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I. Introduction

Signal transduction is one of the most widely studied areas in biology. Extracellular information perceived at the surface of a cell must be translated into an intracellular response that involves a complex network of interwoven signaling cascades. These signaling events regulate cellular responses like proliferation, differentiation, secretion and apoptosis. Signal transduction cascades are generally triggered by the binding of ligands, such as growth factors, cytokines, neurotransmitters, or hormones, to a receptor. These receptors transmit the stimulus to the interior of the cell, where the signal is amplified and directed through a signaling pathway.

The propagation of the primary signal involves a wide array of enzymes with specialized functions. Many of these signaling enzymes propagate the signal by post-translationally modifying other proteins. Protein phosphorylation, one of the most common post-translational modifications, plays a dominant role in almost all signaling events and involves the transfer of a phosphate group from adenosine triphosphate (ATP) to the target protein (van der Geer *et al*. 1994). In general, phosphorylation either activates or inactivates a given protein to perform a certain function. Protein kinases and phosphatases are the enzymes responsible for determining the phosphorylation state of cellular proteins and, thus, whether a signal gets transduced within a cell. Changes in the level, subcellular localization and activity of kinases and phosphatases have consequences for normal cell function and maintenance of cellular homeostasis (De Meyts, 1995; Denton and Tavare, 1995).

The human genome is reported to contain 518 protein kinases that are involved in phosphorylation of 30% all cellular proteins (Manning *et al*. 2002). Taken together, genes for protein kinases and phosphatases represent five percent of the human genome (Cohen, 2001). Many other phosphotransferases play equally important roles in cellular reactions that use ATP as substrate but are not classified as protein kinases. These include PI3-kinases (Shears, 2004), lipid kinases such as sphingosine kinases (French *et al*. 2003) and sugar kinases such as glucokinase (Grimsby *et al*. 2003). Changes in the level, activity or localization of these kinases, phosphotransferases and phosphatases greatly influence the regulation of key cellular processes. Because of the role that these enzymes play in cellular functions and in various pathologies, they represent important drug targets (Cohen, 2002). By 2002, more than twenty-six small molecule inhibitors of protein kinases alone were either approved for clinical use or in phase I, II or III clinical trials (Cohen, 2002; Pearson and Fabbro, 2004).

This chapter describes the tools available for investigating the activities of kinases and phosphatases that are involved in signaling cascades. We describe a variety of technologies including luminescent and fluorescent assays for kinase and phosphatases. The phosphorylation state of the substrates of kinases can also be informative when studying cell signaling. We describe a variety of antibodies for detecting the phosphorylated forms of some kinase substrates as well as kinase substrates and inhibitors that can be used as tools to analyze kinase activities in samples.

These signaling pathways are complex and intertwined with each other. An interactive cell signaling application that presents the PI3K/mTOR and MAPK/ERK pathways is available as a free download for the iPad here.

A. The MAPK Pathways

The Mitogen-Activated Protein Kinase (MAPK) signaling pathways play an important role in signal transduction in eukaryotic cells where they modulate many cellular events including mitogen-induced cell cycle progression through G1 phase, embryonic development, cell movement, apoptosis and cell differentiation. MAPK pathways can be activated through diverse mechanisms including G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKS), Ser/Thr membrane receptors, inflammatory cytokines and environmental stresses including osmotic shock and ionizing radiation (Kyriakis and Avruch, 2001).

Because MAPK signaling is integral to key cellular and developmental processes, disruption of MAPK signaling or its regulation leads to a host of pathologies including cancers, neurodegenerative diseases, and developmental disorders (Murphy and Blenis, 2006). MAPK pathways are organized in three tiers of kinases consisting of a MAP kinase (MAPK); an activator of MAP kinase (MAP Kinase kinase or MEK) and a MAP Kinase Kinase kinase (MEKK, MAP3K or MEK kinase; Kyriakis and Avruch, 2001; Figure 7.1). There are several distinct MAPK pathways, including the extracellular signal-related kinases (ERK1/2 pathway) and three stress-activated pathways (p38 MAPK; SAPK/JNK 1,2,3; and ERK5/BMK1; Kyriakis and Avruch, 2001; Pimenta and Pascual, 2007).

Activated ERKs phosphorylate many targets including members of the 90 kDa ribosomal S6 kinases (RSKs ; Murphy and Blenis, 2006). Activated ERK1 and 2 can translocate into the nucleus, where they phosphorylate transcription factors such as cAMP-response element-binding protein (CREB), and ELK1, among others, to regulate expression of genes controlling the cell cycle and cell survival. (Murphy and Blenis, 2006). Aberrant activation of the MAPK/ERK pathway can play roles at several stages of tumorigenesis. Inappropriate phosphorylation of targets like myosin light chain kinase, calpain, focal adhesion kinase and paxilin promote cell migration (Kim and Choi, 2010). Because the ERK pathway also induces matrix metalloproteinase expression, constitutive activation can aid tissue invasion by tumor cells (Kim and Choi, 2010). ERK1/2 signaling also regulates some proapoptotic protein activities and in conjuction with PI3K-mTOR signaling can promote the survival of cancer cells (Mendoza *et al*. 2011; Roberts and Der, 2007). An animated presentation highlighting some of the events during MAPK signaling is available.

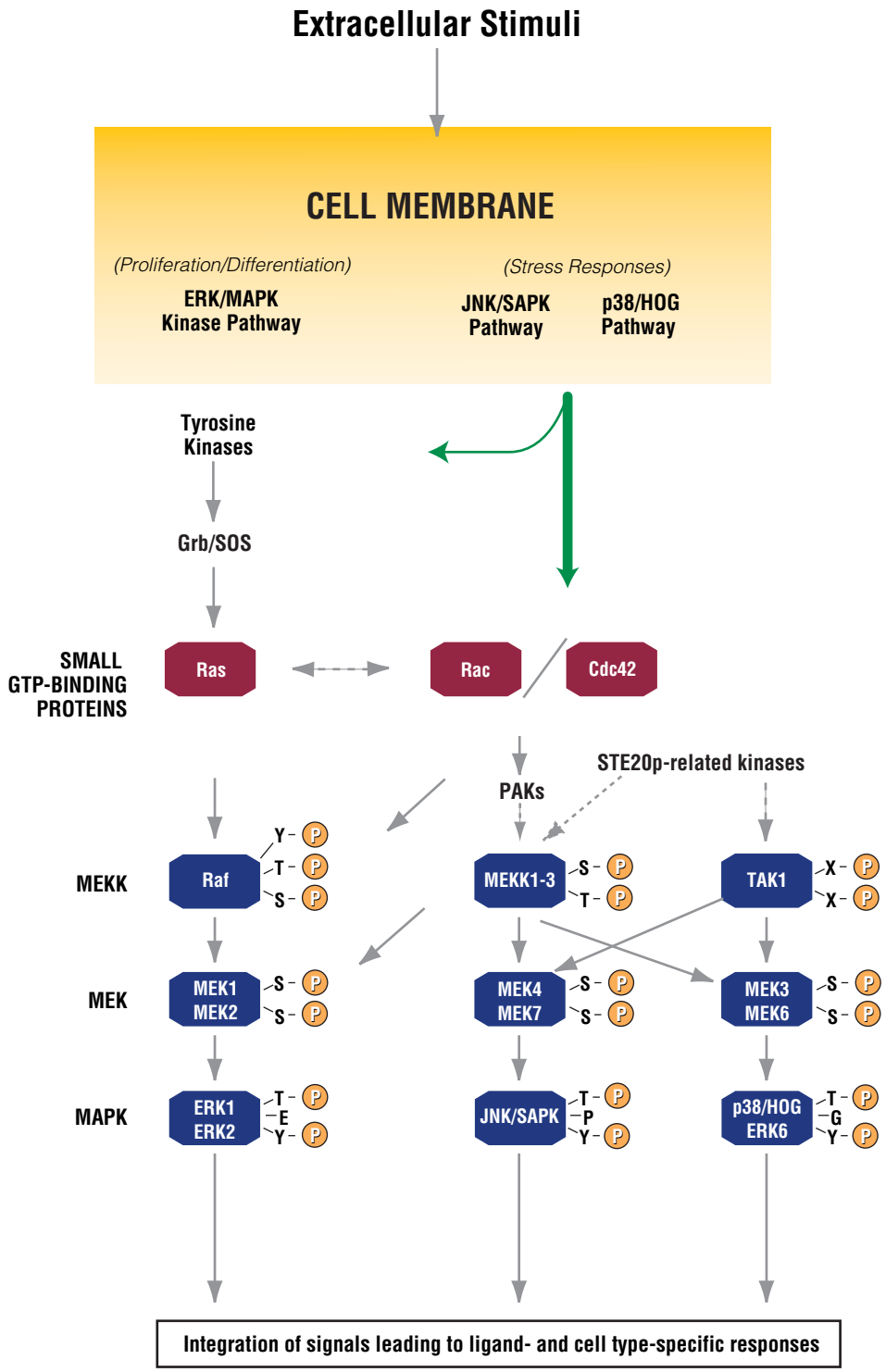


Figure 7.1. Activation of different MAPK signaling cascades by different extracellular stimuli. The ERK, JNK and p38 cascades all contain the same series of three kinases. A MEK Kinase (MEKK) phosphorylates and activates a MAP Kinase Kinase (MEK), then MEK phosphorylates and activates a MAP Kinase (MAPK).

Signal transduction cascades involving ERK/MAPK enzymes are also regulated by the activities of protein phosphatases. Several dual-specificity protein phosphatases have been identified that can differentially dephosphorylate MAPK, JNK or p38 enzymes (Neel and Tonks, 1997; Ellinger-Ziegelbauer *et al.* 1997). In addition, individual Ser/Thr (e.g., PP2A) or Tyr (e.g., PTP1) phosphatases also appear to regulate the activity of the ERK/MAPK enzymes

by dephosphorylating either core residue (Hunter, 1995; Keyse, 1995; Alessi, 1995; Doza, 1995). Thus, the cell can tightly regulate the activity of the ERK/MAPK enzymes by judicious use of different combination of MEKS, mono- and dual-specificity protein phosphatases and the subcellular localization of each enzyme to elicit the appropriate physiological response (Payne, 1991; Zhang, 2001).

Products Suitable for Studying the MAPK/ERK Pathway

ADP-Glo™ Kinase Assay (Cat.# V9101)

ADP-Glo™ Max Kinase Assay (Cat.# V7001)

Receptor Tyrosine Kinase Assay Systems

CMGC Kinase Enzyme Systems (including CDK, MAPK, GSK3 and CLK families)

AGC Kinase Assay Systems

Kinase-Glo® Luminescent Kinase Assay (Cat.# V6711)

Kinase-Glo® Plus Luminescent Kinase Assay (Cat.# V3771)

Kinase-Glo® Max Luminescent Kinase Assay (Cat.# V6071)

U0126 MEK inhibitor (Cat.# V1121)

PD98059 (Cat.# V1191)

EGF Receptor (Cat.# V5551)

Anti-ACTIVE®s MAPK pAb, Rabbit (pTEpY), Rabbit (Cat.# V1141)

Anti-ERK1/2 pAb, Rabbit (Cat.# V1141)

Anti-pT183 MAPK pAb, Rabbit (Cat.# V8081)

B. The PI3K/mTOR Pathway

Phosphoinositol 3-Kinases (PI3Ks) catalyze the transfer of the gamma phosphate group from ATP to the -OH group at the 3′ position of three different substrates: phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PI4P), and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2 or PIP_2). Signaling through PI3K activity modulates many cellular processes including cell growth, gluconeogenesis and glycolysis, motility, and differentiation. Mutations of genes that encode proteins within the PI3K signaling pathway have been implicated in a host of diseases including cancer and neurodegenerative disease.

There are three classes of PI3Ks. Class I PI3Ks catalyze conversion of PIP_2 to (phosphatidylinositol-3,4,5-bisphosphate) PIP_3 , and can be further subdivided based on the pathway through which they are activated. Class IA PI3Ks are activated through receptor tyrosine kinases (RTKs), and Class 1B PI3Ks are activated through G protein-coupled receptors (GPCRs) (Chaloub and Baker, 2009). Class II PI3Ks are associated with clathrin-coated vesicles and may help regulate membrane trafficking (Chaloub and Baker, 2009). Class III PI3-Ks are the only class conserved in lower and higher eukaryotes. These kinases produce PI(3)P as their product and are required for autophagy (Chaloub and Baker, 2009).

Class I PI3Ks consist of two subunits: one regulatory and one catalytic. The regulatory subunit binds to phosphotyrosine residuses on the intracellular domains of RTKs or adaptor proteins; this binding relieves intramolecular inhibition of the catalytic subunit and localizes the catalytic subunit near the inner leaflet of the plasma membrane and its substrate, PI(4,5)P_2 (Chaloub and Baker, 2009). Alternatively PI3K can also be stimulated by activated Ras, which binds directly to the catalytic subunit (Mendoza *et al*. 2011).

The PI3K product propagates the signal from the RTK by binding to specific regions of downstream target proteins, such as the FYVE zinc-finger, pleckstrin homology (PH) and Phox-homology (PX) domains (Courtney *et a*l. 2010). One such target protein is the kinase, AKT, which must be dually phosphorylated by PDK1 and mTORC2 (mTOR complex 2) for complete activation (Mendoza *et al*. 2011) .

Once AKT is activated it interacts with with the GTPase activation protein (GAP) tuberous sclerosis complex 2 and suppresses its GAP activity to release the GTPase Ras homolog enriched in brain (RHEB) from inhibition, activating mTORC1 (mTOR complex 1), leading to the phosphorylation of the 4E Binding Protein and initiation of translation and ribosomal S6 kinase (S6K1), leading to ribosome biogenesis and lipid synthesis (Mendoza *et al*. 2011, Russel *et al*. 2011).

The production of PIP_2 from PIP_3 is regulated by the lipid phosphatase PTEN, which serves as a negative regulator of PI3K signaling (Chaloub and Baker, 2009). PTEN has been identified as a major tumor suppressor, and loss-of-function of this gene is associated with increased incidence of cancer (Chaloub and Baker, 2009). Indeed resistance of certain breast cancer tumors to therapeutic agents such as trastuzumab is often associated with mutations in the the PI3K pathway (Berns *et al*. 2007), and the cross-talk between the MAPK/ERK and PI3K mTOR pathway has illustrated the for therapeutic strategies that target both pathways simultaneously (Courtney *et al*. 2010, Mendoza *et al*. 2011). An animated presentation that shows some events associated with the PI3-K pathway is available.

Products Suited for Studying PI3K/mTOR signaling

ADP-Glo™ Kinase Assay (Cat.# V9101)

ADP-Glo™ Max Kinase Assay (Cat.# V7001)

AGC Kinase Enzyme Systems

Kinase-Glo® Luminescent Kinase Assay (Cat.# V6711)

Kinase-Glo® Plus Luminescent Kinase Assay (Cat.# V3771)

Kinase-Glo® Max Luminescent Kinase Assay (Cat.# V6071)

LY 294002 (Cat.# V1201)

C. Signaling Pathway Cross-Talk

No cell signaling pathway works in isolation, and many pathways even share common core signaling molecules. Understanding the interactions or cross-talk among pathways can be important for understanding the mechanisms of action or inefficacy of pharmaceuticals.

Because of pathway cross-talk cancer therapies often need to inhibit multiple pathways simultaneously (Rosen *et al*. 2010; Rozengurt *et al*. 2010).

Cross-talk among cell signaling pathways can occur at the level of core signaling molecules or pathways can converge on common effectors (Mendoza *et al*. 2011). Mendoza *et al*. (2011) describe four types of interactions among pathways: (1) negative feedback loop, a downstream molecule of a pathway inhibits the activity an upstream molecule of the same pathway; (2) cross-inhibition, a core molecule of one pathway inhibits a core member of another pathway; (3) cross-activation, a core member of one pathway upregulates an upstream core member of another pathway; and (4) pathway convergence, two or more signaling pathways act directly on the same protein. To complicate matters, any two pathways can have all four of these types of interactions operating.

Cross-Talk between MAPK/ERK and PI3K/mTOR Pathways

Both the MAPK/ERK and PI3K/mTOR pathways can be activated through receptor tyrosine kinase (RTK) or by G-protein coupled receptors (GPCR) and cross-talk can occur at the receptor level (Mendoza *et al*. 2011; Courtney *et al*. 2010).

Negative Feedback: Both pathways are subject to negative feedback from their own downstream core components. For instance in the MAPK/ERK pathway, activated ERK can phosphorylate and inhibit the upstream players SOS, Raf, and MEK (Mendoza *et al*. 2011). The PI3K-mTOR pathway is subject to negative feedback by S6K phosphorylation of IRS, which downregulates IGF-1 receptor signaling, and RICTOR, which reduces mTORC1 signaling (Courtney et al. 2010; Mendoza *et al*. 2011).

Cross-inhibition: When the MAPK/ERK pathway is blocked using a small-molecule inhibitor, enhanced EGF-induced AKT activation is often observed (Mendoza *et al*. 2011). This suggests that the MAPK pathway is cross-inhibiting the PI3K pathway. Conversely cross-inhibition between AKT and Raf has been described upon strong IGF-1 stimulation of AKT/mTOR signaling (Mendoza *et al*. 2011).

Cross-activation: The MAPK/ERK pathway can activate the PI3K pathway at several points. Ras-GTP can bind directly to PI3K and activate it; activated p90rsk and ERK can phosphorylate TSC2 and promote mTORC1 activity as a consequence (Mendoza *et al*. 2011).

Pathway convergence: Several of the core kinase components of the MAPK/ERK pathway and the PI3K/mTOR pathway affect the same downstream effectors. For instance ERK phosphorylates the transcription factor FOXO3A as does AKT (Mendoza *et al*. 2011). Several other effectors including BAD, c-Myc, and GSK3 are also targets in both pathways (Mendoza *et al*. 2011).

II. Kinase/ATPase Activity Assays

A. Luminescent ATP/ADP Detection Assays

ADP-Glo™ Kinase Assay Family

The ADP-Glo™ Kinase Assay (Cat.# V9101) is a luminescent kinase assay that measures ADP formed from a kinase reaction; ADP is converted into ATP, which is converted into light by Ultra-Glo™ Luciferase. The luminescent signal positively correlates with kinase activity. The assay is well suited for measuring the effects chemical compounds have on the activity of a broad range of purified kinases, making it ideal for both primary screening as well as kinase selectivity profiling. The ADP-Glo™ Kinase Assay can be used to monitor the activity of virtually any ADP-generating enzyme (e.g., kinase or ATPase) using up to 1mM ATP. The ADP-Glo™ Max Assay (Cat.# V7001) can be used when higher concentrations (up to 5mM) are required.

The assay is performed in two steps; first, after the kinase reaction, an equal volume of ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the remaining ATP. In the second step, the Kinase Detection Reagent is added, which simultaneously converts ADP to ATP and allows the newly synthesized ATP to be measured using a coupled luciferase/luciferin reaction (Figure 7.2).

The ADP-Glo™ Kinase Assay has a high dynamic range and produces a strong signal at low ATP to ADP conversion, making it well suited for screening low activity kinases such as growth factor receptor tyrosine kinases. The assay produces minimal false hits and Z′ values of greater than 0.7.

The assay can be performed over a wide range of ATP concentrations (low micromolar to millimolar). This allows detection of small concentrations of ADP in the presence of large amounts of ATP (Figure 7.3), producing very high signal-to-background (SB) ratios (Figure 7.3). The robustness of the ADP-Glo™ Kinase Assay and suitability for high-throughput applications is evidenced by high Z′-factor values reported in previous studies (Tai *et al*. 2011). The ADP-Glo™ Kinase Assay is as sensitive as radioactivity-based methods and more sensitive than fluorescence-based technologies (Tai *et al*. 2011; Zegzouti *et al*. 2009; Vidugiriene *et al*. 2009). In order to lower the background and further improve the sensitivity of the assay, we increased the purity of our ATP to have less ADP contamination. To assess the importance of ATP purity on ADP-Glo™ assay sensitivity, we compared the signal-to-background ratios generated in an ADP-Glo™ assay using Promega Ultra Pure ATP and ATP from other suppliers. The Promega ATP outperforms ATP from other sources by greatly improving ADP-Glo™ assay sensitivity with SB ratios that are 2–3 times higher than those produced using other commercial preparations (Zegzouti *et al*. 2011).

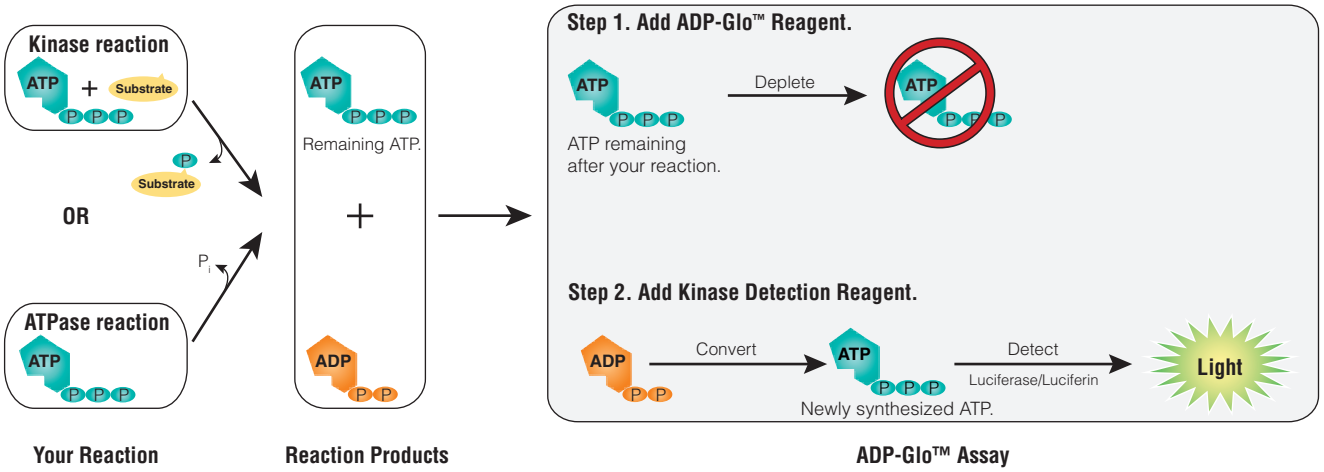


Figure 7.2. The ADP-Glo™ Assay Principle. The assay is composed of two steps. After the kinase or ATPase reaction, the first step is performed by addition of the ADP-Glo™ Reagent that terminates the kinase reaction and depletes any remaining ATP (40-minute incubation time). Addition of a second reagent converts ADP to ATP and generates light from the newly synthesized ATP using a luciferase/luciferin reaction (incubation is 30–60 minutes depending on the ATP concentration used in the kinase reaction). The light generated is proportional to ADP present and, consequently, kinase or ATPase activity. The assay is performed at room temperature and is compatible with automation.

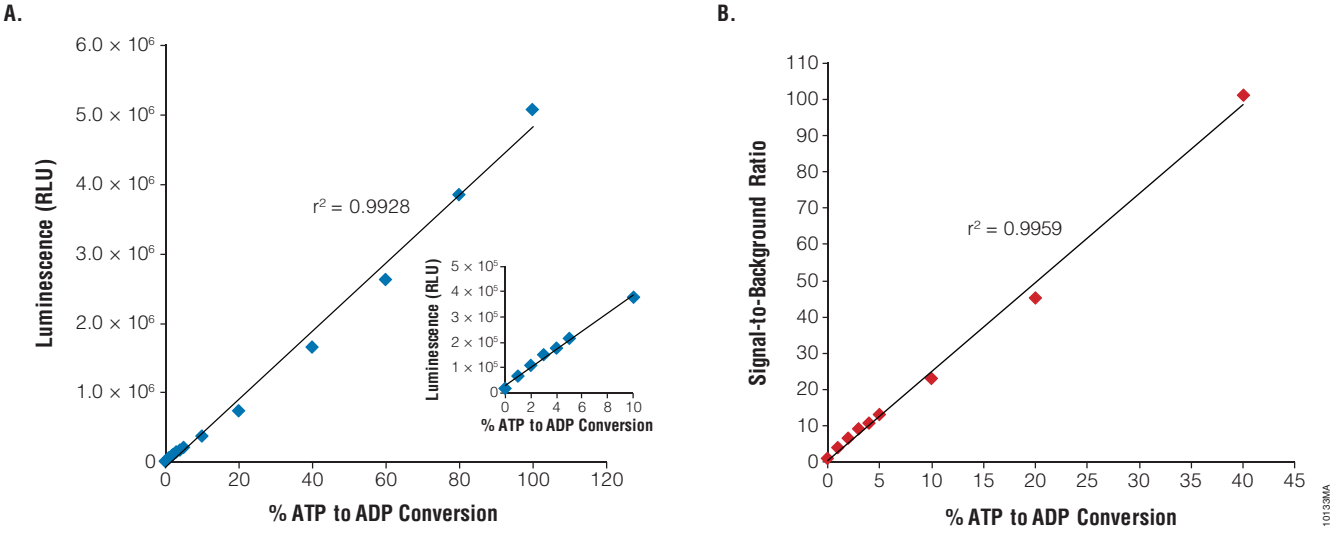


Figure 7.3. Linearity and sensitivity of the ADP-Glo™ Kinase Assay. The 1mM ATP-to-ADP percent conversion curve (standard curve) was prepared in 1X reaction buffer A (40mM Tris [pH 7.5], 20mM $MgCl_2$, and 0.1mg/ml BSA) without kinase present as described in Technical Manual #TM313. The standards were created by combining the appropriate volumes of ATP and ADP 1mM stock solutions. Five microliters of each ATP + ADP standard was transferred to a white, opaque 384-well plate. The ADP-Glo™ Kinase Assay was performed by adding 5µl of ADP-Glo™ Reagent and 10µl of Kinase Detection Reagent at room temperature to each well. ADP-Glo™ assay reagents were dispensed in 384-well plates using Multidrop® Combi nL liquid dispenser (Thermo Fisher Scientific). Luminescence values represent the mean of four replicates (RLU = relative light units). **Panel A.** Linearity of the assay up to 1mM ADP. **Panel B.** Sensitivity of the assay is shown as signal-to-background ratios (SB) over a wide range of % ATP-to-ADP conversion.

Before You Begin

Materials Required:

- ADP-Glo™ Assay (Cat.# V9101, V9102, V9103) or ADP-Glo™ Max Assay (Cat.# V7001, V7002) and appropriate Protocol (Technical Manual #TM313 or TM343).
- solid white multiwell plates (do not use black plates)
- multichannel pipet or automated pipetting station
- plate shaker
- luminometer capable of reading multiwell plates
- appropriate substrate
- ADP-producing enzyme (e.g., ATPase or kinase)

General Instructions for Preparing Detection Buffer

1. Thaw the Detection Buffer at room temperature, and look for any precipitate.
2. If a precipitate is present, incubate the Detection Buffer at 37°C with constant swirling for 15 minutes.

General Instructions for Detection Reagent Preparation

1. Equilibrate the Detection Buffer and the Detection Substrate to room temperature before use.
2. Transfer the entire volume of Detection Buffer into the amber bottle containing the Detection Substrate to reconstitute the lyophilized substrate. This forms the Detection Reagent.
3. Mix by genetly votexing, swirling or inverting the contents to obtain an homogeneous solution. The Detection Substrate should go into solution in less than one minute.
4. The Detection Reagent should be used immediately or dispensed into aliquots and stored at –20°C.

Generating a Standard Curve for the Conversion of ATP to ADP

1. To estimate the amount of ADP produced in the reaction, we recommend creating a standard curve that represents the luminescence corresponding to the conversion of ATP to ADP (the "ATP-to-ADP" conversion curve") based on the ATP concentration used in the kinase or ATPase reaction. These standard curves represent the amounts of ATP and ADP available in a reaction at the specified conversion percentage (Table 7.1). The standard samples used to generate an ATP-to-ADP standard are created by combining the appropriate volumes of ATP and ADP stock solutions. For more information on generating standard curves see Technical Manual TM313 for the ADP-Glo™ Kinase Assay or Technical Manual TM343 for the ADP-Glo™ Max Assay and the Kinase Titration and Determination of SB10 (Part A) below.

General Overview of ADP-Glo™ Kinase Assay Protocol

1. Perform a kinase reaction using 1X kinase buffer. (See appropriate Technical Manual for details.)
2. Add ADP-Glo™ Reagent to stop the kinase reaction and deplete the unconsumed ATP, leaving only ADP and a very low background of ATP.
3. Incubate at room temperature for 40 minutes.
4. Add Detection Reagent to convert ADP to ATP and introduce luciferase and luciferin to detect ATP.
5. Incubate at room temperature for 30–60 minutes.
6. Measure the luminescence with a plate-reading luminometer or charge-coupled device (CCD) camera.

This is a general protocol. Please see the appropriate Technical Manuals for specific details and notes. To screen for inhibitors or to determine IC_{50} values of kinase inhibitors using the ADP-Glo™ Kinase Assay Systems, see Technical Manual TM313 for the ADP-Glo™ Kinase Assay or Technical Manual TM343 for the ADP-Glo™ Max Assay.

Sample Protocol for a Kinase Inhibitor (Staurosporine) Dose-Response Curve Using the ADP-Glo™ Assay

A kinase titration will be performed in order to determine the optimal amount of enzyme to use in subsequent inhibitor does-response curve determination. To estimate the amount of ADP produced in a kinase reaction, create an ADP standard curve, named "ATP-to-ADP Conversion Curve". This curve represents the luminescence (RLU) corresponding to each % conversion of ATP-to-ADP based on the ATP concentration used in the kinase reaction. The standard samples used to generate an ATP-to-ADP conversion curve are created by combining the appropriate volumes of ATP and ADP stock solutions. **Kinase Titrations** and **ATP-to-ADP conversion curves** for similar ATP concentrations will be performed in one plate.

The percent ADP produced by each amount of enzyme is calculated using the reference RLUs from the conversion curves. By titrating the kinase, we will determine *SB10 value*, which corresponds to the amount of the kinase needed to generate a percent conversion reflecting the initial rate of the reaction. Usually we choose 5–10% conversion, as the signal-to-background ratio generated is higher than tenfold.

Using the SB10 amount of the kinase, we will perform a kinase inhibitor (staurosporine) dose response curve to calculate the IC_{50} and to check for any ATPase contaminating activity that will not be inhibited.

Reaction Buffers Needed Using 5X Reaction Buffer A:

5X Reaction Buffer A: 200mM Tris [pH 7.5], 100mM $MgCl_2$ and 0.5mg/ml BSA

4X Kinase Buffer: 4X Reaction Buffer A + 200µM DTT + (4X of any cofactors, e.g. $MnCl_2$)

4X Kinase Buffer D made accordingly by adding 4% DMSO

1X Kinase Buffer made by diluting the 4X Kinase Buffer

1X Kinase Buffer D made by diluting 4X Kinase Buffer D

1X Kinase Buffer (5% DMSO) made by diluting the 4X Kinase Buffer and adding 5% DMSO

Note: All volumes described here are for duplicate samples. If you need to perform more than two replicates per sample, recalculate the volumes accordingly.

All steps are performed at room temperature (22–25°C).

Part A: Kinase Titration and Determination of SB10 Generation of ATP-to-ADP Conversion Curves

1. In a 96-well plate, generate the ATP-ADP series needed by diluting in 1X Kinase Buffer D the samples from a 10X concentrated ATP + ADP ranges as described below.
2. **Preparing 10X Conversion Curve Standards:** Prepare 10X ADP/ATP stock plates in water as described in the tables below to make 100µl stock solutions of ATP/ADP standards (Table 7.2). You will need 1ml of your 10X

Table 7.1. Percent Conversion of ATP to ADP Represented by the Standard Curve

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
%ADP	100	80	60	40	20	10	5	4	3	2	1	0
%ATP	0	20	40	0	80	90	95	96	97	98	99	100

ATP and 500µl of the 10X ADP **Note:** If you are working with only one ATP concentration, make only the corresponding 10X stocks.

3. **ATP Stock Solution Preparation (starting with a 1mM solution)**

Final conc. desired	Prepare this 10X stock	ATP (µl)	Water (µl)
1µM	10µM	10	990
5µM	50µM	50	950
10µM	100µM	100	900

ADP Stock Solution Preparation (starting with a 1mM solution)

Final conc. desired	Prepare this 10X stock	ADP (µl)	Water (µl)
1µM	10µM	5	495
5µM	50µM	25	475
10µM	100µM	50	450

ATP Stock Solution Preparation (starting with a 10mM solution)

Final conc. desired	Prepare this 10X stock	ATP (µl)	Water (µl)
100µM	1.0mM	100	900
250µM	2.5mM	250	750
500µM	5mM	500	500

ADP Stock Solution Preparation (starting with a 10mM solution)

Final conc. desired	Prepare this 10X stock	ADP (µl)	Water (µl)
100µM	1.0mM	50	450
250µM	2.5mM	125	375
500µM	5mM	250	250

4. After you have prepared your ATP and ADP stock solutions, create a conversion curve 10X by transfering the amounts of each solution as described in Table 7.2.

5. **Important Note:** Use the remaining 100% ATP from your conversion curve plate to run the kinase reaction in order to have similar background levels.

6. Preparing a *1X* ADP/ATP working dilution plate in 1X kinase reaction buffer: Mix 105µl of 4X Kinase Buffer D with 273µl of water. Transfer 27µl/well to a 96-well plate, then transfer 3µl of the 10X ATP/ADP curve to each respective well in the dilution plate. This will give a final volume of 30µl, sufficient for 4 replicates.

Preparation of Kinase Titration Components:

1. Prepare the kinase titrations at the same ATP concentrations as ATP-to-ADP conversion curves.

2. **Substrate Mix Preparation:** For each kinase, prepare 200µl of 2.5X ATP/Substrate Mix in a 1.5ml tube. Use the 10µM examples below for a guideline. **Note:** Use ATP from the same 10X ATP that you used for the conversion curve.

Substrate Mix Preparation (10µM example)

Component	Amount
4X Kinase Buffer D	50µl
100µM ATP (10X)	50µl
Substrate (1mg/ml)	100µl

Substrate Mix Preparation: If the substrate is MBP, Casein or Histone H1:

Component	Amount
4X Kinase Buffer D	50µl
100µM ATP (10X)	50µl
Water	50µl
Substrate (1mg/ml)	50µl

3. Transfer 14µl of 2.5X ATP/Substrate Mix to odd numbered wells (1,3,5...23) of a 384-well plate in **Row X**. This is your *ATP/Substrate preparative row.*

4. **Enzyme Dilution Preparation:** Add 10µl of 1X Kinase Buffer D to odd numbered wells, starting with well 3 (3, 5, 7...23) of the 384-well plate in **Row Y**. Do not add buffer to well 1. This is your *Kinase Dilution preparative row*.

5. Prepare 20µl kinase solution as described in the table below (3µl/reaction/well). This will give 200ng kinase/3µl starting concentration.

Kinase Solution Preparation

Component	Volume
Water	1.67µl
4X Kinase Buffer D	5µl
Kinase (100ng/µl)	13.33µl

6. Add 20µl of Kinase Solution to well 1 of the *Kinase Dilution prepartive row Y.* From there, prepare a 1:1 serial dilution of the kinase as shown in Figure 7.4. Mix well after each dilution by pipetting before transferring 10µl to the next well. Do not continue the serial dilution after well 21.**Note:** Do not create bubbles while preparing the dilution series.

7. **Kinase Reaction and Conversion Curve Experiment:** Transfer 5µl of the diluted ATP-ADP series in replicates from your *1X ADP/ATP working dilution plate* into the wells of your 384-well assay plate that are designated for the conversion curve.

Table 7.2. 10X Conversion Curve Preparative Plate

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
% Conversion	100	80	60	40	20	10	5	4	3	2	1	0
ADP (µl)	100	80	60	40	20	10	5	4	3	2	1	0
ATP (µl)	0	20	40	0	80	90	95	96	97	98	99	100

Well #	Kinase	Starting Volume of Each Well	Volume to Transfer
1	200ng	20µl	10µl
3	100ng	10µl	10µl
5	50ng	10µl	10µl
7	25ng	10µl	10µl
9	12.5ng	10µl	10µl
11	6.25ng	10µl	10µl
13	3.12ng	10µl	10µl
15	1.56ng	10µl	10µl
17	0.78ng	10µl	10µl
19	0.39ng	10µl	10µl
21	0.1953ng	10µl	0µl
23	0	10µl	No transfer, buffer only

Figure 7.4. Performing serial 1:1 dilutions of kinase.

8. Transfer 3µl of kinase samples in duplicates from the wells of the *kinase titration preparative, Row Y* to the wells of the assay plate designated for the kinase reactions.

9. Transfer 2µl of the corresponding 2.5X ATP/Substrate Mix from the wells of the *ATP/Substrate preparative Row X* to the same assay rows where the kinase dilutions are.

10. Spin the plate. Mix with a plate shaker for 2 minutes. Incubate the reaction at room temperature for 60 minutes or the desired time.

11. **ADP detection with ADP-Glo™ Kinase Assay:** After the kinase reaction incubation is complete, add 5µl of ADP-Glo™ Reagent to all wells in your assay plate. Mix for 2 minutes and incubate at room temperature for 40 minutes.

12. Add 10µl of kinase detection reagent to all wells in your assay plate. Mix for 2 minutes and then incubate at room temperature for 30–60 minutes.

13. Measure the luminescence (integration time, 0.5 second).

14. Calculate the *SB10 value* (ng or nM). *SB10* is the amount needed to generate a 5–10% ATP to ADP conversion (usually this kinase amount generates a signal-to-background ratio of greater than tenfold).

Part B: Staurosporine Inhibitor Dose Response Curve

1. **Preparation of inhibitor titration components:** Add 50µl of 1X Kinase Buffer (with 5% DMSO) to wells A2–B12 of a 96-well plate. These are your *inhibitor titration preparative rows*. **Note:** Do not add buffer to well A1.

2. Prepare 100µl of 50µM staurosporine solution (will be 5% DMSO) as described in the table below (final 1µl/reaction/well). This will give 10µM staurosporine (1% DMSO) starting concentration in the assay.

Staurosporine Solution Preparation

Component	Volume
Water	70µl
4X Kinase Buffer	25µl
Staurosporine in DMSO (1mM)	5µl

3. Add 100µl of staurosporine solution to well A1 of the *inhibitor titration preparative rows.* Prepare a 1:1 serial dilution of the inhibitor as shown in Figure 7.5. Mix well after each dilution by pipetting before transferring into the next well. **Note:** Do not create bubbles while preparing the diution series.

4. **Preparation of Reaction Components, 10µM ATP example:** For each kinase prepare 200µl of 2.5X ATP/Substrate Mix as described in the tables below.

Well #	Final Inhibitor Concentration	Starting Volume of Each Well	Volume to Transfer
A1	10,000nM	100µl	50µl
A2	5,000nM	50µl	50µl
A3	2,500nM	50µl	50µl
A4	1,250nM	50µl	50µl
A5	625nM	50µl	50µl
A6	312.5nM	50µl	50µl
A7	156.3nM	50µl	50µl
A8	78.1nM	50µl	50µl
A9	39.1nM	50µl	50µl
A10	19.5nM	50µl	50µl
A11	9.8nM	50µl	50µl (transfer to B1)
A12	0	50µl	Buffer Only
B1	4.88nM	50µl	50µl
B2	2.44nM	50µl	50µl
B3	1.22nM	50µl	50µl
B4	0.61nM	50µl	50µl
B5	0.31nM	50µl	50µl
B6	0.15Nm	50µl	50µl
B7	0.08nM	50µl	50µl
B8	0.04nM	50µl	50µl
B9	0.02nM	50µl	50µl
B10	0.01nM	50µl	0µl
B11	0	50µl	Buffer Only
B12	No enzyme	50µl	Buffer Only

Figure 7.5. Performing serial 1:1 dilutions of inhibitor.

Substrate Mix Preparation

Component	Amount
4X Kinase Buffer	50µl
100µM ATP (10X)	50µl
Substrate (1mg/ml)	100µl

Substrate Mix Preparation: If the substrate is MBP, Casein or Histone H1:

Component	Amount
4X Kinase Buffer	50µl
100µM ATP (10X)	50µl
Water	50µl
Substrate (1mg/ml)	50µl

5. Transfer 14µl of 2.5X ATP/Substrate Mix to odd numbered wells (1,3,5...23) of a 384-well plate in **Row X**. This is your *ATP/Substrate preparative row.*

6. Prepare 140µl of kinase solution (excess amount of 70 reactions at 2µl/reaction/well) as described in the table below. this will give SB10ng of kinase/reaction.

Kinase Solution Preparation

Component	Volume
Water	Yµl = 105µl- X
4X Kinase Buffer	35µl
Kinase (100ng/µl)	Xµl = (70 × SB10/100)

7. Add 12µl of the kinase solution to odd numbered wells (1,3,5...21) and 8µl to well 23 of a 384-well plate **Row Y**, as a *kinase preparative row*.

8. **Kinase Reaction Experiment:** Transfer 2µl kinase samples in duplicate from the wells of the *kinase preparative row* to wells A1 through B22 of a 384-well plate. **Note:** Add only 2µl of 1X Kinase Buffer to wells B23-B24 for the no-enzyme control.

9. Transfer 1µl inhibitor samples in duplicate from the wells of the *inhibitor titration preparative rows* to the corresponding wells of the *assay rows* (Well A1 from the 96-well plate to well A1 and A2 of the 384-well plate, etc.)

10. Mix and incubate at room temperature for 10 minutes.
11. Transfer 2µl of the corresponding 2.5X ATP/Substrate Mix from the wells of the *ATP/Substrate preparative row* to the same *assay rows* where the kinase/inhibitor mixes are present.
12. Spin the plate. Mix for 2 minutes and then incubate the kinase reaction at room temperature for 60 minutes, or the desired time.
13. **ADP detection with ADP-Glo™ Kinase Assay:** After the kinase reaction incubation, add 5µl of ADP-Glo™ Reagent to all wells in your assay plate. Mix for 2 minutes and incubate the reaction at room temperature for 40 minutes.
14. Add 10µl of Kinase Detection Reagent to all the wells in your assay plate. Mix for 2 minutes and incubate the reaction at room temperature for 30–60 minutes.
15. Measure the luminescence (integration time 0.5 second).
16. **Calculating Percent Enzyme Activity:** First substract the signal of the negative control (no enzyme and no staurosporine) from all the samples signal. Then use the 0% kinase activity (neither compound nor enzyme) and the 100% kinase activity (no compound) to calculate the other percent enzyme activities remaining in the presence of the different dilutions of staurosporine.

Additional Resources for ADP-Glo™ Kinase and ADP-Glo™ Max Assays

Technical Bulletins and Manuals

TM313 *ADP-Glo™ Kinase Assay Technical Manual*

TM343 *ADP-Glo™ Max Assay*

Promega Publications

Protocol for Kinase Inhibitor Dose Response Curve

Screening and Profiling Kinase Inhibitors with a Luminescent ADP Detection Platform

Citations

Tai, A.W. *et al.* (2011) A homogeneous and nonisotopic assay for phosphatidylinositol 4-kinases *Anal. Biochem.* **417**, 97–102.

The authors of this study evaluated the ADP-Glo™ Assay technology for use in high-throughput screening applications for inhibitors of all four known mammalian PI 4-kinases. They found that K_m values, IC_{50} values of known inhibitors, and dose-response curves were comparable to values reported in the literature or those obtained using the standard isotopic assay. Z′-factor values for the assay in a low-volume, 384-well format were 0.72 and 0.74, indicating that the assay would be suitable for screening activities in 384- or 1536-well formats.

PubMed Number: 21704602

Balzano, D. *et al.* (2011) A general framework for inhibitor resistance in protein kinases *Chemistry and Biology* **19**, 966–75.

The authors of this paper investigated mutations affecting the hinge loop of protein kinases that appear to confer resistance to both Type I and Type II inhibitors. They introduced individual amino acid substitutions into the hinge region of six distantly related protein kinases and determined the inhibitor sensitivity of these kinases. The ADP-Glo™ Kinase Assay was used to asses the activity of the Haspin and c-Src kinases and the engineered mutants in this study.

PubMed Number: 21867912

Ohana, R.F. *et al.* (2010) HaloTag-based purification of functional human kinases from mammalian cells *Protein Expression and Purification* **76**, 154–64.

The authors of this paper demonstrate the utility of the HaloTag® protein purification system for purifying functional proteins from mammalian cells. To this end five kinases were cloned into HaloTag® vectors, expressed in and purified from HEK293T cells. To demonstrate functionality of the purified recombinant kinases, activity was measured using the ADP-Glo™ Kinase Assay.

PubMed Number: 21129486

The Kinase-Glo® Universal Kinase Assays

Kinases are enzymes that catalyze the transfer of a phosphate group from ATP to a substrate. The depletion of ATP as a result of kinase activity can be monitored in a highly sensitive manner through the use of the Kinase-Glo®, Kinase-Glo® Plus, and Kinase-Glo® Max Reagents, which use luciferin, oxygen and ATP as substrates in a reaction that produces oxyluciferin and light (Figure 7.6).

The Kinase-Glo® Reagents rely on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable "glow-type" luminescent signal. The reagents are prepared by combining the Kinase-Glo® or Kinase-Glo® Plus or Kinase-Glo® Max Buffer with the lyophilized substrate provided with each system.

The protocol for both systems involves a single addition of an equal volume of Reagent to a completed kinase reaction that contains ATP, purified kinase and substrate. The plate is mixed and luminescence read. The luminescence is directly proportional to the ATP present in the kinase reaction, and kinase activity is inversely correlated with luminescent output.

The Kinase-Glo® Luminescent Kinase Assay (Cat.# V6711) and the Kinase-Glo® Plus Luminescent Kinase Assay (Cat.# V3771) and Kinase-Glo Max Assay (Cat.# V6071) can be used with virtually any kinase and substrate combination. The Kinase-Glo® Assay is extremely sensitive and is linear from 0 to 10µM ATP. It routinely provides Z′-factor values greater than 0.8 in both 96-well and 384-well formats

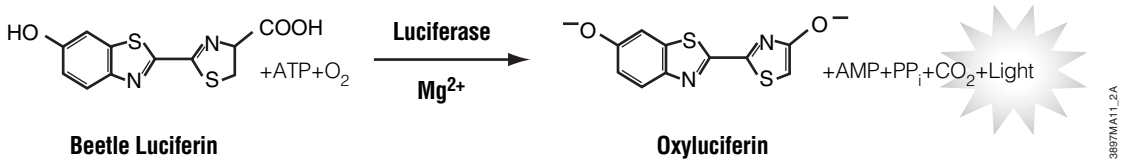


Figure 7.6. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen and produces one photon of light per turnover.

(Figure 7.7). Z′-factor is a statistical measure of assay dynamic range and variability; a Z′-factor greater than 0.5 is indicative of a robust assay (Zhang *et al*. 1999).

We have demonstrated the utility of the Kinase-Glo® Assay for high-throughput screening (Somberg *et al.* 2003; Goueli *et al*. 2004a). We tested the Kinase-Glo® Assay using a commercially available Library of Pharmacologically Active Compounds (LOPAC) to determine if the assay could score true kinase hits in that library. When we screened the LOPAC collection for inhibitors of PKA using the manual protocol, we found six wells in which we could detect kinase inhibition (Somberg *et al.* 2003). The same six wells also showed detectable kinase inhibition when we tested the Kinase-Glo® Assay in low-volume 384 and 1536-well formats (Goueli *et al.* 2004b; Figure 7.8). The Kinase-Glo® Assay can also be used to determine IC_{50} values for kinase inhibitors. The IC_{50} values for one of the six hits from the LOPAC library were determined using the Kinase-Glo® Assay. The Kinase-Glo® Assay gave values similar to values reported in the literature, further establishing the utility of the Kinase-Glo® Assay for high-throughput screening (Goueli *et al*. 2004b).

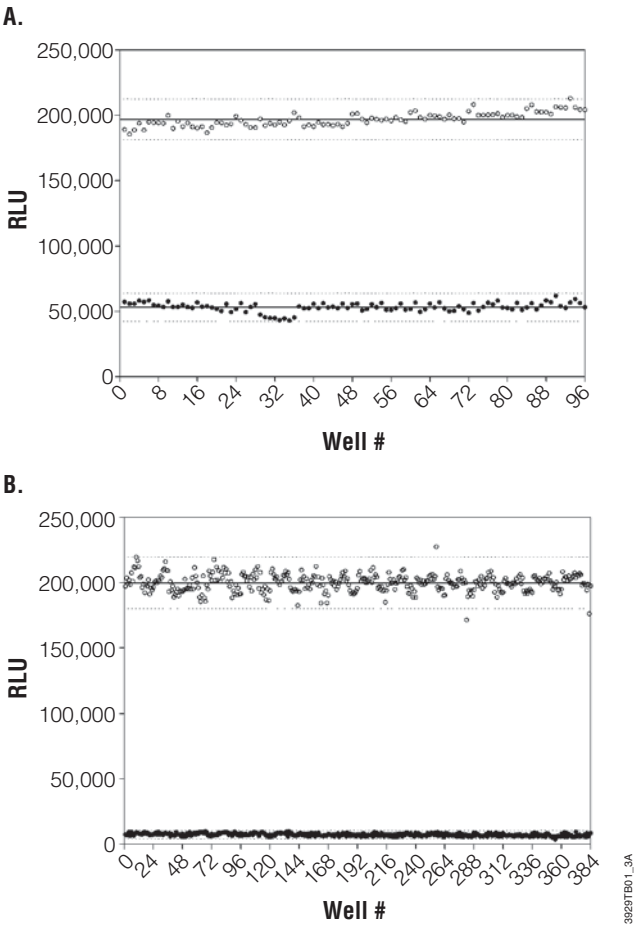


Figure 7.7. Determining Z′-factor for the Kinase-Glo® Assay. Panel A. The reaction was performed using 0.25 units/well PKA (solid circles) or no PKA (open circles) in 100µl volume. PKA was diluted in 50µl kinase reaction buffer (40mM Tris [pH 7.5], 20mM $MgCl_2$ and 0.1mg/ml BSA), containing 5µM Kemptide Substrate (Cat.# V5161) and 1µM ATP. The kinase reaction was run for 20 minutes at room temperature. **Panel B.** The 384-well plate assay was performed using 0.05 units/well (solid circles) or no PKA (open circles) in 20µl volume. Solid lines indicate mean, and dotted lines indicate ± 3 S.D. Z′-factor values were ~0.8 in both formats.

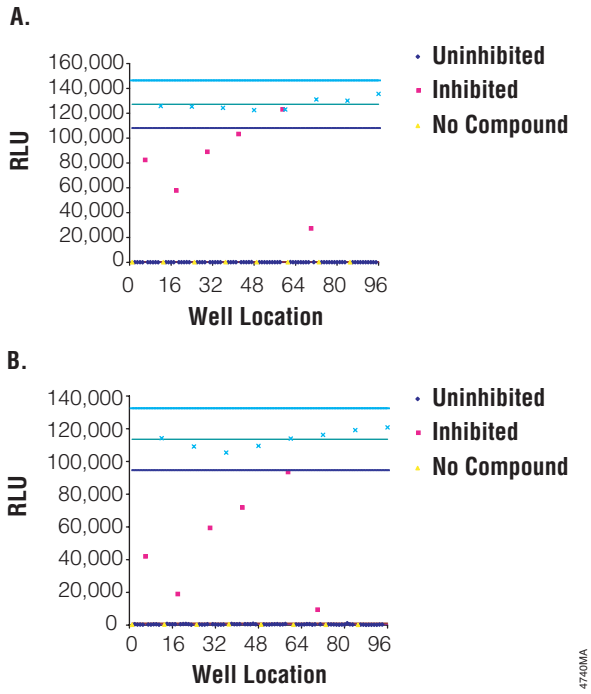


Figure 7.8. Compound screen using Plate 6 of the LOPAC (Sigma-RBI) performed in LV384- (Panel A) and 1536-well (Panel B) formats. Compounds were screened at 10µM. See Goueli *et al.* 2004a for percent inhibition of compounds that inhibited kinase activity.

The Kinase-Glo® Plus Assay not only allows users to detect kinase inhibitors, but also to distinguish between ATP competitive and noncompetitive inhibitors. Because the concentration of ATP in cells is fairly high, inhibitors of protein kinases that are not ATP-competitive are more desirable as therapeutic agents than ATP-competitive kinase inhibitors. Because the catalytic domains and active sites of protein kinases have been evolutionarily conserved, inhibitors that are not only ATP non-competitive, but also selective toward the target kinase are most desireable. The Kinase-Glo® Plus Assay is optimized to work at ATP concentrations that more closely reflect cellular ATP concentrations and is linear up to 100µM ATP.

Materials Required:

- Kinase-Glo® Assay System (Cat.# V6711, V6712, V6713, V6714) or Kinase-Glo® Plus Assay System (Cat.# V3771, V3772, V3773, V3774) or Kianse-Glo® Max Assay System (Cat.# V6071, V6072, V6073, V6074) and Protocol (Technical Bulletin #TB372).
- solid white multiwell plates
- multichannel pipet or automated pipetting station
- plate shaker
- luminometer capable of reading multiwell plates
- ATP
- appropriate kinase substrate
- appropriate kinase reaction buffer

Figure 7.9 provides an overview of the Kinase-Glo® Assay Protocol. The Kinase-Glo® Plus and Max Assays follow the same format.

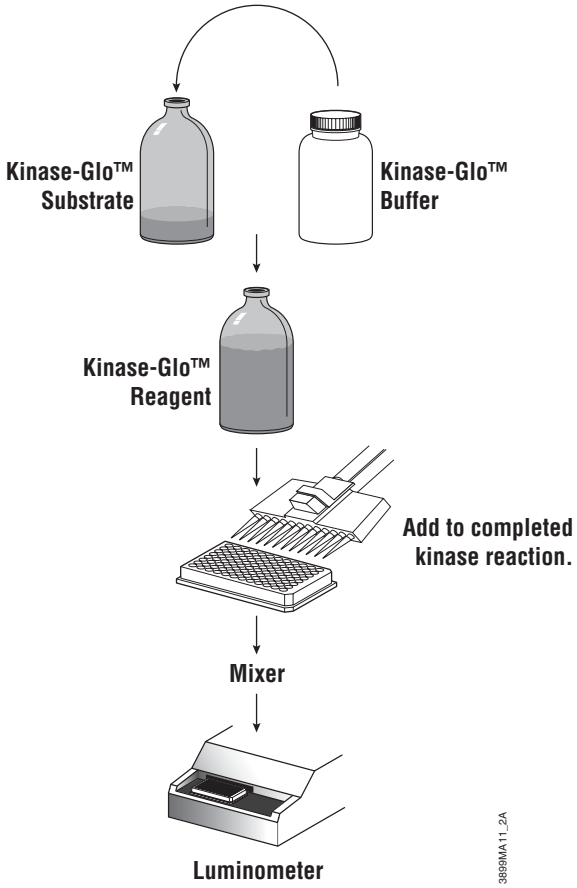


Figure 7.9. Schematic diagram of the Kinase-Glo® Assay protocol.

Additional Resources for Kinase-Glo® and Kinase-Glo® Plus Luminescent Kinase Assays

Technical Bulletins and Manuals

TB372 *Kinase-Glo® Luminescent Kinase Assay Platform*

Promega Publications

Screen for kinase modulators in a high-throughput format with Promega kinase reagents

Optimizing Kinase Assays for ultrahigh-throughput profiling using the Kinase-Glo® Plus Assay

Citation Note: Measuring LPS-induced PKC activity in U937 cells

High-throughput screening using a universal luminescent kinase assay

Citations

Kannan, S. *et al.* (2008) Cholesterol-rich membrane rafts and Lyn are involved in phagocytosis during *Pseudomonas aeruginosa* infection. *J. Immunol.* **180**, 2396–408.

The authors of this study investigated the role of Lyn, a Src-family tyrosine kinase, in regulating the formation of

the phagosome in alveolar macrophages in response to *Psuedomonas aeruginosa* (PA) infection. The Kinase-Glo® Assay was used to assess Lyn activity, using acid-denatured enolase as the substrate. The authors found that Lyn kinase activity was increased following infection with PA.

PubMed Number: 18250449

B. Fluorescent Kinase Assays

The ProFluor® Kinase Assays measure PKA (Cat.# V1240, V1241) or PTK (Cat.# V1270, V1271) activity using purified kinase in a multiwell plate format and involve "add, mix, read" steps only. The user performs a standard kinase reaction with the provided bisamide rhodamine 110 substrate. The provided substrate is nonfluorescent. After the kinase reaction is complete, the user adds a Termination Buffer containing a Protease Reagent. This simultaneously stops the reaction and removes amino acids specifically from the nonphosphorylated R110 Substrate, producing highly fluorescent rhodamine 110. Phosphorylated substrate is resistant to protease digestion and remains nonfluorescent. Thus, fluorescence is inversely correlated with kinase activity (Figure 7.10).

We tested the ability of several tyrosine kinases to phosphorylate the peptide substrate provided in the ProFluor® Src-Family Kinase Assay using protease cleavage and fluorescence output as an indicator of enzyme activity. The PTK peptide substrate served as an excellent substrate for all of the Src-family PTKs such as Src, Lck, Fyn, Lyn, Jak and Hck and the recombinant epidermal growth factor receptor (EGFR) and insulin receptor (IR). The fluorescence decreases with increasing concentrations for four Src family enzymes tested (Goueli *et al*. 2004a). The amount of enzyme required to phosphorylate 50% of the peptide (EC_{50}) was quite low (EC_{50} for Src, Lck, Fyn, Lyn A and Hck were 14.0, 1.38, 4.0, 4.13 and 1.43ng, respectively). As low as a few nanograms of Lck could be detected using this system.

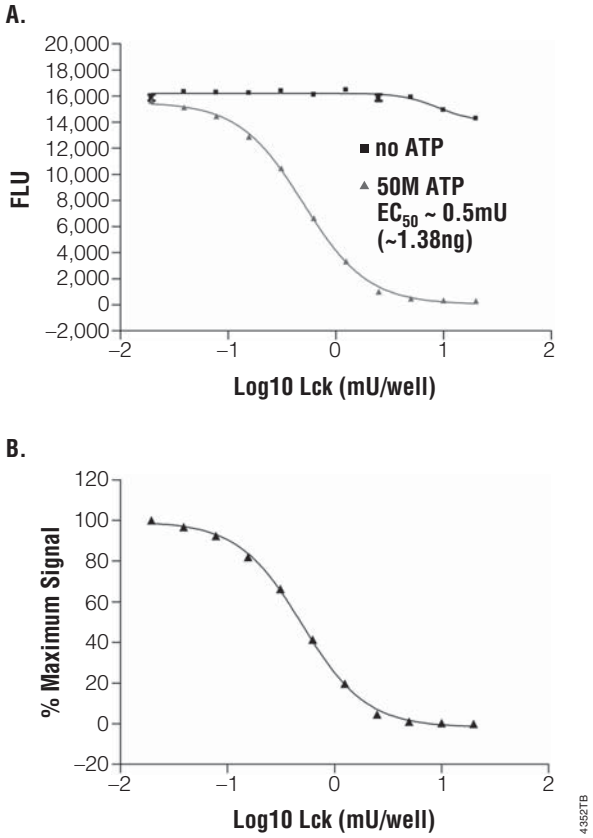


Figure 7.11. Kinase activity is inversely correlated with R110 fluorescence. Results of titration curves performed according to the protocol in Technical Bulletin #TB331 in solid black, flat-bottom 96-well plates. Panels A and B show the results of a Lck titration (Upstate Biotech Cat.# 14-442). **Panel A** shows the data collected (actual R110 FLU) with or without ATP. Data points are the average of 4 determinations. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose response (variable slope) software. The r^2 value is 0.99, EC_{50} is 0.5mU/well, and the maximum dynamic range in the assay is ~50- to ~60-fold. Normalizing the data allows quick determination of the amount of kinase required for the percent conversion desired (**Panel B**).

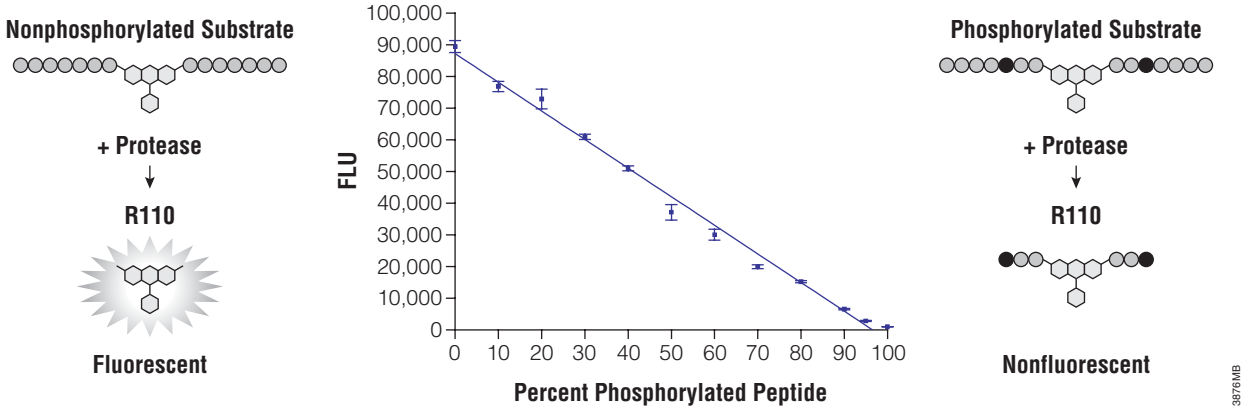


Figure 7.10. Schematic graph demonstrating that the presence of a phosphorylated amino acid (black circles) blocks the removal of amino acids by the protease. The graph shows the average FLU (n = 6) obtained after a 30-minute Protease Reagent digestion using mixtures of nonphosphorylated PKA R110 Substrate and phosphorylated PKA R110 Substrate. (FLU = Fluorescence Light Unit, excitation wavelength 485nm, emission wavelength, 530nm, $r^2 = 0.992$). As the concentration of the phosphopeptide increases in the reaction, FLU decreases.

ProFluor® Kinase Assays
Materials Required:

- ProFluor® PKA Assay (Cat.# V1240, V1241) or ProFluor® Src-Family Kinase Assay (Cat.# V1270, V1271) and protocol (Technical Bulletin #TB315 or #TB331, respectively)
- black-walled multiwell plates (e.g., Microfluor 2, black 96-well plate; ThermoElectron Cat.# 7805)
- multichannel pipet or automated pipetting station
- plate shaker (e.g., DYNEX MICRO-SHAKER® II)
- plate-reading fluorometer with filters capable of reading R110 and AMC fluorescence
- protein kinase

We highly recommend performing a kinase titration to determine the optimal amount of kinase to use for screening and to determine whether or not the enzyme preparation contains components that negatively affect the performance of the assay. Please see Technical Bulletins #TB315 or #TB331 for additional information.

Additional Resources for ProFluor® Kinase Assays

Technical Bulletins and Manuals

TB315 *ProFluor® PKA Assay Technical Bulletin*

TB331 *ProFluor® Src-Family Kinase Assay Technical Bulletin*

Promega Publications

ProFluor® PKA Assay: Excellent Z′-factor values mean reliable results

Assay protein tyrosine kinase and protein tyrosine phosphatase activity in a homogeneous, non-radioactive, high-throughput format

Citations

Watanabe, R. *et al.* (2010) Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity *J. Immunol.* **184**, 4801–4809.

B cells purified from single-cell mouse splenocyte suspensions were resuspended in medium and then stimulated with goat anti-mouse IgM Ab F(ab′)$_2$ fragments and then lysed. Lysates were either incubated with phosphospecific antibodies (Anti-ACTIVE® ERK or JNK Ab) or analyzed for tyrosine kinase activity using the ProFluor™ Src-Family Kinase Assay.

PubMed Number: 20368271

C. Radioactive Kinase Assays

SAM² ® Biotin Capture Membrane

The SAM² ® Biotin Capture Membrane (Cat.# V2861, Cat.# V7861; Figure 7.12) is a proprietary technology that relies on the high-affinity streptavidin:biotin interaction for the capture and detection of biotinylated molecules regardless of their sequence. The unique features of the SAM² ® Membrane compared to other membranes or substrates (e.g., P81 phosphocellulose or streptavidin-coated plates), are the high density of covalently linked streptavidin per square centimeter and the selective mode of capture. This high-density streptavidin matrix efficiently captures biotinylated molecules or substrates, providing high signal-to-noise ratios even in assays using low enzyme concentrations or crude cell extracts. The SAM² ® Biotin Capture Membrane offers superior assay performance by providing high binding capacity, low nonspecific binding, sequence-independent capture and the flexibility of multiple format configurations. The SAM² ® Membrane is available as a sheet containing 96 numbered and partially cut squares. This format is used in the SignaTECT® Kinase Assay Systems. The SAM² ® Membrane is also available as a 7.6 × 10.9cm solid sheet, which can be used for high-throughput applications. The membrane can be analyzed by autoradiography, PhosphofImager® analysis, or scintillation counting.

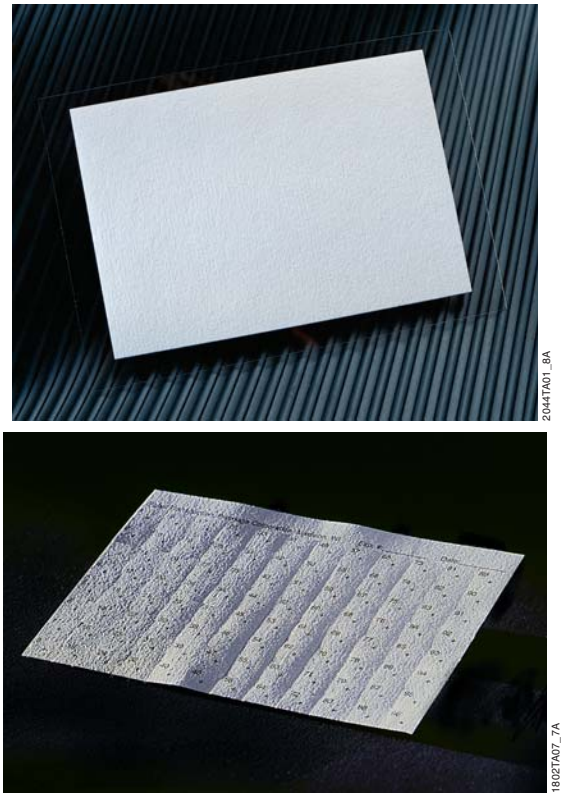


Figure 7.12. SAM² ® Biotin Capture Membrane shown as a 7.6 × 10.9cm sheet (top) and in a 96-square format (bottom).

Additional Resources for SAM² ® Membrane

Technical Bulletins and Manuals

TB547 *SAM² ® Biotin Capture Membrane Technical Bulletin*

Promega Publications

From one to 9,000 samples: Using high-density streptavidin-coated membranes for kinase detection

Advances in SAM2 ® Membrane technology: High-throughput biotin capture systems for use in rapid screening

Protein kinases as drug targets in high-throughput systems

Citations

Xuei, X. *et al.* (2003) Use of SAM2 ® Biotin Capture Membrane in microarrayed compound screening (μARCS) format for nucleic acid polymerization assays *J. Biol. Mol. Screening* **8**, 273–82.

This study evaluated the feasibility of using the μARCS technology for nucleic acid polymerization assays. To ensure the efficient capture of the nucleic acid polymerization reaction and to minimize the nonspecific binding, the authors used a SAM2 ® Biotin Capture Membrane in the assay. In both studies, the nucleic acid substrate was biotinylated on one end and was bound to the SAM2 ® Membrane.

PubMed Number: 12857381

SignaTECT® Protein Kinase Assay Systems

The SignaTECT® Protein Kinase Assay Systems use biotinylated peptide substrates in conjunction with the streptavidin-coated SAM2 ® Biotin Capture Membrane. The binding of biotin to the streptavidin is rapid and strong, and the association is unaffected by rigorous washing procedures, denaturing agents, wide extremes in pH, temperature and salt concentration. High signal-to-noise ratios are generated even with complex samples, while the high substrate capacity allows optimum reaction kinetics. The systems can be used to measure protein kinase activities using low femtomole levels of purified enzyme or crude cellular extracts. SignaTECT® Assays are available to measure protein tyrosine kinases (Cat.# V6480), cdc2 kinase (Cat.# V6430), cAMP-dependent protein kinase (Cat.# V7480), protein kinase C (Cat.# V7470), DNA-dependent protein kinase (Cat.# V7870) and calmodulin-dependent protein kinase (Cat.# V8161).

As outlined in Figure 7.13, the assay steps and analysis of results are straightforward and require only common laboratory equipment. Following phosphorylation and binding of the biotinylated substrate to the numbered and partially cut squares of SAM2 ® Biotin Capture Membrane, unincorporated [γ-^{32}P]ATP is removed by a simple washing procedure. This procedure also removes nonbiotinylated proteins that have been phosphorylated by other kinases in the sample. The bound, labeled substrate is then quantitated by scintillation counting or PhosphorImager® analysis. Typical results generated using the SignaTECT® Assays are presented in Figure 7.14.

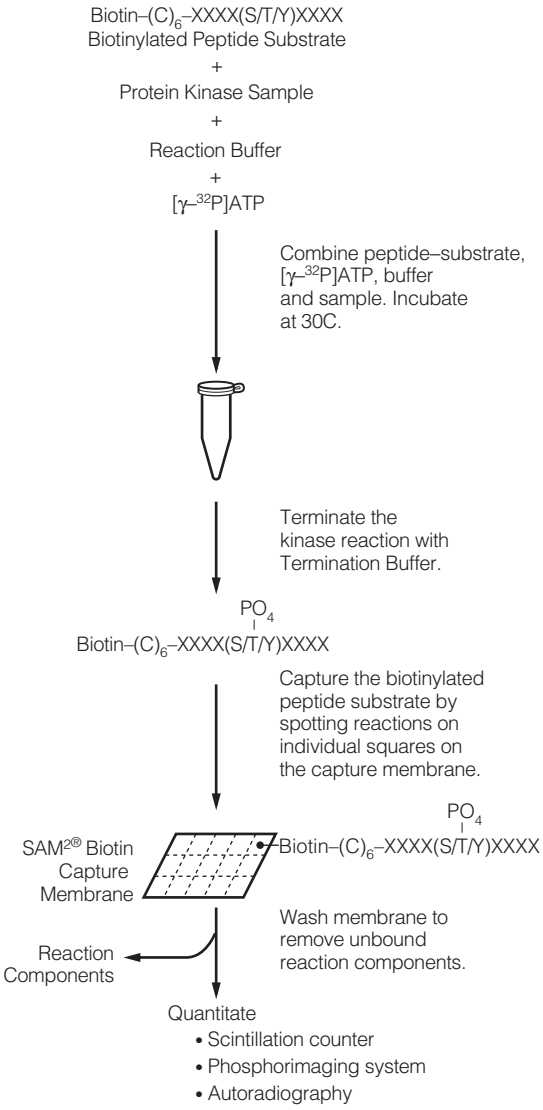


Figure 7.13. The SignaTECT® Protein Kinase Assay protocol.

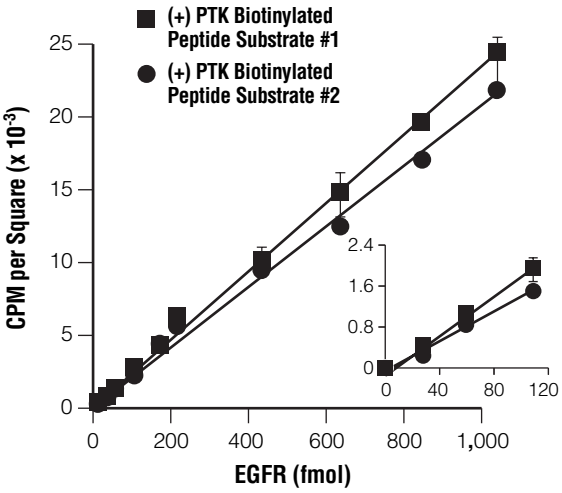


Figure 7.14. Linear detection of EGFR kinase activity with the SignaTECT® PTK Assay System. EGFR (Cat.# V5551) activity was measured in the presence of PTK Biotinylated Peptide Substrate 1 or PTK Biotinylated Peptide Substrate 2, provided with the SignaTECT® PTK System (Cat.# V6480). Inset: enlargement of the data using 120fmol of EGFR.

Additional Resources for the SignaTect® Kinase Assay Systems

Technical Bulletins and Manuals

TB211	*SignaTECT® Protein Tyrosine Kinase (PTK) Assay System Technical Bulletin*
TB227	*SignaTECT® cdc2 Protein Kinase Assay System Technical Bulletin*
TB241	*SignaTECT® cAMP-Dependent Protein Kinase Assay System Technical Bulletin*
TB242	*SignaTECT® Protein Kinase C (PKC) Assay System Technical Bulletin*
TB250	*SignaTECT® DNA-Dependent Protein Kinase Assay System Technical Bulletin*
TB279	*SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System Technical Bulletin*

Promega Publications

Store operated calcium entry activates at the GVBD stage of *Xenopus* meiosis

SAM2 ® Biotin Capture Membrane and SignaTECT® Protein Kinase Assay Systems

Detection and quantitation of protein tyrosine kinases

SignaTECT® DNA-Dependent Protein Kinase Assay System

Tools to study the activation of CaM KII in neuronal functions

Citations

Chau, K.M. and Cornwall, G.A. (20ll) Reduced fertility in vitro in mice lacking the cystatin CRES (Cystatin-Related Epididymal Spermatogenic): Rescue by Exposure of Spermatozoa to Dibutyryl cAMP and Isobutylmethylxanthine *Biology of Reproduction* **84**, 140-152.

The cystatin CRES (cystatin-related epididymal spermatogenic) is encoded by the *Cst8* gene in mice and represents a new, secreted subgroup of the family 2 cystatins. It is synthesized and secreted in the mouse epididymidis and is found in germ cells and spermatozoa. The authors of this paper investigated the potential role of CRES in cAMP-mediated signaling events necessary for fertilization. Protein kinase A activity was compared between *Cst8*$^{-/-}$ and *Cst8*$^{+/+}$ mouse spermatozoa during capacitation using the SignaTECT® PKA Assay System.

PubMed Number: 20811015

Ramana, K.V. *et al.* (2007) Aldose reductase-regulated tumor necrosis factor-alpha production is essential for high glucose-induced vascular smooth muscle cell growth *Endocrinology* **148**, 4371–84.

Inflammation may be a key contributor to the cardiovascular diseases including heart attack and stroke that are associated with diabetes, and markers associated with inflammation, such as TNFα, that are associated with elevated cytokines are elevated in both Type 1 and Type 2 diabetes. The authors of this study looked at the response of rat vascular smooth muscle cells (VSMCs) to high glucose, and found that serum-starved VSMCs exposed to high glucose secrete TNFα. They used the SignaTECT® PKC Assay to show that the expression of the TNFα gene in response to high glucose was preceded by an increase in PKC activity.

PubMed Number: 14966294

Other Kinase Assay Formats (non-radioactive)

The PepTag® Protein Kinase Assays are fast and quantitative nonradioactive alternatives to [γ-^{32}P]ATP-based assays for measuring protein kinase C (Cat.# V5330) and cAMP-dependent protein kinase (Cat.# V5340) activity. The assays use fluorescently-tagged peptide substrates with a net positive charge. Phosphorylation changes the charge of the peptide to a net negative, which influences the migration of the peptide in an agarose gel. This is the basis for detecting changes in phosphorylation via a rapid, 15-minute agarose gel separation (Figure 7.15).

General PepTag® Assay Protocol

Materials Required:

- PepTag® Non-Radioactive PKC Assay (Cat.# V5330) or PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay (Cat.# V5340) and protocol (#TB132)
- PKA or PKC dilution buffer
- horizontal agarose gel apparatus
- glycerol, 80%
- Tris-HCl, 50mM (pH 8.0)
- agarose, 0.8% in 50mM Tris-HCl (pH 8.0)
- probe sonicator

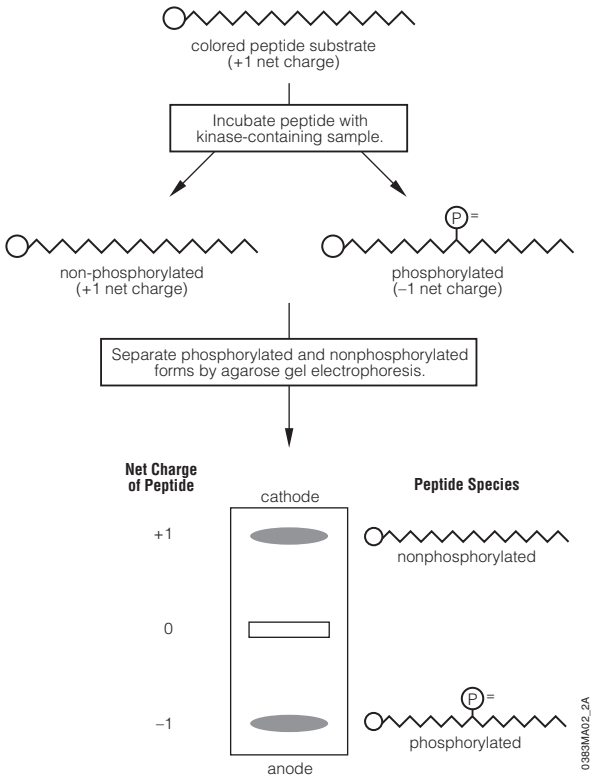


Figure 7.15. Schematic diagram of the PepTag® Non-Radioactive Protein Kinase Assay procedure.

Additional Resources for the PepTag® Non-Radioactive Protein Kinase Assay

Technical Bulletins and Manuals

TB132 *PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase Technical Bulletin*

Citations

Christian, F. *et al.* (2011) Small molecule AKAP-protein kinase A (PKA) interaction disruptors that activate PKA interfere with compartmentalized cAMP signaling in cardiac myocytes *J. Biol. Chem.* **286**, 9079–96.

PKA and other signaling proteins achieve some "compartmentalization" and specification of signaling through the action of tethering proteins known as A-kinase anchoring proteins (AKAPs). The authors of this study investigated the effect of the small molecule, 3,3′-diamino-4,4′-dihydroxydiphylmethane (FMP-API-1) and its derivatives, on compartmentalized PKA signaling in cardiac myocytes. Rat neonatal cardiac myocytes were plated, serum starved overnight and then treated with the small molecule inhibitors of AKAP interactions. The cells were lysed and assayed for PKA activity in the absence of added cAMP using the PepTag® Non-Radioactive Protein Kinase Assay.

PubMed Number: 21177871

III. Kinase Enzyme Systems

The human kinome is composed of more than 500 protein kinase genes that can be grouped together based on sequence homology. The group abbreviations are as follows: *AGC:* Containing PKA, PKG, PKC families; *CAMK:* Calcium/calmodulin-dependent protein kinase; *CK1:* Casein kinase 1; *CMGC*: Containing CDK, MAPK, GSK3, CLK families; *STE:* Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases; *TK:* Tyrosine kinase; *TKL:* Tyrosine kinase-like. Click on each individual group for more detailed information on the kinase members of that group. Promega offers Kinase Enzyme Systems for a number of protein kinases which include: enzyme, preferred substrate, buffer and other components to have you up and running in no time. The Kinase Enzyme Systems are optimized for use with our ADP-Glo™ Kinase Assay and can be ordered together. The ADP-Glo™ Kinase Assay (Cat.# V9101) is a luminescent kinase assay that measures ADP formed from a kinase reaction; ADP is converted into ATP, which is converted into light by Ultra-Glo™ Luciferase. The luminescent signal positively correlates with kinase activity. The assay is well suited for measuring the effects chemical compounds have on the activity of a broad range of purified kinases, making it ideal for both primary screening as well as kinase selectivity profiling.

Receptor Tyrosine Kinase Enzyme Systems

Nonreeceptor Tyrosine Kinase Enzyme Systems

AGC Kinase Enzyme Systems

CAMK Kinase Enzyme Systems

CMGC Kinase Enzyme Systems

STE Kinase Enzyme Systems

Miscellaneous Enzyme Systems

IV. Phosphorylation-Specific Antibodies

The Anti-ACTIVE® phosphorylation-specific antibodies were developed to provide an accurate measure of enzyme activation. These antibodies specifically recognize the active, phosphorylated form of a given kinase. The Anti-ACTIVE® Antibodies are raised against phosphorylated peptide sequences present in the activating loop of a number of protein kinases. Whether used in Western analysis, immunocytochemistry or immunohistochemical staining, the Anti-ACTIVE® MAPK, JNK, p38 and CaM KII Antibodies will recognize only the active form of the enzyme.

A. Phosphorylation-Specific Antibodies in MAPK Signaling Pathways

Anti-ACTIVE® MAPK, pAb, Rabbit, (pTEpY)
This antibody is an affinity purified polyclonal antibody that specifically recognizes the dually phosphorylated, active form of MAPK. The antibody is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active ERK enzyme and recognizes the active forms of ERK1, ERK2 and ERK7.

Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)
Anti-ACTIVE® JNK pAb is an affinity purified polyclonal antibody that recognizes the dually phosphorylated, active form of cJun N-terminal protein Kinase (JNK).
Anti-ACTIVE® JNK pAb is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active JNK enzyme. The antibody recognizes the active forms of JNK1, JNK2, and JNK3 isoforms.

Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)
Anti-ACTIVE® p38 Ab, Rabbit, is an affinity purified polyclonal antibody that recognizes the active form of p38 kinase. The Anti-ACTIVE® p38 pAb is raised against the dually phosphorylated peptide sequence representing the catalytic core of the active p38 enzyme. The Anti-ACTIVE® p38 pAb recognizes the active forms of p38α, γ, and δ isoforms.

Western Blot Analysis with Anti-ACTIVE® MAPK, JNK and p38 pAbs
Materials Required:

- Anti-ACTIVE® MAPK (Cat.# V8031), JNK (Cat.# V7931), or p38 (Cat.# V1211) pAb
- Anti-ACTIVE® Qualified Donkey Anti-Rabbit IgG (H+L), HRP (Cat.# V7951) Secondary Antibodies
- protein sample transfered to nitrocellulose or PVDF membrane
- bovine serum albumin, 1%
- TBS buffer
- TBST or PVDF buffer
- shaking platform

See Figure 7.16 for a sample Western blot protocol.

Immunocytochemistry with Anti-ACTIVE® MAPK, JNK and p38 pAbs
The following method is for preparing and immunostaining PC12 cells stimulated by either nerve growth factor to activate MAP kianses or soribitol to activate JNK and p38 kinases. For additional information see Technical Bulletin #TB262

Materials Required:

- Anti-ACTIVE® Qualified Donkey Anti-Rabbit IgG (H+L), HRP (Cat.# V7951) Secondary Antibodies
- LabTek® 4-chambered slides (Fisher Cat.# 12-565-21)
- rat-tail collagen (Collaborative BioScience Products)
- RPMI 1640 with 25mM HEPES, 300mg/l L-glutamine, 10% horse serum, 5% fetal bovine serum and 0.5mM EGTA
- NGF (Cat.# G5141) or sorbitol
- PBS
- 10% paraformaldehyde
- methanol, –20°C
- blocking buffer
- donkey anti-rabbit Cy®3 conjugate (Jackson ImmunoResearch Cat.# 741-165-152)

Preparation and Activation of PC12 Cells

1. Coat 4-chambered slides with rat tail collagen (6µg/cm^2 in sterile PBS) for one hour.
2. Grow PC12 cells in chambers at 37° in 5% CO_2 in medium containing RPMI 1640 with 25mM HEPES, 300mg/L L-glutamine, 10% horse serum, 5% fetal bovine serum and 0.5mM EGTA. The medium should be changed every other day until the cells reach 80% confluence.
3. Activate the cells in 2 chambers as described below. Use the cells in the remaining 2 chambers as untreated controls.

   NGF: The day before immunocytochemistry, add fresh medium with serum. The next day add 200ng/ml NGF in RPMI. Incubate for 5 minutes at 37°C.

   Sorbitol: The day before immunocytochemistry, add fresh medium without serum. The next day add sorbitol to a final concentration of 1M. Incubate for 30 minutes at 37°C.
4. Proceed with staining as outlined in Figure 7.17.

Additional Resources for the Anti-ACTIVE® Antibodies

Technical Bulletins and Manuals

TB262 *Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates*

Promega Publications

New Anti-ACTIVE® MAPK and 'pan ERK 1/2' antibodies for Western analysis

Technically speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways

Demonstration of immunohistochemical staining using Promega Anti-ACTIVE® and apoptosis Aatibodies

Citations

Hsu, C.Y. *et al.* (2004) Characterization of active mitogen-activated protein kinase in ovarian serous carcinomas *Clin. Can. Res.* **10**, 6432–6.

The Anti-ACTIVE® MAPK polyclonal antibody was used to immunohistochemically stain and type patient ovarian serous carcinomas using paraffin-fixed tissue sections on tissue microarrays. Western blots were also performed on tissue lysates using a 1:3,000 dilution of the antibody.

PubMed Number: 15475429

Le'Negrate, G. *et al.* (2003) Downregulation of caspases and Fas ligand expression, and increased lifespan of neutrophils after transmigration across intestinal epithelium *Cell Death Differ.* **10**, 153–62.

Anti-ACTIVE® JNK pAb was used in immunoblot analysis of human polymorphonuclear leukocyte protein lysates.

PubMed Number: 12700643

Aballay, A. *et al.* (2003) *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway *Curr. Biol.* **13**, 47–52.

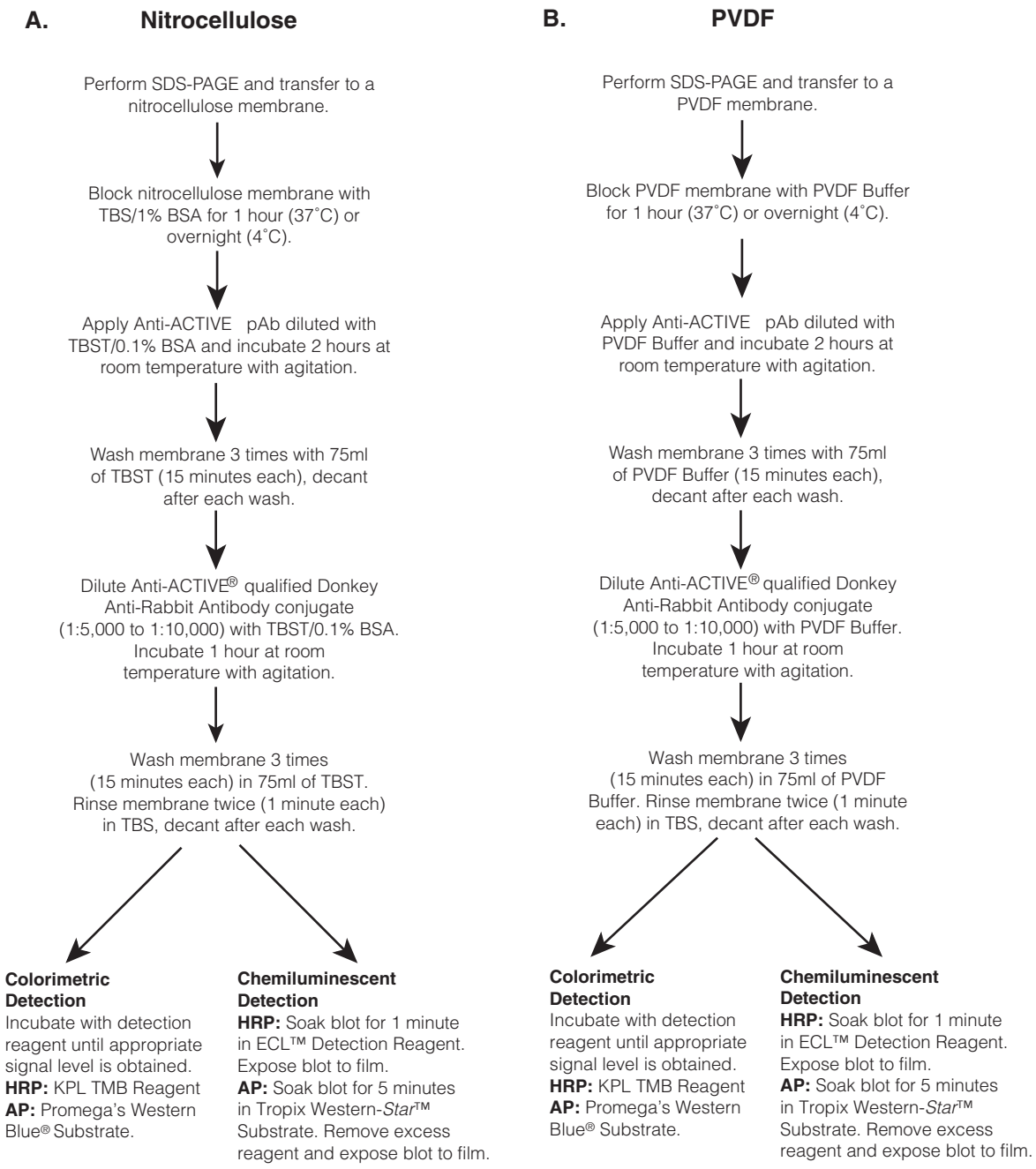
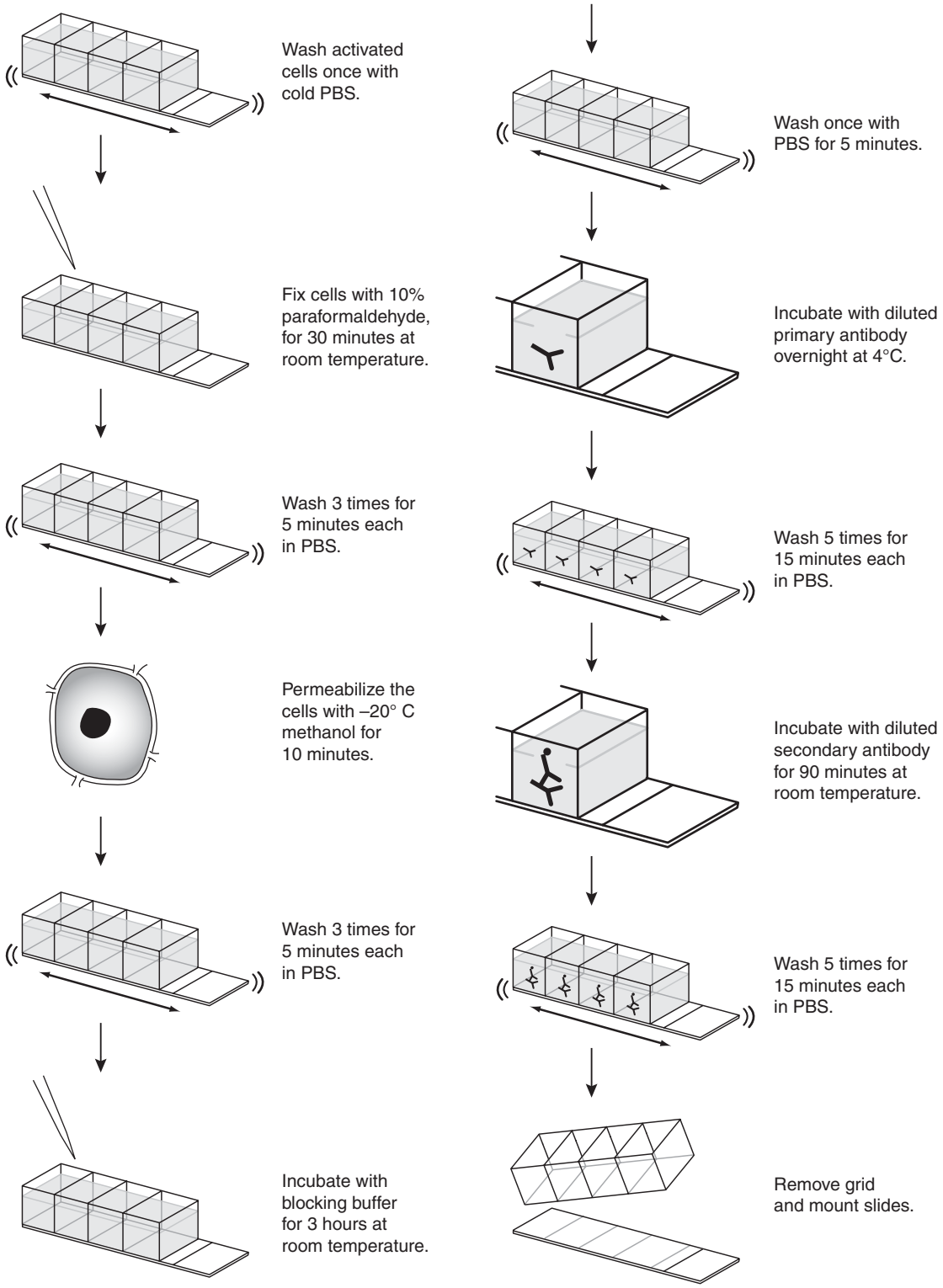


Figure 7.16. This schematic diagram illustrates the use of nitrocellulose and PVDF membranes in Western blot analysis with Anti-ACTIVE® pAbs. Protocols for use with nitrocellulose (**Panel A**) and PVDF (**Panel B**) membranes. The recommended dilutions of the Anti-ACTIVE® pAbs are 1:5,000 for Anti-ACTIVE® MAPK pAb, 1:2,000 for Anti-ACTIVE® p38 pAb, 1:5,000 for Anti-ACTIVE® JNK pAb and 1:5,000 to 1:10,000 for the Anti-ACTIVE® Donkey Anti-Rabbit IgG (H+L) secondary antibodies (HRP-conjugated). KPL is an abbreviation for Kirkegaard and Perry Laboratories. See Technical Bulletin #TB262 for more information about this protocol. You may need to determime the optimal dilutions of primary and secondary antibodies for your system. If you use secondary antibodies other than those available from Promega, you may need to perform additional experiments to determine optimal conditions.

Activation of the p38 homolog in the worm was monitored by Western analysis using the Anti-ACTIVE® p38 pAb.
PubMed Number: 12526744



(continued in next column)

Figure 7.17. Immunostaining of activated PC12 cells. This protocol is for immunostaining of activated PC12 cells and may need to be optimized for your particular experimental system. Incubation times and antibody dilutions will need to be empirically determined for optimal results.

B. Phosphorylation-Specfic CaM KII Antibody

This antibody recognizes calcium/calmodulin-dependent protein kinase CaM KII that is phosphorylated on threonine 286. The Anti-ACTIVE® CaM KII pAb (Cat.# V1111) was raised against the phosphothreonine-containing peptide derived from this region.

Additional Information for the Anti-ACTIVE® CaM KII pAb

Technical Bulletins and Manuals

TB264 *Anti-ACTIVE® CaM KII pAb, (pT286) and Anti-ACTIVE® Qualified Secondary Antibody Conjugates Technical Bulletin*

Promega Publications

Anti-ACTIVE® Antibody for specific detection of phosphorylated CaM KII protein kinase

Citations

Matsumoto, Y. and Maller, J.L. (2002) Calcium, calmodulin and CaM KII requirement for initiation of centrosome duplication in *Xenopus* egg extracts *Science* **295**, 499–502.

CaM KII(281-309) was added to metaphase-arrested extracts. After adding calcium, the extracts were incubated at room temperature. Anti-ACTIVE® CaM KII pAb and Anti-ACTIVE® Qualified HRP secondary antibodies were used to probe immunoblots for phospho-T286 CaM KIIα.

PubMed Number: 11799245

V. Kinase Inhibitors

A. MEK Inhibitor U0126

MEK Inhibitor U0126 (Cat.# V1121) inhibits the activity of MAP Kinase Kinase (MEK 1/2) and thus prevents the activation of MAPK. U0126 inhibits MEK 1 with an IC_{50} of 0.5µM in vitro (Favata *et al*. 1998). U0126 inhibits phosphorylation activated MEK 1 and MEK 2 as well as constitutively active MEK 1 and MEK 2 mutants (Favata *et al.* 1998; Goueli *et al*. 1998). U0126 is noncompetitive with respect to the MEK substrates ATP and ERK (Favata *et al.* 1998; Tolwinski *et al*. 1999).

Additional Resources for MEK Inhibitor U0126

Technical Bulletins and Manuals

9PIV112 *MEK Inhibitor U0126 Promega Product Information*

Promega Publications

Frequently asked questions: Kinase inhibitors and activators

U0126: A novel, selective and potent inhibitor of MAP Kinase Kinase (MEK)

Citations

Cancedda, L. *et al.* (2003) Patterned vision causes CRE-mediate gene expression in the visual cortex through PKA and ERK. *J. Neurosci.* **23**, 7912–20.

MEK Inhibitor U0126 was used to demonstrate specific CRE-mediated gene expression through Erk activation in mouse brains.

PubMed Number: 12904462

B. PD 98059

PD 98059 (Cat.# V1191) inhibits MEK activation (Alessi *et al*. 1995; Dudley *et al*. 1995; Favata *et al*. 1998). PD 98059 inhibits MEK 1 but is an inefficient inhibitor of MEK 2. (Alessi *et al*. 1995; Dudley *et al*. 1995). It inhibits activation of MEK 1 by Raf with an IC_{50} of 5µM and of the active MEK 1 mutant with an IC_{50} of 10µM (Alessi *et al*. 1995; Dudley *et al*. 1995).

Additional Resources for PD 98059

Promega Publications

Frequently asked questions: Kinase inhibitors and activators

Citations

Schmidt, H. *et al.* (2000) Involvement of mitogen-activated protein kinase in agonist-induced phosphorylation of the mu-opioid receptor in HEK 293 cells *J. Neurochem.* **74**, 414–22.

HEK 293 cells stably expressing the mu-opioid receptor respond to agonists by MAPK phosphorylation. Activation of the MAPK was completely inhibited by the MEK Inhibitor U0126 at 100nM as judged by immunocytochemistry. The PD 98059 required 20µM for the same inhibitory effect.

PubMed Number: 10617147

C. SB 203580

SB 203580 (Cat.# V1161) is a specific, cell-permeant inhibitor of the stress and inflammatory cytokine-activate MAP kinase homologues p38α, β and β2. It acts as a competitive inhibitor of ATP binding to the kinase. Reported IC_{50} values range from 21nM to 1µM. SB 203580 has no significant effect on the activities of ERKs, JNKs, p38γ or p38δ.

Promega Publications

Frequently asked questions: Kinase inhibitors and activators

Citations

Wyttenbach, A. *et al.* (2002) Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* **11**, 1137–51.

SB 203580 MAP kinase p38α, p38β and p38β2 inhibitor was used in both COS-7 and SK-N-SH huntingtin exon 1-transfected cell cultures. Decreased nuclear fragmentation was reported when 1 or 10µM SB 203580 inhibitor was added to the transfected cell cultures.

PubMed Number: 11978772

D. PI3 Kinase Inhibitor LY 294002

LY 294002 (Cat.# V1201) is a potent and specific cell-permeant inhibitor of phosphatidylinositol 3-kinases (PI3-K) with an IC_{50} value in the 1–50µM range. LY 294002 competitively inhibits ATP binding to the catalytic subunit of PI3-Ks and does not inhibit PI4-Kinase, DAG-kinase, PKC, PKA, MAPK, S6 kinase, EGFR or c-src tyrosine kinases and rabbit kidney ATPase (Rameh and Cantley, 1999; Fruman *et al.* 1998). LY 294002 has improved stability and specificity compared to Wortmannin, which is an irreversible inhibitor that covalently interacts with PI3-Ks.

Additional Resources for LY 294002

Promega Publications

Frequently asked questions: Kinase inhibitors and activators

Citations

Yamaguchi, K. *et al.* (2004) Identification of nonsteroidal anti-inflammatory drug-activated gene (NAG-1) as a novel, downstream target of phosphatidylinositol 3-kinase/Akt/GSK-3B pathway. *J. Biol. Chem.* **279**, 49617–23.

The authors used inhibition of PI3-Kinase by LY 294002 to determine that NAG-1 expression in human colorectal cancer cells is regulated by a PI3-kinase pathway.

PubMed Number: 15377673

E. cAMP-Dependent Protein Kinase (PKA) Peptide Inhibitor

The cAMP-Dependent Protein Kinase Inhibitor (Cat.# V5681), also known as PKI, TTYADFIASGRRNAIHD, inhibits phosphorylation of target proteins by binding to the protein-substrate site of the catalytic subunit of PKA. It corresponds to the region 5–24 of the naturally occurring PKI.

Additional Resources for the PKA Peptide Inhibitor

Citations

Zhang, B. *et al.* (2004) Rac1 inhibits apoptosis in human lymphoma cells by stimulating Bad phosphorylation on Ser-75. *Mol. Cell. Biol.* **24**, 6205–14.

Researchers used the cAMP-Dependent Protein Kinase Peptide Inhibitor to demonstrate that BAD kinase is phosphorylated through a cAMP-Dependent Protein Kinase (PKA) dependent pathway in Burkitt's lymphoma BL-41 cells.

PubMed Number: 15226424

F. InCELLect® AKAP St-Ht31 Inhibitor Peptide

The InCELLect® AKAP St-Ht31 Inhibitor Peptide (Cat.# V8211) and the InCELLect® Control Peptide (Cat.# V8221) can be used for in vivo studies of PKA activation. The Inhibitor Peptide is a stearated (St) form of the peptide Ht31 derived from the human thyroid AKAP (A-kinase anchoring protein). The presence of the hydrophobic stearated moiety enhances the cellular uptake of the peptides through the lipophilic microenvironment of the plasma membrane.

Additional Resources for InCELLect® AKAP St-Ht31 Inhibitor Peptide

Promega Publications

InCELLect® cell-permeable, stearated peptides to probe cAMP-dependent protein kinase-mediated cellular signaling reactions in vivo.

G. Myristoylated Protein Kinase C Peptide Inhibitor

Myristoylated Protein Kinase C Peptide Inhibitor (Cat.# V5691) specifically inhibits calcium- and phospholipid-dependent protein kinase C. It is based on the pseudosubstrate region of PKC-α and PKC-β (Eicholtz, 1993).

Additional Resources for Myristoylated Protein Kinase C Peptide Inhibitor

Citations

Spyridopoulos, I. *et al.* (2002) Divergence of angiogenic and vascular permeability signaling by VEGF inhibition of protein kinase C suppresses VEGF-induced angiogenesis but promotes VEGF-induced, NO-dependent vascular permeability. *Ateriosclerosis. Thromb. Vasc. Biol.* **22**, 901–6.

The Myristoylated Protein Kinase C Peptide Inhibitor and cAMP-Dependent Protein Kinase Peptide Inhibitor were used in cell and animal studies to help specifically identify Protein Kinase A and C activities.

PubMed Number: 12067896

H. Olomoucine cdc2 Protein Kinase Inhibitor

Olomoucine is a chemically synthesized inhibitor that is specific for $p34^{cdc2}$ and related protein kinases. Its molecular weight is 298, and its molecular formula is $C_{15}H_{18}N_6O$.

Additional Resources for Olomoucine cdc2 Protein Kinase Inhibitor

Citations

Yan, X. *et al.* (2003) Human Nudel and NudE as regulators of cytoplasmic dynein in poleward protein transport along the mitotic spindle *Mol. Cell. Biol.* **23**, 1239–50.

Mitotic extracts were prepared from HEK293T cells transfected with plasmids encoding FLAG/Nudel fusion protein. Kinase assays were performed on the immunoprecipitated mitotic extracts in the presence or absence of olomoucine.

PubMed Number: 12556484

VI. Phosphatase Assays

Protein phosphorylation plays a key role in signal transduction, and genes for protein kinases and phosphatases represent a large portion of the human genome (Goueli *et al.* 2004b; Cohen, 2001). They are the opposing partners to the kinases in the cell, catalyzing the dephosphorylation of molecules involved in cellular

pathways. Protein phosphatases can be divided into three general categories: a) protein tyrosine phosphatases, which remove phosphate from phosphotyrosine-containing proteins, b) protein serine/threonine phosphatases, which remove phosphate from phosphoserine- or phosphothreonine-containing proteins, and c) dual-specificity phosphatases, which can remove phosphate from phosphotyrosine, phosphothreonine, and phosphoserine (Hunter, 1995).

A. Fluorescent Phosphatase Assays

We have developed the ProFluor® Phosphatase Assays to overcome safety issues associated with radioactive assays while maintaining sensitivity and specificity. The ProFluor® Phosphatase Assays use bisamide R110-linked phosphopeptides that serve as substrates for PTPases. Phosphorylation of the peptide substrate renders it resistant to cleavage by the Protease Reagent that is included with these assay systems, reducing the fluorescence generated. However, when the phosphoryl moiety is removed by a phosphatase, the peptides become cleavable by the protease, releasing the highly fluorescent, free R110 molecule (Figure 7.18).

The ProFluor® PPase Assays offer the simplicity, sensitivity and specificity required for screening chemical libraries for novel inhibitors of protein phosphatases. These assays are robust with Z′ factor values routinely greater than 0.8 (Figure 7.19; Goueli *et al*. 2004b)

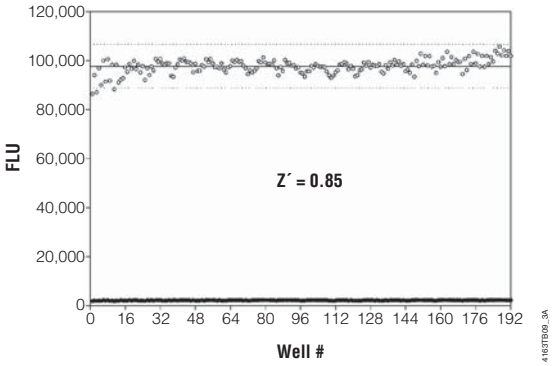


Figure 7.19. Z′ factor values obtained in 384-well plates for the ProFluor® S/T PPase Assay. The assay was performed manually according to the protocol provided in Technical Bulletin #TB324 using solid black, flat-bottom plates with phosphatase (open circles) and without phosphatase (solid circles). Solid lines indicate the mean, and the dotted lines indicate ±S.D. 6.25milliunits/well PP1 (Calbiochem Cat.# 539493) was used. Z′ factor was 0.85).

Z′ factor is a statistical description of the dynamic range and variability of an assay. Z′ factor values >0.5 are indicative of a robust assay (Zhang *et al.* 1999). These fluorescent assays can be performed in single tubes, 96-well plates or 384-well plates, giving the user flexibility in format. The signal-to-noise ratio is very high, and the generated signal is stable for hours.

General Protocol for the ProFluor® Phosphatase Assays
Materials Required:

- ProFluor® Ser/Thr Phosphatase Assay (Cat.# V1260, V1261) or ProFluor® Tyrosine Phosphatase Assay (Cat.# V1280, V1281) and protocol (Technical Bulletin #TB324 or TB334, respectively)
- opaque-walled multiwell plates
- multichannel pipet or automated pipetting station
- plate shaker (DYNEX MICRO-SHAKER® or equivalent)
- plate-reading fluorometer with filters for reading R110 and AMC fluorescence
- protein tyrosine phosphatase or S/T protein phosphatase
- okadaic acid (for PP1 and PP2A)
- calmodulin (for PP2B)

1. Dilute the phosphatase in Reaction Buffer and add to wells.
2. Dilute the PTPase R110 Substrate and the Control AMC Substrate in Reaction Buffer and add to wells.
3. Mix the contents of the plate for 15 seconds and incubate at room temperature (10 minutes for PP1 and PP2A; 30 minutes for PP2B; 60 minutes for tyrosine PPase).
4. Add Protease Solution.
5. Mix the contents of the plate briefly and incubate at room temperature (90 minutes for PP2A, PP2B or PP1; 30 minutes for tyrosine PPase).
6. Add Stabilizer Solution.
7. Mix the contents of the plate and read fluorescence.

Additional Resources for ProFluor® Phosphatase Assays

Technical Bulletins and Manuals

TB324 *ProFluor® Ser/Thr PPase Assay Technical Bulletin*

TB334 *ProFluor® Tyrosine Phosphatase Assay Technical Bulletin*

Promega Publications

Monitor purified phosphatase activity with a homogeneous non-radioactive high-throughput fluorogenic assay

Assay protein tyrosine kinase and protein tyrosine phosphatase activity in a homogeneous, non-radioactive high-throughput format

Citations

Gagne, J.M. and Clark, S.E. (2011) The *Arabidopsis* stem cell factor POLTERGEIST is membrane localized and phospholipid stimulated *The Plant Cell* **22**, 729–43.

Two protein phosphatase type 2C (PP2C) proteins, POL and PLL1, are required for asymmetric divisions associated with stem cell maintenance and differentiation in *Arabidopsis* at both the root and shoot meristems. The authors of this paper expressed recombinant Maltose

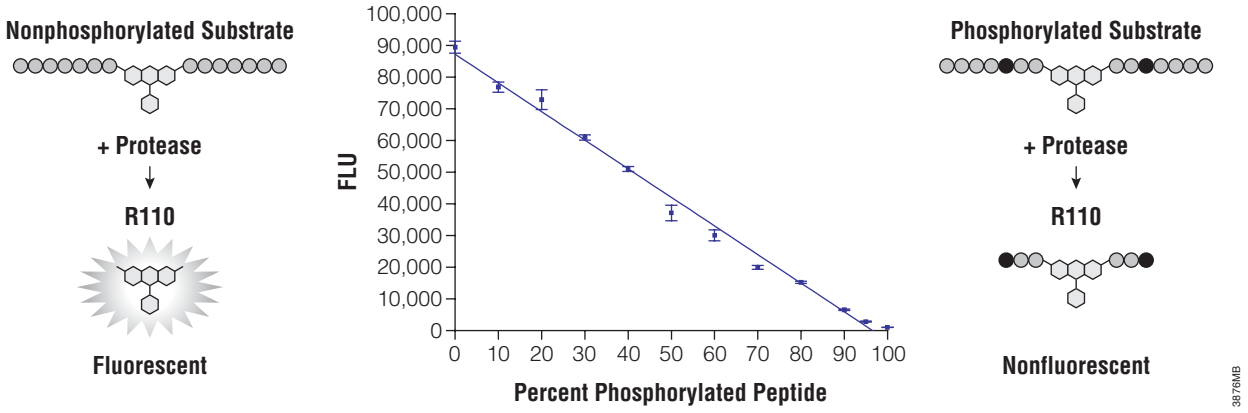


Figure 7.18. Schematic and graph demonstrating that Rhodamine 110 is essentially nonfluorescent in the bisamide form and that the presence of a phosphorylated amino acid (dark circle) blocks the removal of amino acids by the protease. The graph shows the average FLU obtained after a 30-minute protease reagent digestion using mixtures of nonphosphorylated R1110 PKA Substrate and phosphorylated R110 PKA Substrate as indicated (n = 6).

Binding Protein-POL fusion proteins, and used the ProFluor™ Ser/Thr Phosphatase Assay to assess the nature of the phosphatase activity associated with POL and determine the effect of various phospholipids on it. The assays showed that known PP2C protein phosphatase inhibitors also inhibited the MBP-POL activity. They also showed that PI(3)P, PI(4)P and PI(5)P also stimulated POL phosphatase activity.

PubMed Number: 20348433

Kupcho, K. *et al.* (2004) A homogeneous, nonradioactive high-throughput fluorogenic protein phosphatase assay. *J. Biomol. Screen.* **9**, 223–31.

This article describes the use of the ProFluor® Phosphatase Assays to measure the activity of protein phosphatases at low concentrations.

PubMed Number: 15140384

B. Colorimetric Phosphatase Assays

Both the Tyrosine Phosphatase (Cat.# V2471) and the Serine/Threonine Phosphatase (Cat.# V2460) Assay Systems detect the release of phosphate from specific peptide substrates by measuring the appearance of a phosphate complex of molybdate:malachite green. For assays of crude extracts, endogenous phosphate and other inhibitory molecules are first removed by a simple 20-minute procedure using Spin Columns that are supplied with each system. This step is unnecessary for assays using pure or partially purified enzyme preparations. Each system includes ready-to-use, specific substrates: the Tyrosine Phosphatase System provides two phosphotyrosine-containing peptides; the Serine/Threonine Phosphatase Assay System provides a phosphothreonine-containing peptide. Other phosphopeptides or phosphoproteins can be used as substrates to increase specificity or to use natural substrates. The simple assay procedure is outlined in Figure 7.20.

Materials Required:

- Serine/Threonine Phosphatase Assay System (Cat.# V2460) or Tyrosine Phosphatase Assay System (Cat.# V2471) and protocol (Technical Bulletin # TB218 or #TB212, respectively)
- 50ml disposable conical centrifuge tubes (e.g., Corning Cat.# 25330-50)
- appropriate storage buffer (see TB212 or TB218)
- Sephadex® G-25 storage buffer (for storing column)

Additional Resources for Serine/Threonine and Tyrosine Phosphatase Assay Systems

Technical Bulletins and Manuals

TB218	*Serine/Threonine Phosphatase Assay System Technical Bulletin*
TB212	*Tyrosine Phosphatase Assay System Technical Manual*

Citations

Bandyopadhyay, J. *et al.* (2002) Calcineurin, a calcium/calmodulin-dependent protein phosphatase, is involved in movement, fertility, egg laying, and growth in *Caenorhabditis elegans*. *Mol. Biol. Cell* **13**, 3281–93.

Purified fusion proteins created *C. elegans* that resemble insect and mammalian calcineurin were tested in the Serine/Threonine Phosphatase Assay System

PubMed Number: 12221132

Liu, T *et al.* (2004) Reactive oxygen species mediate virus-induced STAT activation: Role of tyrosine phosphatases. *J. Biol. Chem.* **279**, 2461–9.

The Tyrosine Phosphatase Assay System was used to assess the level of tyrosine phosphatase activity in human alveolar type II-like epithelial cells (the A549 cell line).

PubMed Number: 14578356

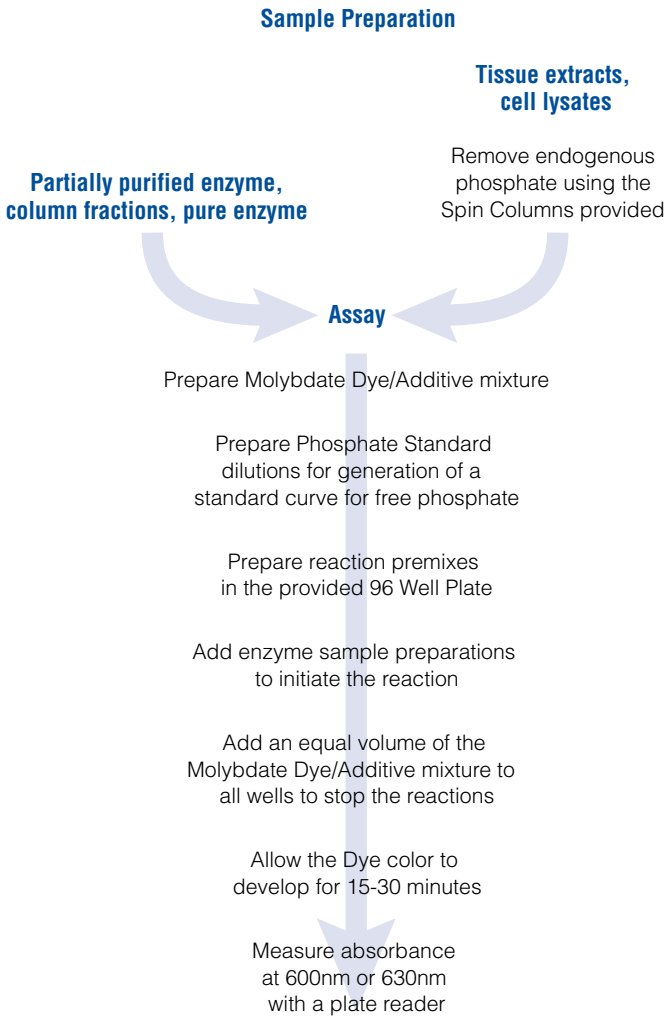


Figure 7.20. Steps required for measuring phosphatase activity using the Serine/Threonine or the Tyrosine Phosphatase Assay System. These systems can be used to measure phosphatase activity from partially purified enzyme preparations and tissue extracts or cell lysates.

VII. References

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