DuoSet® IC

Human Active PTP1B

Catalog Number DYC1625-2
DYC1625-5

For the development of a phosphopeptide substrate assay to measure protein tyrosine phosphatase 1B (PTP1B) activity in cell lysates.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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PRINCIPLE OF THE ASSAY

This DuoSet® IC activity assay contains the basic components required for the development of capture assays to measure the activity of protein tyrosine phosphatase 1B (PTP1B), also known as PTPN1 and placental protein tyrosine phosphatase, in cell lysates. An immobilized capture antibody specific for PTP1B binds both active and inactive PTP1B. After washing away unbound material, a synthetic phosphopeptide substrate is added that is dephosphorylated by active PTP1B to generate free phosphate and unphosphorylated peptide. The free phosphate is detected by a sensitive dye-binding assay using malachite green and molybdic acid. By calculating the rate of phosphate release, the activity of PTP1B is determined.

MATERIALS PROVIDED

Store the unopened kit at 2 - 8° C. Do not use past kit expiration date.

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC1625-2**	**Cat. # DYC1625-5**
PTP1B Capture Antibody	841377	2 - 8° C	1	2
Tyrosine Phosphatase Substrate I	841378	2 - 8° C	1	2
Phosphate Standard	892809	2 - 8° C	1	1
Malachite Green Reagent A	895855	2 - 8° C	1	2
Malachite Green Reagent B	895856	2 - 8° C	1	2

DYC1625-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*

DYC1625-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General Assay Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

If additional reagents are required, Tyrosine Phosphatase Substrate I (Catalog # ES006) and a Malachite Green Phosphate Detection Kit (Catalog # DY996) are available from R&D Systems.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Sigma # L8511)
- Pepstatin (Sigma # P4265)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium orthovanadate (Na_3VO_4) (Sigma # S6508)
- Dithiothreitol (DTT) (Sigma # D0623)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Deionized or distilled water
- Pipettes and pipette tips
- 30° C water bath or incubator
- Plate reader capable of measurements at a wavelength of 620 nm
- Centrifuge capable of reaching 12,000 x g
- Rocking platform
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems, Catalog # DY990) are suggested]. For measurement of very low phosphatase activity, use 1/2 area microplates with a suitable plate reader [Costar EIA Plates (Catalog # 3690) are recommended]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2 - 7.4, 0.2 μm filtered.

TBS - 25 mM Tris, 150 mM NaCl, pH 7.5.

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2 - 7.4 (R&D Systems, Catalog # WA126).

Block Buffer - 1% BSA,* in PBS, pH 7.2 - 7.4, 0.2 μm filtered.

IC Diluent #10 - 50 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 120 mM NaCl, 0.5% NP-40 Alternative, pH 7.5. Store at 2 - 8° C.

IC Diluent #11 - 10 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 0.5% BSA, 1 mM DTT, pH 7.5. Prepare fresh just before use.
Note: *The presence of DTT is essential to measure phosphatase activity.*

Lysis Buffer #8 - 50 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 120 mM NaCl, 0.5% NP-40 Alternative (pH 7.5), 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, 2 μg/mL Aprotinin, 1 mM PMSF. Prepare fresh just before use.

*The use of R&D Systems Reagent Diluent Concentrate 2 (Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2 - 8° C.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

PTP1B Capture Antibody (Part 841377) - Each vial contains 1440 μg/mL of mouse anti-human PTP1B antibody when reconstituted with 200 μL of PBS. After reconstitution, store at 2 - 8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Tyrosine Phosphatase Substrate I (Part 841378) - Each vial contains 13.0 mg/mL of DADEY(PO_3)LIPQQG when reconstituted with 0.25 mL of deionized water. After reconstitution, store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Phosphate Standard (Part 892809) - 1 mL of 1 M Phosphate ($KHPO_4$). A seven point curve using 2-fold serial dilutions and a high standard of 100 μM is recommended. Store at room temperature for up to 3 months.*

*Provided this is within the expiration date of the kit.

PREPARATION OF SAMPLES

Note: *Immediately before harvesting cells, prepare Lysis Buffer #8 and store it on ice until use.*

Cell Lysates - Rinse cells two times with TBS, making sure to remove any remaining TBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #8 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in IC Diluent #10.

PRECAUTION

The Malachite Green Reagent A, Malachite Green Reagent B, and Phosphate Standard supplied with this kit are acidic solutions. Wear eye, hand, face, and clothing protection when using this material.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC activity assay should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- This kit uses a highly sensitive phosphate detection method. Many soaps and dish detergents contain phosphate and will leave a residue that will increase the background absorbance of the assay. Containers should be rinsed extensively with distilled or deionized water before use.
- It is recommended that all standards and samples be assayed in duplicate or triplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2 - 8° C or be prepared fresh daily.

GENERAL ASSAY PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 8.0 μg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

   Note: *The Phosphate Standard used in this assay does not require antibody-coated wells.*

2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for 1 - 2 hours.

4. Repeat the aspiration/wash as in step 2 using IC Diluent #10. The plates are now ready for sample addition.

Recommended Controls

The following controls may be included with samples being assayed.

	Control	1 mM Sodium Orthovanadate	Purpose
1	No Lysate	–	To determine non-enzymatic substrate hydrolysis and phosphate contamination.
2	Lysate	+	To determine phosphatase activity due to non-protein tyrosine phosphatases.

Assay Procedure

1. Thaw lysate and keep on ice until ready for use.

2. Dilute the lysate with IC Diluent #10. The degree of dilution will vary with the cell line used. For initial experiments, several different dilutions should be tested.

3. Add 100 μL of the diluted cell lysate to the appropriate wells. Do not add cell lysate to Control 1. Add only 100 μL of IC Diluent #10.

4. Place the plate on a rocking platform at 30 rpm for 3 hours at 2 - 8° C.

5. Aspirate lysates from wells. Add 400 μL of IC Diluent #10. Aspirate to remove IC Diluent #10. Repeat twice more for a total of three washes. Remove residual IC Diluent #10 by inverting the plate and blotting it against clean paper towels.

6. Add 40 μL of IC Diluent #11 to all wells. To Control 2, also add 5 μL of 10 mM sodium orthovanadate.

   Note: *The presence of DTT in IC Diluent # 11 is essential to measure phosphatase activity.*

7. Dilute the Tyrosine Phosphatase Substrate I 10-fold with IC Diluent #11.

8. Add 10 µL of diluted Tyrosine Phosphatase Substrate I to each well. The final concentration is 200 µM. Incubate for 30 minutes at 30° C.

9. During the 30 minute incubation in step 8, prepare the Phosphate Standard. A seven point standard curve using 2-fold serial dilutions and a high standard of 100 µM is recommended.

   Note: *The 1 M Phosphate Standard will precipitate in the presence of divalent cations such as calcium, magnesium or manganese. If divalent cations have been added to IC Diluent #11, dilute the Phosphate Standard to 10 mM with distilled water and further dilute with IC Diluent # 11. See Appendix for additional interfering substances.*

   Prepare six 1:2 serial dilutions of the 100 µM Phosphate Standard in IC Diluent #11 (see Table 1).

   Table 1: Dilution of standards for microplate assay.

Standard	Phosphate	nmol/well	Volume of IC Diluent #11	Volume of Standard
——	10 mM	——	990 µL	10 µL of 1 M Phosphate
1	100 µM	5	990 µL	10 µL of 10 mM Phosphate
2	50 µM	2.5	200 µL	200 µL of 100 µM Phosphate
3	25 µM	1.25	200 µL	200 µL of 50 µM Phosphate
4	12.5 µM	0.625	200 µL	200 µL of 25 µM Phosphate
5	6.25 µM	0.313	200 µL	200 µL of 12.5 µM Phosphate
6	3.13 µM	0.156	200 µL	200 µL of 6.25 µM Phosphate
7	1.56 µM	0.078	200 µL	200 µL of 3.13 µM Phosphate
Std. Blank	——	0	200 µL	——

10. Use 50 µL of each standard in duplicate or triplicate to construct a standard curve.

11. After the 30 minute incubation with Tyrosine Phosphatase Substrate I, add 10 µL of Malachite Green Reagent A to each sample, standard or blank. Mix thoroughly by tapping the side of the plate and incubate for 10 minutes at room temperature.

12. Add 10 µL of Malachite Green Reagent B to each well. Mix thoroughly by tapping the side of the plate and incubate for 20 minutes at room temperature. This allows time for the yellow background color to bleach out.

13. Measure A_{620nm} on a microplate reader.

    Note: *The blue color will slowly fade over time. For best interassay consistency, read plates within 5 minutes of completing the incubation in step 12.*

CALCULATION OF RESULTS

Average the A_{620nm} readings of each standard or sample. Subtract the assay blank from all standards and unknown samples. Plot nmol/well of Phosphate Standard vs. A_{620nm} (see Figure 1). Use computer software capable of simple linear regression or 4 parameter logistic curve fitting to create a standard curve. The phosphate content of unknown samples can then be calculated from the standard curve.

In the graph below, the Phosphate Standard was diluted in IC Diluent #11 and assayed in triplicate.

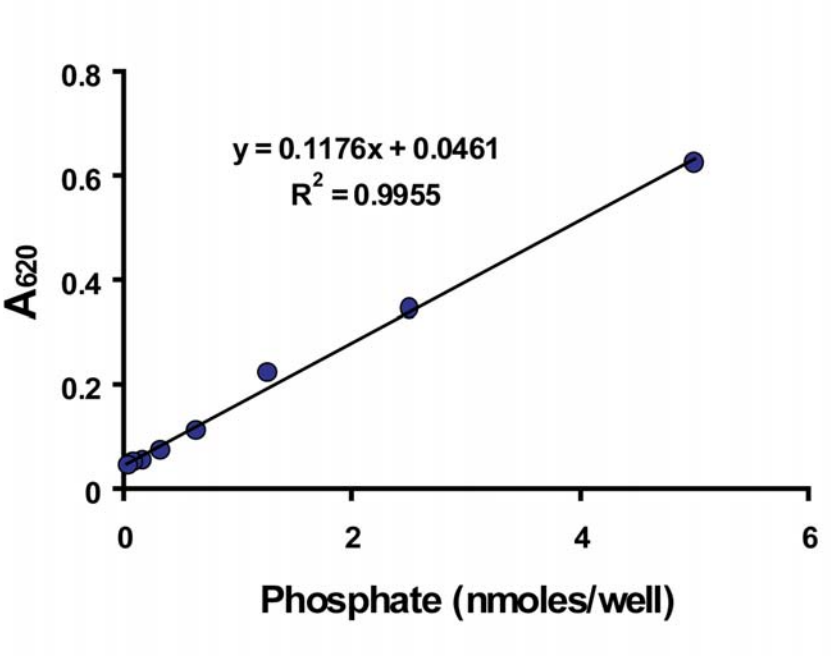

Figure 1: Assay for inorganic phosphate.

SPECIFICITY

This DuoSet IC activity assay is specific for PTP1B. Specificity was demonstrated by Western blot analysis of protein bound by the capture antibody supplied in the kit. Only one band was observed corresponding to PTP1B. In addition, phosphatase activity in this assay was inhibited by peroxyvanadate, further demonstrating its specificity for protein tyrosine phosphatases.

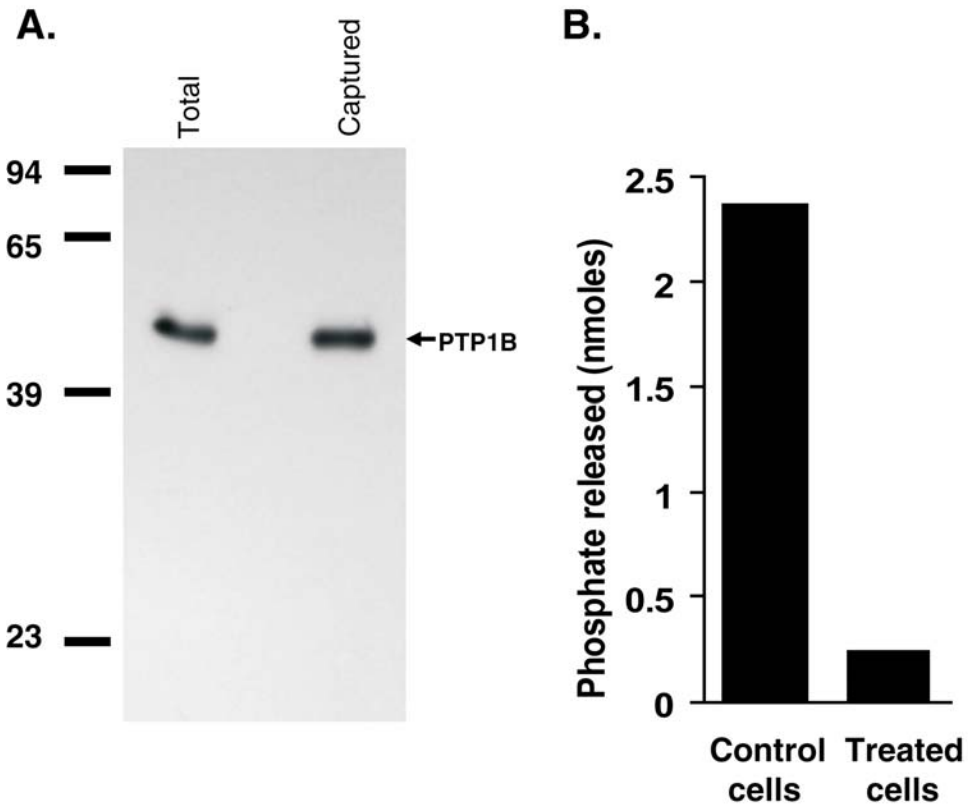

Figure 2:

A. Lysates from MCF-7 cells were incubated in wells coated with PTP1B Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to an Immobilon-P (Millipore) membrane, and immunoblotted with a rabbit anti-PTP1B antibody (R&D Systems, Catalog # AF1366). The lysate and bound proteins were also blotted with antibodies against TC-PTP (54% homology) and SHP-2 (19% homology). These proteins were present in the lysates but not in the bound proteins (data not shown).

B. MCF-7 cells were incubated without (Control) or with 100 μM peroxyvanadate for 10 minutes (Treated). Peroxyvanadate is a cell-permeable covalent inhibitor of tyrosine protein phosphatases. The cells were then lysed and analyzed in this assay. The results indicate that peroxyvanadate treatment reduced PTP1B activity to 6% of the amount measured in the untreated cells.

QUANTIFICATION

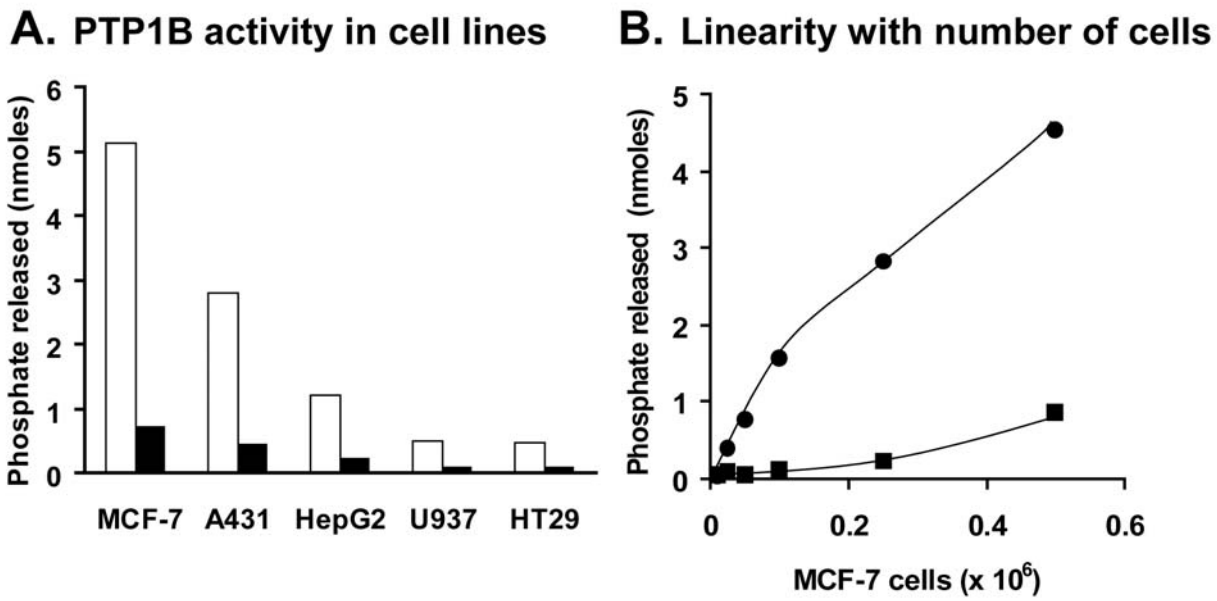

Figure 3:

A. PTP1B activity in lysates prepared from MCF-7, A431, HepG2, U937, and HT29 cells was quantified in this assay. The lysates were prepared at 1 x 10^7 cells per mL and activity was measured. To determine non-specific activity, 1 mM sodium orthovanadate was added to the assay buffer in some of the wells. The levels of activity were normalized to 2 x 10^5 cells per well. Open bars indicate total activity. Closed bars indicate activity in the presence of 1 mM sodium orthovanadate.

B. An MCF-7 cell lysate was prepared at 1 x 10^7 cells/mL and 2-fold serially diluted prior to the assay. To determine non-specific phosphatase activity, 1 mM sodium orthovanadate was added to the assay buffer in some of the wells. Circles represent total activity, and squares represent activity in the presence of 1 mM sodium orthovanadate.

Note: *If high activity samples are anticipated, a shorter reaction time or greater sample dilution is advised. When substrate dephosphorylation exceeds 1.8 nmoles per 30 minutes (18% of the total substrate), the slope of the curve decreases. A correction curve can also be generated either by assaying dilutions of a cell lysate or with recombinant PTP1B (R&D Systems, Catalog # 1366-PT). Although this recombinant PTP1B can be assayed in the well, it cannot be captured by the antibody supplied in this kit.*

APPENDIX

Substances tested for interference with phosphate determination

Buffers[1]	Compatible Concentrations	Adverse Effect at Higher Levels
Tris-HCl, pH 9.0	100 mM	—
HEPES, pH 7.5	100 mM	—
MOPS, pH 7.0	100 mM	—
Imidizole, pH 7.0	100 mM	—
MES, pH 5.5	100 mM	—
Detergents[2]		
Triton X-100	0.3%	Increased Background
Tween 20	0.1%	Reduces Sensitivity
NP-40	1%	—
CHAPS	1%	—
Deoxycholate	0.01%	Precipitates & Increases Background
SDS	0.01%	Increases Background
Common Reagents[2]		
Glycerol	5%	Reduces Sensitivity
DMSO	10%	Reduces Sensitivity
Ethanol	25%	Reduces Sensitivity
BSA	0.03 mg/mL	Reduces Sensitivity
EDTA	10 mM	—
Dithiothreitol	3 mM	Reduces Sensitivity
β-mercaptoethanol	10 mM	—
Na_3VO_4	1 mM	Reduces Sensitivity
NaF	10 mM	—
NaCl	100 mM	—
KCl	100 mM	—
$CaCl_2$	10 mM	—
Citric acid	10 mM	Reduces Sensitivity
ATP	100 µM	Increases Background Reduces Sensitivity

[1]Tested by microplate assay with or without 1 nmol of KH_2PO_4

[2]Tested by microplate assay in 25 mM Tris-HCl buffer (pH 7.5) with or without 1 nmol of KH_2PO_4