# ASSAY PROTEIN TYROSINE KINASE AND PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN A HOMOGENEOUS, NON-RADIOACTIVE HIGH-THROUGHPUT FORMAT

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We have developed homogeneous non-radioactive assay systems for monitoring the activity of purified tyrosine kinases and phosphatases. The assays show excellent reproducibility and flexibilty and are suitable for high-throughput screening activities.

## Abstract

Genes for protein kinases and phosphatases represent a large portion of the human genome (5%) and have been identified by many pharmaceutical companies as valid targets for drug discovery. To facilitate drug discovery, sensitive, homogenous, non-radioactive and cost-effective assays are needed. We present the ProFluor™ Src-Family Kinase and the ProFluor™ Tyrosine Phosphatase Assays. The assays can be performed as individual reactions or in 96- and 384-well plates. The signal-to-noise ratio is very high (>40) with a Z´ factor value >0.7. The signal is stable for at least four hours (<10% increase), and the assay is easily adapted to a robotic system for large-scale screening.

## Targeting Protein Kinases and Phosphatases

Protein kinases and phosphatases are implicated in a variety of cellular processes such as proliferation, differentiation and apoptosis. Over one third of the proteins in the human proteome are phosphoproteins, and the families of protein kinases and phosphatases represent up to 5% of the human genome (1). These enzymes increase or suppress the activity of other enzymes, mark proteins for destruction, allow proteins to move from one subcellular compartment to another, or enhance or impede protein:protein interactions. Any change in the level, activity, or localization of these enzymes greatly influences the regulation of key processes. Because of the role that protein kinases and phosphatases play in cellular functions, they represent important drug targets (2).

The search for orally active protein kinase inhibitors proved successful with the FDA approval of STI-571 (Gleevec), a c-Abl tyrosine kinase inhibitor, to treat chronic myelogenous leukemia and the approval in Japan of Fasudil, a Rho kinase inhibitor, to treat cerebral vasospasm (2,3). Because of the promise of these enzymes as therapeutic targets, researchers need assay systems that monitor the activity of these enzymes under a variety of experimental conditions and to develop selective inhibitors of these enzymes for therapeutic applications. The ProFluor™ Src-Family Kinase Assay[a] and the ProFluor™ Tyrosine Phosphatase Assay[a] are designed to meet this need.

## Evaluating PTK Activity

Current technologies for determining the activity of these enzymes include assays based on radioactivity, fluorescence polarization (FP) (4,5), ELISA using antibodies with reporters, fluorescence resonance energy transfer (FRET) (6,7), and metal-based reagents that interact with phosphopeptides.

> We consistently obtain Z´-factor values greater than 0.7 with the Src-Family PTKs, indicating a highly predictive assay.

Each of these assays has advantages and disadvantages. For example, although the signal-to-noise ratio (S:N) is high for the Scintillation Proximity Assay, the assay requires centrifugation or washing steps and is radioactive ([$\gamma$-$^{33}$P]-ATP), and its efficiency depends on the energy of the isotope used ([$\gamma$-$^{33}$P]-ATP is preferred over [$\gamma$-$^{32}$P]-ATP). A major drawback of FRET is that its dual-label format requires close proximity of the two fluorescent molecules. On the other hand, FP uses only one labeled fluorescent molecule, and it is ratiometric, minimizing interference by sample quenching, but it has low S:N ratio, in some cases is less than 1.5, depending on the assay. It also requires high affinity and selective antibodies. Optimizing the assay may be difficult because predicting the appropriate location and spatial configuration for the fluorescent label is difficult. Other available high-throughput formats are based on the differential electrophoretic mobility of the fluorescent peptide substrate and its phosphorylated product using capillary electrophoresis. This type of assay requires specialized high-throughput capillary electrophoresis setup and equipment (8).

## Evaluating PTPase Activity

Non-radioactive phosphatase assays were based on the quantitation of liberated phosphate and the formation of a colored complex with malachite green and acidified phosphomolybdate (9). For a special group of phosphatases such as phosphotyrosine phosphatases, assays using p-nitrophenyl phosphate (p-NPP) can be employed (10). More recently, a sensitive fluorogenic substrate for protein tyrosine phosphatases

using 3,6-fluorescein diphosphate as a substrate was reported (11). Each of these assays has limitations that make it inadequate for high-throughput screening of a large number of compounds against protein tyrosine phosphatases (PTPases). For example, the malachite green approach suffers from a lower detection limit, requiring a large amount of enzyme and high peptide substrate concentrations. The presence of contaminating inorganic phosphates in samples increases the background significantly. The use of p-NPP as substrate does not represent a true phosphatase phosphopeptide substrate (12). The substrate 3,6-diphosphofluorescein is not only dephosphorylated by PTPases, but it can also be hydrolyzed by nonprotein phosphatases and other unrelated enzymes such as esterases and hydrolases. Thus, there is a need for better phosphatase assay techniques.

## ProFluor™ PTK and PTPase Substrate Chemistry

Rhodamine 110 (R110) is a highly fluorescent molecule, but when it is 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 essentially nonfluorescent bisamide substrate is converted to the highly fluorescent free R110. Rhodamine 110 exhibits spectral properties similar to fluorescein, with peak excitation and emission wavelengths of 496nm and 520nm, respectively and stable fluorescence from pH 3.0–9.0 (13). Free R110 has intense visible absorption ($\varepsilon$496nm ~80,000cm$^{-1}$M$^{-1}$ in pH 6.0 solution).

## ProFluor™ PTK and PTPase Assay Principle

We synthesized bisamide R110-linked peptides that serve as substrates for PTKs and phosphopeptides that serve as substrates for PTPases. Phosphorylation of the peptide substrate renders it resistant to cleavage by the Protease Reagent that is included with the assay system reducing the fluorescence generated. However, when the phosphoryl moiety is removed by a phosphatase, the peptides become cleavable by the protease, releasing the highly fluorescent, free R110 molecule (Figure 1). The fluorescence intensity is directly proportional to the activity of the phosphatase and negatively correlated with the activity of the kinase.

## Use Less Enzyme with the ProFluor™ PTK Assays

We tested the ability of several kinases to phosphorylate the peptide substrate, using protease cleavage and fluorescence output as an indicator of enzyme activity. The PTK peptide substrate served as an excellent substrate for all the Src-Family of PTKs such as Src, Lck, Fyn, Lyn, and Hck and the recombinant epidermal growth factor receptor (EGFR) and insulin receptor (IR). The fluorescence output decreases with increasing concentrations of four Src family enzymes tested. The amount of enzymes required to phosphorylate 50% ($EC_{50}$) of the peptide by each enzyme was quite low ($EC_{50}$ for Src, Lck, Fyn, Lyn A, and Hck were 14.0, 1.38, 4.0, 4.13, and 1.43ng, respectively). As low as a few nanograms of Lck could be detected using this system (Figure 2).

## Get High Sensitivity with ProFluor™ PTPase Assays

We also tested the ability of several PTPases to dephosphorylate the phosphopeptide substrate again using protease cleavage and fluorescence output as an indicator of enzyme activity. The PTPase phosphopeptide substrate served as an excellent substrate for all the PTPases tested such as PTP1B, CD45, LAR, Yop51 and T-cell PTPases. As shown in Figure 3, the fluorescence output increases with increasing concentrations of any of the PTPases tested (i.e., proportional increase in fluorescence with increasing concentration of the PTPase in the reaction). The amount of enzymes required to dephosphorylate 50% ($EC_{50}$) of the peptide by each enzyme was

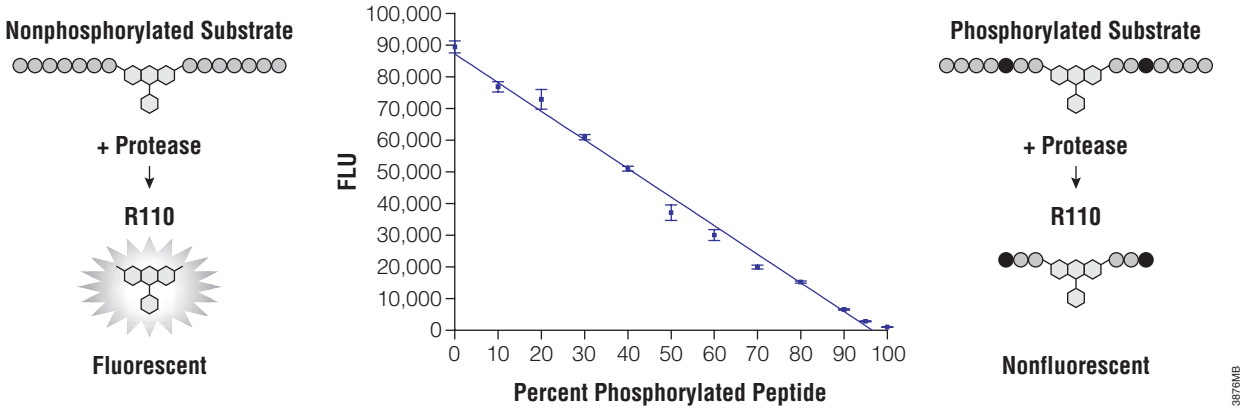


**Figure 1. Schematic and graph demonstrating that Rhodamine 110 is essentially nonfluorescent in the bisamide form and that the presence of a phosphorylated amino acid (red circle) blocks the removal of amino acids by the protease.** The graph is the average FLU obtained after a 30-minute protease reagent digestion using mixtures of nonphosphorylated R110 PKA Substrate and phosphorylated R110 PKA Substrate as indicated (n = 6). The total amount of peptide was 5µM in 50µl of Reaction Buffer A to which 25µl of protease reagent diluted in Termination Buffer A was added.

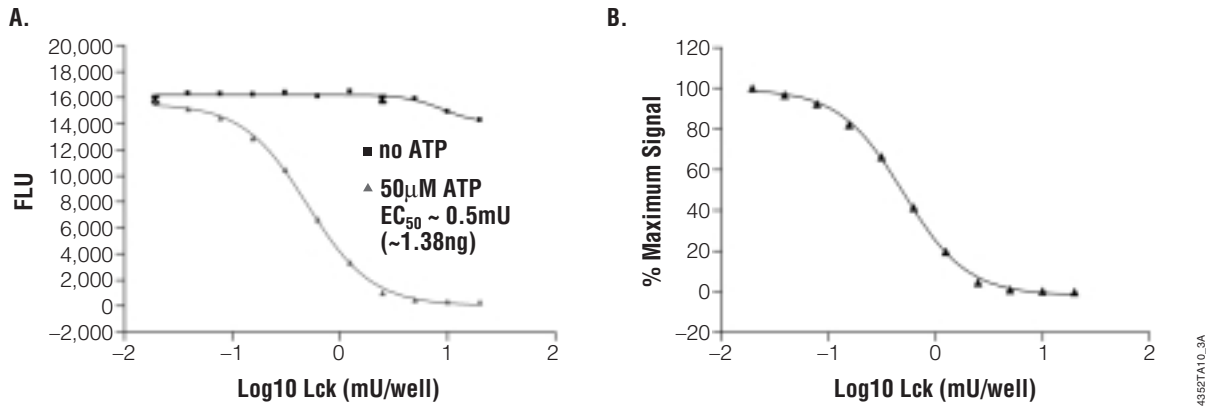


**Figure 2. Kinase activity is inversely correlated with R110 fluorescence output.** Results of titration curves that were performed according to the kinase titration protocol (Technical Bulletin #TB331) in solid black, flat-bottom 96-well plates. **Panels A** and **B** show the results of a Lck titration (Upstate Biotech Cat.# 14-442). **Panel A** is the data collected (actual R110 FLU units) with or without ATP from the plate. Data points are the average of 4 determinations. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose response (variable slope) software. The $R^2$ value is 0.99, $EC_{50}$ is 0.5mU/well, and the maximum dynamic range in the assay is ~50- to 60-fold. Normalizing the data allows for a quick determination of the amount of kinase required for the percent conversion desired (**Panel B**).

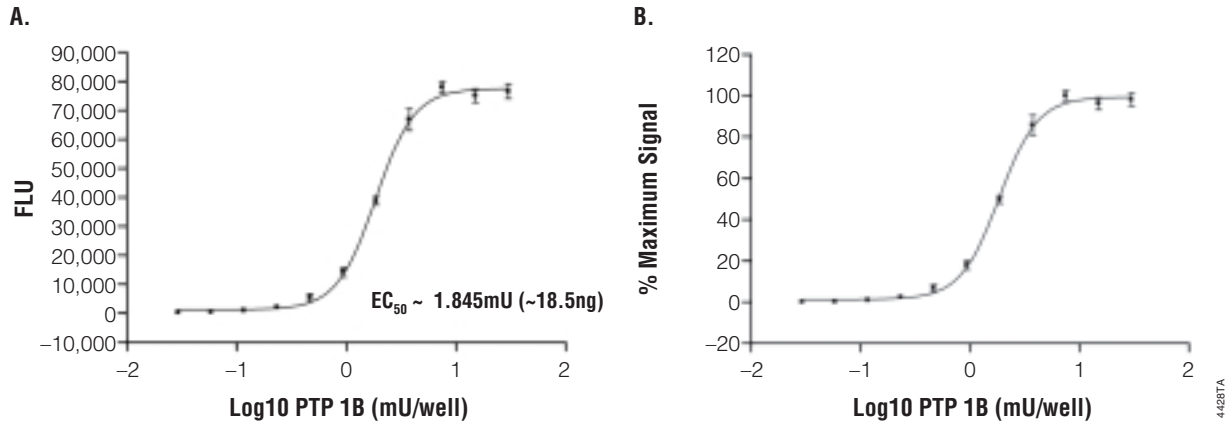


**Figure 3. PTPase activity is directly correlated with R110 fluorescence output.** Results of titration curves that were performed according to the phosphatase titration protocol (Technical Bulletin #TB334) in solid black, flat-bottom 96-well plates. **Panels A** and **B** show the results of a PTP-1B titration (Calbiochem Cat.# 539735). **Panel A** shows the data collected (actual R110 FLU units) from the plate. Data points are the average of 8 determinations, and error bars are ± S.D. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose-response (variable slope) software. The $R^2$ value is 0.99, $EC_{50}$ is 2mU/well (~ 20ng), and the maximum dynamic range in the assay is ~1,000-fold. Normalizing the data allows for a quick determination of the amount of phosphatase required for the percent conversion (85–90% recommended) desired (**Panel B**).

quite low: $EC_{50}$ for PTP1B, CD45 PTPase, LAR PTPases, T-cell PTPase, and Yop51 were 20, 37, 46, 7.8, and 25.5ng, respectively (15).

## Obtain Excellent Reproducibility and Flexibility

The ProFluor™ Src-Family Kinase Assay and ProFluor™ Tyrosine Phosphatase Assay are versatile and are 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 also determined Z´-factor values for the assays. 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 (16). We consistently obtain Z´-factor values greater than 0.7 with the Src Family PTKs (Figure 4), indicating a highly predictive assay. We obtain similar values with any of the PTPases tested (Figure 5).

In both the PTK and PTPase assays, 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.

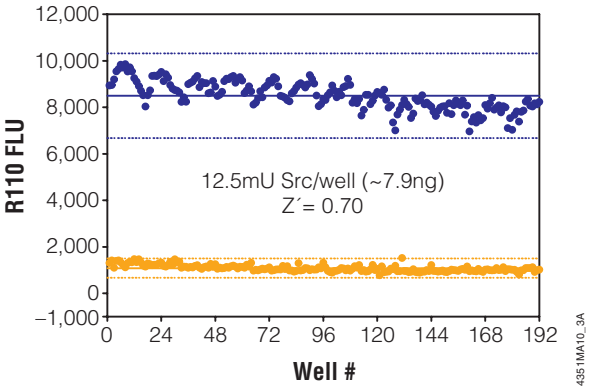


**Figure 4. Z´-factor value obtained in a 384-well plate (kinase assay).** Results of a Z´ analysis are shown for a 384-well plate using the indicated amount of Src/well. The assay was performed manually according to Technical Bulletin #TB331 in a solid black, flat-bottom plate with 50µM ATP (red) or without ATP (blue). Solid lines indicate the mean, and the dotted lines are ± 3 S.D. The Z´ factor for the assay under those conditions is 0.70 as calculated by the equation from reference 14.

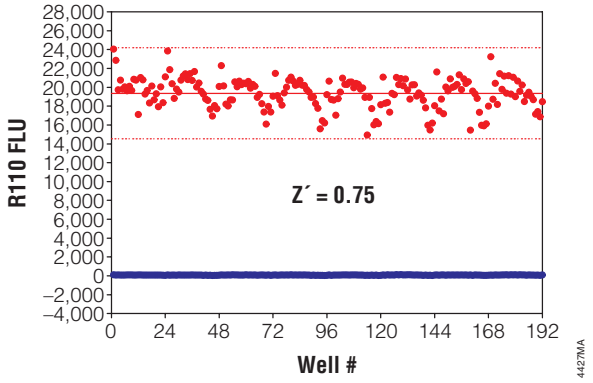


**Figure 5. Z´-Factor value obtained in a 384-well plate (phosphatase assay).** The assay was performed manually according to Technical Bulletin #TB334 in a solid black, flat-bottom plate with 0.2 units LAR PTPase (red) and without enzyme (blue). Solid lines indicate the mean, and the dotted lines are ± 3 S.D. The Z´ factor for the assay under those conditions is 0.75 as calculated by the equation from reference 14.

## Produce Accurate and Reliable $IC_{50}$ Values

To verify that the enzyme activity observed is specific for the enzyme under investigation, we tested the validity of our assay using selective enzyme inhibitors. If the activity of a kinase or a phosphatase is a reflection of its enzyme activity, then including a selective inhibitor of the kinase or the phosphatase should reverse the signal (i.e., increase the signal in the kinase reaction and decrease the signal in the phosphatase reaction). Figure 6 shows that the $IC_{50}$ value (concentration of compound needed to inhibit the kinase activity by 50%) determined for staurosporine, a kinase inhibitor, was 38nM. This value corresponds well with those reported in the literature for staurosporine against Src-family PTK (17).

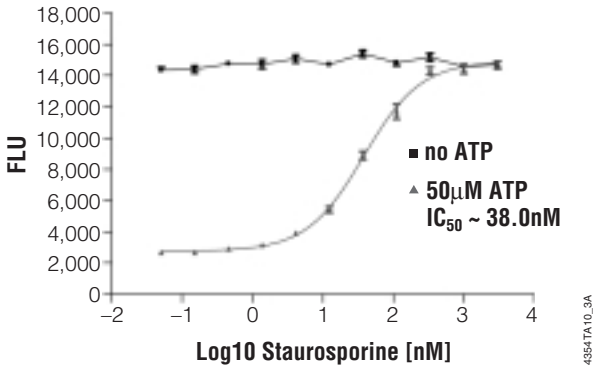


**Figure 6. Accurate $IC_{50}$ values (kinase assay).** Results of a staurosporine titration in a solid black, flat-bottom 96-well plate using 1.25mU Lck/well (Upstate Biotech Cat.# 14-442 and 50µM ATP). The assay was performed as described in Technical Bulletin #TB331. Data points are the average of 4 determinations, and error bars are ± S.D. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose-response (variable slope) software.

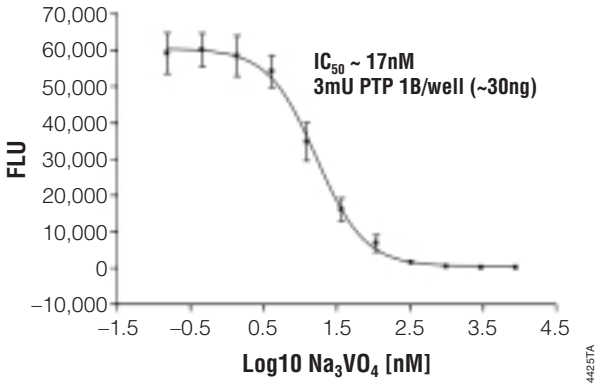


**Figure 7. Accurate $IC_{50}$ values (phosphatase assay)**. Results of a sodium vanadate titration in a solid black, flat-bottom 96-well plate using 3.75mU PTP1B/well (Calbiochem Cat.# 539735). The assay was performed as described in Technical Bulletin #TB334. Data points are the average of 8 determinations, and error bars are ± S.D. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose-response (variable slope) software.

We also tested the effect of vanadate, a well-known inhibitor of PTPases. As shown in Figure 7, an $IC_{50}$ value of 17nM was obtained, which is similar to those reported for this group of enzymes (18).

## Specific Results with Low False-Hit Rate

One of the most important features in a screening technology is the ability to detect false hits. Reliable assays should enable the investigators to recognize those hits when they arise. We included another fluorogenic peptide, alanine-alanine-phenylalanine linked to 7-amino-4-methyl-coumarin (AAF-AMC). It is devoid of amino acid residues that can be subjected to phosphorylation or dephosphorylation but is cleavable by the

**Table 1. Possible Outcomes of the ProFluor™ PTK Assay.**

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

Up arrows (⇧) indicate high fluorescent values; down arrows (⇩) indicate low fluorescent values.

**Table 2. Possible Outcomes of the ProFluor™ PTPase Assay.**

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

Up arrows (⇧) indicate high fluorescent values; down arrows (⇩) indicate low fluorescent values.

protease. Any compound that interferes with the activity of the protease can be distinguished from those inhibiting the kinase or phosphatase. The cleavage of this peptide by the protease used in the assay releases free AMC that can be monitored and quantified by excitation at 360nm and emission at 460nm, which is different from R110.

Figure 8 shows a representative example of the expected results when screening for phosphatase inhibitors. 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. A summary of the expected results when testing for the effect of compounds on kinases, phosphatases, or the protease is shown in Tables 1 and 2. Including the AAF-AMC peptide in the phosphatase or the kinase reactions does not affect the activity profile of any of the phosphatases or kinases tested under the conditions specified in the Technical Bulletins.

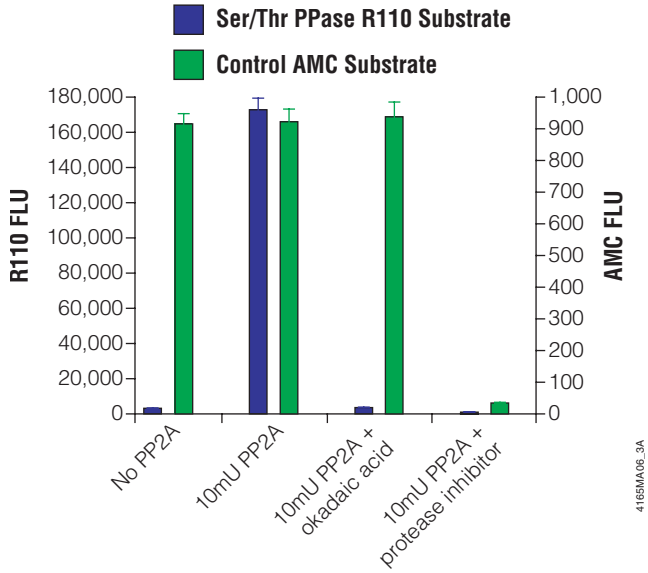


**Figure 8. Distinction between the effect of compounds on the activity of phosphatases and proteases by using control peptide.** Blue bars indicate fluorescence using excitation at Ex485 and emission at Em530 (R110 peptide substrate), and green bars indicate fluorescence using excitation at Ex355 and emission at Em460 (control peptide). The assay was performed as described in the *ProFluor™ Ser/Thr Phosphatase Assay[a] Technical Bulletin*, #TB324 in solid black, flat-bottom 96-well plates using the conditions indicated above. Data points are the average of 8 determinations, and error bars are ± S.D. The results demonstrate that a phosphatase-only inhibitor will produce a decrease in R110 fluorescence but not AMC fluorescence, while a protease inhibitor will decrease both fluorescence signals.

## Summary

The ProFluor™ Assays offer the simplicity, sensitivity and the specificity required for screening chemical libraries for novel inhibitors of protein kinases and phosphatases (19,20). These assays are robust as shown by the high Z′ value; furthermore, they are non-radioactive, do not require antibodies, and do not suffer from the presence of high concentrations of ATP in the kinase reaction. The assays can be performed in single tubes, 96-well plates or 384-well plates in as little as 1.5 hours for tyrosine phosphatases or 2 hours for Src-family kinases, and they require only two reagent additions. The S:N ratio is very high (S:N = 40) compared to other assays (S:N = 2–3 for FP), and the generated signal is stable for hours. We have validated these assays with known selective inhibitors of protein kinases and phosphatases. ■

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## Protocols

*ProFluor™ Src-Family Kinase Assay Technical Bulletin* #TB331
(www.promega.com/tbs/tb331/tb331.html)

*ProFluor™ Tyrosine Phosphatase Assay Technical Bulletin* #TB334
(www.promega.com/tbs/tb334/tb334.html)

## Ordering Information

| Product | Size | Cat.# |
|---|---|---|
| ProFluor™ Src-Family Kinase Assay[a] | 4 plate | V1270 |
| | 8 plate | V1271 |
| ProFluor™ Tyrosine Phosphatase Assay[a] | 4 plate | V1280 |
| | 8 plate | V1281 |

[a] Patent Pending.



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