects. If a compound inhibits the phosphatase but not the protease, users will observe high fluorescence for the AMC-containing substrate (460nm) and low fluorescence for the R110-containing substrate (527nm). A compound that inhibits the protease will exhibit low fluorescence at both 527 and 460nm, indicating a false positive. Figure 5 shows the possible results from this assay.

Using the AAF-AMC control substrate with the phosphatase substrate in the same reaction or using the control substrate to screen for protease inhibition after screening against the phosphatase in separate reactions will ensure the validity of the positive hits. Including the AAF-AMC peptide in the phosphatase reaction does not affect the activity profile of any of the phosphatases tested under the conditions specified in the Technical Bulletin #TB324.

Summary

When compared to other high-throughput assays for protein phosphatases, the ProFluor™ Ser/Thr Phosphatase Assay is convenient, fast and non-radioactive. It does not require costly highly specific antibodies, and it is specific for PPases and will not be affected by hydrolases or esterases. The exquisite performance of the system is indicated by its high Z´ values, even with low concentrations of enzyme. The simplicity of the assay system makes it amenable to screening a large number of compounds in a rapid and efficient manner. ■

References

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Web site

www.promega.com/techserv/apps/sigtran

Protocol

ProFluor™ Ser/Thr Phosphatase Assay Technical Bulletin, #TB324
(www.promega.com/tbs/tb324/tb324.html)

Ordering Information

Product	Size	Cat.#
ProFluor™ Ser/Thr PPase Assay[a,b]	4-plate	V1260
	8-plate	V1261

[a]Patent Pending.

[b]This product is covered by U.S. Pat. Nos. 4,557,862 and 4,640,893 and is sold for research use only. All other uses, including but not limited to use as a clinical diagnostic or therapeutic, require a separate license. Please contact Promega Corporation for details relating to obtaining a license for such other use.