

Phosphatases

The phosphorylation state of a variety of proteins has been implicated in a multitude of biological processes and in the control of many basic cellular events, including: differentiation, cell division, metabolism, contractility, fertilization and memory (for reviews, see references 1–17). Based on results from the Human Genome Sequencing Initiative, the human genome could encode for approximately 2,000 protein kinases. Since kinases tend to have multiple functions in vivo, potentially tens of thousands of proteins could be substrates for these kinases. The capacity for removing these phosphates resides with an impressive array of phosphatases.

Protein phosphatases are classified into two main families: those that dephosphorylate phosphotyrosine residues and those that dephosphorylate phosphoserine residues and/or phosphothreonine residues. Some phosphatases have dual specificity, such as the vaccinia virus phosphatase H1 (18) and *puckered*, a *Drosophila* phosphatase involved in control of MAPK signaling cascades (19). Phosphatases can be subdivided further depending on their regulation by protein modulators (e.g., calmodulin), their requirement for metal cations (e.g., Mg²⁺ and Ca²⁺) and by their sensitivity to various phosphatase inhibitors (20,21; Table 9.1).

Table 9.1. 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					
	Phospho-tyrosine-Containing Peptide	Phospho-threonine-Containing Peptide	Vanadate	NaF	EDTA (No Mg ²⁺)	EGTA (No Ca ²⁺)	Okadaic Acid (50nM)	Trifluoperazin
PTPases	++++	–	–	++++	++++	++++	++++	++++
PPTase-2A	–	++++	++++	–	++++	++++	–	++++
PPTase-2B	++	+++	++	–	++++	–	++++	–
PPTase-2C	–	++++	++++	–	–	++++	++++	++++

KEY

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

Kinases have achieved specificity by using very specific catalytic domains that recognize particular sequence motifs in target substrates. Phosphatases, on the other hand, have highly conserved catalytic domains that gain specificity through the formation of heteromultimeric holoenzymes (15), which localize phosphatases to specific areas of the cell where their activity is required (22). This localization is facilitated through specific protein-protein interactions. For example, the tyrosine phosphatases, Shp-1 and Shp-2, contain SH2 (src-homology) domains, which target the phosphatases to sites of tyrosyl phosphorylation, such as receptor tyrosine kinases (23,24). Protein phosphatase-1 (PP-1) is localized to different cellular compartments depending on the cell type. In liver cells, a “glycogen-targeting subunit” anchors PP-1 to glycogen particles. In skeletal muscle, a different subunit anchors PP-1 to the sarcoplasmic reticulum (22). Protein Phosphatase-2A is a heterotrimer with a catalytic subunit, a structural subunit and a regulatory subunit. The regulatory subunit interacts with various subcellular structures (e.g., centrosomes, endoplasmic reticulum, golgi and nucleus) to compartmentalize the enzyme (22). Protein Phosphatase-2B also interacts with subcellular structures and associates with PKA and PKC on the A-kinase-anchoring protein, AKAP79 (25).

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Protocol

Tyrosine Phosphatase Assay System.....**TB212**

Publications

Promega's Non-Radioactive Phosphatase Assay Systems (1995) *Neural Notes* **1**(3), 12.

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Enzyme Assay Systems

Non-Radioactive Phosphatase Assay Systems

Product	Size	Catalog #
Tyrosine Phosphatase Assay System	96 reactions	V2471

Description: Promega's Non-Radioactive Tyrosine Phosphatase Assay System provides a fast, convenient and flexible alternative to measuring protein tyrosine 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. The Tyrosine Phosphatase Assay System contains two chemically synthesized phosphopeptides, END(pY)INASL (4) and DADE(pY)LIPQQG (5), which serve as substrates for many protein tyrosine phosphatases. The effective range for the detection of phosphate released during an assay using Promega's Phosphatase Assay Systems is 50-2,000pmol of phosphate. In addition to measuring phosphatase activity in partially fractionated and purified samples, Promega's Phosphatase Assay Systems can also 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, a unique Molybdate Dye Additive that is combined with the Molybdate Dye Solution aids in the solubilization of proteins exposed to the acid conditions of the Molybdate Dye Solution, which alone could potentially cause precipitation of the proteins.

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1. Van Veldhoven, P.P. and Mannaerts, G.P. (1987) Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **161**, 45.
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Features

- **Non-Radioactive:** No need to prelabel substrate. The substrate provided is phosphorylated and is stable for months.
- **Accurate:** 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.

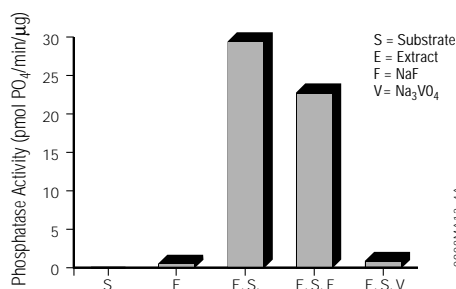


Figure 9.1. Tyrosine phosphatase activity in cell lysate. A431 cell lysate was passed once through the supplied Sephadex® G-25 Spin Column to remove free phosphate. The samples were incubated for 45 minutes with or without substrate (100µM Tyr phosphopeptide-1), 50mM sodium fluoride (NaF) and/or 1mM sodium vanadate. Phosphatase activity was substrate-dependent and was relatively insensitive to high concentrations of the serine/threonine phosphatase inhibitor, sodium fluoride but was inhibited by the protein tyrosine phosphatase inhibitor, sodium vanadate.

Non-Radioactive Phosphatase Assay Systems

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

Description: Promega's Non-Radioactive Serine/Threonine Phosphatase Assay System provides a fast, convenient and flexible alternative to 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. The Serine/Threonine Phosphatase Assay System contains the chemically synthesized phosphopeptide, RRA(pT)VA, a peptide substrate that is compatible with several serine/threonine phosphatases, such as the Protein Phosphatases 2A, 2B, and 2C. **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 Promega's Phosphatase Assay Systems is 50-2,000pmol of phosphate. In addition to measuring phosphatase activity in partially fractionated and purified samples, Promega's Phosphatase Assay Systems can also 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, a unique Molybdate Dye Additive that is combined with the Molybdate Dye Solution aids in the solubilization of proteins exposed to the acid conditions of the Molybdate Dye Solution, which alone could potentially cause precipitation of the proteins.

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1. Van Veldhoven, P.P. and Mannaerts, G.P. (1987) Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **161**, 45.
2. Ekman, P. and Jager, O. (1993) Quantification of subnanomolar amounts of phosphate bound to seryl and threonyl residues in phosphoproteins using alkaline hydrolysis and malachite green. *Anal. Biochem.* **214**, 128.
3. Harder, K.W. *et al.* (1994) Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase beta (HPTP beta) using synthetic phosphopeptides. *Biochem. J.* **298**, 395.

Features

- **Non-Radioactive:** No need to prelabel substrate. The substrate provided is phosphorylated and is stable for months.
- **Accurate:** 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.

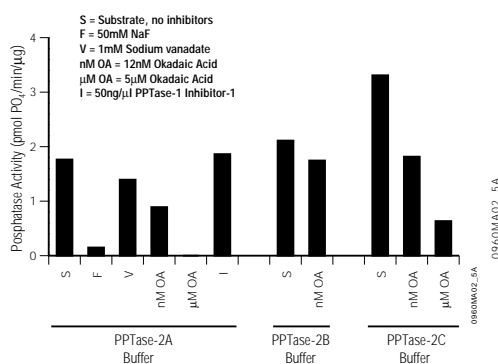


Figure 9.2. Serine/threonine phosphatase activity in HeLa cell lysate. HeLa cell lysate (250μl) was passed once through a Sephadex® G-25 Spin Column to remove free phosphate. The lysate and Ser/Thr Phosphopeptide Substrate (100μM) were incubated for 30 minutes with or without 50mM sodium fluoride (NaF), 1mM sodium vanadate, okadaic acid (12nM or 5μM, as indicated) and/or 50ng/μl PPTase-1 Inhibitor-1 (Cat.# V6321). PPTase-2A activity was relatively insensitive to high concentrations of the protein tyrosine phosphatase inhibitor, sodium vanadate, but was inhibited by the serine/threonine phosphatase inhibitors, sodium fluoride and okadaic acid.

Promega Product Citations

Berry, M. and Gehring, W. (2000) Phosphorylation status of the SCR homeodomain determines its functional activity: Essential role for protein phosphatase 2A,B'. *EMBO J.* **19**, 2946.

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Serine/Threonine Phosphatase Assay System**TB218**

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Promega's Non-Radioactive Phosphatase Assay Systems (1995) *Neural Notes* **1**(3), 12.

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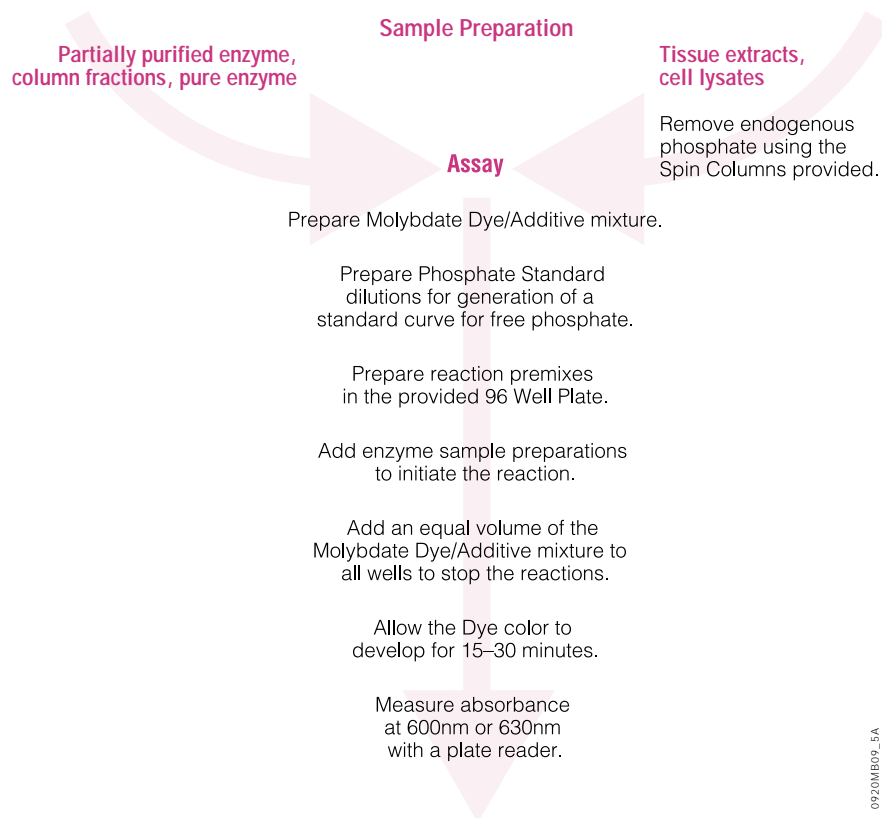


Figure 9.3. Overview of the steps required for measuring phosphatase activity using the Serine/Threonine Phosphatase Assay System or the Tyrosine Phosphatase Assay System. These systems can be used to measure phosphatase activity from partially purified enzyme preparations and tissue extracts/cell lysates.

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Enzymes and Inhibitors

Protein Phosphatase-2A Catalytic Subunit

Product	Size	Catalog #
PPase-2A Catalytic Subunit	25 units	V6311

Description: Protein Phosphatase-2A (PPase-2A) is a serine/threonine phosphatase that was purified based on its ability to dephosphorylate the α -subunit of phosphorylase kinase (1). With its 36–38kDa catalytic subunit, PPase-2A has broad substrate specificity and may play a regulatory role in DNA replication, transcription, protein synthesis, mitosis and glycogen metabolism (2). PPase-2A is stimulated in vitro by basic proteins such as protamine, histones and polylysine (1). The enzyme is inhibited by several environmental toxins and tumor promoters such as okadaic acid (3) and microcystin-LR (4). Promega's PPase-2A Catalytic Subunit is isolated from rabbit skeletal muscle.

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1. Tung, H.Y., Alemany, S. and Cohen, P. (1985) The protein phosphatases involved in cellular regulation. 2. Purification, subunit structure and properties of protein phosphatases-2A0, 2A1, and 2A2 from rabbit skeletal muscle. *Eur. J. Biochem.* **148**, 253.
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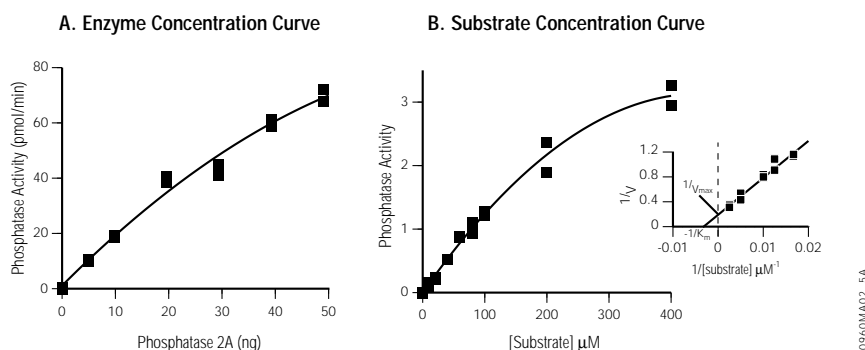


Figure 9.4. Protein phosphatase-2A activity. Purified Protein Phosphatase 2A (Cat.# V6311) was reacted for 10 minutes at 30°C with (Panel A) 200 μ M Ser/Thr Phosphopeptide in PPase-2A buffer and (Panel B) various concentrations Ser/Thr Phosphopeptide in PPase-2A buffer. The insert in Panel B shows a kinetic analysis of the data in the form of a Lineweaver-Burk plot with a determined K_m of 310 μ M and a V_{max} of 5.3nmol/min/ μ g. Phosphatase activity was measured with the Non-Radioactive Serine/Threonine Phosphatase Assay System (Cat.# V2460).

Promega Product Citations

Protein Phosphatase-2A

Berry, M. and Gehring, W. (2000) Phosphorylation status of the SCR homeodomain determines its functional activity: essential role for protein phosphatase 2A,B'. *EMBO J.* **19**, 2946.

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Protein Phosphatase-2A Catalytic Subunit.....**TB537**

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Protein Phosphatase-2B

Banke, T.G. *et al.* (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *J. Neurosci.* **20**, 89.

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Protein Phosphatase-2B..**TB534**

Protein Phosphatase-2B

Product	Size	Catalog #
PPase-2B	10 units	V6361

Description: Protein Phosphatase-2B (PPase-2B) is a heterodimeric enzyme composed of a 19kDa calmodulin-like subunit and a catalytic subunit (61kDa) that binds calmodulin. PPase-2B was originally identified based on its calcium- and calmodulin-dependent activity towards phosphorylase kinase and inhibitor-1. PPase-2B, also known as the brain protein calcineurin, constitutes up to 1% of the total brain protein (1). The immunosuppressive drugs FK-506 and cyclosporin A inhibit PPase-2B activity in immune cells, implicating a role for this enzyme in regulation of the immune system. PPase-2B also plays a major role in regulating secretory functions of a variety of cells (1).

PPase-2B is less sensitive to okadaic acid than PPase-2A and PPase-1, requiring micromolar concentrations of okadaic acid for inhibition. It is not inhibited by inhibitor-1 or inhibitor-2. Promega's PPase-2B is isolated from bovine brain.

Reference

1. Shenolikar, S. (1994) Protein serine/threonine phosphatases--new avenues for cell regulation. *Ann. Rev. Cell. Biol.* **10**, 55.

Protein Phosphatase-1 Inhibitor-1

Product	Size	Catalog #
PPase-1 Inhibitor-1	5µg	V6321

Description: PPase-1 Inhibitor-1 (molecular weight: 26kDa) is a heat-stable inhibitor of type 1 protein phosphatases and is isolated from rabbit skeletal muscle. It does not inhibit type 2 protein phosphatases.

Protein Phosphatase-1 Inhibitor-2

Product	Size	Catalog #
PPase-1 Inhibitor-2	10µg	V6331

Description: PPase-1 Inhibitor-2 (molecular weight: 31kDa) is a heat-stable inhibitor of type 1 protein phosphatases and is isolated from rabbit skeletal muscle. It does not inhibit type 2 protein phosphatases.

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