SIGMA

ProductInformation

PROTEIN TYROSINE PHOSPHATASE ASSAY KIT
Non Radioactive

Product Number **PTP-101**
Store at 2-8°C, DO NOT FREEZE.

TECHNICAL BULLETIN

Product Description
Protein phosphorylation and dephosphorylation are central mechanisms that mediate signal transduction events involved in a wide range of cellular processes. Protein phosphatases are considered to play a crucial role in the regulation of protein phosphorylation by reversing the action of protein kinases. Protein phosphatases are present in all eukaryotic cells and regulate several cellular processes. Among them are cell-cycle progression, transcriptional regulation, cell growth, differentiation and apoptosis. The protein phosphatases can be divided into two main groups: protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PPs) which remove phosphate from proteins/peptides containing phosphotyrosine (pTyr) or phosphoserine/phosphothreonine (pSer/pThr), respectively. An additional group consists of dual specificity pTyr and pSer/pThr phosphatases, which includes the MAP Kinase Phosphatases.
Of special importance among the phosphatases, is the role of the PTPs in controlling cell growth, differentiation and oncogenesis. Several of the PTPs are known to control the function of growth factor receptors, many of which are tyrosine kinases encoded by oncogenes. PTPs can be further subdivided into receptor transmembrane-type PTPs and non-receptor, intracellular PTPs. The receptor PTPs (e.g. LAR, CD45, PTP α,β,δ,μ,κ, etc.) contain a general structure of membrane receptor with an extracellular domain, a single transmembrane domain and one or two tandem repeats of a conserved PTP catalytic domain (250 amino acid residues). The extracellular domain may contain functional domains such as IgG-like and fibronectin type III (Fn-III) repeats. The non-receptor intracellular PTPs (e.g. PTP1B, cdc25, SH-PTP1, SH-PTP2, MEG, PTP-Bas, etc.) contain a conserved PTP catalytic domain (250 amino acid residues) and additional domains such as SH2 domain. The phosphatases can be further subdivided on the basis of their cellular localization, requirement for Ca^{2+} or Mg^{2+}, and sensitivity to specific inhibitors.

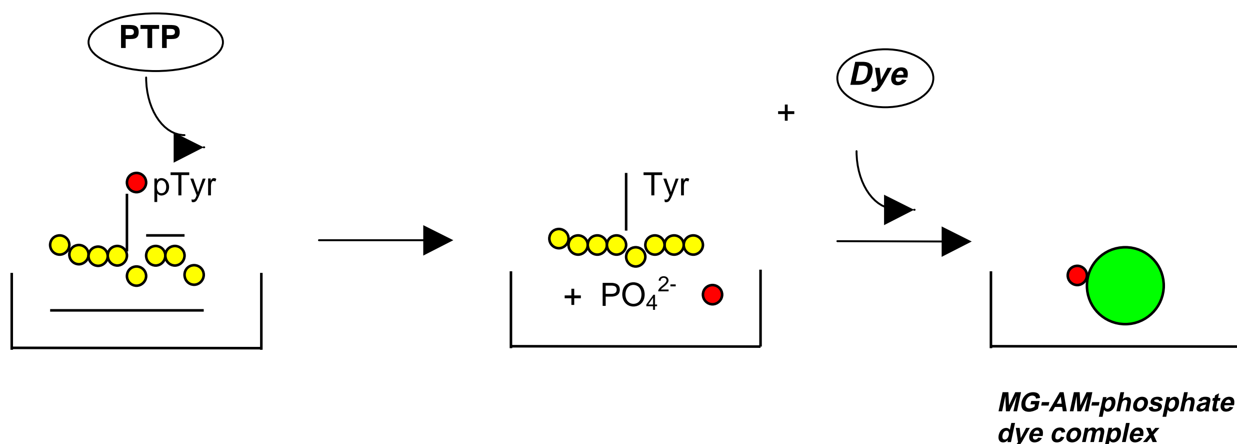
Measurement of PTP activity has become a basic requirement in the following areas of signal transduction research:

- Purification and characterization of protein tyrosine phosphatases (natural and recombinant).
- Elucidation of PTP biological function.
- Development of specific PTP inhibitors: *in vitro* High-throughput screening (HTS) of PTP inhibitors.

Principle of the Assay
The PTP Assay Kit is based on the *in vitro* colorimetric determination of protein tyrosine phosphatase (PTP) activity. It is based on the determination of free phosphate, generated in the dephosphorylation reaction of the PTP (phosphotyrosine peptide) substrate, using Malachite Green/Ammonium Molybdate reagent.

- Synthetic phosphorylated peptide substrate (PTP substrate) and phosphatase reaction buffer are added to microtiter plates.
- Dephosphorylation reaction of phosphorylated peptide susbstrate is initiated by protein tyrosine phosphatase (tissue or cell extracts, control protein tyrosine phosphatase, e.g., LAR PTP, etc.) in the reaction buffer.
- For tissue and cell extracts, phosphate salts must be removed by a buffer exchange step using gel filtration columns.
- The reaction is terminated by the addition of Malachite Green/ Ammonium Molybdate complex to the reaction mixture.
- The inorganic phosphate formed in the reaction complexes with Malachite Green/Ammonium Molybdate complex to generate a color product (brilliant green).

- The color is quantitated by spectrophotometry at 620 nm (using an ELISA reader) and reflects the total amount of free, inorganic phosphate in the sample and thus the relative amount of tyrosine phosphatase activity in the sample (qualitative).
- A standard phosphate curve can be constructed using phosphate standard solutions and by plotting the absorbance at 620 nm vs nmoles phosphate.
- A standard phosphatase activity curve can be constructed using PTP (LAR phosphatase, Sigma Product No. L0907) by plotting LAR phosphatase activity (units) vs nmoles phosphate.
- Assay time : Protein tyrosine phosphatase enzymatic reaction and assay, 1 hour.



MG-AM-phosphate dye complex

Advantages of the PTP Assay Kit
Radioactive methods using ^{32}P-labeled ATP for labeling peptide/substrate are labor intensive, produce large amounts of hazardous radioactive waste, require constant supply of radioactive labeled ATP and enzymatic pre-labeling of the substrate. In addition, the half-life of the ^{32}P isotope is relatively short (14.3 days).
The Protein Tyrosine Phosphatase Assay Kit totally avoids the use of radioactive reagents and has several advantages over conventional radioactive techniques:

- ♦ High purity (>95%) of PTP peptide substrates, because they are synthetically prepared tyrosine phosphorylated peptides.
- ♦ High specificity. No cross-reactivity of phosphotyrosine vs. phosphothreonine and phosphoserine PPs, because synthetic peptides have no Ser or Thr residues.
- ♦ Easy and reliable calibration.
- ♦ Fast performance (1 hour, hands-on assay).
- ♦ Convenient component sizes for easy handling.

Components
Reagents and materials supplied in the PTP Assay Kit are sufficient for at least 96 reactions. The phosphotyrosine peptide substrates supplied are sufficient for at least 120-140 PTP assays.

M7427 Malachite Green-Molybdate Dye Solution, 25 ml/bottle
D7309 Dye Additive Solution, Stabilizer for M7427, 1 ml/vial
P4601 10 mM Phosphate Standard Solution, 1 ml/vial
P6462 Protein Tyrosine Phosphatase Monophosphate Substrate (PTP Substrate 1) [pTyr^{1146}] Insulin Receptor (1142-1153). Lyophilized, 1.7 mg/vial.
P4726 Protein Tyrosine Phosphatase Monophosphate Substrate (PTP Substrate 2) [pTyr^{1018}] EGF Receptor (1014-1024). Lyophilized, 1.6 mg/vial.
W1136 Water, Phosphate-free, 25ml/ bottle.
M7302 96-wells Microtiter Plate A1/2 with Plate Cover, 1 each.
B7533 Sephadex G-25 Column, Prepacked 3 ml Columns, 5 each.

Precautions and Disclaimer
Malachite Green-Molybdate Dye Solution contains malachite green, ammonium molybdate and 7% hydrochloric acid. Caution: Toxic. Refer to MSDS.

Preparation Instructions
General Notes:
All components of this kit must be used and/or diluted in plastic vials (Eppendorf vials, etc.). Do not to use glassware since some type of glass may contain high levels of free inorganic phosphates.
Components added to the assay system (buffers, detergents, proteins) may interfere with the assay. For example, **phosphate buffers are not compatible with this assay**, and buffers containing phosphate derivatives (e.g., phosphoglycerate) or detergents such as SDS above 0.1% may interfere with this assay and should not be used. In addition, some proteins are not soluble in strong acidic conditions and may precipitate upon addition of the Dye Reagent Mixture. It is recommended to test a component's compatibility with the PTP Assay Kit by performing a small trial assay. **The supplied Phosphate-free Water should be used for the preparation of all the buffers required.** Highly purified deionized water may also be used, however it must be pretested by the standard phosphate assay. Refer to the Phosphate Standard Assay to ascertain that the phosphate levels are in the acceptable range.

Reagents and Equipment Required but not Provided
1. Glycerol
2. Reagent containers (e.g. 50 ml graduated conical tubes with screw cap).
3. Microcentrifuge and vials
4. Precision pipettes (2-20, 20-100 and 200-1000 μl) with disposable tips.
5. Protein assay reagent (optional) for determination of protein content in samples (e.g. cell lysates).
6. Lysis Buffer for preparation of cell extracts.

A. Preparation of Cell Lysates
   Different cells and tyrosine phosphatases (membrane type, cytosolic etc.) may require different lysis buffer compositions (with detergents, protease inhibitors etc.) and conditions (e.g. lysis time etc.). The lysis buffer and conditions described here are provided only as a guideline. For a particular application the lysis buffer and lysis conditions must be optimized.
   Unless noted otherwise, the following steps are carried out rapidly, on ice, using the appropriate ice cold lysis buffer.
   1. Prepare lysis buffer.
      Lysis Buffer: 50 mM Hepes buffer, pH 7.4, containing 0.5% Triton X-100, 10% glycerol. Add protease inhibitors: 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A or a protease inhibitor cocktail (i.e., Sigma Product No. P8340), 10 μl/ml cell suspension.
   2. Grow cells in appropriate culture medium.
   3. For attached cells, wash by rinsing with ice cold saline (0.9% NaCl or other buffers without phosphate), (2 x 10 ml). For cells in suspension, wash by centrifuging at 800 x g for 5 min. at room temperature with ice cold saline (0.9% NaCl or other buffers without phosphate), (2 x 10 ml). Remove supernatant.
   4. Add to attached cells freshly prepared lysis buffer at 4°C. For cells in suspension, add lysis buffer to washed cell pellet in a 15 ml conical tube.
   5. Transfer cell lysate to an eppendorf tube and leave for 10-20 min. at 4°C.
   6. Centrifuge lysate at 10,000 x g for 15 min at 4°C.
   7. Collect supernatant containing lysate fraction. Discard the pellet containing nuclear and cell debris.
   8. Remove a 0.1 ml aliquot of lysate for a protein determination.
   9. Label B7533, a Sephadex G-25 column, with the lysate name, date etc. Support the column over a suitable container.
   10. Remove the cap from the top of the column. Remove the lower tip of the column and let the excess liquid flow through. Under normal gravity, the column is designed not to run dry.
   11. Equilibrate the column with 10 ml (2 x 5 ml) of an appropriate enzyme dilution buffer.
   12. Apply 0.2 - 0.4 ml of lysate to the top of the gel bed and collect the flow-through.
   13. Elute the column with 2 - 4 ml of appropriate enzyme dilution buffer and collect each 0.2 - 0.4 ml fraction in a separate tube. Keep fractions on ice until completion of assay.
   14. Remove a 0.1 ml aliquot of lysate for a protein determination.
   15. Fractions of the lysate passed through the column may be tested in the PTP assay (See Procedure). It is recommended to test different lysate dilutions to obtain an optimal signal.

16. Where applicable, add to samples of cell lysate and fractions, glycerol to a final concentration of 50%. Freeze and store samples at −70°C.

B. Preparation of Dye Reagent
Add 10 µl of D7309, Dye Additive Solution, to 1 ml of M7427, Malachite Green-Molybdate Dye Solution. Mix well. The dye reagent must be freshly prepared. Do not store this solution.
The Dye Reagent (Malachite Green- Molybdate Dye Solution/Dye Additive Solution mixture) supplied in this system is sufficient for 500 tests in the 96 well format.

C. Preparation of Phosphate Standard
1. High Range Phosphate (500-4000 pmol): Mix 20 µl of 10 mM P4601, Phosphate Standard, in 2 ml W1136, Phosphate-free Water, to make a 100 µM Phosphate Standard. Mix well.
   Prepare further phosphate standard dilutions using this solution. (Refer to Table 1 for selection of required range of pmoles PO_4^{2-}).
2. Low Range Phosphate (25-200 pmol): Mix 0.1 ml of the 100 µM Phosphate Standard prepared in step 1 above in 0.9 ml W1136, Phosphate-free Water, to a give a final concentration of 10 µM Phosphate Standard. Mix well.
   Prepare further phosphate standard dilutions using this solution. (Refer to Table 1 for selection of required range of pmoles PO_4^{2-}).

Note: The phosphate standard dilutions must be freshly prepared. Do not store phosphate standard dilutions. Prepare enough volume to perform duplicate assays.

Table 1 Phosphate Standard Assay - Dilution Table

High Range Phosphate					**Low Range Phosphate**					**Blank**	**Phosphate Standard**
3000	2000	1500	1000	500	200	150	100	50	25	0	**pmoles Phosphate/ well (50µl/well)**
--	--	--	--	--	160	120	80	40	20	0	**LOW RANGE µl of 10 µM phosphate**
240	160	120	80	40	--	--	--	--	--	0	**HIGH RANGE µl of 100 µM phosphate**
160	240	280	320	360	240	280	320	360	380	50	**Phosphate-free Water (µl)**

Note: To obtain the pmoles of phosphate/50 µl/well (first row), mix the corresponding stated volume of 10 µM phosphate (2nd row) or 100 µM phosphate (3rd row) with the corresponding volume of phosphate-free water (4th row).

D. Protein Tyrosine Phosphatase (PTP) Substrates
1. Peptide Stock Solutions: Reconstitute each of the supplied PTP substrates, (P6462 and P4726), with W1136, Phosphate-free Water to a final concentration of 1 mM. This concentration is equivalent to 10 nmole peptide/10 µl. Follow the information provided in Table 2.
   Store the reconstituted peptide substrate stock solutions at −20°C.

Table 2 Preparation of Phosphotyrosine Peptide Substrates Stock Solutions

Protein Tyrosine Phosphatase Substrate	MW	Net Peptide/ vial	Add Phosphate-Free Water (W1136)	Stock Final Concen-tration
P6462 **PTP Substrate 1** [pTyr^{1146}] Insulin Receptor (1142-1153)	1702.7	1.7 mg	1.0 ml	1mM
P4726 **PTP Substrate 2** [pTyr^{1018}] EGF Receptor (1014-1024)	1328.3	1.6 mg	1.2 ml	1mM

2. Peptide Working Dilutions: The peptides may be used undiluted (stock solution) or further diluted from the 1 mM stock solution with W1136, Phosphate free-Water (e.g., 1:1 for 0.5 mM) as required.

Notes:
Peptides, in general, are highly hygroscopic and may acquire moisture during shipment and storage. This does not affect the activity of the peptide. If required, the peptide may be dried using a SpeedVac centrifuge at room temperature prior to reconstitution.
Other substrates such as histone, casein or any specific tyrosine phosphatase substrate may be used. Preliminary testing is recommended, as some proteins have limited solubility in strong acid. Substrates containing phosphates (e.g., prepared in buffers like PBS), usually will require a preliminary dialysis or desalting step to remove free phosphate before use.

E. Protein Tyrosine Phosphatase (PTP)
   1. Dilute PTP (e.g. fractionated or purified enzyme) with enzyme dilution buffer to the optimal assay dilution. If the activity of the enzyme is not known, a preliminary assay should be performed, testing several enzyme dilutions and buffer compositions.
   2. Throughout the assay, keep the enzyme solution on ice. Do not store enzyme dilutions.
   3. LAR Phosphatase (Sigma Product No. L0907) may be used as a positive PTP control with PTP Substrate 1 (Figures 3 and 4). Dilute enzyme with enzyme dilution buffer to optimal recommended dilution for assay, e.g., 40 units/1.5 ml. Keep enzyme solution on ice until used. Do not store enzyme dilutions.

F. PTP Reaction Buffer and Enzyme Dilution Buffer
   1. The required phosphatase reaction buffer (not supplied) must be freshly prepared. A 4X concentrated PTP reaction buffer is required for use in the assay.
      It is recommended to determine the optimal working dilution of the supplied phosphotyrosine peptides when using a particular PTP/cell extract in the assay. In addition, protein phosphatases may work optimally under diverse conditions (e.g. pH, buffer composition, temperature etc.). The pH optimum of phosphatases is also substrate dependent, reflected in varying Vmax and Km values. **Therefore, a universal buffer system cannot be provided.** It is recommended to perform a pH profile of activity on the phosphatase of interest between pH 5.0 and 8.0 using the supplied phosphotyrosine peptide substrates.
   2. Examples of commonly used PTP reaction buffers (e.g., for LAR Phosphatase) (Sigma Product No. L0907):
      - Phosphatase Reaction Buffer:
        4X Reaction Buffer 100 mM Imidazole, pH 7.0, containing 10 mM EDTA, 200 mM NaCl, 20 mM DTT.
      - Enzyme Dilution Buffer:
        10 mM Imidazole, pH 7.0.

Storage/Stability
The 96-well Microtiter Plate, M7302, and the Sephadex G-25M Columns, B7533, should be stored at room temperature. Do not freeze B7533, Sephadex G-25 columns.
After reconstitution, the peptide substrate solutions P6462 and P4726 should be stored at −20°C.
All other components should be stored at 2-8°C.
The phosphate standard dilutions must be freshly prepared. Do not store phosphate standard dilutions.

Procedure

Reagents and Equipment Required but not Provided

1. ELISA Reader capable of measuring absorbance of 96-well plates at wavelength of 620-630 nm, or spectrophotometer (UV/Visible).
2. Incubator 30°C (If required).
3. Precision pipettes (2-20, 20-100 and 200-1000 µl) with disposable tips.
4. Protein Tyrosine Phosphatase (PTP) reaction buffer (See Preparation Instructions, Step F).
5. Enzyme Dilution Buffer (See Preparation Instructions, Step F).

Phosphate Standard Assay

Note: It is recommended to perform this assay in duplicates for each concentration in the standard curve.

1. For a blank, (without phosphate), add to a well of the 96-well plate
   - 50 µl of W1136, Phosphate-free Water
   - 50 µl of Dye Reagent, Malachite Green- Molybdate Dye Solution/Dye Additive Solution mixture.
   - Final assay volume is 100 µl.
2. For the standards, add to each well of the 96-well plate the following reagents: In duplicate,
   - 50 µl of Phosphate Standard dilutions (Table 1, Low and/or High Range) and
   - 50 µl of Dye Reagent, Malachite Green- Molybdate Dye Solution/Dye Additive Solution mixture.
   - Final assay volume is 100 µl.
3. Incubate 10 min. at RT.
4. Read absorbance at 620 nm.

Protein Tyrosine Phosphatase Assay

It is recommended to perform a Phosphate Standard Assay in parallel to the PTP assay. Perform the PTP assay in duplicates for each enzyme concentration or cell lysate. For inhibition tests with phosphatase inhibitors, preincubate the test sample with the phosphatase inhibitor for the required period of time prior to addition to the assay.

1. For a blank test (without enzyme or lysate), add to a well of the 96-well plate the following reagents:
   - 10 µl of appropriate phosphatase reaction buffer,
   - 10 µl PTP Substrate stock solution (1 mM) or other required working dilution,
   - Add 30 µl enzyme dilution buffer
2. Add to each well of the 96-well plate the following reagents:
   - 10 µl of appropriate phosphatase reaction buffer,
   - 10 µl PTP Substrate stock solution (1 mM) or other required working dilution,
   - 30 µl Enzyme Preparation, Protein tyrosine phosphatase (e.g. approximately 1unit LAR phosphatase, Sigma Product No. L0907) or 30 µl cell lysate in appropriate enzyme dilution buffer.
3. Incubate 10-30 min. at optimal temperature for enzyme under investigation (e.g., for LAR phosphatase, Sigma L0907, incubate 10 min. at 30°C).
4. Add 50 µl of freshly prepared Dye Reagent, Malachite Green-Molybdate Dye Solution/Dye Additive Solution mixture. Final assay volume is 100 µl.
5. Incubate 10 min. at room temperature.
6. Read absorbance at 620 nm.

Notes:

If an ELISA reader is unavailable, larger volumes of the Dye reagent may be used (e.g. 500 µl). A disposable spectrophotometer cuvette may be used for this purpose. The Dye Reagent (Malachite Green- Molybdate Dye Solution/Dye Additive Solution mixture) supplied in this system is sufficient for 50 tests in a 500 µl format.

The 96 well plate supplied in this system (1/2 area, flat bottom, Costar Code No. 3690) has reduced well area and volume (190 µl). Due to the well shape, the 96 well plate provides higher absorbance measurements than standard microtiter plates. This feature is particularly useful in the determination of low phosphate concentrations in the assay.

Do not discard the 96 well plate until all wells have been used. Do not reuse the wells that are stained with the dye.

Results

Phosphate Standard Assay
Prepare a standard curve by plotting the absorbance at 620 nm versus the phosphate standard (pmoles). Refer to Figures 1 and 2 for a typical phosphate standard curve.

Protein Tyrosine Phosphatase Assay
Determine the PTP activity (pmoles PO_4^{2-}/min) of the sample (e.g., enzyme, cell lysate) from the Phosphate Standard curve by extrapolating the absorbance obtained at 620 nm to the pmoles of phosphate and dividing it by the actual reaction time.

Figure 1. Phosphate Standard Assay Curve- Low-High Range (50-3000pmol PO_4^{2-})

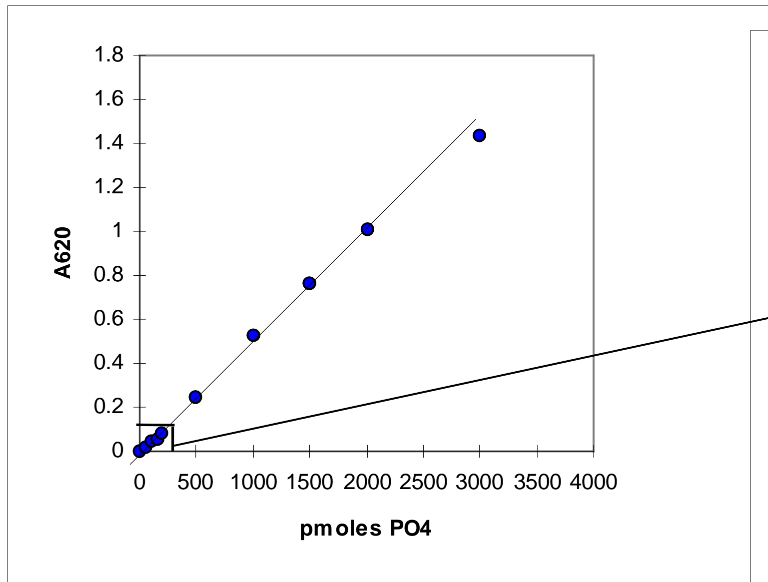


Figure 2. Phosphate Standard Assay Curve-Low Range (50-200 pmole PO_4^{2}

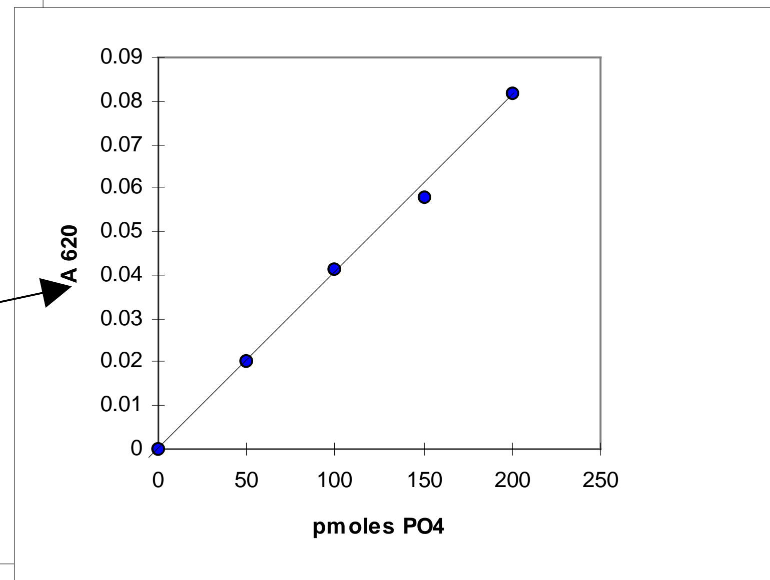
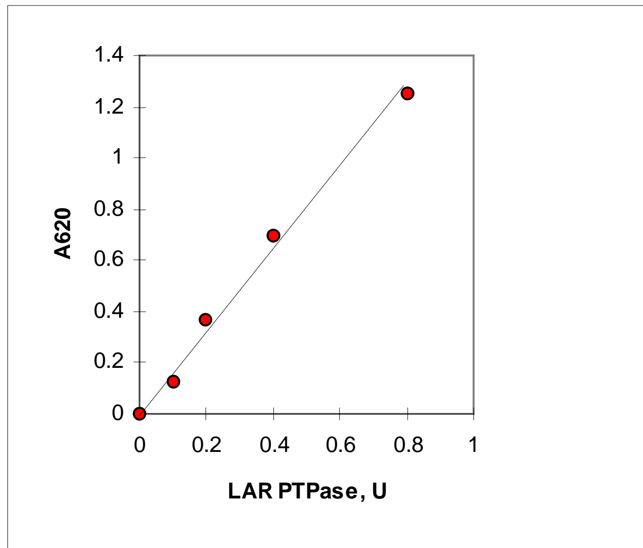
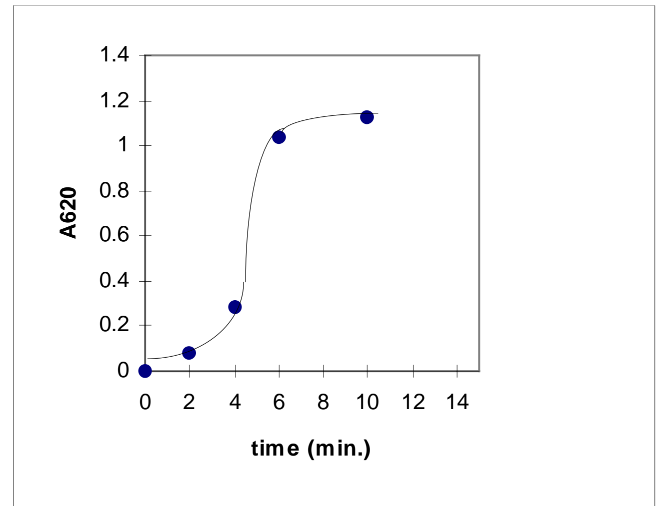


Figure 3. Phosphatase activity of LAR PTP



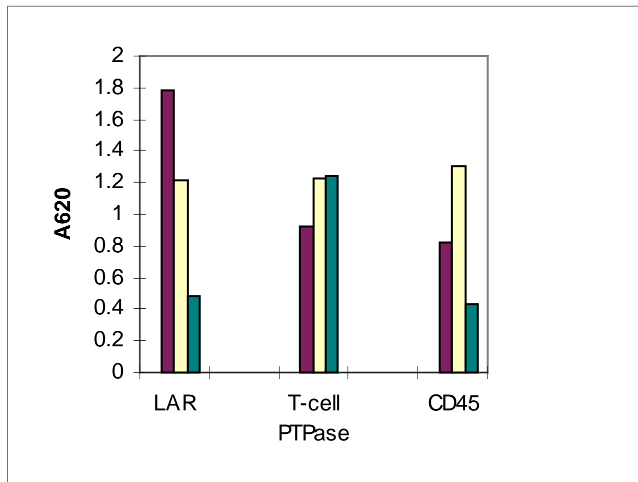
Protein tyrosine phosphatase, LAR PTP, 0.1-0.8U (Sigma L0907), was incubated for 10 min at 30°C with PTP substrate 1 (6 nmole).

Figure 4. Time course of PTP activity



Protein tyrosine phosphatase, LAR PTP, 0.8U (Sigma L0907), was incubated for different time periods at 30°C with PTP substrate 1 (6 nmole).

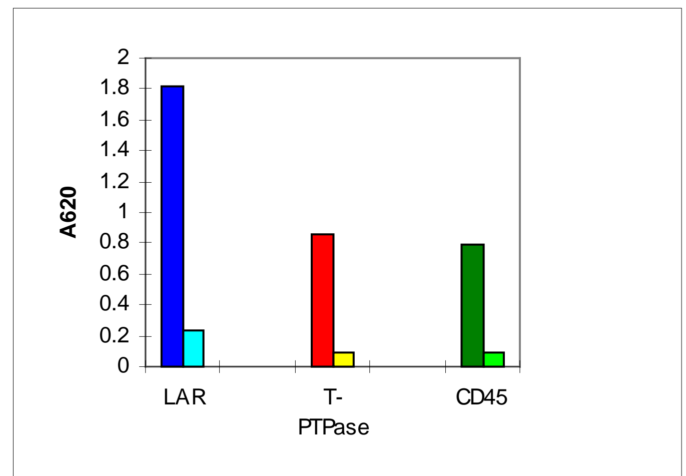
Figure 5. Specificity of different PTPs toward PTP substrates



Protein tyrosine phosphatases, LAR PTP, 1U (Sigma L0907), T-cell PTP, 1U (Calbiochem) and CD45, 1U (Calbiochem) were incubated for 10 min at 30°C with different phosphotyrosine peptides :

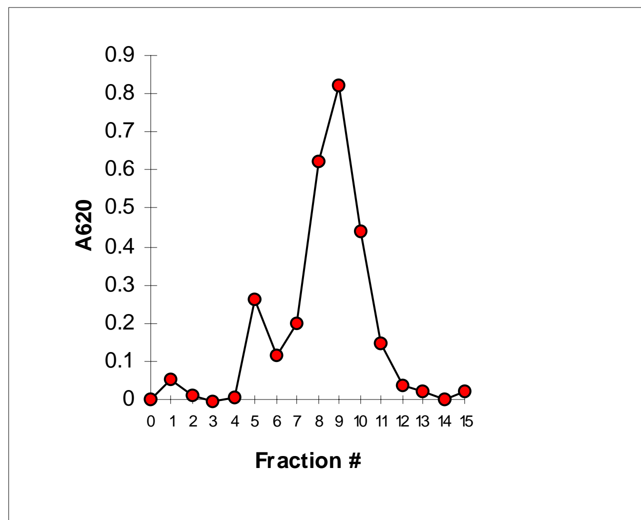
- PTP substrate 1 (8 nmole),
- PTP substrate 2 (10 nmoles) and
- ENDpYINASL substrate (10 nmoles).

Figure 6. Inhibition of purified PTPs activity



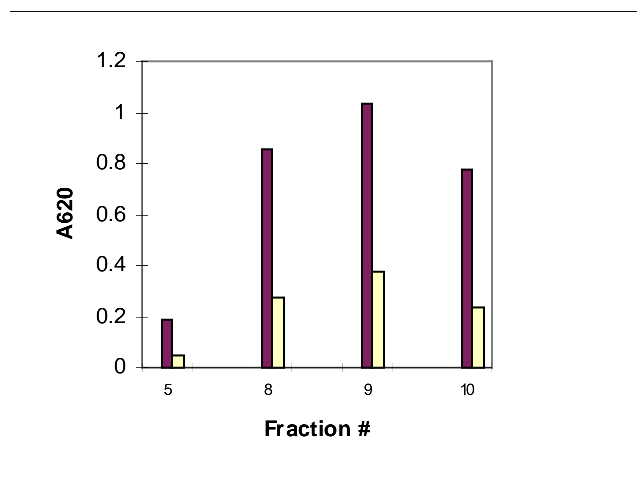
Protein tyrosine phosphatases, LAR PTP, 1U (Sigma L0907), T-cell PTP, 1U (Calbiochem 539732) and CD45, 1U (Calbiochem 217614) were preincubated with or without PTP inhibitor sodium orthovanadate 1.5µM for 10min on ice and assayed with PTP substrate 1 (8 nmole) as described under Figure 5.

Figure 7. PTP activity of A431 cell lysate



Lysate from A431 human carcinoma cells (0.3ml) was desalted on a Sephadex G25M column in 10 mM Imidazole buffer pH 7.0. Fractions (0.3ml) were collected and incubated for 10 min at 30°C with PTP substrate 1 (6 nmole).

Figure 8. Inhibition of PTP activity in A431 cell lysate



Selected fractions with PTP activity from Sephadex G25 column (see Figure 7) were preincubated with or without sodium orthovanadate 1.5 mM for 15 min on ice and assayed with PTP substrate 1 as described under Figure 7.

References

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Related Reagents

Sources of PTP

1. LAR Protein Tyrosine Phosphatase, Human, Recombinant, Expressed in *E. coli*, Sigma Product No. L0907.
2. Protein Tyrosine Phosphatase (Cytosolic PTP, EC 31.1.3.48), from rabbit kidney, Sigma Product No. P4569.

Protein Phosphatase Inhibitors

3. Sodium orthovanadate, Sigma Product No. S6508.
4. Okadaic acid, Sigma Product No. O4511.
5. Microcystin LR, from *Microcystis aeruginosa*, Sigma Product No. M2912.

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