Promega

Technical Bulletin

ProFluor® Src-Family Kinase Assay

INSTRUCTIONS FOR USE OF PRODUCTS V1270 AND V1271.

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ProFluor® Src-Family Kinase Assay

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1. Description

The ProFluor® Src-Family Kinase Assay[a] measures the enzymatic activity of the Src-family tyrosine kinases (1) such as Src, Lck, Fyn, Lyn and Hck using purified enzymes in a multiwell-plate format and involves "add, mix and read" steps only (Table 1). The assay begins with a standard kinase reaction performed in the provided reaction buffer with the provided rhodamine 110 peptide substrate (Src-Family Kinase R110 Substrate). In this configuration, the substrate is nonfluorescent (Figure 1, reference 2). Following the kinase reaction, addition of a termination buffer that contains the Protease Reagent simultaneously stops the kinase reaction and removes amino acids specifically from the nonphosphorylated substrate, resulting in the production of highly fluorescent rhodamine 110. The phosphorylated substrate, however, is resistant to digestion by the Protease Reagent and remains nonfluorescent. Thus, the measured fluorescence intensity is inversely correlated with kinase activity (Figure 3). The fluorescent signal is very stable (<15% change of fluorescence intensity over 4 hours), allowing batch-plate reading (Figure 2). The assay produces Z´-factor values greater than 0.7 in 96-well (data not shown) or 384-well plate formats (Figure 4) and easily identifies known kinase inhibitors in a screen of library compounds (Figure 5). The assay produces IC_{50} values for known inhibitors that are comparable to those reported in the literature (Figure 6, references 3,4).

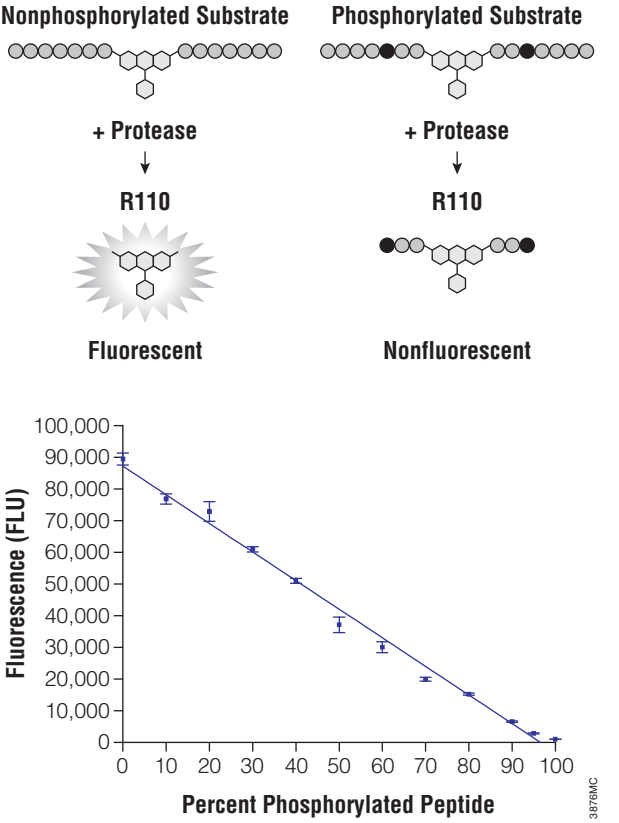


Figure 1. Schematic diagram and graph demonstrating that rhodamine 110 is essentially nonfluorescent in the bisamide form and that the presence of a phosphorylated amino acid (dark circle) blocks the removal of amino acids by the protease. The graph shows the average fluorescence obtained after a 30-minute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

2. Product Components and Storage Conditions

Product	Size	Cat.#
ProFluor® Src-Family Kinase Assay	4 plate	V1270

Each system contains sufficient reagents for 4 × 96 assays at 100µl per assay in 96-well plates or 5 × 384 assays at 20µl per assay in 384-well plates. Includes:

- 12µl Src-Family Kinase R110 Substrate, 4mM
- 12µl Control AMC Substrate
- 250µl rATP, 10mM
- 1ml $MnCl_2$, 300mM
- 200µl Sodium Vanadate, 100mM
- 6ml 5X Reaction Buffer A
- 5ml 5X Termination Buffer A
- 2 × 240µl Protease Reagent
- 12µl Stabilizer Reagent

Product	Size	Cat.#
ProFluor® Src-Family Kinase Assay	8 plate	V1271

Each system contains sufficient reagents for 8 × 96 assays at 100µl per assay in 96-well plates or 10 × 384 assays at 20µl per assay in 384-well plates. Includes:

- 24µl Src-Family Kinase Substrate R110, 4mM
- 24µl Control AMC Substrate
- 500µl rATP, 10mM
- 1ml $MnCl_2$, 300mM
- 200µl Sodium Vanadate, 100mM
- 12ml 5X Reaction Buffer A
- 10ml 5X Termination Buffer A
- 2 × 480µl Protease Reagent
- 24µl Stabilizer Reagent

Storage Conditions: For long-term storage, store the system at –20°C. Protect the Src-Family Kinase R110 Substrate and Control AMC Substrate from light. Avoid multiple freeze-thaw cycles or exposure to frequent temperature changes. These fluctuations can greatly alter product stability.

3. Protocols for the ProFluor® Src-Family Kinase Assay

Materials to Be Supplied by the User

- opaque-walled multiwell plates (e.g., Microfluor 2, Black 96-well; Thermo Electron Cat.# 7805)
- multichannel pipette or automated pipetting station
- plate shaker (DYNEX Micro-Shaker® II or equivalent)
- fluorometer capable of reading multiwell plates
- Src-family protein tyrosine kinase

3.A. Assay Overview

The ProFluor® Src-Family Kinase Assay works with purified Src, Lck, Lyn, Fyn and Hck tyrosine kinases in 96- or 384-well plates and involves "add, mix and read" steps only. The 1X Reaction Buffer A contains the following components: 40mM Tris-HCl (pH 7.5), 20mM $MgCl_2$ and 0.1mg/ml BSA. Reaction Buffer A may also be supplemented with the provided $MnCl_2$ and the selective tyrosine phosphatase inhibitor Sodium Vanadate (optional). The amount of ATP can be adjusted above or below 50µM, the value recommended in the protocol.

The assay begins with a kinase reaction containing 2µM of Src-Family Kinase R110 Substrate. In this configuration, R110 demonstrates very little fluorescence. Following phosphorylation by a kinase, a protease reaction is initiated by adding a buffer that also terminates the kinase reaction. After completion of the protease reaction, the plate can be read immediately or stabilized by adding the Stabilizer Reagent, which terminates the protease reaction.

When there is no kinase activity, the peptide substrate remains nonphosphorylated, and the protease will remove all amino acids from the peptide substrate and liberate the highly fluorescent rhodamine 110. However, in the presence of active kinase, the peptide substrate will be phosphorylated, effectively blocking the protease activity and resulting in low fluorescence of the Src-Family Kinase R110 Substrate. Thus, increasing amounts of kinase activity result in a change from high fluorescence to low fluorescence. The provided Control AMC Substrate (5µM of AAF-AMC), which has different excitation and emission wavelengths than the R110 substrate, will identify any false negatives in the assay (i.e., protease inhibitors that might conceal the presence of true kinase inhibitors on fluorescence output). The control substrate can be added directly to the kinase reaction, or hits can be rescreened against the protease with the control substrate.

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

Step	Description	Volume of Prepared Reagent Per Well: 96-Well	384-Well
1.	Add kinase and peptide substrate in 1X Reaction Buffer A	25µl	5µl
2.	Start the reaction by adding ATP in 1X Reaction Buffer A	25µl	5µl
3.	Mix plate, and incubate 60 minutes at room temperature		
4.	Add Protease Reagent in 1X Termination Buffer A	25µl	5µl
5.	Mix plate, and incubate 60 minutes at room temperature		
6.	Add Stabilizer Reagent in 1X Termination Buffer A	25µl	5µl
7.	Mix plate, and read R110 and AMC fluorescence		

We highly recommend performing a kinase titration to determine the optimal amount of kinase to use for screening and also to determine whether or not the enzyme preparation contains components that negatively affect assay performance. Minimize the use of reducing reagents when performing this assay. However, concentrations of up to 100µM of DTT or β-mercaptoethanol have only minimal effects on protease activity in this assay. We have tested the assay in reactions containing up to 2% DMSO, a common compound vehicle, and have observed only minimal effects on performance.

3.B. Kinase Titration (one 96- or 384-well plate)

Reagent Preparation

Prepare the following solutions before performing the assay.

Component	96-Well	384-Well
Kinase Solution		
5X Reaction Buffer A	600µl	480µl
Control AMC Substrate	3µl	2.4µl
Src-Family Kinase R110 Substrate	3µl	2.4µl
$MnCl_2$, 300mM	20µl	16µl
Sodium Vanadate, 100mM	6µl	4.8µl
NANOpure® water to a volume of	**3ml**	**2.4ml**
ATP Solution		
5X Reaction Buffer A	400µl	320µl
10mM ATP	20µl	16µl
NANOpure® water to a volume of	**2ml**	**1.6ml**
Control Buffer		
5X Reaction Buffer A	400µl	320µl
NANOpure® water to a volume of	**2ml**	**1.6ml**

3.B. Kinase Titration (continued)

Component	96-Well	384-Well
Protease Solution		
5X Termination Buffer A	600µl	480µl
Protease Reagent	120µl	96µl
NANOpure® water to a volume of	**3ml**	**2.4ml**
Stabilizer Solution		
5X Termination Buffer A	600µl	480µl
Stabilizer Reagent	3µl	2.4µl
NANOpure® water to a volume of	**3ml**	**2.4ml**

96-Well Plate Protocol

1. Add 25µl of Kinase Solution to columns 1 through 11 of a 96-well plate.

   Note: Solid-black plates provide the best signal-to-noise ratios, although solid-white plates can also be used.

2. Dilute kinase in 0.5ml Kinase Solution. Mix. Add 50µl to wells in column 12. Serially dilute with a multichannel pipette by transferring 25µl to column 11, pipetting up and down to mix and transferring 25µl to column 10. Repeat for columns 9 through 2. Discard the final 25µl, and do not dilute the kinase into column 1; this column is the no-enzyme control. All wells should now have 25µl.

3. Add 25µl of Control Buffer to rows A through D. Add 25µl of ATP Solution to rows E through H. Adding ATP initiates the kinase reaction. All wells should now have 50µl.

   Note: The protease reaction is temperature sensitive. We strongly recommend performing this reaction at 22–25°C. Increasing or decreasing the temperature will change the dynamic range of the assay.

4. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).

5. Add 25µl of Protease Solution to all wells. All wells should now have 75µl.

6. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).

7. Add 25µl of Stabilizer Solution to all wells. All wells should now have 100µl.

8. Mix plate, and read fluorescence at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal). Read plate at an excitation wavelength of 355nm and an emission wavelength of 460nm (AMC signal).

384-Well Plate Protocol

1. Add 5µl of Kinase Solution to columns 1 through 11, rows A through H, of a 384-well plate.

   Note: Solid-black plates provide the best signal-to-noise ratios, although solid-white plates can also be used.

2. Dilute kinase in 0.5ml Kinase Solution. Mix. Add 10µl to wells in column 12. Serially dilute with a multichannel pipettor by transferring 5µl to column 11, pipetting up and down to mix and transferring 5µl to column 10. Repeat for columns 9 through 2. Discard the final 5µl, and do not dilute the kinase into column 1; this column is the no-enzyme control. All wells should now have 5µl.

3. Add 5µl of Control Buffer to rows A through D. Add 5µl of ATP Solution to rows E through H. Adding ATP initiates the kinase reaction. All wells should now have 10µl.

   Note: The protease reaction is temperature sensitive. We strongly recommend performing this reaction at 22–25°C. Increasing or decreasing the temperature will change the dynamic range of the assay.

4. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).

5. Add 5µl of Protease Solution to all wells. All wells should now have 15µl.

6. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).

7. Add 5µl of Stabilizer Solution to all wells. All wells should now have 20µl.

8. Mix plate, and read fluorescence at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal). Read plate at an excitation wavelength of 355nm and an emission wavelength of 460nm (AMC signal).

3.C. Determining Z´-Factor (one 96- or 384-well plate)

Based on your kinase titration (Section 3.B), choose an amount of enzyme that results in approximately 20% of the maximum signal in the assay (80% phosphorylation). Using less enzyme is possible; however, the dynamic range of the assay will narrow.

Reagent Preparation

Prepare the following solutions before performing the assay.

Component	96-Well	384-Well
Kinase Solution		
5X Reaction Buffer A	600µl	480µl
Control AMC Substrate	3µl	2.4µl
Src-Family Kinase R110 Substrate	3µl	2.4µl
$MnCl_2$, 300mM	20µl	16µl
Sodium Vanadate, 100mM	6µl	4.8µl
Kinase (determined by titration)	Xµl	Xµl
NANOpure® water to a volume of	**3ml**	**2.4ml**
ATP Solution		
5X Reaction Buffer A	400µl	320µl
10mM ATP	20µl	16µl
NANOpure® water to a volume of	**2ml**	**1.6ml**
Control Buffer		
5X Reaction Buffer A	400µl	320µl
NANOpure® water to a volume of	**2ml**	**1.6ml**
Protease Solution		
5X Termination Buffer A	600µl	480µl
Protease Reagent	120µl	96µl
NANOpure® water to a volume of	**3ml**	**2.4ml**
Stabilizer Solution		
5X Termination Buffer A	600µl	480µl
Stabilizer Reagent	3µl	2.4µl
NANOpure® water to a volume of	**3ml**	**2.4ml**

Step	96-Well	384-Well
1. Add Kinase Solution to each well.	25µl	5µl
2. Add Control Buffer to rows A through D for a 96-well plate or rows A through H for a 384-well plate.	25µl	5µl
3. Add ATP Solution to rows E through H for a 96-well plate or rows I through P for a 384-well plate.	25µl	5µl
4. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).		
5. Add Protease Solution to all wells.	25µl	5µl
Note: The protease reaction is temperature sensitive. We strongly recommend performing this reaction at 22–25°C. Increasing or decreasing the temperature will change the dynamic range of the assay.		
6. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).		
7. Add Stabilizer Solution to all wells.	25µl	5µl
8. Mix plate, and read fluorescence at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal). Read plate at an excitation wavelength of 355nm and an emission wavelength of 460nm (AMC signal).		

3.D. Screening for Src-Family Tyrosine Kinase Inhibitors (one 96- or 384-well plate)

Based on your kinase titration (Section 3.B), choose an amount of enzyme that results in approximately 20% of the maximum signal in the assay (80% phosphorylation). Using less enzyme is possible; however, the dynamic range of the assay will narrow.

Reagent Preparation

Prepare the following solutions before performing the assay.

Component	96-Well	384-Well
Kinase Solution (1.25X)		
5X Reaction Buffer A	600µl	480µl
Control AMC Substrate	3µl	2.4µl
Src-Family R110 Substrate	3µl	2.4µl
$MnCl_2$, 300mM	20µl	16µl
Sodium Vanadate, 100mM	6µl	4.8µl
Kinase (determined by titration)	Xµl	Xµl
NANOpure® water to a volume of	**2.4ml**	**1.9ml**
ATP Solution		
5X Reaction Buffer A	600µl	480µl
10mM ATP	30µl	24µl
NANOpure® water to a volume of	**3ml**	**2.4ml**
Control Buffer		
5X Reaction Buffer A	50µl	40µl
NANOpure® water to a volume of	**0.25ml**	**0.2ml**
Protease Solution		
5X Termination Buffer A	600µl	480µl
Protease Reagent	120µl	96µl
NANOpure® water to a volume of	**3ml**	**2.4ml**
Stabilizer Solution		
5X Termination Buffer A	600µl	480µl
Stabilizer Reagent	3µl	2.4µl
NANOpure® water to a volume of	**3ml**	**2.4ml**

Step	96-Well	384-Well
1. Add test compound to each well, except for 16 control wells that receive only the vehicle (vehicle-only control).	5µl	1µl
2. Add Kinase Solution to each well.	20µl	4µl
3. Add ATP Solution to each well, except for 8 control wells that receive Control Buffer.	25µl	5µl
4. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).		
5. Add Protease Solution to all wells.	25µl	5µl
Note: The protease reaction is temperature sensitive. We strongly recommend performing this reaction at 22–25°C. Increasing or decreasing the temperature will change the dynamic range of the assay.		
6. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).		
7. Add Stabilizer Solution to all wells.	25µl	5µl
8. Mix plate, and read fluorescence at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal). Read plate at an excitation wavelength of 355nm and an emission wavelength of 460nm (AMC signal).		

3.E. Determining IC_{50} Values (one 96- or 384-well plate)

Based on your kinase titration (Section 3.B), choose an amount of enzyme that results in approximately 20% of the maximum signal in the assay (80% phosphorylation). Using less enzyme is possible, but the dynamic range for the assay will narrow.

1. Perform threefold serial dilutions of 10X concentrated inhibitor in a separate 96-well round-bottom plate in 10% DMSO (or equivalent vehicle), leaving one well as a vehicle-only control with no inhibitor. The initial 10X inhibitor should be in a solution of DMSO or equivalent vehicle.

   Example: Add 66µl of 10% DMSO to columns 1–11 in one row of the plate. Add 99µl of the highest concentration of 10X inhibitor to be tested into column 12. Serially dilute with a pipette by transferring 33µl to column 11, pipetting up and down to mix, and transferring 33µl to column 10. Repeat for columns 9 through 2. Discard the final 33µl, and do not dilute the inhibitor into column 1; this column is the vehicle-only control. All wells should now have 66µl.

3.E. Determining IC_{50} Values (continued)

2. **For 96-well plate:** Transfer 5µl of the appropriate concentration of diluted inhibitor (from dilution series performed in Step 1) to the appropriate wells of a solid-black, 96-well plate (columns 1–12, rows A–H). Column 1 should be your vehicle-only control, with columns 2–12 being your dilution series of inhibitor and column 12 being the highest concentration.

   Note: The final concentration of DMSO (or equivalent vehicle) will be 1%. The final concentration of inhibitor in each well will be 1X in a 50µl reaction.

   For 384-well plate: Transfer 1µl of the appropriate concentration of diluted inhibitor (from dilution series performed in Step 1) to the appropriate wells of a solid-black, 384-well plate (columns 1–12, rows A–H). Column 1 should be your vehicle-only control, with columns 2–12 being your dilution series of 10X inhibitor and column 12 being the highest concentration.

   Note: The 384-well protocol uses only one half of the plate.

   Note: The final concentration of DMSO (or equivalent vehicle) will be 1%. The final concentration of inhibitor in each well will be 1X in a 10µl reaction.

Reagent Preparation

3. Prepare the following solutions before performing the assay.

Component	96-Well	384-Well
Kinase Solution (1.25X)		
5X Reaction Buffer A	600µl	480µl
Control AMC Substrate	3µl	2.4µl
Src-Family Kinase R110 Substrate	3µl	2.4µl
$MnCl_2$, 300mM	20µl	16µl
Sodium Vanadate, 100mM	6µl	4.8µl
Kinase (determined by titration)	Xµl	Xµl
NANOpure® water to a volume of	**2.4ml**	**1.9ml**
ATP Solution		
5X Reaction Buffer A	400µl	320µl
10mM ATP	20µl	16µl
NANOpure® water to a volume of	**2ml**	**1.6ml**
Control Buffer		
5X Reaction Buffer A	400µl	320µl
NANOpure® water to a volume of	**2ml**	**1.6ml**
Protease Solution		
5X Termination Buffer A	600µl	480µl
Protease Reagent	120µl	96µl
NANOpure® water to a volume of	**3ml**	**2.4ml**

Component	96-Well	384-Well
Stabilizer Solution		
5X Termination Buffer A	600µl	480µl
Stabilizer Reagent	3µl	2.4µl
NANOpure® water to a volume of	**3ml**	**2.4ml**

Step	96-Well	384-Well
4. To the inhibitor titration series previously added in Steps 1 and 2 above, add Kinase Solution to each well in columns 1–12, rows A–H.	20µl	4µl
5. Add ATP Solution to wells in columns 1–12, rows A–D. Add Control Buffer to wells in columns 1–12, rows E–H.	25µl	5µl
6. Mix plate, and incubate for 60 minutes at room temperature (22–25°C).		
7. Add Protease Solution to all wells.	25µl	5µl

Note: The protease reaction is temperature sensitive. We strongly recommend performing this reaction at 22–25°C. Increasing or decreasing the temperature will change the dynamic range of the assay.

8. Mix plate, and incubate for 60 minutes at room temperature.		
9. Add Stabilizer Solution to all wells.	25µl	5µl
10. Mix plate, and read fluorescence at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal). Read plate at an excitation wavelength of 355nm and an emission wavelength of 460nm (AMC signal).		

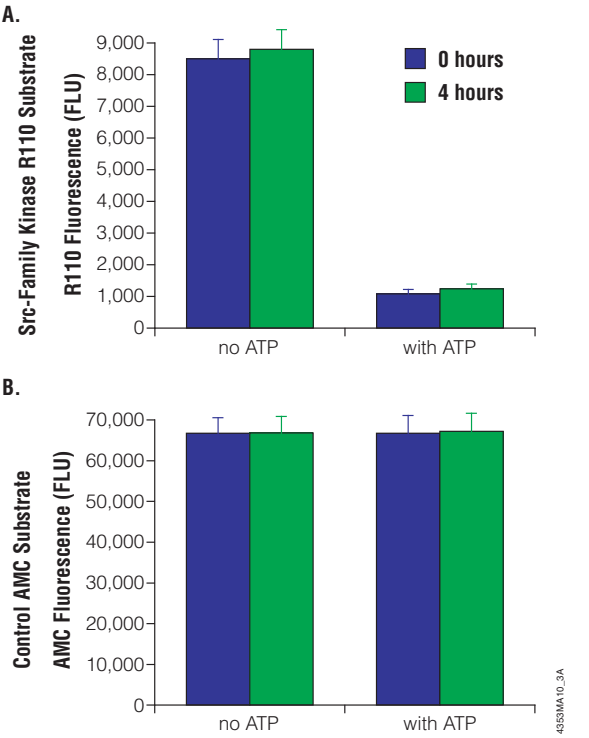


Figure 2. Stable fluorescent signal allows high-throughput batch processing. The bar chart shows averages of ± standard deviations of FLU values (n = 192) collected from an assay performed in a solid-black, flat-bottom 384-well plate using 12.5mU (~7.9ng) Src per well (Invitrogen Cat.# P3044) as described in Section 3.D, with and without 50µM ATP, at time 0 and 4 hours later. The signal increased less than 15% in four hours.

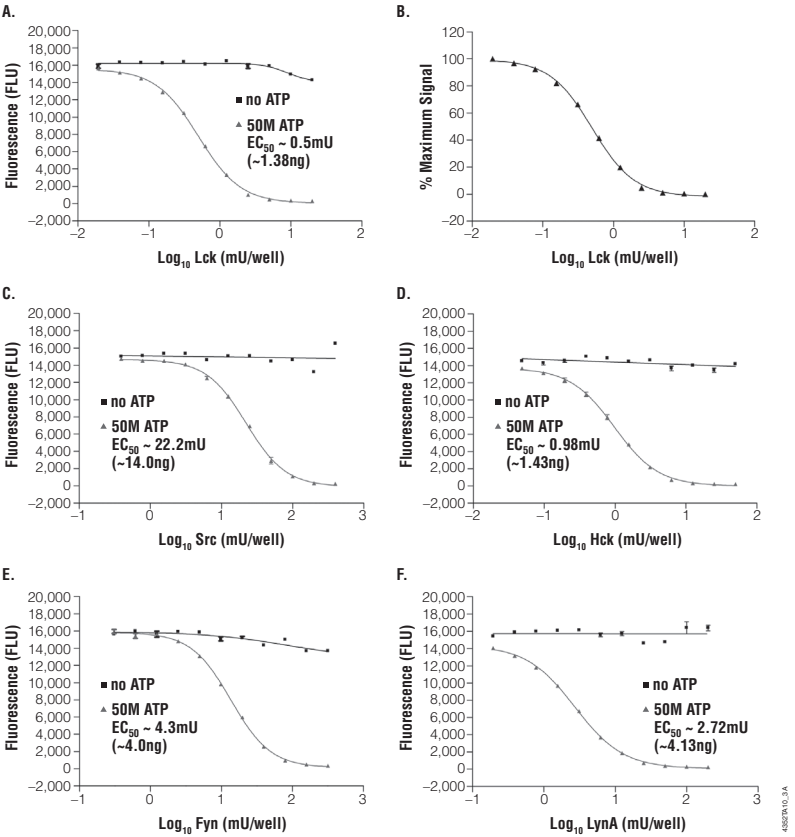


Figure 3. Kinase activity is inversely correlated with R110 fluorescence output. Results of titration curves that were performed according to the kinase titration protocol (Section 3.B) in solid-black, flat-bottom 96-well plates. **Panels A and B.** Results of a Lck titration (Upstate Biotech Cat.# 14-442). **Panel A.** Data collected (actual R110 FLU units) from the plate. Data points are the average of 4 determinations, and error bars are ± standard deviations. 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 per well. **Panel B.** Normalizing the data allows quick determination of the amount of kinase required for the desired percent conversion. **Panels C, D, E, and F**. Kinase titrations with Src (Invitrogen Cat.# P3044), Hck (Invitrogen Cat.# P2908), Fyn (Upstate Biotech Cat.# 14-441), and LynA (Calbiochem Cat.# 44021), respectively. **Note:** Due to low baseline, the software program displays a negative Y-axis value.

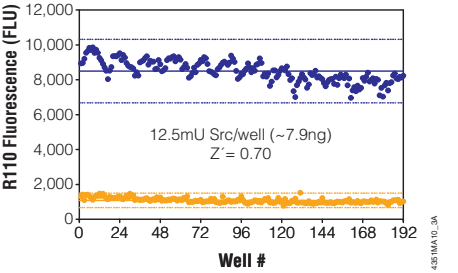
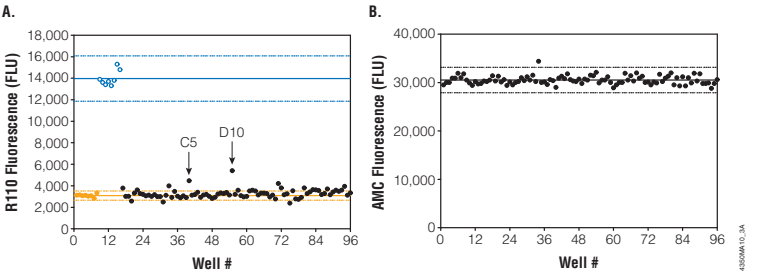


Figure 4. Z´-Factor value obtained in a 384-well plate. Results of a Z´-factor analysis are shown for a 384-well plate using the indicated amount of Src per well. The assay was performed manually according to Section 3.A in a solid-black, flat-bottom plate with 50µM ATP (light gray circles) or without ATP (black circles). Solid lines indicate the mean, and the dotted lines are ± 3 standard deviations. The Z´-factor for the assay under these conditions is 0.70 as calculated by the equation from Zhang, Chung and Oldenburg (6).



Well #	Compound	Description	% Inhibition
C5	Typhostin AG 494	Protein Tyr kinase inhibitor	12.7
D10	Tamoxifen citrate	Anti-Estrogen, PKC inhibitor	21.2

Figure 5. Results of a single 80-compound screen of plate 6 from the LOPAC library (RBI). The assay was performed as described in Section 3.D (except that the ATP concentration was 20µM) in a solid-black, flat-bottom 96-well plate with 100µM compounds in 10% DMSO. Final concentrations in the kinase reaction were 10µM compound and 1% DMSO. **Panel A** shows the R110 fluorescent signal using 1.25mU (~ 3.44ng) Lck per well (Upstate Biotech Cat.# 14-442). The light gray circles (n = 8) indicate wells without compound (1% DMSO only) in the presence of 20µM ATP, and the 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 20µM ATP. The compounds scored as hits are defined in the table below Panel A. **Panel B** shows the AMC fluorescent signal of the same plate. Notice that none of the compounds inhibited the protease.

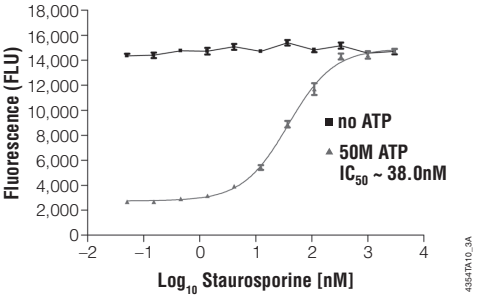


Figure 6. Accurate IC_{50} values. Results of a staurosporine titration in a solid black, flat-bottom 96-well plate using 1.25mU Lck per well (Upstate Biotech Cat.# 14-442). The assay was performed as described in Section 3.E. Data points are the average of 4 determinations, and error bars are ± standard deviations. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose-response (variable slope) software. These results are comparable to staurosporine IC_{50} values reported in the literature: 6.5nM for $p60^{src}$ (3) and 33nM for Lck at 5µM ATP; 95nm for Lck at 20µM ATP (4). IC_{50} values depend on the assay conditions and amount of enzyme used.

4. General Considerations

Temperature: Environmental factors that affect the rates of the protease and kinase reactions can influence fluorescence intensity. Temperature is one factor that affects the protease reaction rate and thus the fluorescence in the assay. For consistent results, equilibrate assay plates and reagents to a constant temperature prior to performing the assay. Insufficient equilibration may result in a temperature gradient effect between the wells in the center and on the edge of the plates. The temperature gradient pattern may also depend on the position of the plate in the stack. Incubating the plate outside of the 22–25°C range may result in changes in the dynamic range of the assay.

Chemicals: The chemical environment of the protease reaction will affect the enzymatic rate and thus fluorescence intensity. Some solvents used for the chemical compounds tested may interfere with the protease reaction. Interference with the protease reaction can be detected by assaying a parallel set of control wells without kinase and measuring the AMC signal. We have tested dimethylsulfoxide (DMSO), a commonly used vehicle to solubilize organic chemicals, at final concentrations up to 2% in the assay and found that it had minimal effect on fluorescence.

Standard kinase preparations are another potential source of interfering chemicals. Performing a kinase titration without ATP will determine if there is interference with the protease reaction. We have tested 100µM β-mercaptoethanol, 100µM DTT and 1% glycerol in the assay, and none had a significant effect on fluorescence (Table 2).

4. General Considerations (continued)

Table 2. Effect of Solvents and Additives on Fluorescence.

Chemical	Concentration in Kinase Reaction	Reduction in Signal
DMSO	2%	<25%
DTT	100µM	<15%
β-mercaptoethanol	100µM	<3%
glycerol	1%	<3%

Plate Recommendations: We recommend using standard opaque-walled multiwell plates suitable for fluorescence measurements. Black plates provide a better signal-to-noise ratio, while white plates provide greater fluorescence. Round-bottom plates also provide greater fluorescence than flat-bottom plates.

Specific Results with Low False-Negative Rate: Minimizing the number of false hits is important, and we have addressed this issue in the ProFluor® Kinase Assays. There are four possible outcomes from the combined effect of compounds on kinase and protease using the ProFluor® Src-Family Kinase Assay (Table 3). Compounds that inhibit the kinase but not the protease will result in high fluorescence, indicating a positive hit. Compounds that inhibit both the kinase and protease will result in low R110 fluorescence and will be missed as hits, but such compounds will not be desirable because they lack specificity. Compounds that do not inhibit the kinase or protease will show low R110 fluorescence. Compounds that do not inhibit the kinase but do inhibit the protease will show low fluorescence also.

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

Protease Inhibition: The Control AMC Substrate allows users to differentiate kinase from protease effects. If a compound inhibits the kinase but not the protease, users will observe high fluorescence for the AMC-containing substrate (460nm) and high fluorescence for the R110-containing substrate (527nm). A compound that inhibits the protease will exhibit low fluorescence at both 527 and 460nm. Using the Control AMC Substrate and kinase substrate in the same reaction or using the control substrate to screen for protease inhibition after screening against the kinase in separate reactions will ensure the validity of the positive hits.

Table 3. Possible Outcomes of the ProFluor® Src-Family Kinase 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.

5. References

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6. Composition of Buffers and Solutions

5X Reaction Buffer A

200mM	Tris-HCl (pH 7.5)
100mM	$MgCl_2$
0.5mg/ml	BSA

5X Termination Buffer A

200mM	Tris-HCl (pH 7.5)
500mM	EDTA
0.5mg/ml	BSA

7. Related Products

Protein Kinase Assay Systems

Product	Size	Cat.#
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241

SignaTECT® Protein Kinase Assay Systems

Product	Size	Cat.#
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT® Protein Tyrosine Kinase (PTK) Assay System	96 reactions	V6480
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System	96 reactions	V8161
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430

PepTag® Non-Radioactive Protein Kinase Assays

Product	Size	Cat.#
PepTag® Non-Radioactive PKC Assay	120 reactions	V5330
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay	120 reactions	V5340

Non-Radioactive Phosphatase Assay Systems

Product	Size	Cat. #
ProFluor® Ser/Thr PPase Assay	4 plate	V1260
	8 plate	V1261
ProFluor® Tyrosine Phosphatase Assay	4 plate	V1280
	8 plate	V1281
Serine/Threonine Phosphatase Assay System	96 reactions	V2460
Tyrosine Phosphatase Assay System	96 reactions	V2471

Protein Phosphatases

Product	Size	Cat.#
PPase-2A	25 units	V6311
PPase-2B	10 units	V6361

[a]U.S. Pat. No. 7,195,884 has been issued to Promega Corporation for methods and kits for transferases. Other patents are pending.

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