Technical Bulletin

ProFluor® PKA Assay

INSTRUCTIONS FOR USE OF PRODUCTS V1240 AND V1241.

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ProFluor® PKA Assay

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1. Description

The ProFluor® PKA Assay[a] measures protein kinase A activity using purified kinase 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 a provided Bisamide Rhodamine 110 peptide substrate (PKA R110 Substrate). In this configuration, the PKA R110 Substrate is nonfluorescent (Figure 1; Reference 1). Following the kinase reaction, addition of a Termination Buffer, which contains a Protease Reagent, simultaneously stops the kinase reaction and removes amino acids specifically from the nonphosphorylated PKA R110 Substrate, resulting in the production of highly fluorescent Rhodamine 110. The phosphorylated PKA R110 Substrate, however, is resistant to digestion by the Protease Reagent and remains nonfluorescent. Thus, the fluorescence intensity measured in the assay is inversely correlated with kinase activity (Figure 3). The assay produces Z´ values greater than 0.8 in either 96- or 384-well plate formats (Figure 4) and easily distinguishes known PKA inhibitors from other compounds (Figure 5). The amount of kinase used per well is very low (ng/well), and the assay produces IC_{50} values for known inhibitors that are comparable to those currently reported in the literature (Figure 6). The fluorescence signal is very stable (≤10 percent change of fluorescence intensity in 4 hours), allowing batch plate reading (Figure 2).

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

Step	Description	96-Well	384-Well
1.	Dilute Kinase and PKA R110 Substrate in 1X Reaction Buffer.	25µl	5µl
2.	Dilute ATP in 1X Reaction Buffer.	25µl	5µl
3.	Mix plate 15 seconds and incubate 20 minutes at room temperature.		
4.	Dilute Protease Reagent in 1X Termination Buffer.	25µl	5µl
5.	Mix plate and incubate for 30 minutes at room temperature.		
6.	Dilute Stabilizer Reagent in 1X Termination Buffer.	25µl	5µl
7.	Mix plate and read at 485/530nm.		

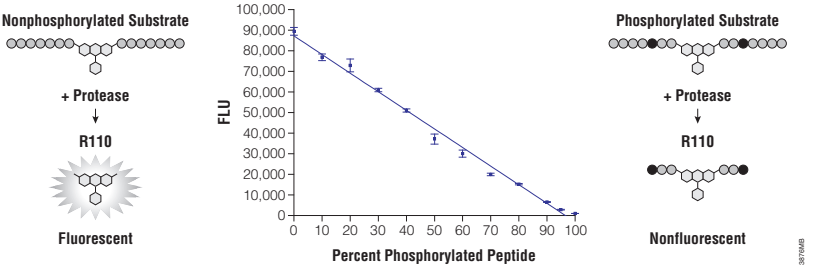

Figure 1. Schematic and graph demonstrating that the presence of a phosphorylated amino acid (black circles) blocks the removal of amino acids by the protease. The graph shows the average FLU (n = 6) obtained after a 30-minute Protease Reagent digestion using mixtures of nonphosphorylated PKA R110 Substrate and phosphorylated PKA R110 Substrate as indicated. The total peptide concentration was 5µM in 50µl of Reaction Buffer A to which 25µl of Protease Reagent diluted in Termination Buffer A was added. (FLU = Fluorescence Light Unit, excitation wavelength 485nm, emission wavelength 530nm; $r^2 = 0.992$).

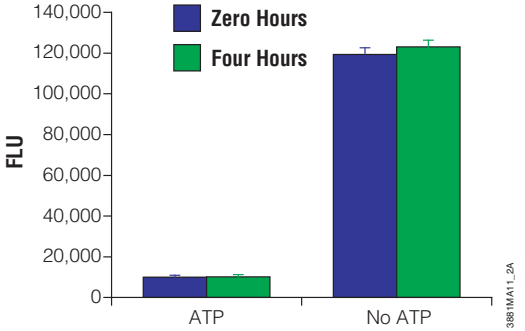

Figure 2. Stable fluorescence signal allows for high-throughput batch processing. The bar chart shows averages ± S.D. of FLU values (n = 48) collected from an assay performed in a solid black, flat-bottom 96-well plate using 0.25 units/well of PKA (Cat.# V5161) as described in Section 3, with and without ATP, at time 0 and 4 hours later. The average of 5 such determinations on each plate indicated that the signal increased less than 10% in four hours.

2. Product Components and Storage Conditions

Product	Size	Cat. #
ProFluor® PKA Assay	4 plate	V1240

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 PKA R110 Substrate, 10mM
- 250µl rATP, 10mM
- 240µl Protease Reagent
- 12µl Stabilizer Reagent
- 6ml 5X Reaction Buffer A
- 5ml 5X Termination Buffer A

Product	Size	Cat. #
ProFluor® PKA Assay	8 plate	V1241

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

- 24µl PKA R110 Substrate, 10mM
- 500µl rATP, 10mM
- 480µl Protease Reagent
- 24µl Stabilizer Reagent
- 12ml 5X Reaction Buffer A
- 10ml 5X Termination Buffer A

2. Product Components and Storage Conditions (continued)

Storage Conditions: The entire contents of the kit should be stored at –20°C. The PKA R110 Substrate should be protected from light. For best results, make solutions fresh and use immediately. Kit components should be thawed on ice and returned to –20°C as soon as possible. The PKA R110 Substrate is provided in 100% DMSO and therefore requires thawing at room temperature.

3. Protocols for ProFluor® PKA Assay

Materials to Be Supplied by the User

- opaque-walled multiwell plates
- multichannel pipettor or automated pipeting station
- plate shaker (DYNEX MICRO-SHAKER® II or equivalent)
- fluorometer capable of reading multiwell plates
- purified protein kinase A

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

3.A. Kinase 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 solution and buffer recipes as appropriate.

Reagent Preparation

1. Prepare the following solutions before performing the assay.

	Component	96-Well Plate	384-Well Plate
Kinase Dilution Solution	5X Reaction Buffer	600µl	480µl
	PKA R110 Substrate	3µl	2.4µl
	NANOpure® water to a volume of:	**3ml**	**2.4ml**
ATP Solution	5X Reaction Buffer A	400µl	320µl
	rATP	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	60µl	48µl
	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

If processing a 384-well plate, go to Step 11.

2. Add 25µl Kinase Dilution Solution to columns 2 through 12.

3. Dilute PKA into 0.5ml of Kinase Dilution Solution. Mix. Add 50µl to the wells in column 1. Serially dilute with a multichannel pipettor by transferring 25µl to column 2. Mix by pipetting. Transfer 25µl to column 3. Mix by pipetting. Repeat for columns 4 though 12. The 25µl removed from column 12 should be discarded. All wells should now contain 25µl.

   Note: Promega cAMP-Dependent Protein Kinase, Catalytic Subunit (Cat.# V5161) activity will vary from lot to lot. We recommend a starting dilution that contains 0.08 units/µl.

4. Add 25µl Control Buffer to Rows A through D.

5. Add 25µl ATP Solution to Rows E through H.

6. Mix the plate for 15 seconds and incubate for 20 minutes at room temperature.

7. Add 25µl Protease Solution to all wells.

8 Mix plate and incubate for 30 minutes at room temperature.

9. Add 25µl Stabilizer Solution to all wells.

10. Mix plate and read at an excitation wavelength of 485nm and an emission wavelength of 530nm.

    Note: Fluorescent signal shows minimal change over 4 hours or more, allowing for batch plate processing.

384-Well Plate Protocol

11. Add 5µl Kinase Dilution Solution to columns 2 through 12.

12. Dilute PKA into 0.5ml of Kinase Dilution 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. Transfer 5µl to column 3. Mix by pipetting. Repeat for columns 4 through 12. The 5µl removed from column 12 should be discarded. All wells should now contain 5µl.

    Note: Promega cAMP-Dependent Protein Kinase, Catalytic Subunit (Cat.# V5161) activity will vary from lot to lot. We recommend a starting dilution that contains 0.08 units/µl.

13. Add 5µl Control Buffer to Rows A through D.

14. Add 5µl ATP Solution to Rows E though H.

15. Mix the plate for 15 seconds and incubate for 20 minutes at room temperature.

3.A. Kinase Titration (continued)

384-Well Plate Protocol (continued)

16. Add 5µl Protease Solution to all wells.
17. Mix the plate and incubate for 30 minutes at room temperature.
18. Add 5µl Stabilizer Solution to all wells.
19. Mix the plate and read at an excitation wavelength of 485nm and an emission wavelength of 530nm.

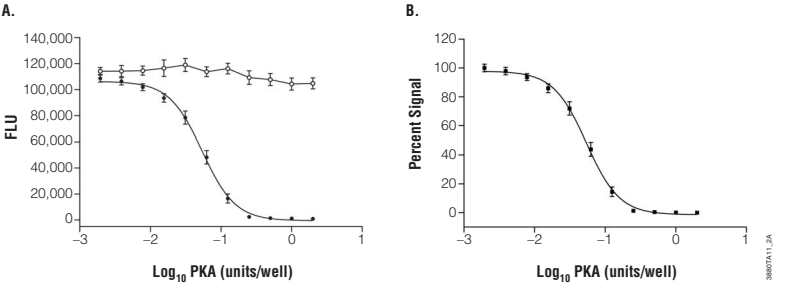

Figure 3. Amount of kinase activity is inversely correlated with fluorescent output. Results of an assay that was performed according to the kinase titration protocol (Section 3.A) in a solid black, flat-bottom 96-well plate with ATP (solid circles) and without ATP (open circles) are shown in **Panel A**. Data points are the average of 4 determinations and error bars are ± S.D. Curve fitting was performed using GraphPad Prism® 3.0 sigmoidal dose-response (variable slope). The R^2 value is 0.99, EC_{50} is 0.05 units/well PKA (Cat.# V5161). Normalizing the data allows for a quick determination of the amount of PKA required to provide the percent conversion desired (**Panel B**).

Note: EC_{50} is the amount of kinase needed to achieve 50% of maximum signal. This generally equates to 50% phosphorylation.

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 solution and buffer recipes as appropriate.

Choose an amount of kinase from the kinase titration protocol that results in approximately 20% of maximum FLU (80% of maximum phosphorylation). Using less kinase is possible, but the dynamic range of the assay will decrease.

Reagent Preparation

1. Prepare the following solutions before performing the assay:

	Component	96-Well Plate	384-Well Plate
Kinase Solution	5X Reaction Buffer A	600µl	480µl
	PKA R110 Substrate	3µl	2.4µl
	PKA (determined by titration)	Xµl	Xµl
	NANOpure® water to a volume of:	**3ml**	**2.4ml**
ATP Solution	5X Reaction Buffer A	400µl	320µl
	rATP	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	60µl	48µl
	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 Plate	384-Well Plate
2.	Add Kinase Solution to each well.	25µl	5µl
3.	Add Control Buffer to Rows A through D.	25µl	5µl
4.	Add ATP Solution to Rows E through H.	25µl	5µl
5.	Mix plate and incubate for 20 minutes at room temperature.	25µl	5µl
6.	Add Protease Solution to all wells.	25µl	5µl
7.	Mix plate and incubate for 30 minutes at room temperature.	25µl	5µl
8.	Add Stabilizer Solution to all wells.	25µl	5µl
9.	Mix plate and read at an excitation wavelength of 485nm and emission wavelength of 530nm.	–	5µl

Note: Fluorescent signal shows minimal change over 4 hours or more, allowing for batch plate processing.

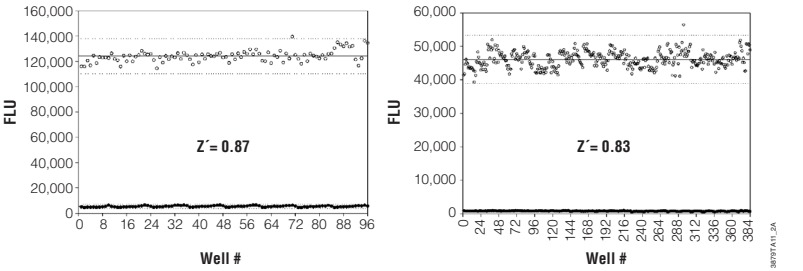

Figure 4. Z´ values obtained in both 96- and 384-well plates. Results of Z´ analysis are shown for 96-well plates (**Panel A**) and 384-well plates (**Panel B**). The assay was performed manually according to Section 3.B in solid black, flat-bottom plates with ATP (solid circles) and without ATP (open circles). Solid lines indicate the mean, and the dotted lines indicate ±3 S.D. Amount of PKA (Cat.# V5161) used was 0.125 units/well in 96-well plates and 0.05 units/well in 384-well plates.

3.C. Screening

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 solution and buffer recipes as appropriate.

Choose an amount of kinase from the Kinase Titration Protocol that results in approximately 20% of maximum FLU (80% of maximum phosphorylation). Using less kinase is possible, but the dynamic range of the assay will decrease.

Reagent Preparation

1. Prepare the following solutions before performing the assay:

	Component	96-Well Plate	384-Well Plate
Kinase Solution	5X Reaction Buffer A	600µl	480µl
	PKA R110 Substrate	3µl	2.4µl
	PKA (determined by titration)	Xµl	Xµl
	NANOpure® water to a volume of:	**2.4ml**	**1.9ml**
ATP Solution	5X Reaction Buffer A	600µl	480µl
	rATP	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	60µl	48µl
	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 Plate	384-Well Plate
2.	Add compound to each well, except for the 16 control wells that should receive only the vehicle (vehicle-only control).	5µl	1µl
3.	Add Kinase Solution to each well.	20µl	4µl
4.	Add ATP Solution to each well, except 8 of the control wells that should receive Control Buffer.	25µl	5µl
5.	Mix plate and incubate for 20 minutes at room temperature.	25µl	5µl
6.	Add Protease Solution to all wells.	25µl	5µl
7.	Mix plate and incubate for 30 minutes at room temperature.	–	–
8.	Add Stabilizer Solution to all wells.	25µl	5µl
9.	Mix plate and read at an excitation wavelength of 485nm and emission wavelength of 530nm.	–	5µl

Note: Fluorescent signal shows minimal change over 4 hours or more, allowing for batch plate processing.

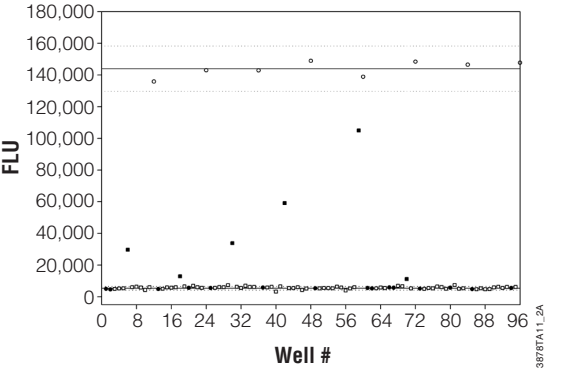

Figure 5. Easy identification of PKA inhibitors. Results from a single 72-compound screen of plate 6 from the LOPAC library (RBI). The assay was performed as described in Section 3.C in a solid black, flat-bottom 96-well plate with 100µM compounds in 10% DMSO. Final concentrations in the kinase reaction were 10µM compound and 1% DMSO. Solid circles represent wells without compounds (n = 16), open circles represent wells without ATP (n = 8). Solid lines indicate means, and dotted lines indicate ± 3 standard deviations of these populations. Squares are wells containing library compounds. Solid squares were scored as hits and are defined in Table 2 below.

Table 2. "Hits" Identified by Solid Squares in Figure 5.

Well #	Compound	% Inhibition
6	HA-1004	17.6
18	H-7	5.5
30	H-8	20.6
42	H-9	38.8
59	U-73122	71.9
70	GW5074	4.2

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 Solution and Buffer recipes as appropriate.

Reagent Preparation

1. Prepare the following solutions before performing the assay:

	Component	96-Well Plate	384-Well Plate
Kinase Solution	5X Reaction Buffer A	600µl	480µl
	PKA R110 Substrate	3µl	2.4µl
	PKA (determined by titration)	Xµl	Xµl
	NANOpure® water to a volume of:	**3ml**	**2.4ml**
ATP Solution	5X Reaction Buffer A	600µl	480µl
	rATP	30µl	24µl
	NANOpure® water to a volume of:	**3ml**	**2.4ml**
Protease Solution	5X Termination Buffer A	600µl	480µl
	Protease Reagent	60µl	48µl
	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

2. Add 25µl of Kinase Solution to columns 2–12.
3. Dilute inhibitor into Kinase Solution. Mix. Add 50µl to the wells in column 1. Serially dilute with a multichannel pipettor by transferring 25µl to column 2. Mix by pipeting. Transfer 25µl to column 3. Repeat for columns 4–12. The 25µl removed from column 12 should be discarded. All wells should now contain 25µl.
4. Add 25µl of ATP Solution to all wells.
5. Mix plate and incubate for 20 minutes at room temperature.
6. Add 25µl of Protease Solution to all wells.
7. Mix the plate and incubate for 30 minutes at room temperature.

   Note: Fluorescent signal shows minimal change over 4 hours or more, allowing for batch plate processing.
8. Add 25µl of Stabilizer Solution to all wells.
9. Mix plate and read at an excitation wavelength of 485nm and emission wavelength of 530nm.

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

384-Well Plate Protocol

10. Add 5µl Kinase Solution to columns 2–12.
11. Dilute inhibitor into Kinase Solution. Mix. Add 10µl to the wells in column 1. Serially dilute with a multichannel pipettor by transferring 5µl to column 2. Mix by pipeting. Transfer 5µl to column 3. Repeat for columns 4–12. The 5µl removed from column 12 should be discarded. All wells should now contain 5µl.
12. Add 5µl ATP Solution to all wells.
13. Mix plate and incubate for 20 minutes at room temperature.
14 Add 5µl Protease Solution to all wells.
15. Mix plate and incubate for 30 minutes at room temperature.

    Note: Fluorescent signal shows minimal change over 4 hours or more, allowing for batch plate processing.
16. Add 5µl Stabilizer Solution to all wells.
17. Mix plate and read at an excitation wavelength of 485nm and emission wavelength of 530nm.

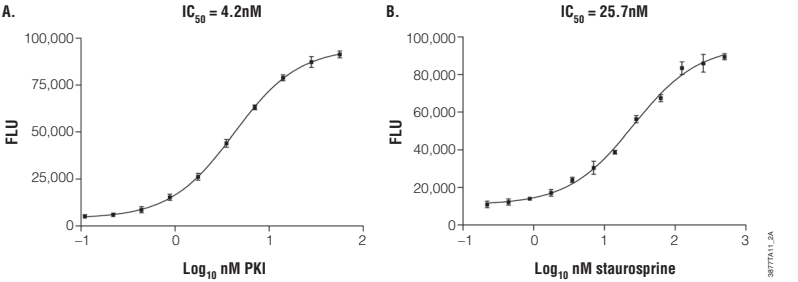

Figure 6. Accurate IC_{50} values. Results of inhibitor titrations for PKA Peptide Inhibitor (Cat.# V5681, **Panel A**) and staurosporine (**Panel B**). The assay was performed as described in Section 3.D in solid black, flat-bottom 96-well plates using 0.25 units/well PKA (Cat.# V5161) and the indicated amount of inhibitor. Data points are the average of 4 determinations, and error bars are ± standard deviation. The assay produced IC_{50} results in accordance with the published literature IC_{50} values of 3.0nM for PKI (2) and 18nM for staurosporine (3). Curve fitting was performed using GraphPad Prism® 3.0 sigmoidal dose-response (variable slope).

Note: IC_{50} is the inhibitor amount required to achieve 50% of maximum signal.

4. General Considerations

Temperature: Environmental factors that affect the rate of the protease and kinase reaction will result in a change in the intensity of fluorescence. Temperature is one factor that affects the rate of the protease and kinase activity and thus the fluorescence signal 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.

Solvents: 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. Interference with the protease reaction can be determined by assaying a parallel set of control wells without the kinase. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and found to have a minimal effect on fluorescence, less than 25% change.

Standard kinase preparations are potentially another source of interfering chemicals. Performing a kinase titration experiment without ATP will determine if there is any interference with the protease reaction. We have tested 100µM β-mercaptoethanol, 100µM DTT, and 1% glycerol in the kinase reaction and none had a significant effect on fluorescence (Table 3).

Table 3. Effect of Solvents on Fluorescence.

Chemical	Concentration in Kinase Reaction	Effect
DMSO	2%	≤25%
DTT	100µM	≤15%
β-ME	100µM	≤3%
Glycerol	1%	≤3%

These data are based on three determinations.

Plate Recommendations: Standard opaque-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.

Protease Inhibition and Assay Results: Compounds that only inhibit the kinase will result in increased fluorescence and are easily distinguishable from compounds that only inhibit protease activity, which decrease fluorescence. Compounds that inhibit both the protease and kinase may increase, decrease or have no effect on fluorescence, depending on the level of inhibition directed toward the kinase and protease. Titration of screening hits will allow for the determination of accurate IC_{50} values.

5. References

1. Leytus, S.P., Mehado, L.L. and Mangel, W.F. (1983) Rhodamine-based compounds as fluorogenic substrates for serine proteases. *Biochem. J.* **209**; 299–307.
2. Walsh, D.A. and Glass, D.B. (1991) Utilization of the inhibitor protein of adenosine cyclic monophosphate dependent protein kinase, and peptides derived from it, as tools to study adenosine cyclic monophosphate-mediated cellular processes. *Meth. Enzymol.* **201**, 304–16.
3. Tamaoki, T. (1991) Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Meth. Enzymol.* **201**, 340–7.
4. Flockhart, D.A. and Corbin, J.D. (1982) Regulatory mechanisms in the control of protein kinases. *CRC Crit. Rev. Biochem.* **12**, 133–86.

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. ATP Titration Curve

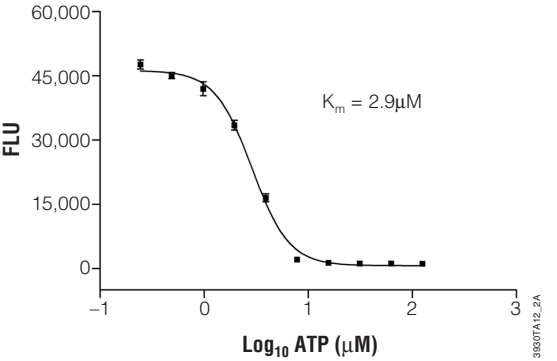

Figure 7. ATP Titration. The assay was performed using a modification of the protocol described in Section 3.D in solid-white, flat-bottom, 96-well plates using 0.5 units/well PKA (Cat.# V5161) and the indicated amount of ATP. Data points are the average of 4 determinations and error bars are ± S.D. The assay produced a K_m for ATP of 9.2µM in accordance with the published literature K_m value for PKA of 3-15µM (4). Curve fitting was performed using GraphPad Prism® 3.0 software sigmoidal dose-response (variable slope).

8. Related Products

Protein Kinase A Assays

Product	Size	Cat. #
SignaTECT® cAMP-Dependent Protein Kinase Assay System	96 reactions	V7480
PepTag® Non-Radioactive PKC Assay	120 reactions	V5330
PepTag® Non-Radioactive cAMP-Protein Kinase Assay	120 reactions	V5340

Protein Kinase A

Product	Size	Cat.#
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500u	V5161

Protein Kinase A Inhibitors and Substrates

Product	Size	Cat.#
InCELLect® AKAP St-Ht31 Inhibitor Peptide	150µl	V8211
InCELLect® St-Ht31P Control Peptide	150µl	V8221
cAMP-Dependent Protein Kinase Peptide Inhibitor	1mg	V5681
Kemptide (PKA) Peptide Substrate	1mg	V5601

Universal Kinase Assay

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

[a]Patent Pending.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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