

ProFluor™ Src-Family Kinase Assay

Description

The ProFluor™ Src-Family Tyrosine Kinase Assay measures the activity of purified protein from members of the Src-Family of Tyrosine Kinases (such as Src, Lck, Fyn, Lyn, and Hck) in a multiwell plate format that involves “add, mix, and read” steps only. The assay begins with a standard kinase reaction performed in the provided reaction buffer with the provided bisamide rhodamine 110 peptide substrate (Src-Family Tyrosine Kinase R110 Substrate) and Control AMC Substrate (Amino Methyl Coumarin fluorophore peptide conjugate) that serves as a control for compounds that may inhibit the protease resulting in false hits. In this configuration, both the kinase R110 Substrate and Control AMC Substrate are nonfluorescent. Following the kinase reaction, addition of a protease solution simultaneously stops the kinase reaction and completely digests the non-phosphorylated kinase R110 Substrate and the Control AMC substrate, producing highly fluorescent rhodamine 110 and AMC. Phosphorylated kinase R110 Substrate, however, is resistant to protease digestion and remains nonfluorescent (Figure 1). Thus, the R110 fluorescence intensity measured in the assay is inversely correlated with kinase activity, while the AMC fluorescence intensity is a monitor of protease activity. The assay produces excellent Z' values (> 0.7) in either 96- or 384-well plate formats and easily distinguishes known kinase inhibitors from other compounds.

A compound that only inhibits the kinase will increase the R110 fluorescent signal in the assay and not affect the AMC signal (Figure 4). A protease inhibitor will affect both the AMC and R110 signals. To distinguish protease inhibitors from kinase inhibitors, the AMC fluorescence signal should be examined carefully when an increase in R110 fluorescence is observed.

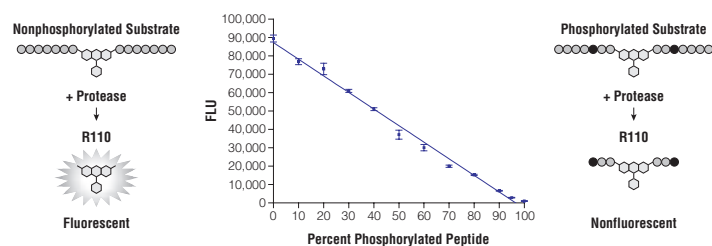


Figure 1. Effect of phosphopeptide content on fluorescence intensity in the ProFluor™ Kinase Assays. Schematic demonstrates the principle behind the ProFluor™ Src-Family Assay where the unphosphorylated peptide substrate is readily digested by a protease reagent leading to the release of highly fluorescent rhodamine 110. Phosphorylated substrate (indicated by dark circles), however, is resistant to digestion by the protease and does not contribute to the fluorescent signal. The graph shows fluorescence intensity as a function of percent phosphorylated substrate following a protease digestion (PKA Peptide Substrate data shown) to mimic a kinase titration. FLU = fluorescence light units.

Features

- **Screen more compounds:** The micromolar concentration of substrate used in the assay combined with the rhodamine 110 fluorophore produce fluorescent signals much higher than the intrinsic fluorescence of most problematic test compounds.
- **Homogeneous and Nonradioactive:** Simple “add, mix and read” format with none of the safety and disposal costs associated with radiometric assays.
- **Low False-Positive Rate:** The Protease Reagent in the ProFluor™ Src-Family Kinase Assay was tested against the Library of Pharmacologically Active Compounds (LOPAC- Sigma-RBI). None of the 640 LOPAC library compounds interfered with the Protease Reagent (data not shown). To further ensure against picking up false-positives a control peptide (AAF-AMC) is included that is used to monitor protease activity (Figure 4).
- **Sensitive:** Requires just nanogram quantities of enzyme per well (Figure 2).
- **Highly Predictive Results:** Signal-to-background ratios of $>50:1$ and low well-to-well variability result in Z' values of 0.7 or greater, leading to highly predictive results (Figure 3).

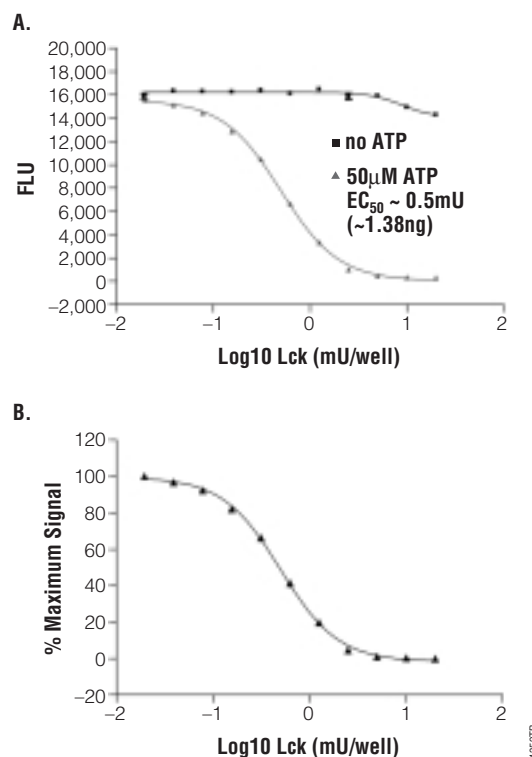


Figure 2. Kinase activity is inversely correlated with R110 fluorescence output. Results of titration curves that were performed in solid black, flat-bottom 96-well plates. **Panels A and B** show the results of a Lck titration. **Panel A** represents the data collected (actual R110 FLU units) from the plate. Data points are the average of 4 determinations, and error bars are \pm S.D. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose response (variable slope) software. Normalizing the data allows quick determination of the amount of kinase required for the desired percent conversion (**Panel B**). FLU = Fluorescence Light Units.

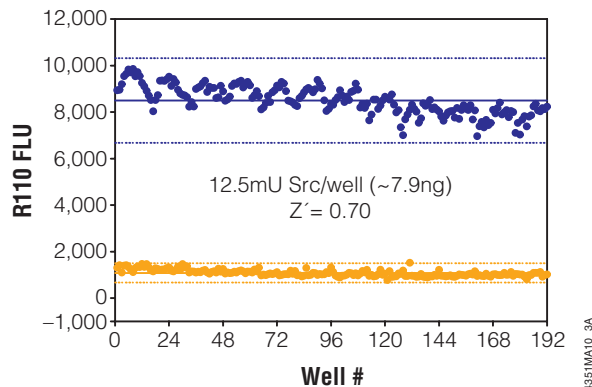


Figure 3. Z'-factor values of greater than 0.7 are routinely obtained using the ProFluor™ Src-Family Kinase Assay. Results of a Z'-factor analysis are shown for a 384-well plate using the indicated amount of Src kinase/well. The assay was performed manually according to Section III.B of the *ProFluor™ Src-Family Kinase Assay Technical Bulletin* (#TB331) in a solid black, flat-bottom plate with 50 μM ATP (gold circles) or without ATP (blue circles). Solid lines indicate the mean, and the dotted lines are ± 3 S.D. FLU = Fluorescence Light Units. R110 = Rhodamine 110.

Ordering Information

Product	Size	Cat. #
ProFluor™ Src-Family Kinase Assay ^(a)	4 Plate	V1270
	8 Plate	V1271

Component listing for V1270:

- 12 μl Src-Family Kinase R110 Substrate, 4mM
- 12 μl Control AMC Substrate, 10mM
- 250 μl ATP, 10mM
- 1ml MnCl₂, 300mM
- 2 × 240 μl Protease Reagent
- 12 μl Stabilizer Reagent
- 6ml 5X Reaction Buffer A
- 5ml 5X Termination Buffer A
- 200 μl Sodium Vanadate

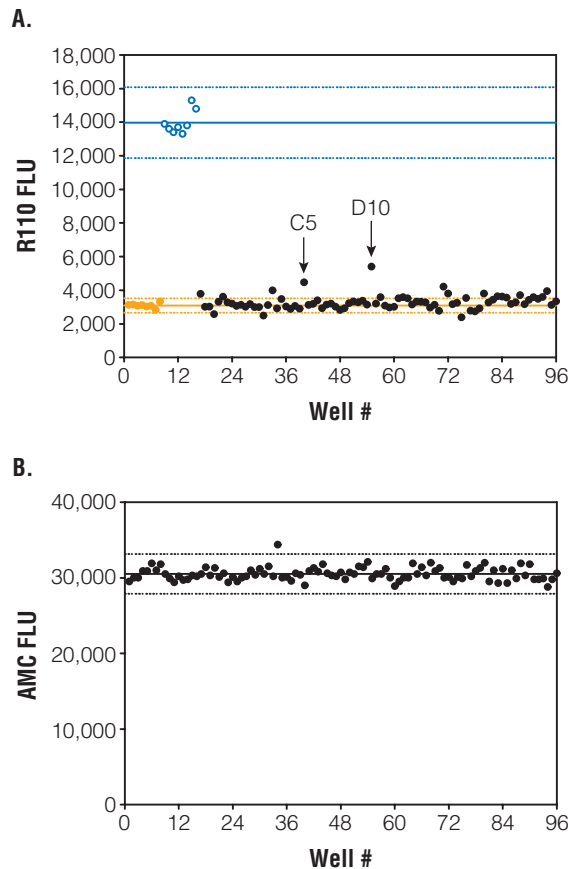
Component listing for V1271:

- 24 μl Src-Family Kinase R110 Substrate, 4mM
- 24 μl Control AMC Substrate, 10mM
- 500 μl ATP, 10mM
- 1ml MnCl₂, 300mM
- 2 × 480 μl Protease Reagent
- 24 μl Stabilizer Reagent
- 12ml 5X Reaction Buffer A
- 10ml 5X Termination Buffer A
- 200 μl Sodium Vanadate

ProFluor is a trademark of Promega Corporation.

GraphPad Prism is a registered trademark of Graphpad Software, Inc.

^(a) Patent Pending.



Well #	Compound	Description	% Inhibition
C5	Typhostin AG 494	Protein Tyr Kinase Inhibitor	12.7
D10	Tamoxifen Citrate	Anti-Estrogen, PKC Inhibitor	21.2

Figure 4. Results of a single 80-compound screen of plate 6 from the LOPAC library (Sigma-RBI). The assay was performed as described in Section III.D of the *ProFluor™ Src-Family Kinase Assay Technical Bulletin* (#TB331 - except that the ATP concentration was 20 μM) in a solid-black, flat-bottom 96-well plate with 100 μM compounds in 10% DMSO. Final concentrations in the kinase reaction were 10 μM compound and 1% DMSO. **Panel A** shows the R110 fluorescent signal using 1.25mU Lck/well. The gold solid circles (n = 8) indicate wells without compound (1% DMSO only) in the presence of 20 μM ATP and the blue open circles (n = 8) indicate wells without compound (1% DMSO only) in the absence of ATP. The solid lines indicate the means, and the dotted lines indicate ± 3 standard deviations of these populations. The black circles indicate compounds screened in the presence of 20 μM ATP. The compounds scored as hits are defined in the table above. **Panel B** shows the AMC fluorescent signal of the same plate. Note: None of the LOPAC compounds inhibited the protease (as measured in the AMC channel). FLU = Fluorescence Light Units. R110 = Rhodamine 110.

