



# Protein Tyrosine Phosphatase (PTP) Assay System

Preparation of Phosphorylated Protein Substrate  
and Radioactive Assay of Protein Phosphatases

**I n s t r u c t i o n   M a n u a l**

Catalog #P0785S  
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## **The PTP System Includes:**

### **■ Sufficient Reagents for 150 Assays:**

Abl Protein Tyrosine Kinase (Abl)/Myelin Basic Protein (MyBP) Mix

ATP (unlabeled)

10X Abl Buffer

Substrate Solubilization Buffer

10% Brij 35

10X Protein Phosphatase Buffer

BSA Purified (10 mg/ml)

T-Cell Protein Tyrosine Phosphatase (TC PTP)

Instruction Manual

## Introduction:

Protein phosphatases comprise several families of enzymes that catalyze the dephosphorylation of intracellular phosphoproteins, thereby reversing the action of protein kinases. Reversible protein phosphorylation is the basis for the regulation of many diverse cellular processes that include metabolism, contractility, transport, cell division, differentiation and development, learning and memory. In eukaryotic cells regulatory proteins are phosphorylated on their serine, threonine and tyrosine residues (there is also evidence for histidine phosphorylation).

Protein phosphatases are classified according to their substrate specificities: protein serine/threonine phosphatases (PSPs), which are specific for phosphoserine/phosphothreonine-containing proteins, protein tyrosine phosphatases (PTPs), which are specific for phosphotyrosine-containing proteins, and the dual specificity phosphatases, which dephosphorylate all three phosphoamino acid residues.

PSPs have been classified into two groups; type 1 (protein phosphatase 1) and type 2 (protein phosphatases 2A, 2B and 2C), which can be distinguished on the basis of differences in substrate specificity, divalent cation dependency, and regulatory properties (using specific inhibitors and activators) (1).

While protein kinases have been relatively easy to study measuring the incorporation of radioactive phosphate from [ $\gamma$ - $^{32}\text{P}$ ] ATP into proteins or specific peptide substrates, characterization of protein phosphatases is a more difficult task. Before one can assay activity of a protein phosphatase a suitably purified phosphorylated substrate must be prepared.

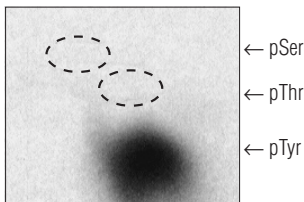
Protein Phosphatase Assay Systems provide highly purified reagents and protocols for the preparation of a broad specificity protein substrate, which can be phosphorylated exclusively on serine/threonine (PSP Assay System), or exclusively on tyrosine residues (PTP Assay System). The Protein Phosphatase Assay Systems employ a radiolabeled substrate that allows the highest sensitivity detection of protein phosphatases.

## Description

The Protein Tyrosine Phosphatase (PTP) Assay System is designed for the preparation of the phosphotyrosyl protein substrate and the radioactive assay of the protein tyrosine phosphatase activity of purified protein phosphatases or cell extracts (1, 2).

The PTP Assay System includes the commonly-used model protein substrate, Myelin Basic Protein (MyBP), which can be used for assay of protein tyrosine phosphatases, protein serine/threonine phosphatases, and dual specificity protein phosphatases.

The  $^{32}\text{P}$  or  $^{33}\text{P}$ -labeled MyBP is prepared by phosphorylation on multiple tyrosine residues with Abl Protein Tyrosine Kinase (Abl) in the presence of  $[\gamma\text{-}^{32}\text{P}$  or  $^{33}\text{P}]$  ATP (3). The reaction is terminated by adding trichloroacetic acid (TCA)



**Figure 1:** Phosphoamino acid analysis of MyBP phosphorylated by Abl using PTP Assay System. MyBP was treated with the kinase in the presence of  $[\gamma\text{-}^{33}\text{P}]$  ATP, TCA-precipitated phosphoprotein was subjected to partial acid hydrolysis. Phosphoamino acids were separated by two-dimensional thin layer electrophoresis, internal standard markers were stained with ninhydrin, and  $^{33}\text{P}$ -labeled phosphoamino acids were detected by autoradiography.

to precipitate the phosphoprotein, inactivate the protein kinase, and remove the excess ATP. Labeled MyBP is purified from residual ATP by washing with TCA and dialysis. MyBP is phosphorylated by Abl on tyrosine residues to high stoichiometries (routinely ~1–2 mol phosphate/mol) (Figure 1).

The protein tyrosine phosphatase activity can then be determined by measuring the release of inorganic phosphate (TCA-soluble radioactivity) from labeled protein. T-Cell Protein Tyrosine Phosphatase (TC PTP) is included as a positive control (4).

The PTP Assay System contains the reagents and protocols for preparation of  $^{32}\text{P}$  or  $^{33}\text{P}$ -labeled MyBP and for approximately 150 protein phosphatase assays.

### **Quality Controls**

The PTP Assay System is functionally tested using protein tyrosine phosphatases such as TC PTP, LAR, YOP, and a dual specificity protein phosphatase,  $\lambda$ -PPase.

### **Note**

Labeled nucleotide is not included in the PTP Assay System.

## The PTP Assay System Components:

Abl (20,000 units/ml)/MyBP (18.5 mg/ml) Mix	0.04 ml
Supplied in 50 mM Tris-HCl (pH 7.5 @ 25°C), 100 mM NaCl, 0.1 mM Na <sub>2</sub> EDTA, 1 mM DTT, 0.01% Brij 35, and 50% glycerol	
Adenosine 5'-Triphosphate (ATP) (10 mM)	0.02 ml
Supplied in purified water, pH 7.5	
10X Abl Buffer	0.1 ml
1X Abl Buffer: 50 mM Tris-HCl (pH 7.5 @ 25°C) 10 mM MgCl <sub>2</sub> 1 mM EGTA 2 mM DTT 0.01% Brij 35	
Substrate Solubilization Buffer	2.5 ml
50 mM Tris-HCl (pH 8.5 @ 25°C) 0.1 mM Na <sub>2</sub> EDTA 2 mM DTT 0.01%Brij 35	
10% Brij 35 (v/w)	2.5 ml
10X Protein Phosphatase Buffer	3 ml
1X Protein Phosphatase Buffer: 50 mM Tris-HCl (pH 7.0 @ 25°C) 1 mM Na <sub>2</sub> EDTA 5 mM DTT 0.01% Brij 35	
BSA Purified (10 mg/ml)	3 ml
T-Cell Protein Tyrosine Phosphatase (TC PTP) (0.1 mg/ml)	0.02 ml
Supplied in 50 mM Tris-HCl (pH 7.0 @25°C), 100 mM NaCl, 2 mM Na <sub>2</sub> EDTA, 5 mM DTT, 0.01% Brij 35, and 50% glycerol	

## **Preparation of $^{32}\text{P}$ or $^{33}\text{P}$ -labeled MyBP (Protocol 1):**

### **Materials Supplied with the PTP Assay System:**

Abl/MyBP Mix

ATP (unlabeled)

10X Abl Buffer

Substrate Solubilization Buffer

10X Protein Phosphatase Buffer

10% Brij 35 (for Dialysis Buffer)

### **Other Materials Required:**

$[\gamma\text{-}^{32}\text{P}$  or  $^{33}\text{P}]$  ATP

100% and 20% TCA (w/v)

Spectra/Por 1 dialysis membrane tubing, molecular weight cut-off 6,000 to 8,000 (Spectrum Catalog # 132650)

Dialysis Buffer:

25 mM Tris-HCl (pH 7.5 @ 25°C)

0.1 mM  $\text{Na}_2\text{EDTA}$

2 mM DTT

0.01% Brij 35

*Note:  $[\gamma\text{-}^{33}\text{P}]$  ATP is recommended: the half-life of  $^{33}\text{P}$  is longer (25.4 days) than that of  $^{32}\text{P}$  (14.3 days), and at sub-millicurie amounts  $^{33}\text{P}$  can be safely manipulated on an open benchtop without any special shielding. Whenever handling  $[\gamma\text{-}^{32}\text{P}$  or  $^{33}\text{P}]$  ATP on an open bench though, the eyes should be shielded with safety glasses.*

## Method:

1. Mix together the following components in a 1.5 ml microcentrifuge tube, adding ATP and the radioactive ATP last (for a 200  $\mu$ l reaction):

Abl/MyBP Mix	40 $\mu$ l
10X Abl Buffer	20 $\mu$ l
ATP (10 mM)	20 $\mu$ l
[ $\gamma$ - $^{32}$ P or $^{33}$ P] ATP	5 $\mu$ l
water	115 $\mu$ l

*Note: If a different amount of label is added, adjust the volume to 200  $\mu$ l with an appropriate amount of water.*

*The final concentration of ATP is 1 mM. This is a 5-fold excess over MyBP (0.2 mM) to ensure that phosphorylation goes to completion. The specific activity of ATP will be ~500 cpm/pmol if for example [ $\gamma$ - $^{33}$ P] ATP (DuPont NEN Catalog # NEG/602H; 10 mCi/ml; 2000 Ci/mmol) is used. A higher specific activity of ATP will enable lower levels of protein phosphatases to be detected.*

2. Incubate overnight at 30°C.
3. Take a sample (2–5  $\mu$ l), make a 1/100 dilution in water, and count 2 x 10  $\mu$ l aliquots in a scintillation counter to determine specific activity of the ATP. Note time and date, and refer to a  $^{32}$ P or  $^{33}$ P decay chart to calculate the ATP specific activity at the time of use. Alternatively keep these aliquots and count them to redetermine the specific activity each time an assay is performed.

The calculation for the ATP specific activity is:

$$\text{Specific Activity (cpm/pmol)} = \text{Aliquot cpm} \times \frac{1}{10} \times 100 \times 200 \times \frac{1}{200,000} \quad (1)$$

For the calculation (1) above, 10 is the volume of the aliquot in  $\mu$ l, 100 is the dilution, 200 is the volume of the reaction in  $\mu$ l, and 200,000 is the amount of pmol ATP in the 200  $\mu$ l reaction.

4. Terminate a reaction by adding 1/9 volume of 100% TCA. Leave on ice for 30 minutes then centrifuge at 12,000 x g for 10 minutes at 4°C (~90% of the radioactive ATP are removed on this step).

Remove the supernatant with a pipet tip or a plastic transfer pipet, without touching the pellet (it is very sticky). Repeat this step after each wash.

Wash the pellet three times (by vortexing) with 1 ml of 20% TCA, centrifuging at 12,000 x g for 5 minutes after each wash.

5. Add 0.5 ml of Substrate Solubilization Buffer to the tube. Carefully tap the tube with your finger several times. The protein pellet will dissolve in approximately 5–15 minutes. Transfer the substrate solution into a dialysis bag. Wash the tube with an additional 0.5 ml of the same buffer and transfer into the bag.
6. Dialyze for ~16 hours at 4°C against 2 x 1 liter of Dialysis Buffer to remove residual ATP.
7. Count duplicate 5 µl aliquots to determine the radioactivity. Calculate the incorporated phosphate concentration (µM) by referring to the original ATP specific activity (Step 3):

$$\text{Phosphate Concentration } (\mu\text{M}) = \frac{\text{Aliquot cpm}}{\text{Specific Activity}} \times \frac{1}{5} \times 10^6 \times 10^{-6} \quad (2)$$

For the calculation (2) above, 5 is the volume of the aliquot in the µl, 10<sup>6</sup> is to convert the result for 1 liter rather than 1 µl, and 10<sup>-6</sup> is to convert the amount of phosphate from pmoles to µmoles.

*Note: The specific activity of the labeled MyBP should correspond to 1–2 moles of phosphotyrosine per mole of MyBP (18.5 kDa). It can be calculated after the measurement of protein concentration (the concentration of MyBP should be ~0.4 mg/ml using Bio-Rad Protein Assay and BSA as a standard).*

8. Dilute labeled MyBP in 1X Protein Phosphatase Buffer to a concentration of 25  $\mu\text{M}$  (5X concentrated) phosphotyrosine. Substrate prepared in this way may be used for approximately two half-lives of  $^{32}\text{P}$  or  $^{33}\text{P}$ .
9. Store phosphorylated MyBP at 4°C.

## **Assay of PTP Activity (Protocol 2):**

### **Materials Supplied with the PTP Assay System:**

10X Protein Phosphatase Buffer

BSA Protease Free (10 mg/ml)

### **Other Materials Required:**

$^{32}\text{P}/^{33}\text{P}$ -labeled MyBP (Protocol 1)

20% TCA (w/v)

Activator/Inhibitor (if required)

### **Method:**

1. Make Assay Buffer using 10X Protein Phosphatase Buffer and BSA (activators/inhibitors if required):
  - 50 mM Tris-HCl (pH 7.0 @ 25°C)
  - 1 mM  $\text{Na}_2\text{EDTA}$
  - 5 mM DTT
  - 0.01% Brij 35
  - 1 mg/ml BSA
2. Dilute the protein phosphatase sample (purified enzyme or crude extract) in Assay Buffer just before use.

*Note: To estimate the protein phosphatase activity accurately it is essential to ensure linear kinetics of dephosphorylation. Therefore the dilution of the protein phosphatase sample should be determined empirically for each enzyme, but typically the extent of dephosphorylation should be kept below ~30%.*

3. Set up the following mixture on ice in a 1.5 ml microcentrifuge tube, and preincubate for ~2–5 min at 30°C:

- Assay Buffer (containing inhibitor/activator if required)	30 $\mu$ l
- Protein phosphatase, diluted in Assay Buffer	10 $\mu$ l

Also carry out a blank reaction in which the protein phosphatase is replaced by Assay Buffer. Ideally, counts released in the blank should be less than 2% of those released in the assay itself.

4. Start the reaction by adding 10  $\mu$ l of substrate and incubate for 10 minutes at 30°C.
5. Terminate the reaction by adding 200  $\mu$ l of cold 20% TCA, vortex and place on ice for 5–10 minutes.
6. Spin at 12,000 x g in a microcentrifuge for 5 minutes.
7. Very carefully remove 200  $\mu$ l of the TCA supernatant with the pipet tip. Do not touch the protein pellet with the tip. Add the TCA supernatant to 2 ml of aqueous-compatible scintillation fluid and count.
8. Determine the total radioactivity in the assay by counting 10  $\mu$ l of the substrate.

**Calculation:**

One unit of protein phosphatase activity releases 1 nanomole phosphate from MyBP (5  $\mu$ M with respect to incorporated phosphate) per minute in the standard assay (50  $\mu$ l).

The calculation for a 10 minute assay is:

$$\text{Released cpm} = \text{Sample cpm} - \text{Blank cpm} \quad (3)$$

$$\text{Activity (unit/ml)} = \frac{\text{Released cpm}}{\text{Total cpm} - \text{Blank cpm}} \times \frac{0.25}{10} \times 20 \times \frac{250}{200} \quad (4)$$

For calculation (4) above, 0.25 is the number of nanomoles of incorporated phosphates in the assay (Protocol 1, step 8), 10 is the incubation time in minutes, 20 is to convert the results for 1 ml rather than 50  $\mu$ l of reaction, and 250/200 corrects for the fraction of the TCA supernatant that is counted.

If the protein concentration of the enzyme or crude extract is known, the activity may be expressed as specific activity (unit/mg).

## Assay of T-Cell Protein Tyrosine Phosphatase (TC PTP) (Protocol 3):

### **Materials Supplied with the PTP Assay System:**

TC PTP (0.1 mg/ml)

10X Protein Phosphatase Buffer

BSA Protease Free (10 mg/ml)

### **Other Materials Required:**

$^{32}\text{P}$  or  $^{33}\text{P}$ -labeled MyBP (Protocol 1 )

20% TCA (w/v)

### **Method:**

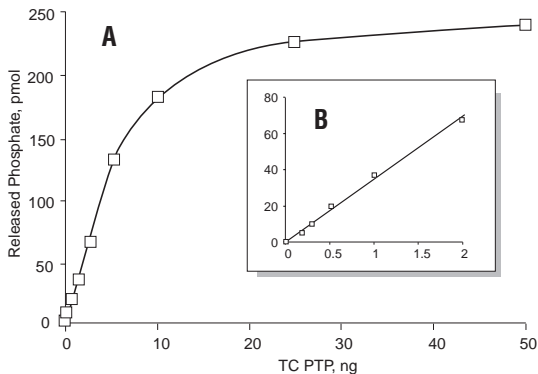
1. Make TC PTP Assay Buffer (using 10X Protein Phosphatase Buffer and BSA):
  - 50 mM Tris-HCl (pH 7.0 @ 25°C)
  - 1 mM  $\text{Na}_2\text{EDTA}$
  - 5 mM DTT
  - 0.01% Brij 35
  - 1 mg/ml BSA
2. Make 20-, 40-, 100-, 200-, 500-, 1000-, 2000-, 4000 and 8000-fold dilutions of TC PTP in Assay Buffer just before use.

*Note: TC PTP (NEB #P0752) employed in the PTP Assay System is the recombinant truncated form of the human T-Cell protein tyrosine phosphatase (4).*

3. Perform steps 3 through 8 of Protocol 2.

## Sample Calculation:

The experimental data of the TC PTP Assay shown in Figure 2 demonstrates that dephosphorylation (up to ~30%) of phosphotyrosyl-MyBP is linear with enzyme concentration (0.125 – 2.0 ng TC PTP in reaction; Figure 2 B). At higher enzyme concentrations, >90% dephosphorylation is reached (Figure 2 A).



**Figure 2:** Various concentrations of TC PTP were reacted with  $5 \mu\text{M}$   $^{33}\text{P}$ -Tyr-MyBP (with respect to incorporated phosphate; MyBP was phosphorylated by Abl with incorporation of  $\sim 1.5$  mol phosphate/mol using the PTP Assay System) under standard reaction conditions. Reactions were terminated by addition of TCA, and TCA-soluble radioactivity (inorganic phosphate released from the substrate) was measured (A). The linear range of dephosphorylation of  $^{33}\text{P}$ -Tyr-MyBP by TC PTP from the same experiments is shown in panel B.

The TC PTP specific activity is best calculated while the reaction is in the linear range.

For 1/1000 dilution (1 ng TC PTP in reaction), the Blank cpm was 72, the Sample cpm was 10,995 and the Total cpm of <sup>33</sup>P-labeled MyBP was 94,657.

The calculation for a 10 minute assay is:

$$\text{Released cpm} = 10,995 - 72 = 10,923 \quad (5)$$

$$\text{Activity (unit/ml)} = \frac{10,923}{94,657 - 72} \times \frac{0.25}{10} \times 20 \times \frac{250}{200} = 0.072 \quad (6)$$

$$\text{Concentration of TC PTP (mg/ml)} = 0.1 \times \frac{1}{1000} \times \frac{10}{50} = 0.00002 \quad (7)$$

$$\text{Specific Activity (unit/mg)} = \frac{0.072}{0.00002} = 3,600 \quad (8)$$

For calculation (7) above, 0.1 is the concentration of undiluted TC PTP in mg/ml, 1/1000 is the dilution, and 10/50 is the dilution of 10  $\mu$ l TC PTP in 50  $\mu$ l reaction.

## **References:**

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3. Foulkes, J.G., Chow, M., Gorka, C., Frackelton, A.R. and Baltimore, D. (1985) *J. Biol. Chem.* 260, 8070–8077.
4. Zander, N.F., Lorenzen, J.A., Cool, D.E., Tonks, N.K., Daum, G., Krebs, E.G. and Fischer, E.H. (1991) *Biochemistry* 30, 6964–6970.

## **Protein Phosphatase Assay Systems:**

Protein Serine/Threonine Phosphatase (PSP) Assay System

#P0780S            150 assays

Protein Tyrosine Phosphatase (PTP) Assay System

#P0785S            150 assays

### **Companion Products:**

Abl Protein Tyrosine Kinase (Abl)

#P6050S            2,000 units

#P6050L            10,000 units

cAMP-dependent Protein Kinase (PKA), catalytic subunit

#P6000S            100,000 units

#P6000L            500,000 units

T-Cell Protein Tyrosine Phosphatase (TC PTP)

#P0752S            200 units

#P0752L            1,000 units

LAR Protein Tyrosine Phosphatase (LAR)

#P0750S            200 units

#P0750L            1,000 units

YOP Protein Tyrosine Phosphatase (YOP)

#P0751S            2,000 units

#P0751L            10,000 units

Lambda Protein Phosphatase ( $\lambda$ -PPase)

#P0753S 20,000 units

#P0753L 100,000 units

Protein Phosphatase 1 (PP1)

#P0754S 100 units

#P0754L 500 units

Protein Phosphatase Inhibitor 2 (I-2)

#P0755S 20  $\mu$ g

#P0755L 100  $\mu$ g









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