Technical Bulletin

ProFluor® Ser/Thr PPase Assay

INSTRUCTIONS FOR USE OF PRODUCTS V1260 AND V1261.

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ProFluor® Ser/Thr PPase Assay

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1. Description

The ProFluor® Ser/Thr PPase Assay[a] measures purified serine/threonine protein phosphatase activity in a multiwell plate format and involves "add, mix and read" steps only (Table 1). The assay works with protein phosphatase 1 (PP1), PP2A, PP2B, and PP2C. The assay begins with a standard phosphatase reaction performed in the reaction buffer with the provided phosphorylated bisamide rhodamine 110 peptide substrate (S/T PPase R110 Substrate) and Control AMC Substrate that serves as a control for compounds that may inhibit the protease. In this configuration, both the S/T PPase R110 Substrate and Control AMC Substrate are nonfluorescent (Figure 1; 1). Following the phosphatase reaction, adding a protease solution simultaneously stops the phosphatase reaction and completely digests the dephosphorylated S/T PPase R110 Substrate and the Control AMC Substrate, producing highly fluorescent rhodamine 110 and AMC. Phosphorylated S/T PPase R110 Substrate, however, is resistant to protease digestion and remains nonfluorescent. Thus, the R110 fluorescence intensity measured in the assay is correlated with phosphatase activity in the presence of active protease (Figure 3), and the AMC fluorescence intensity is an indication of protease activity. A compound that only inhibits the phosphatase will decrease the R110 fluorescent signal as will a compound that only inhibits the protease. To prevent protease inhibitors from being picked as "hits" during a screen, the AMC fluorescence signal should be examined carefully where a decrease in R110 fluorescence is observed.

The assay produces Z´ values greater than 0.8 in both 96- and 384-well plate formats (Figure 4) and produces IC_{50} values for known inhibitors that are comparable to those currently reported in the literature (Figure 5). The amount of phosphatase used per well is low (ng/well), and the fluorescence signal is stable (approximately 10 percent change of fluorescence intensity in 4 hours), allowing batch-plate reading (Figure 2).

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

Step	Description	96-Well	384-Well
1	Ser/Thr PPase diluted in Phosphatase Solution added	25µl	5µl
2	S/T PPase R110 Substrate and Control AMC Substrate added	25µl	5µl
3	Mix plate 15 seconds and incubate at room temperature (22–25°C) for 10 minutes (**PP1** and **PP2A**) or for 30 minutes (**PP2B** and **PP2C**)		
4	Protease Solution added	25µl	5µl
5	Mix plate and incubate for 90 minutes at room temperature (22–25°C)		
6	Stabilizer Solution added	25µl	5µl
7	Mix plate and read fluorescence at 485/530nm (R110) and 360/460nm (AMC)		

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.

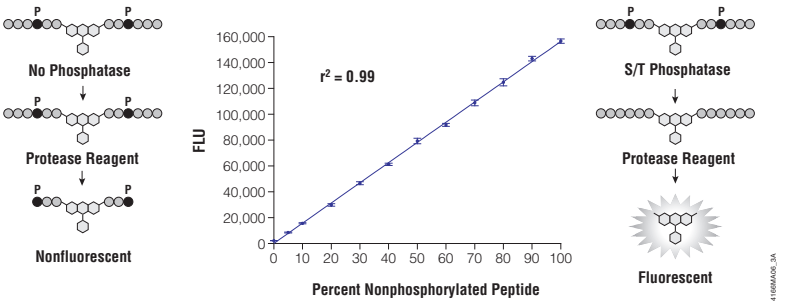

Figure 1. Effect of phosphopeptide content on fluorescence intensity. The graph shows the average FLU (n = 8) obtained after a 90-minute Protease Reagent digestion using mixtures of nonphosphorylated S/T PPase R110 Substrate and phosphorylated S/T PPase R110 Substrate as indicated to mimic a phosphatase 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 cartoons represent phosphorylated amino acids.

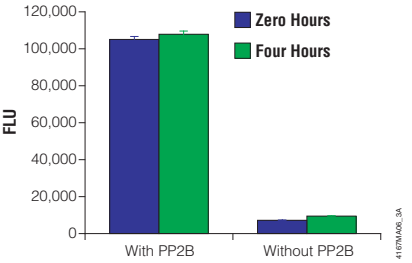

Figure 2. Stable fluorescence signal allows for high-throughput batch processing. The bar chart shows averages ± S.D. of FLU values (n = 48) collected from an assay performed in a solid black, flat-bottom 96-well plate using 0.2µg/well PPase-2B (Cat.# V6361) as described in Section 3, with and without PP2B, at time 0 and 4 hours later. The signal increased less than 10% in four hours.

2. Product Components and Storage Conditions

Product	Size	Cat. #
ProFluor® Ser/Thr PPase Assay	4 plate	V1260

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

- 12µl S/T PPase R110 Substrate, 10mM
- 12µl Control AMC Substrate, 10mM
- 240µl Protease Reagent
- 1ml $MgCl_2$, 1M
- 1ml $MnCl_2$, 20mM
- 1ml $CaCl_2$, 100mM
- 1ml $NiCl_2$, 100mM
- 12µl Stabilizer Reagent
- 6ml 5X Reaction Buffer B
- 5ml 5X Termination Buffer B

Product	Size	Cat. #
ProFluor® Ser/Thr PPase Assay	8 plate	V1261

Each system contains sufficient reagents for 8 × 96 assays at 100µl/assay in 96-well plates or 5 × 384 assays at 20µl/assay in 384-well plates. Includes:

- 24µl S/T PPase R110 Substrate, 10mM
- 24µl Control AMC Substrate, 10mM
- 480µl Protease Reagent
- 1ml $MgCl_2$, 1M
- 1ml $MnCl_2$, 20mM
- 1ml $CaCl_2$, 100mM
- 1ml $NiCl_2$, 100mM
- 24µl Stabilizer Reagent
- 12ml 5X Reaction Buffer B
- 10ml 5X Termination Buffer B

Storage Conditions: The entire contents of the system should be stored at –20°C. The S/T PPase R110 Substrate and Control AMC Substrate should be protected from light. For best results, make solutions fresh and use immediately. System components should be thawed on ice and returned to –20°C as soon as possible. The S/T PPase R110 Substrate is provided in 100% DMSO and therefore requires thawing at room temperature. Vortex each component before use.

3. Protocols for ProFluor® Ser/Thr PPase Assay

Materials to Be Supplied by the User

- black-walled multiwell plates
- multichannel pipettor or automated pipetting station
- plate shaker (DYNEX MICRO-SHAKER® II or equivalent)
- plate-reading fluorometer with filters capable of reading R110 and AMC fluorescence
- purified protein phosphatases
- okadaic acid (sodium salt recommended) (for PP1 and PP2A)
- calmodulin (for PP2B)

3.A. Phosphatase Titration

The volumes provided in this protocol are intended for a single 96-well plate or a single 384-well plate. To perform the assay with more than one plate simultaneously, scale up the solutions.

Reagent Preparation

Prepare the following solutions before performing the assay.

	Component	96-Well Plate	384-Well Plate
Phosphatase Dilution Solution			
	5X Reaction Buffer B	600µl	480µl
Additives for:			
PP1	$MgCl_2$, 1M	6µl	4.8µl
	$MnCl_2$, 20mM	60µl	48µl
PP2A	None		
PP2B	$CaCl_2$, 100mM	60µl	48µl
	$NiCl_2$, 100mM	60µl	48µl
	Calmodulin, 0.5mM*	12µl	9.6µl
PP2C	$MgCl_2$, 1M	120µl	96µl
	NANOPure® Water to a volume of:	**3ml**	**2.4ml**
*Provided by the user.			
Peptide Solution			
	5X Reaction Buffer B	600µl	480µl
	S/T PPase R110 Substrate, 10mM	3µl	2.4µl
	Control AMC Substrate, 10mM	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**

	Component	96-Well Plate	384-Well Plate
Protease Solution			
	5X Termination Buffer B	600µl	480µl
	Protease Reagent	60µl	48µl
Additives for:			
PP1	okadaic acid, 0.3mM*	30µl	24µl
PP2A	okadaic acid, 0.3mM*	3µl	2.4µl
PP2B	None		
PP2C	None		
	NANOPure® Water to a volume of:	**3ml**	**2.4ml**
*Provided by the user.			
Stabilizer Solution			
	5X Termination Buffer B	600µl	480µl
	Stabilizer Reagent	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**

96-Well Plate Protocol

1. Add 25µl Phosphatase Dilution Solution to columns 2 through 12.
2. Dilute Phosphatase into 0.5ml of Phosphatase Dilution Solution. Mix. Add 50µl to the wells in column 1. Serially dilute twofold with a multichannel pipettor by transferring 25µl to column 2. Mix by pipetting up and down. Transfer 25µl to column 3. Mix by pipetting up and down. Repeat for columns 4 though 11. The 25µl removed from column 11 should be discarded. Column 12 should have no enzyme as a control. All wells should now contain 25µl.
3. Add 25µl of Peptide Solution to all wells to start the reaction.
4. Mix the plate for 15 seconds, and incubate at room temperature (22–25°C) for 10 minutes (**PP1** and **PP2A**) or for 30 minutes (**PP2B** and **PP2C**).
5. Add 25µl Protease Solution to all wells.
6. Mix the plate, and incubate for 90 minutes at room temperature (22–25°C).
7. Add 25µl Stabilizer Solution to all wells.
8. Mix the plate and read at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal) and at an excitation wavelength of 360nm and an emission wavelength of 460nm (AMC signal).

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.

384-Well Plate Protocol

1. Add 5µl Phosphatase Dilution Solution to columns 2 through 12.
2. Dilute Phosphatase into 0.5ml of Phosphatase Dilution Solution. Mix. Add 10µl to the wells in column 1. Serially dilute twofold with a multichannel pipettor by transferring 5µl to column 2. Mix by pipetting up and down. Transfer 5µl to column 3. Mix by pipetting up and down. Repeat for columns 4 through 11. The 5µl removed from column 11 should be discarded. Column 12 should have no enzyme as a control. All wells should now contain 5µl.
3. Add 5µl Peptide Solution to all wells to start the reaction.
4. Mix the plate for 15 seconds, and incubate at room temperature (22–25°C) for 10 minutes (**PP1** and **PP2A**) or for 30 minutes (**PP2B** and **PP2C**).
5. Add 5µl Protease Solution to all wells.
6. Mix the plate, and incubate for 90 minutes at room temperature (22–25°C).
7. Add 5µl Stabilizer Solution to all wells.
8. Mix the plate, and read at an excitation wavelength of 485nm and an emission wavelength of 530nm (R110 signal) and at an excitation wavelength of 360nm and an emission wavelength of 460nm (AMC signal).

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.

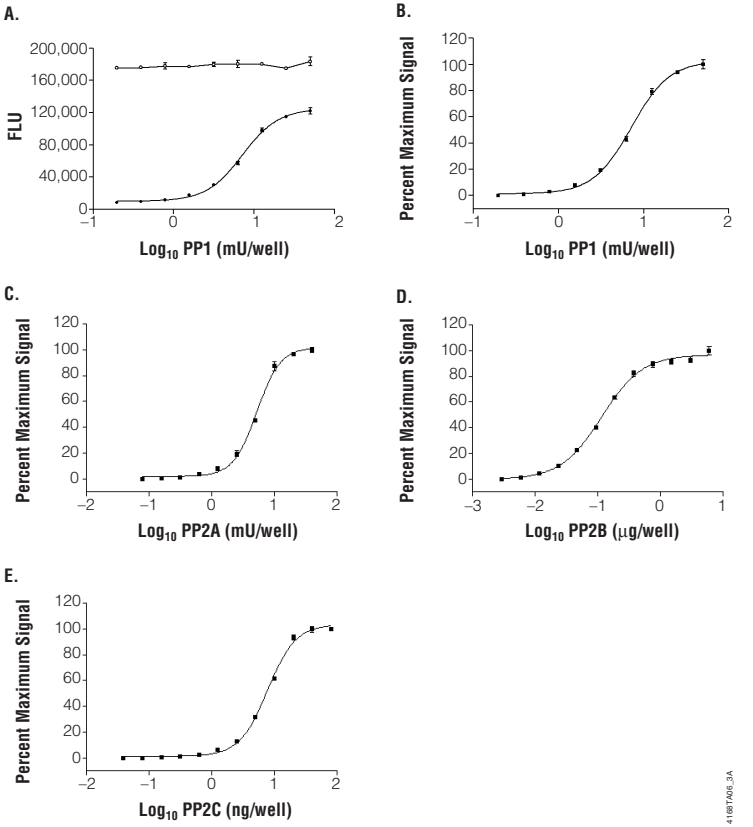

Figure 3. Phosphatase activity is directly correlated with R110 fluorescent output. Results show titration curves that were performed according to the phosphatase titration protocol (Section 3.A) in solid black, flat-bottom 96-well plates. The plates were read using an excitation wavelength of 485nm and an emission wavelength of 530nm (solid circles) and using an excitation wavelength of 360nm and an emission wavelength of 460nm (open circles). **Panels A and B** show the results of a PP1 titration. **Panel A** is data collected from the plate after Stabilizer Reagent was added. Data points are the average of 2 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 7.1 milliunits/well. Normalizing the data allows for a quick determination of the amount of PP1 required to provide the percent conversion desired (**Panel B**). **Panels C**, **D**, and **E** show percent maximum signal for titrations with PP2A (Upstate Biotech Cat.# 14-110), PP2B (Upstate Biotech Cat.# 14-390), and PP2C (Upstate Biotech Cat.#14-218).

3.B. Determining Z´-Factor

The volumes provided in this protocol are intended for a single 96-well plate or a single 384-well plate. To perform the assay with more than one plate simultaneously, scale up the solutions. Choose an amount of phosphatase from the phosphatase titration protocol that results in approximately 80% of maximum FLU (80% of maximum dephosphorylation). Using less phosphatase is possible, but the dynamic range of the assay will decrease.

Reagent Preparation

Prepare the following solutions before performing the assay.

	Component	96-Well Plate	384-Well Plate
Phosphatase Solution			
	5X Reaction Buffer B	600µl	480µl
	phosphatase (determined by titration)	Xµl	Xµl
Additives for:			
PP1	$MgCl_2$, 1M	4µl	3.2µl
	$MnCl_2$, 20mM	40µl	32µl
PP2A	None		
PP2B	$CaCl_2$, 100mM	40µl	32µl
	$NiCl_2$, 100mM	40µl	32µl
	Calmodulin, 0.5mM*	8µl	6.4µl
PP2C	$MgCl_2$, 1M	80µl	64µl
	NANOPure® Water to a volume of:	**2ml**	**1.6ml**

*Provided by the user.

Control Buffer			
	5X Reaction Buffer B	400µl	320µl
Additives for:			
PP1	$MgCl_2$, 1M	4µl	3.2µl
	$MnCl_2$, 20mM	40µl	32µl
PP2A	None		
PP2B	$CaCl_2$, 100mM	40µl	32µl
	$NiCl_2$, 100mM	40µl	32µl
	Calmodulin, 0.5mM*	8µl	6.4µl
PP2C	$MgCl_2$	80µl	64µl
	NANOPure® Water to a volume of:	**2ml**	**1.6ml**

*Provided by the user.

	Component	96-Well Plate	384-Well Plate
Peptide Solution			
	5X Reaction Buffer B	600µl	480µl
	S/T PPase R110 Substrate, 10mM	3µl	2.4µl
	Control AMC Substrate, 10mM	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**
Protease Solution			
	5X Termination Buffer B	600µl	480µl
	Protease Reagent	60µl	48µl
Additives for:			
PP1	okadaic acid, 0.3mM*	30µl	24µl
PP2A	okadaic acid, 0.3mM*	3µl	2.4µl
PP2B	None		
PP2C	None		
	NANOPure® Water to a volume of:	**3ml**	**2.4ml**

*Provided by the user.

Stabilizer Solution		
5X Termination Buffer B	600µl	480µl
Stabilizer Reagent	3µl	2.4µl
NANOpure® Water to a volume of:	**3ml**	**2.4ml**

Step	96-Well Plate	384-Well Plate
1. Add Control Buffer to Rows A through D (for 96-well plate) or Rows A through H (for 384-well plate)	25µl	5µl
2. Add Phosphatase Solution to Rows E through H (for 96-well plate), or Rows I through P (for 384-well plate).	25µl	5µl
3. Add Peptide Solution to each well to start the reaction.	25µl	5µl

4. Mix the plate, and incubate at room temperature (22–25C°)
   for 10 minutes (**PP1** and **PP2A**) or
   for 30 minutes (**PP2B** and **PP2C**).

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.

Step	96-Well Plate	384-Well Plate
5. Add Protease Solution to all wells.	25µl	5µl
6. Mix the plate, and incubate for 90 minutes at room temperature (22–25°C).		
7. Add Stabilizer Solution to all wells.	25µl	5µl
8. Mix the plate, and read at an excitation wavelength of 485nm and emission wavelength of 530nm (R110 signal) and at an excitation wavelength of 360nm and an emission wavelength of 460nm (AMC signal).		

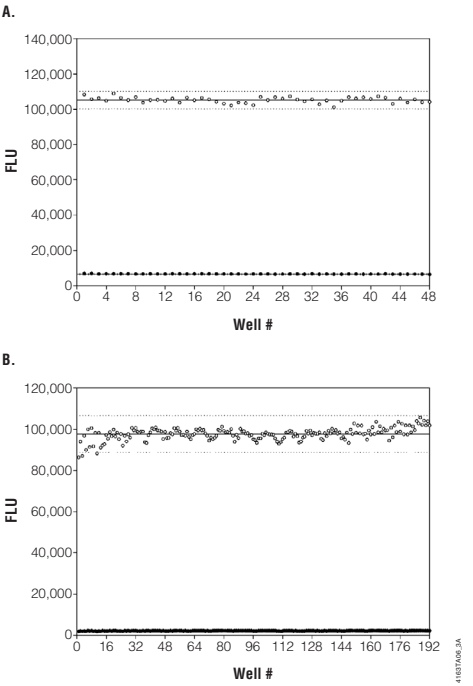

Figure 4. Z´-factor values obtained in both 96- and 384-well plates. Results of Z´ analysis are shown for 96-well plates (**Panel A**) and 384-well plates (**Panel B**). The assay was performed manually according to Section 3.B in solid black, flat-bottom plates with phosphatase (open circles) and without phosphatase (solid circles). Solid lines indicate the mean, and the dotted lines indicate ± 3 S.D. In **Panel A** 200ng/well PP2B (Upstate Biochemicals, Cat# 14-390) was used, and in **Panel B** 6.25 milliunits/well PP1 (Calbiochem Cat.# 539493) was used. Z´-factors were 0.94 and 0.85, respectively.

3.C. Screening for Phosphatase Inhibitors

The volumes provided in this protocol are intended for a single 96-well plate or a single 384-well plate. To perform the assay with more than one plate simultaneously, scale up the solutions. Choose an amount of phosphatase from the Phosphatase Titration protocol (Section 3.A) that results in approximately 80% of maximum FLU (80% of maximum dephosphorylation). Using less phosphatase is possible, but the dynamic range of the assay will decrease.

Reagent Preparation

Prepare the following solutions before performing the assay.

	Component	96-Well Plate	384-Well Plate
Phosphatase Solution			
	5X Reaction Buffer B	600µl	480µl
	phosphatase (determined by titration)	Xµl	Xµl
Additives for:			
PP1	$MgCl_2$, 1M	6µl	4.8µl
	$MnCl_2$, 20mM	60µl	48µl
PP2A	None		
PP2B	$CaCl_2$, 100mM	60µl	48µl
	$NiCl_2$, 100mM	60µl	48µl
	Calmodulin, 0.5mM*	12µl	9.6µl
PP2C	$MgCl_2$, 1M	120µl	96µl
	NANOpure® Water to a volume of:	**2ml****	**1.6ml****

*Provided by the user. **Volumes assume that 96-well plates contain 5µl of inhibitor solution and that 384-well plates contain 1µl of inhibitor solution.

Control Buffer

Same composition as the Phosphatase Solution above, except that no phosphatase is included.

Peptide Solution			
	5X Reaction Buffer B	600µl	480µl
	S/T PPase R110 Substrate, 10mM	3µl	2.4µl
	Control AMC Substrate, 10mM	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**

	Component	96-Well Plate	384-Well Plate
Protease Solution			
	5X Termination Buffer B	600µl	480µl
	Protease Reagent	60µl	48µl
Additives for:			
PP1	okadaic acid, 0.3mM*	30µl	24µl
PP2A	okadaic acid, 0.3mM*	3µl	2.4µl
PP2B	None		
PP2C	None		
	NANOPure® Water to a volume of:	**3ml**	**2.4ml**

*Provided by the user.

Stabilizer Solution			
	5X Termination Buffer B	600µl	480µl
	Stabilizer Reagent	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**

Step	96-Well Plate	384-Well Plate

Note: Volumes assume that 96-well plates contain 5µl of inhibitor solution and that 384-well plates contain 1µl of inhibitor solution.

1. Add compound to each well, except for 16 control wells that should receive only the vehicle (vehicle-only control).	5µl	1µl
2. Add Phosphatase Solution to each well, except for 8 of the control wells that should receive Control Buffer.	20µl	4µl
3. Add Peptide Solution to all wells.	25µl	5µl
4. Mix the plate, and incubate at room temperature (22–25°C) for 10 minutes (**PP1** and **PP2A**) or for 30 minutes (**PP2B** and **PP2C**).		

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.

5. Add Protease Solution to all wells.	25µl	5µl
6. Mix the plate, and incubate for 90 minutes at room temperature (22–25°C).		
7. Add Stabilizer Solution to all wells.	25µl	5µl

8. Mix the plate, and read at an excitation wavelength of 485nm and emission wavelength of 530nm (R110 signal) and at an excitation wavelength of 360nm and an emission wavelength of 460nm (AMC signal).

3.D. Determining IC_{50} Values

The volumes provided in this protocol are intended for a single 96-well plate or a single 384-well plate. To perform the assay with more than one plate simultaneously, scale up the solutions. Choose an amount of phosphatase from the phosphatase titration protocol (Section 3.A) that results in approximately 80% of maximum FLU (80% of maximum dephosphorylation). Using less phosphatase is possible, but the dynamic range of the assay will decrease.

Reagent Preparation

	Component	96-Well Plate	384-Well Plate
Phosphatase Dilution Solution			
	5X Reaction Buffer B	600µl	480µl
	phosphatase (determined by titration)	Xµl	Xµl
Additives for:			
PP1	$MgCl_2$, 1M	6µl	4.8µl
	$MnCl_2$, 20mM	60µl	48µl
PP2A	None		
PP2B	$CaCl_2$, 100mM	60µl	48µl
	$NiCl_2$, 100mM	60µl	48µl
	Calmodulin, 0.5mM*	12µl	9.6µl
PP2C	$MgCl_2$, 1M	120µl	96µl
	NANOPure® Water to a volume of:	**3ml**	**2.4ml**
*Provided by the user.			
Peptide Solution			
	5X Reaction Buffer B	600µl	480µl
	S/T PPase R110 Substrate, 10mM	3µl	2.4µl
	Control AMC Substrate, 10mM	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**

	Component	96-Well Plate	384-Well Plate
Protease Solution			
	5X Termination Buffer B	600µl	480µl
	Protease Reagent	60µl	48µl
Additives for:			
PP1	okadaic acid, 0.3mM*	30µl	24µl
PP2A	okadaic acid, 0.3mM*	3µl	2.4µl
PP2B	None		
PP2C	None		
	NANOPure® Water to a volume of:	**3ml**	**2.4ml**
*Provided by the user.			
Stabilizer Solution			
	5X Termination Buffer B	600µl	480µl
	Stabilizer Reagent	3µl	2.4µl
	NANOpure® Water to a volume of:	**3ml**	**2.4ml**

96-Well Plate Protocol

1. Add 25µl Phosphatase Solution to columns 2–12.
2. Dilute inhibitor into Phosphatase Solution. Mix. Add 50µl to the wells in column 1. Serially dilute with a multichannel pipettor by transfering 25µl to column 2. Mix by pipetting up and down. Transfer 25µl to column 3. Repeat for columns 4–11. The 25µl removed from column 11 should be discarded. Column 12 should have no inhibitor as control. All wells should now contain 25µl.
3. Add 25µl Peptide Solution to all wells to start the reaction.
4. Mix the plate, and incubate at room temperature (22–25°C)
   for 10 minutes (**PP1** and **PP2A**) or
   for 30 minutes (**PP2B** and **PP2C**).

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.

5. Add 25µl Protease Solution to all wells.
6. Mix the plate, and incubate for 90 minutes at room temperature (22–25°C).
7. Add 25µl Stabilizer Solution to all wells.
8. Mix the plate, and read at an excitation wavelength of 485nm and emission wavelength of 530nm (R110 signal) and at an excitation wavelength of 360nm and an emission wavelength of 460nm (AMC signal).

384-Well Plate Protocol

1. Add 5µl Phosphatase Solution to columns 2–12.
2. Dilute inhibitor into Phosphatase Solution. Mix. Add 10µl to the wells in column 1. Serially dilute with a multichannel pipettor by transferring 5µl to column 2. Mix by pipetting up and down. Transfer 5µl to column 3. Repeat for columns 4–11. The 5µl removed from column 11 should be discarded. Column 12 should have no inhibitor as control. All wells should now contain 5µl.
3. Add 5µl Peptide Solution to all wells to start the reaction.
4. Mix the plate, and incubate at room temperature (22–25°C)
   for 10 minutes (**PP1** and **PP2A**) or
   for 30 minutes (**PP2B** and **PP2C**).

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.

5. Add 5µl Protease Solution to all wells.
6. Mix the plate, and incubate for 90 minutes at room temperature (22–25°C).
7. Add 5µl Stabilizer Solution to all wells.
8. Mix the plate, and read at an excitation wavelength of 485nm and emission wavelength of 530nm (R110 signal) and at an excitation wavelength of 360nm and an emission wavelength of 460nm (AMC signal).

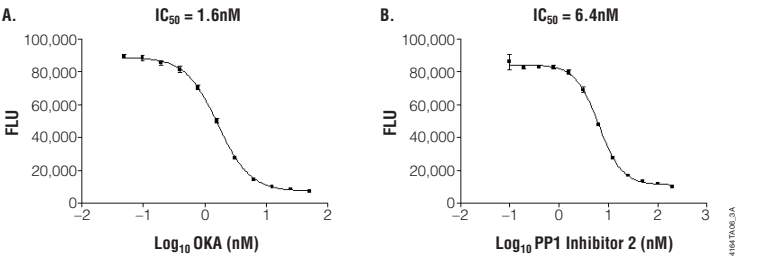

Figure 5. Accurate IC_{50} values. Results of inhibitor titrations for PP2A (Upstate Biochemicals Cat.# 14-111) using okadaic acid (LC Laboratories, Cat.# O-5857, **Panel A**) and PP1 (Calbiochem, Cat.# 539493) using PP1 Inhibitor 2 (**Panel B**). The assay was performed as described in Section 3.D in 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. The assay produced IC_{50} results in accordance with the published literature IC_{50} values of 0.6nM for okadaic acid for PP2A and 3.0nM for PP1 Inhibitor 2 for PP1 (2). Curve fitting was performed using GraphPad Prism® 3.0 sigmoidal dose-response (variable slope) software.

4. General Considerations

Temperature: Environmental factors that affect the rate of the protease and phosphatase reaction will result in a change in the intensity of fluorescence. Temperature is one factor that affects the protease and phosphatase activity and thus the maximum fluorescence signal and the dynamic range of 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 the wells at the edge of the plates. Incubating the plate outside of the 22–25°C range may result in changes in the dynamic range of the assay.

Additives: The chemical environment of the protease reaction will affect the enzymatic activity and thus fluorescence intensity. It is possible that solvents used for the various chemical compounds tested may interfere with the protease reaction. The protease will not completely digest the substrate (S/T PPase R110 Substrate) unless it is dephosphorylated by the phosphatase. To detect solvent interference, incubate the protease with or without solvent in the presence of the Control AMC Substrate.

Dimethylsulfoxide (DMSO), which is commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the phosphatase reaction and found to reduce fluorescence by less than 25%. Standard phosphatase preparations are potentially another source of interfering chemicals. We have tested 100µM β-mercaptoethanol (β-ME), 100µM DTT, and 1% glycerol in the phosphatase reaction, and the effects on fluorescence are given in Table 2.

Table 2. Effect of Additives on Fluorescence.

Additive	Final Concentration in Phosphatase Reaction	Effect
DMSO	2%	≤25%
DTT	100µM	≤15%
β-ME	100µM	≤3%
Glycerol	1%	≤3%

Plate Recommendations: Standard black-walled multiwell plates suitable for fluorescence measurements are recommended for this assay. 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.

Phosphatases: We recommend using phosphatase preparations that are protease-free in our ProFluor® Ser/Thr PPase Assay. To determine if the enzyme preparation has protease contamination, perform the phosphatase titration with both substrates (R110 and Control AMC), then read them before adding protease solution. If there is contamination, high fluorescent signal is expected across the enzyme titration before adding protease solution.

Protease Inhibition and Assay Results: A compound that only inhibits the phosphatase will decrease the R110 fluorescent signal as will a compound that only inhibits the protease. To prevent protease inhibitors from being picked as "hits" during a screen, the AMC fluorescence signal should be examined carefully where a decrease in R110 fluorescence was observed. If the AMC fluorescence signal is as strong as control wells that have active phosphatase, then the compound is not inhibiting the protease. However, if the AMC fluorescence signal is decreased, then the compound is inhibiting the protease reagent, and no call can be made with respect to the compound's effect on the phosphatase. Titrating screening hits that decrease R110 fluorescence and have no effect on AMC fluorescence will allow you to determine accurate IC_{50} values.

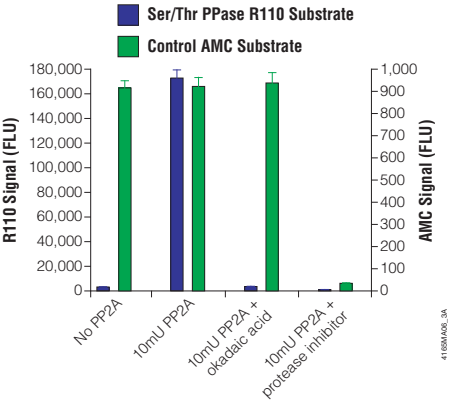

Figure 6. Use of the Control AMC Substrate to distinguish between effects on phosphatase and on protease. Dark bars indicate fluorescence using excitation at 485nm and emission at 530nm (S/T PPase R110 Substrate), and grey bars indicate fluorescence using excitation at 355nm and emission at 460nm (Control AMC Substrate). The assay was performed as described in Section 3.D 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 compound that only inhibits the phosphatase will produce a decrease in R110 fluorescence but not AMC fluorescence, while a protease inhibitor will decrease both fluorescence signals.

5. References

1. Leytus, S.P., Melhado, L.L. and Mangel, W.F. (1983) Rhodamine-based compounds as fluorogenic substrates for serine proteases. *Biochem. J.* **209**, 299–307.

2. Sheppeck, J.E., Gauss, C.M. and Chamberlin, A.R. (1997) Inhibition of the Ser/Thr phosphatases PP1 and PP2A by naturally occuring toxins. *Biorganic. Med. Chem.* **5**, 1739–50.

6. Composition of Buffers and Solutions

5X Reaction Buffer B

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

5X Termination Buffer B

200mM Tris-HCl (pH 8.0)
500mM EDTA
500mM EGTA
0.5mg/ml BSA

7. Related Products

Non-Radioactive Phosphatase Assay Systems

Product	Size	Cat. #
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

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

[a]Patent Pending.