Technical Bulletin

Serine/Threonine Phosphatase Assay System

INSTRUCTIONS FOR USE OF PRODUCT V2460.

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Serine/Threonine Phosphatase Assay System

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1. Description

The non-radioactive Serine/Threonine Phosphatase Assay System provides a fast, convenient and flexible method for measuring protein serine/threonine phosphatase activity. This system determines the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate:malachite green:phosphate complex (1–3). The system allows the use of a variety of buffer conditions and substrates, including naturally phosphorylated proteins or synthetic phosphopeptides.

Protein phosphorylation plays an important role in the regulation of many diverse cellular processes including differentiation, cell division, metabolism, contractility, fertilization and memory (for reviews, see references 4–17). These responses are regulated by a delicate balance between protein kinases and protein phosphatases. While protein kinases have been relatively easy to study by measuring the incorporation of radioactive phosphate into proteins or specific peptide substrates, characterization of protein phosphatases has proved to be more difficult. One approach has involved the use of radiolabeled

substrates for the phosphatases. However, this approach has several disadvantages: prelabeling of protein substrates with radioactive phosphate is time-consuming, the labeled substrate must be made repeatedly, and the resulting substrate rarely can be used at optimal concentrations.

Protein phosphatases can be divided into two main classes: 1) those that remove phosphate from proteins or peptides containing phosphotyrosine, and 2) those that remove phosphate from proteins or peptides containing phosphoserine or phosphothreonine. To distinguish these two phosphatase classes, we have designed two assay systems.

The Tyrosine Phosphatase Assay System (Cat.# V2471) contains two chemically synthesized phosphopeptides, END(pY)INASL (18) and DADE(pY)LIPQQG (19), which serve as substrates for many protein tyrosine phosphatases. The Serine/Threonine Phosphatase Assay System (Cat.# V2460) contains the chemically synthesized phosphopeptide, RRA(pT)VA, a peptide substrate that is compatible with several serine/threonine phosphatases such as protein phosphatases 2A, 2B and 2C (20). **However, the supplied phosphopeptide is a poor substrate for protein phosphatase 1 because of its more stringent structural requirements.**

The effective range for the detection of phosphate released during an assay using the Serine/Threonine Phosphatase Assay System is 100–4,000pmol of phosphate. While the lower end of this range is less sensitive than radioactive assays, the concentration of substrates and the working range of the assay are compatible with the V_{max} and K_m values of commonly used phosphatases using phosphopeptide substrates. This feature more accurately assesses kinetic data, a benefit that cannot be claimed by the so-called more sensitive methods. Working in the higher range also reduces the possibility of detecting aberrant phosphatase activity due to promiscuity of certain phosphatases. At this range of sensitivity, low nanogram levels of phosphatases can be detected.

In addition to measuring phosphatase activity in partially fractionated and purified samples, the Serine/Threonine Phosphatase Assay System also can measure phosphatase activity in crude cell or tissue extracts. For this application, the high concentration of phosphate in these preparations is eliminated prior to performing the assay using the supplied Spin Columns, which rapidly and effectively remove free phosphate and other low-molecular-weight inhibitors from the sample. In addition, the unique Molybdate Dye Additive, which is combined with the Molybdate Dye Solution, aids in the solubilization of proteins exposed to the acidic conditions of the Molybdate Dye Solution, which alone could potentially cause precipitation of the proteins.

Figure 1 provides an overview of the steps required to measure serine/threonine phosphatase activity from partially purified enzyme preparations or cell/tissue extracts using this system.

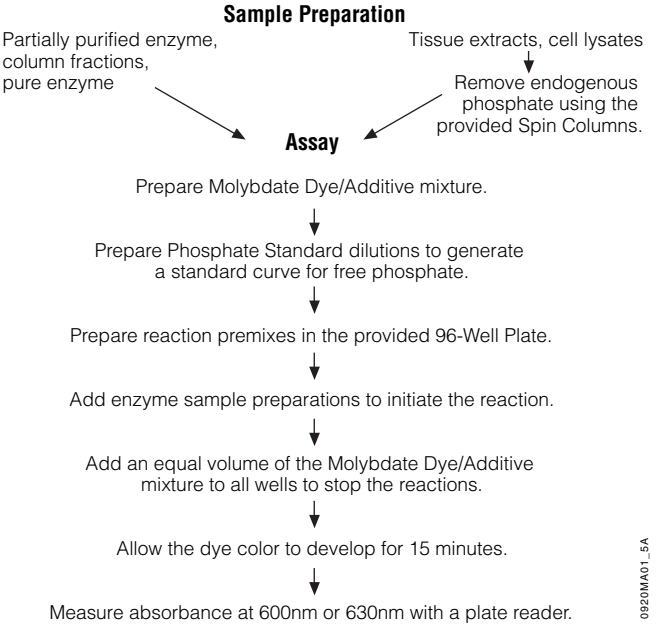

Figure 1. Overview of the steps required for measuring serine/threonine phosphatase activity using the Serine/Threonine Phosphatase Assay System. This system can be used to measure phosphatase activity from partially purified enzyme preparations and tissue extracts/cell lysates.

2. Product Components and Storage Conditions

Product	Size	Cat.#
Serine/Threonine Phosphatase Assay System	96 reactions	V2460

Each system contains sufficient reagents to perform 96 phosphatase reactions. Includes:

- 20ml Molybdate Dye Solution
- 200µl Molybdate Dye Additive
- 1ml Phosphate Standard, 1mM
- 1mg Ser/Thr Phosphopeptide
- 1 96-Well Plate (½ -area, flat-bottom)
- 4 Spin Columns, Reservoirs and Adaptors
- 40ml Sephadex® G-25
- 25ml Phosphate-Free Water

Storage Conditions: Store the system at 4°C. The 96-Well Plate and Spin Columns may be stored at room temperature. Reconstituted Ser/Thr Phosphopeptide (Section 3.B) may be stored at 4°C or –20°C.

3. Before Beginning

3.A. Selection of Assay Format

Phosphatase assays may be performed in the supplied 96-Well Plate (Costar® Cat.# 3690) or in standard 96-well plates with good optical qualities. The 96-Well Plate provided with this system has a reduced well diameter and volume (190µl) that accommodates the standard 50µl reaction plus 50µl of the Molybdate Dye/Additive mixture. Because of the well shape (tall and narrow), the supplied ½ -area 96-Well Plate allows higher absorbance readings than standard 96-well plates. The convenient 96-well format of the supplied plate allows sample analysis under a variety of conditions using several time points.

Do not discard the supplied plate until all wells have been used. **Do not** reuse stained wells.

If you choose to use standard 96-well plates, perform reactions in 50–100µl volumes, and add an equal volume of the Molybdate Dye/Additive mixture as described in Section 4.A.

Standard plates typically have about half the optical density as the supplied 96-Well Plate at any given phosphate concentration and thus have a proportionally lower background for negative control samples. Because standard plates are not as sensitive, higher phosphate concentrations can be measured accurately (up to 4nmol of phosphate can be assayed before the optics of plate readers generate nonlinear responses). A comparison of the two plate types is shown in Figure 2.

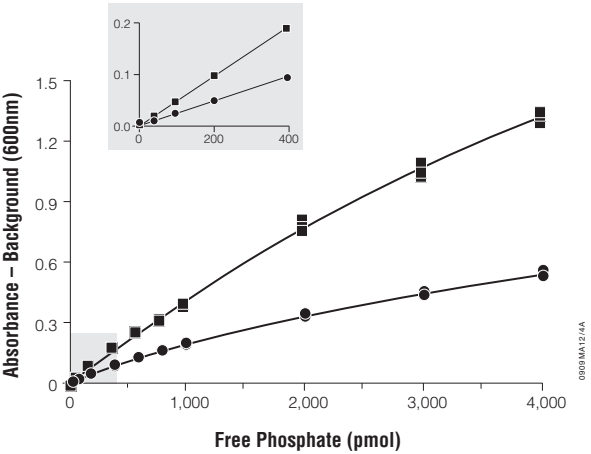

Figure 2. Comparison of absorbance results for the Phosphate Standard when using the supplied 96-Well Plate (½-area, flat-bottom; squares) and standard plates (circles). Samples were measured in triplicate. The inset depicts the lower, linear detection range from the same experiments.

If a plate reader is unavailable, you may use larger volumes (e.g., 400µl of reaction mixture and 400µl of Molybdate Dye/Additive mixture) so that cuvettes and a spectrophotometer can be used. Determine the minimum volume that can be used in a disposable cuvette (the dye will stain cuvettes). Half of this volume must be the Molybdate Dye/Additive mixture (prepared in Section 4.A); and the other half can be reaction mix or a combination of reaction mix and water. The system contains enough Molybdate Dye Solution for about 50 reactions with a final volume of 800µl (reaction mixture plus Molybdate Dye/Additive mixture).

3.B. Substrates

Phosphopeptide

Reconstitute the supplied Ser/Thr Phosphopeptide to 1mM with 1.33ml of the supplied Phosphate-Free Water. For general use, 5µl of this reconstituted substrate in a 50µl reaction is sufficient and will yield 100µM peptide, resulting in the release of up to 5nmol phosphate during the reaction. For kinetic data, perform a substrate concentration analysis by titrating the amount of substrate in the reaction over a range of 10–400µM, depending on the K_m value of the enzyme under study.

Table 1. Physical Characteristics of the Supplied Ser/Thr Phosphopeptide.

Phosphopeptide	Sequence	Molecular Weight	Volume of Water for a 1mM Solution
Ser/Thr Phosphopeptide	RRA(pT)VA	754Da	1.33ml

Phosphoproteins

Other substrates such as histone, casein or a natural substrate of the phosphatase of interest also may be used in this assay. Some of these proteins can be obtained commercially in a partially dephosphorylated state, but they usually require extensive dialysis to remove free phosphate before use. In addition, large proteins, proteins with multiple phosphorylation sites or proteins with limited solubility in strong acid will require some preliminary testing as described in Section 3.D.

3.C. Buffers

As a family of enzymes, phosphatases possess diverse optimal conditions; therefore, a universal buffer system cannot be provided. Suggested buffers for some phosphatases are listed in Section 5.

3.D. Testing Reaction Components

Because the assay measures free phosphate, phosphate buffers are not compatible with this system. Reaction components that contain phosphate (i.e., glycerol phosphate) may interfere with the analysis, depending on their concentration, purity and stability in strong acid. High concentrations of reductants may bleach the dye color over time, resulting in lower sensitivity. A final concentration of 0.02% β-mercaptoethanol has no effect on sensitivity; 0.05% β-mercaptoethanol has only a slight effect, and 0.1% β-mercaptoethanol reduces sensitivity by approximately 20%. Many detergents can be used at or below 0.1%, but higher concentrations may generate high backgrounds. If high concentrations of detergent are required in the reaction, the background can be determined by including the corresponding concentration of the detergent in the phosphate standards (see Note following Step 1 in Section 4.B). Also, some proteins such as casein are not soluble in strong acid and will precipitate when the Molybdate Dye/Additive mixture is added, requiring an additional step (see Note below).

To test the suitability of the various components, add individual or combined components at the highest concentration to be used in a total of 50µl. Add 50µl of Molybdate Dye/Additive mixture (prepared in Section 4.A), and incubate at room temperature for 15 minutes (30 minutes if more than 5µg of protein is present). Prepare a control reaction containing 50µl of the supplied Phosphate-Free Water and 50µl of the Molybdate Dye/Additive mixture. Components that remain yellow and do not cause precipitation are compatible with this system. The optical density at 600–630nm can be obtained to determine exact backgrounds. Backgrounds (plate plus water/Dye Solution) range between 0.06 and 0.13, depending on the plate type and wavelength used.

Note: If a protein precipitate is present, use lower concentrations of protein, or upon completion of the reaction, treat the reaction with 10µg of proteinase K for 5 minutes at 30°C in a buffer containing 5mM $CaCl_2$ (final concentration) before adding the Molybdate Dye/Additive mixture. (The Molybdate Dye/Additive volume is equal to the reaction volume plus proteinase K and buffer volumes.) The standard protocol described in Section 4 allows accurate measurements in the presence of >200µg of BSA (or 75µg of acetylated BSA)

or approximately 50µg of tissue extract protein and should not require subsequent protease digestion.

Important: If a protein precipitate is present, either use lower concentrations of protein, or after the reaction is complete, treat the reaction with proteinase K.

4. Protocols

The following protocol can be used to quantitate protein serine/threonine phosphatase activity in cell lysates, tissue extracts, partially fractionated samples or purified enzymes. For partially purified preparations, no special sample preparation procedures are necessary as long as the free phosphate concentration is below 5µM (assuming 5µl sample size for a 50µl reaction).

This phosphatase assay accommodates a wide variety of buffers, reducing agents, detergents and glycerol. To ensure compatibility, however, pretest all reagents as described in Section 3.D.

4.A. Preparation of Dye

Determine the total reaction volume that you will use based on the particular assay format and sample number. Reactions prepared in the supplied 96-Well Plate or other standard plates require 50µl of the Molybdate Dye/Additive mixture for each 50µl reaction. (Larger reaction volumes may be used.) Reactions to be analyzed in a spectrophotometer require approximately 400µl of the Molybdate Dye/Additive mixture for each 400µl reaction. On the day of the experiment, prepare the Molybdate Dye/Additive mixture (10µl of Molybdate Dye Additive per 1ml of Molybdate Dye Solution).

Do not store the mixed reagents (Molybdate Dye/Additive mixture) for future use. The Molybdate Dye/Additive mixture is relatively unstable; prepare only the amount needed for one day.

Note: Proceed directly to Section 4.C for partially purified samples.

4.B. Preparation of Tissue Extracts or Cell Lysates

Cell lysates and tissue extracts contain millimolar concentrations of free phosphate, which will interfere with the phosphatase assay. In addition, high concentrations of ATP can increase the background and lead to phosphorylation by contaminating kinases. Therefore, you will need to eliminate these components from samples.

The method of extraction may influence both the recovery of phosphatases and the presence of inhibitors of these enzymes.

Sample and Column Preparation

Materials to Be Supplied by the User
(Solution compositions are provided in Section 5.)

- 50ml disposable conical centrifuge tubes (e.g., Corning Cat.# 430290)
- appropriate phosphatase storage buffer (see Note following Step 1)
- Sephadex® G-25 storage buffer (for storing column)

Sample and Column Preparation (continued)

1. Homogenize the tissue at 0–4°C for 30 seconds using 1g of tissue in 3ml of phosphatase storage buffer.

   Note: The choice of phosphatase storage buffer depends on several factors, including whether membrane-associated or cytoplasmic phosphatases are being examined. The phosphatase storage buffer will generally include a reducing agent, a chelator of divalent cations and various protease inhibitors. Up to 1% detergent (e.g., Triton® X-100) can be used to prepare membrane-associated phosphatases if the sample is diluted appropriately in the reaction mix. References 21–24 provide recipes for a variety of phosphatase storage buffers.

2. Centrifuge the homogenized lysate at 100,000 × *g* at 4°C for 1 hour to remove particulate matter.

3. Add 10ml of deionized water to a Spin Column, and allow it to drain. To begin draining the column the first time, apply slight pressure to the Spin Column with a 10ml syringe or shake it briskly. The supplied Adaptor allows centrifugation in most standard 50ml disposable tubes. (A lid is not necessary.) Allow the Spin Column to drain into a waste container.

4. Resuspend the Sephadex® G-25 resin by rocking gently or pipetting with a wide-mouth pipette.

   Do not vortex or stir the resin with a magnetic stir bar.

5. Pipet 10ml of the resuspended Sephadex® slurry into the Spin Column, and allow it to drain by gravity into a spare 50ml tube. Remove the flowthrough liquid from the tube.

6. Add 10ml of cold phosphatase storage buffer to the column.

7. Allow the column to drain by gravity, remove the flowthrough liquid from the tube, and then centrifuge at 600 × *g* for 5 minutes at 4°C using a spare 50ml tube to remove the remaining buffer surrounding the Sephadex® beads.

8. Place the Spin Column with Adaptor in the supplied Reservoir (50ml tube), and add 250µl of tissue extract or cell lysate. (A larger volume of sample may be used; see Notes following Step 9.)

9. Centrifuge at 600 × *g* for 5 minutes at 4°C. The sample lysate in phosphatase storage buffer will be in the bottom of the Reservoir in the original volume.

Notes:

1. The sample flowthrough in the Reservoir should contain 4–10% of the endogenous phosphate. This reduced phosphate level should be low enough to perform most experiments with minimal background. If additional phosphate must be removed, pass the collected sample flowthrough through a second Spin Column. To determine specific activities, measure the protein concentration of the phosphate-reduced sample.

2. Using a larger sample volume in Step 8 will reduce the efficiency with which free phosphate is removed. For example, if 500µl of sample is used in Step 8, approximately 85–90% of the endogenous phosphate will be removed.
3. Spin Columns can be washed with at least 25ml of deionized water or phosphate-free buffer and stored in Sephadex® G-25 storage buffer. Store used Spin Columns wet at 4°C. Spin Columns and Sephadex® G-25 can be reused following the Sample and Column Preparation instructions in Section 4.B.

4.C. Phosphatase Assay Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 5.)

- enzyme-specific 5X reaction buffer (PPase-2A 5X reaction buffer, PPase-2B 5X reaction buffer or PPase-2C 5X reaction buffer)

1. Make appropriate phosphate standards by diluting the 1mM Phosphate Standard with the supplied Phosphate-Free Water. Dilute the standard 1:20 to generate a solution containing 50pmol phosphate per microliter (50µM). Prepare wells containing 0, 100, 200, 500, 1,000 and 2,000pmol free phosphate and 1X reaction buffer in 50µl for use as a standard curve.
2. Prepare appropriate reaction premix solutions directly in the 96-Well Plate, excluding the enzyme sample, as follows: 10µl of PPase 5X reaction buffer (Section 5), 5µl of 1mM phosphopeptide. Do not create bubbles.

   Note: Reaction components may include substrate, phosphatase activators or inhibitors (see Table 2 for information on specificity of inhibitors) and a suitable PPase 5X reaction buffer (Section 5). The final reaction volume depends on the assay format. We recommend 50µl reactions for the supplied ½-area, flat-bottom 96-Well Plate; 50–100µl reactions with standard 96-well plates; and 400µl reactions for standard disposable cuvettes and a spectrophotometer in Step 7 of this section.
3. Place the 96-Well Plate at the desired reaction temperature for 3 minutes.

   Note: One approach to controlling the reaction temperature is to place a glass dish containing a small amount of water in a water bath and allowing it to equilibrate. The 96-Well Plate can be placed in the equilibrated dish, where manipulations can be performed easily.
4. Start the reaction by adding the enzyme sample (1–35µl) to the wells containing the appropriate reaction solution, and incubate the reactions for the desired time. Proper controls include reactions without enzyme sample and reactions without substrate as well as complete reactions (substrate plus enzyme sample) terminated at time zero as described in Step 5.

4.C. Phosphatase Assay Protocol (continued)

5. Stop the reaction by adding 50µl of Molybdate Dye/Additive mixture prepared in Section 4.A. This mixture is a strong acid and will stop the enzymatic reaction so that accurate time points can be taken. **Other termination solutions such as SDS should be used with caution** because these reagents may react with the Molybdate Dye/Additive mixture and generate high backgrounds or lower the sensitivity of the assay.

   Note: Add 50µl of the Molybdate Dye/Additive mixture to wells containing the Phosphate Standard dilutions. If you are using standard 96-well plates, add an equal volume of the Molybdate Dye/Additive mixture as described in Section 4.A.

6. Place the 96-Well Plate at room temperature, and incubate for 15 minutes. Incubate the 96-Well Plate for 30 minutes if more than 5µg of protein is present per reaction because high concentrations of protein delay color development.

   Once color development is complete, the color remains stable for at least 2 hours. Adding dye at various times during a time-course experiment is acceptable as long as the appropriate development time at room temperature is allowed for the last time point.

   Note: Incubate the 96-Well Plate for 30 minutes if more than 5µg of protein is present per reaction. High concentrations of protein delay color development.

7. Read the optical density of the samples using a plate reader with a 630nm or 600nm filter. The 630nm filter will give slightly higher values and higher backgrounds for both experimental and control samples.

Important: Bubbles in the wells or water drops on the bottom of the plate will adversely affect the absorbance readings.

Table 2. General Approaches to the Initial Characterization of Phosphatases in Cell/Tissue Extracts and Column Fractions.

Enzyme	Phosphopeptide Substrate		Enzyme Activity in the Presence of Various Protein Phosphatase Inhibitors					
Protein Phosphatase Class	Phospho-tyrosine-containing peptide	Phospho-threonine-containing peptide	Vanadate	NaF	EDTA (no Mg^{2+})	EGTA (no Ca^{2+})	Okadaic Acid	Trifluor-perazin
PTPases	++++	–	–	++++	++++	++++	++++	++++
PPase-2A	–	++++	++++	–	++++	++++	–	++++
PPase-2B	++	+++	++	–	++++	–	++++	–
PPase-2C	–	++++	++++	–	–	++++	++++	++++

++++ high activity; +++ moderately high activity; ++ moderate activity; + low activity;
– very low to no detectable activity

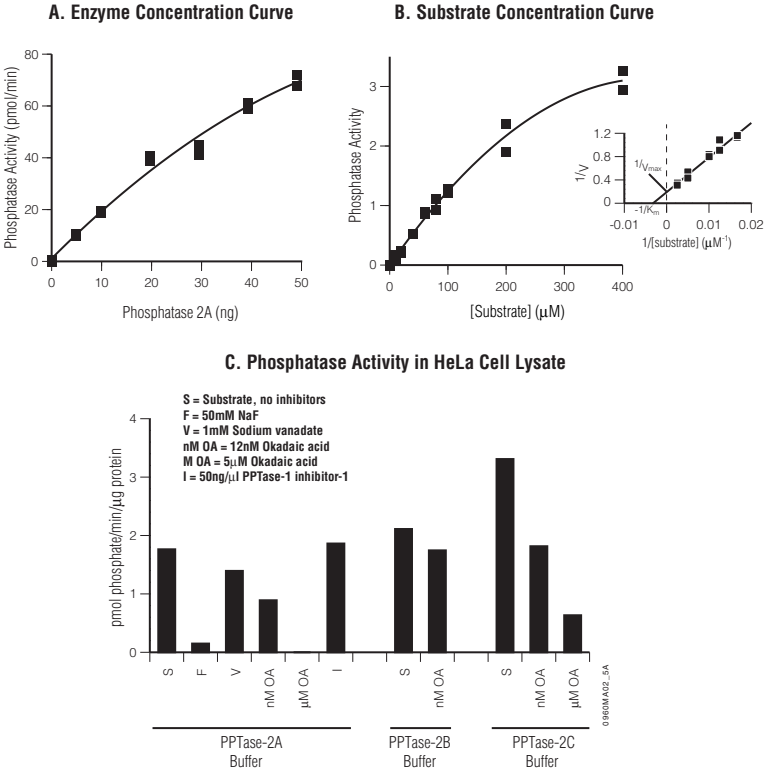

Figure 3. Typical data generated with the provided phosphopeptide. Purified Protein Phosphatase 2A (Cat.# V6311) was reacted for 10 minutes at 30°C with: (**Panel A**) 200µM Ser/Thr Phosphopeptide in PPase-2A buffer, and (**Panel B**) various concentrations Ser/Thr Phosphopeptide in PPase-2A buffer. The insert in Panel B shows a kinetic analysis of the data in the form of a Lineweaver-Burk plot with a determined K_m of 310µM and a V_{max} of 5.3nmol/minute/µg. **Panel C** shows data obtained from HeLa cell lysate (250µl) that was passed once through the supplied Sephadex® G-25 Spin Column to remove free phosphate. The samples in Panel C were incubated for 30 minutes with or without substrate (100µM Ser/Thr Phosphopeptide), 50mM sodium fluoride (NaF), 1mM sodium vanadate, 12nM okadaic acid, 5µM okadaic acid and 50ng/µl PPase-1 inhibitor-1. Phosphatase activity was substrate-dependent and was relatively insensitive to high concentrations of the protein tyrosine phosphatase inhibitor, sodium vanadate, but was inhibited by the serine/threonine phosphatase inhibitor, sodium fluoride. See Table 2 for information on specificity of inhibitors. See Section 5 for the composition of buffers.

5. Composition of Buffers and Solutions

Sephadex® G-25 storage buffer

10mM	Tris (pH 7.5)
1mM	EDTA
0.02%	sodium azide

Phosphate Standard

1mM	KH_2PO_4

PPase-2A 5X reaction buffer

250mM	imidazole (pH 7.2)
1mM	EGTA
0.1%	β-mercaptoethanol
0.5mg/ml	BSA

PPase-2B 5X reaction buffer

250mM	imidazole (pH 7.2)
1mM	EGTA
50mM	$MgCl_2$
5mM	$NiCl_2$
250µg/ml	calmodulin
0.1%	β-mercaptoethanol

PPase-2C 5X reaction buffer

250mM	imidazole (pH 7.2)
1mM	EGTA
25mM	$MgCl_2$
0.1%	β-mercaptoethanol
0.5mg/ml	BSA

6. Related Products

Product	Size	Cat.#
Tyrosine Phosphatase Assay System	96 reactions	V2471

Fluorescent Phosphatase Assay Systems

Product	Size	Cat.#
ProFluor® Ser/Thr PPase Assay	4 plate*	V1260
ProFluor® Tyrosine Phosphatase Assay	4 plate*	V1280

*Available in additional sizes.

Luminescent Universal Kinase Assays

Product	Size	Cat.#
Kinase-Glo® Max Luminescent Kinase Assay	10ml*	V6071
Kinase-Glo® Plus Luminescent Kinase Assay	10ml*	V3771
Kinase-Glo® Luminescent Kinase Assay	10ml*	V6711

*Available in additional sizes.

Fluorescent Kinase Assays

Product	Size	Cat.#
ProFluor® PKA Assay	4 plate*	V1240
ProFluor® Src-Family Kinase Assay	4 plate*	V1270

*Available in additional sizes.

Radioactive Kinase Assays

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

Phosphatases

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

7. References

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