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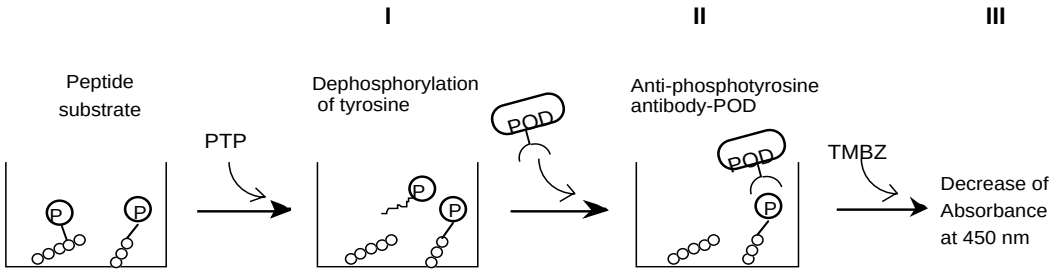
Universal Tyrosine Phosphatase Assay Kit

TaKaRa

1. Description

Protein tyrosine phosphorylation/dephosphorylation plays an important role in the regulation of protein activity during cell proliferation and differentiation. Protein Tyrosine Phosphatase (PTP) is involved in dephosphorylation which keeps the balance of those protein activities. Like Protein Tyrosine Kinase (PTK), PTP is classified into two groups; 1) the one is membrane receptor PTP and 2) the second is cytoplasmic located. As PTP acts on a wide range of substrate, it is also classified into several groups by its substrate; ones involved in signal transduction in differentiation of hematopoietic cells (SH-PTP-1, PTP-β2, CD45, etc.), ones which transduct cells adhesion signal (PTP1B-LP, LAR, PTPμ, PTPκ, etc.), ones involved in differentiation of neuro cells (SHP-1), ones involved in signals from antigen receptor of lymphocyte (PEP, CD45), ones engaged in signal transduction specific to germ cell, and so on.
Universal Tyrosine Phosphatase Assay Kit enables in vitro measurement of the activity of PTP quickly and specifically with non-RI method. As this kit utilizes the mono-peptide substrate (Glu and Tyr phosphorylated by natural kinases) which has broad PTP specificity, it is useful to measure the activity of various PTPs. It can also be used for screening of selective inhibitor of PTP, not only for screening of PTP itself.

2. Principle



I. Into the wells of the microtiter plate which is immobilized with PTP substrate (random peptide including phosphorylated tyrosine), add PTP samples and per form dephosphorylation of tyrosine.
II. Add anti-phosphotyrosine antibody-POD (PY20). The POD-labeled antibody binds only to the substrate which has not been dephosphorylated.
III. Perform color development by adding TMBZ which is the substrate for POD, and measure the absorbance at 450 nm.

The resulting color intensity is a reciprocal proportional measure for the PTP in the samples. High PTP activity results in low absorbance values. The PTP activity of a sample can be quantitated from the standard curve obtained with the supplied PTP control (Vial 5).

3. Features

1. *Safety :*	No radioactive isotopes are required.
2. *Accuracy :*	No cross reactions with Protein Ser / Thr Phosphatase are observed. Background is very low.
3. *Sensitivity :*	Sensitive comparable to radioactive tests
4. *Ease of use :*	Preparation of solutions are very easy. Tyrosine phosphatase standard is included in this kit.
5. *Flexibility :*	Synthesized peptides are designed to possess the wide spectrum for various PTPs. Combining a specific antibody enables to measure PTP activity specifically.

4. Kit components

(For 96 tests)

1. PTP substrate immobilized microplate (8 wells x 12) 1 plate
2. Phosphatase reacting solution 11 ml x 1
3. (None)*
   *The component labeled with 3 is not included in this kit.
4. Lysis buffer 11 ml x 2
5. PTP control (CD45) (lyophilized) 0.5 ml x1
6. Anti-phosphotyrosine (PY20) - Horse Radish Peroxidase (HRP) (lyophilized, for 5.5 ml / H_2O) 5.5 ml x1
7. Blocking solution 11 ml x 1
8. HRP substrate solution (TMBZ) 12 ml x 1

5. Reagents and instruments not supplied in this kit

- Stop solution : 1 N H_2SO_4 11 ml / kit
- Washing buffer : PBS including 0.05% (v/v) Tween20 (PBS-Tablet TaKaRa Code. T900 is recommended.)
- Incubator (37 ˚C)
- Microtiter plate reader with a filter for the use of a wavelength nearby 450 nm
- Distiled water
- Multichannel pipettor (10 - 500 μl)
- 2-mercaptoethanol

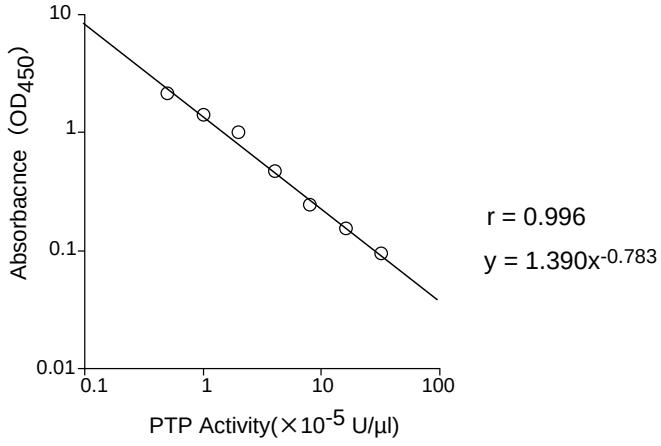
6. Storage

4 ˚C

7. Assay characteristics

7-1. Specificity

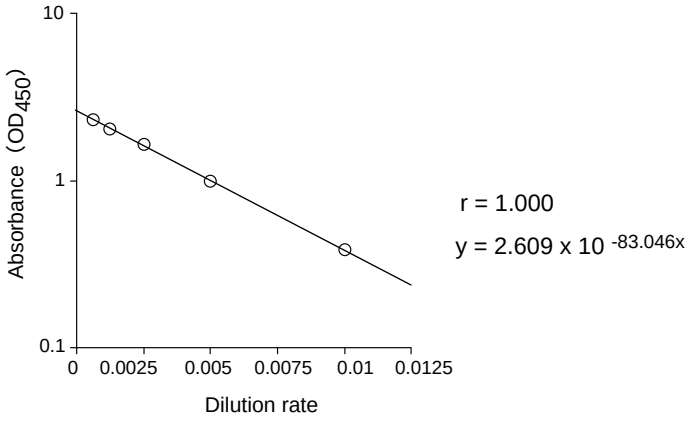
a) PTP activity and absorbance using purified recombinant human CD45 as a sample



PTP activity (x10^{-5}units / μl)	32	16	8	4	2	1	0.5	0
Absorbance (A_{450})	0.095	0.152	0.246	0.472	1.007	1.393	2.143	3.470

b. Correlation between dilution rate and absorbance in use of A431 cells extract as sample

A431 cells were cultured in a ϕ90 mm plate until 100% confluent. After removing the culture supernatant, 1 ml of Lysis buffer was added and the cells were collected with a cell scraper. After centrifugation at 14,500 rpm (10,000 x g), the serial dilution of the obtained supernant was prepared and it was used as the sample.



Dilution rate	1/50	1/100	1/200	1/400	1/800	1/600
Absorbance (A_{450})	0.190	0.386	0.994	1.641	2.043	2.310

7-2. Sensitivity

$\geq 0.125 \times 10^{-5}$ units / μl when using CD45 as a sample

7-3. Definition of activity

One unit (U) of enzyme is defined as the amount needed to dephosphorylate 1 nmol of p-NPP (p-nitrophenyl phosphate) at pH7.0, 30°C for 1 minute.

7-4. Assay time

It usually takes 1.5 - 3 hrs. to perform the whole procedure. Assay time may vary depending on the time of enzyme reaction.

7-5. Variable assay parameters

Since the PTP content in the sample can vary over a wide range depending on its source, the concentration of sample and the phosphorylation reaction time should be adapted according to the properties of the material.

7-6. Substrate

Synthesized oligopeptide (poly (Glu-Tyr)) that possesses a wide spectrum of specificity is immobilized on the well. All tyrosine residues in this substrate are phosphorylated with natual tyrosine kinase.

7-7. Measuring range and phosphopeptide standard curve

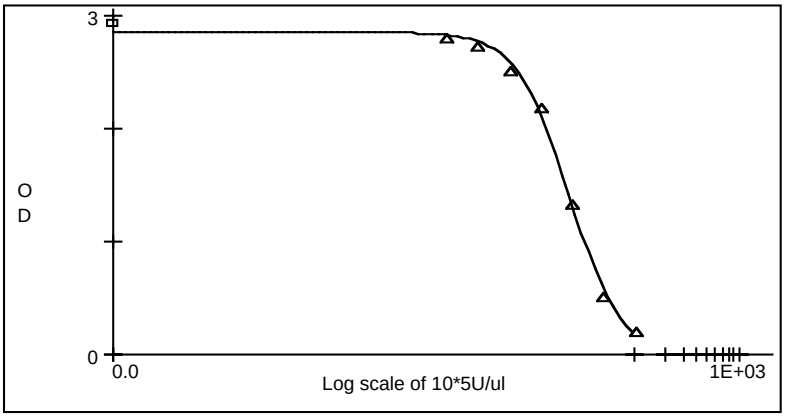
The activity of PTP is determined by comparing its absorbance with that of PTP standard supplied in this kit. The measuring range of this kit is from 0.125 x 10^{-5} units /μl to 90 x 10^{-5} units /μl) .

Typical standard curve
Sample volume: 50 μl
Reaction scale: 50 μl / well,
Enzyme reaction time: 45 min. at 37 ˚C

Curve Fit: 4-Parameter Corr. Coeff: 0.999

y = (A-D)/(1 + (x/C)^B) + D

A = 2.86 B = 1.76 C = 24.0 D = -0.0207



PTK activity (x10^{-5}units / μl)	**104.0**	**52.00**	**26.00**	**13.00**	**6.500**	**3.250**	**1.625**	**0**
Absorbance (A_{450})	**0.217**	**0.514**	**1.333**	**2.189**	**2.526**	**2.740**	**2.816**	**2.943**

7-8. Interference with Tyrosine Kinase in the sample

It was confirmed that substrate peptide would not be phosphorylated as long as further ATP is added during the reaction. Therefore, dephosphorylated tyrosine residues will not be phosphorylated again.

7-9. Non-specific dephosphorylation by Protein Phosphatase (PP) or acid/alkaline phosphatases in the sample

Most prepared PTP samples contain other phosphatases than PTP. In order to avoid dephosphorylation by these phophatases, both Lysis buffer and Phosphatase reaction solution of this kit contain 5 mM EDTA.2Na and 50 mM NaF.

8. Preparation of solutions

1) PTP substrate immobilized microplate, Lysis buffer and Blocking solution can be used without any preparations.

2) Phosphatase reacting solution
2-mercaptoethanol should be added to Phosphatase reacting solution to a final concentration of 50 mM just before use*. As 2-mercaptoethanol is unstable, this solution should be prepared freshly in a required amount in each use.
* For example, add 38 μl of a commercial 2-mercaptoethanol (99%, 14.4 M) into Phosphatase reacting solution (11 ml).

3) Protein Tyrosine Phosphatase control (PTP control)
One vial of lyophilized PTP control is dissolved in 100 μl of distilled water. (In case that all volume of solution is not used in one time, the solution should be stored in aliquots at - 80 ˚C. However, the PTP activity can decrease to 1/2 by one freezing.) Then add 400 μl of Phosphatase reacting solution including 2-mercaptoethanol (prepared at step 2)) to 100 μl of PTP control (5 times dilutions as a result ,total volume is 500 μl). This is the starting concentration for the preparation of the standard curve by serial dilutions (double dilutions) with the Phosphatase reacting solutions prepared at (2). The Phosphatase reacting solution prepared at step (2) can be used as 0 M standard of PTP. Since the activity (unit / μl) of PTP control could be different by lot, the standard curve should be set up in each measurement.

4) Anti-phosphotyrosine (PY20) - HRP
One vial of lyophilized Anti-phosphotyrosine (PY20) - HRP is dissolved in 5.5 ml of distilled water. (In case that all volume of solution is not used in one time, the solution should be stored at - 20 ˚C for one month) Do not repeat freeze-thaw cycles.

5) HRP substrate solution (TMBZ)
Before use, HRP substrate solution (TMBZ) is warmed up to room temperature. Confirm that this solution is not colored with blue. Avoid contacting this solution with metal ions, because it may be colored when it contacts with metal ions. In case that all volume of solution is not used in one time, only the required volume should be taken from the stock bottle before use.

9. Sample preparation (Preparation of cell extracts)

1) Suspension cells
- Centrifuge 1 - 5 x 10^6 cells at 1500 rpm (300 x g) at room temperature for 5 min. and discard the supernatant. Collect the cells as pellet.
- Add 1 ml of Lysis buffer, and suspend the pellet by vortex gently.
- Spin at 4 ˚C for 10 min. at 14,500 rpm (10,000 x g) and use the supernatant as sample.

2)Adherent cells
- Remove the media completely from the dish (1 - 5 x 10^6 cells, ϕ 9 cm dish).
- Add 1 ml of Lysis buffer to the dish and recover the cells cautiously from the dish with Cell Scraper. Then spin at 4 ˚C for 10 min. at 14,500 rpm (10,000 x g) and recover the supernatant as sample.

(3) Recovery of cells cultured in 96 wells plate
- Add 50 - 100 μl of Lysis buffer to each well.
- After taking off the cells by pipetting, then spin at 4 ˚C for 10 min. at 14,500 rpm (10,000 x g), and recover the supernatant as sample.

10. Assay procedure

1) Dilution
   Dilute the prepared sample (as mentioned in 8-1), 2), 3)) with Phosphatase reacting solution by more than 5 times.
   *When extracts of cultured cells are used, dilute by 15 - 100 times.
   Prepared sample is stable at - 80 ˚C for a few months before dilution.
   The diluted sample is unstable and the activity should be measured on the same day.

2) Dephosphorylation
   1. Add 50 μl of serial dilutions of PTP control or samples into each well with micro pipette in duplicate.
   2. Incubate it at 37 ˚C for 15-60 min.
      Addition of samples starts dephosphorylation of tyrosine.

3) Blocking
   1. Remove sample solution and wash the wells 4 times with Washing buffer; between each washing step, empty out the remaining solution by vigorously tapping the microtiter plate onto paper towel, especially after the last washing.
   2. Add 100 μl of Blocking solution into each well and incubate it for 30 min. at 37 ˚C.

4) Addition of antibody
   1. Discard Blocking solution and empty out Washing buffer fully on a paper towel. (On this step washing procedure is not needed.)
   2. Add 50 μl of Anti-phosphotyrosine (PY20) - HRP solution into each well and incubate it for 30 min. at 37 ˚C.

5) Substrate reaction
   1. Discard the antibody solution and wash each well 4 times with Washing buffer.
   2. Empty out washing buffer fully on a paper towel.
   3. Add 100 μl of HRP substrate solution (TMBZ) into each well.
   4. Incubate it at 37 ˚C.
      (Fifteen min. is recommended as standard reaction time.)

6) Stop color development
   Add 100 μl of stop solution (1 N H_2SO_4) into each well in the same order as HRP substrate solution is added.

7) Measurement
   1. Measure the absorbance at 450 nm with a plate reader.
   2. Construct a standard curve by plotting the absorbance on the y-axis against the activity of PTP control on the x-axis.
   3. Calculate PTP activity of sample on the basis of the prepared standard curve.

   Note: When the PTP acitivity is maximum, color development disappears completely. The resulting color intensity is stable for 1 hr. after addition of stop solution at room temperature in a light room.
   In case that the absorbance is more than 3.5, brown precipitate may generate and the measured value may be lower than the actual value.

11. Q & A

Q1: What kind of principle is applied to this kit ?

A1: A conventional measurement method of PTP activity uses phosphate molybdate to quantitate phosphoric acid released during dephosphorylation reacion. This method is laborious since phosphate acid in samples should be removed by desalting before an assay.
In Universal Tyrosine Phosphatase Assay Kit, samples are added into a 96-well microtiter plate immobilized with synthesized peptide substrates that include phosphorylated tyrosine residues, and dephosphorylation is performed. PTP activity is measured specifically by using anti-phophorylated tyrosine antibody labeled with Horse Radish Peroxidase (HRP).

Q2: Is there a possibility to measure the activities of other Phosphatases than PTP ?

A2: There are Ser / Thr Phosphatases other than PTP as Protein Phosphatase. But antibody that is employed for detection in this kit does not react with phosphorylated Ser / Thr. And Poly (Glu-Tyr) is employed as synthesized substrate, so the activities of other protein kinases should not be measured. In addition, non-specific dephosphoryation by the other phosphatases can be prevented by the inhibitor supplied in Lysis buffer and Reacting solution.

Q3: What kind of samples can be available for the assay with this kit ?

A3: Universal Tyrosine Phosphatase Assay Kit is basically designed for the measurement of PTP activities of cultured cells or hematocyte components that are isolated from blood. A few data have been obtained so far using the sample derived from tissue. When using tissue as a sample, prepare the sample for the assay as follows; the tissue is mixed with Lysis buffer, then this mixture is homogenized and spin. The supernatant is used for the assay.

Q4: The specificity of synthesized peptide substrates that are used in Universal Tyrosine Phosphatase Assay Kit

A4: The employed synthesized peptide substrate is Poly (Glu-Tyr) (4:1, 20-50 kDa) which is specific to a wide range of PTP and has been already phosphorylated by a natural kinase. Synthesized substrates specific to each enzyme are employed in other companies' kits, which make the operation laborious, because various kinds of substrates specific to the target PTKs should be prepared respecrively to measure a wide range of PTP. The sensitivity of these kits might not be enough either. Takara's Universal Tyrosine Phosphatase Assay Kit allows highly sensitive assay of the activity of a target PTP with a single substrate, only by combining with a PTP specific antibodies.

Q5: How is the activity defined ?

A5: One unit (U) of PTP is defined as the amount needed to hydrolate 1 nmol of p-NPP (p-nitrophenyl phosphate) in 1 min.

Q6: What is the PTP control ?

A6: PTP control is lyophilized powder prepared from tyrosine phosphatase (CD45) (Calbiochem-Novabiochem Co.) which is a common leucocyte antigen. The unit is defined by each lot.

Q7: The sensitivity and the assay range of this kit

A7: The sensitivity is 0.125×10^{-5} units / μl, and the measurement range is from 0.125×10^{-5} to 90×10^{-5} units / μl.

Q8: How many cells are needed to measure PTP activity with this kit?

A8: The number of needed cell depends on a kind of cell and how they are simulated. For example, regarding K562 cells, more than 500 cells allows detection.

Q9: Can PTP be extracted only by suspending cells with Lysis buffer included in this kit ?

A9: Yes.
The assay results were compared by using the following two samples.
• Sample that was extracted with Takara's Lysis buffer.
• Sample that was prepared with 8 times of 5-sec. sonication.
The result showed that the same level of assay result can be obtianed with these two samples. Therefore, only suspending cells in Lysis buffer is enough to recover most PTP for activity measurement.

Q10: Is the supplied microplate immobilized with the substrate stable at 4°C?

A10: Yes. The thermostability of the plate was confirmed by the warming test at 40°C. In addition, the state of phosphate radical is regularly examined by detection with anti-phosphotyrosine antibody. The plate is stable if it is stored at 4°C in a dry condition. The variation coefficient of phosphate radical number in the substrate is less than 5%.

Q11: Is there any possibility that dephosphorylation could progress during blocking step?

A11: No. The washing procedure with Tween-PBS after reaction can prevent dephosphorylation. Also the blocking soltuion contains phosphate acid to inhibit phosphatase activity.

Q12: What should be kept in mind in measuring tyrosin phosphatase activity in cultured cells or organ tissues?

A12: Since the tyrosine phosphatase in cultured cells or organ tissues is considerably high, serial dilution of samples needs to be prepared when preparing samples by suspending cells or tissues in the Lysis buffer.

Q13: Protocol to assay PTP activity using a specific antibody

A13: Cell extracts can include tyrosine phosphatases which non-specifically bind to Proein A-agarose or Protein G-agarose. Therefore, it is required to perform a complete pre-treatment and to evaluate the background by processing the blank antibody with the same procedure. The following describes the protocol to assay a specific PTP activity through immunoprecipitation of PTP using specific antibodies.

1) Recover K562 cells from the two plates (ϕ 9 cm) of the 100% cofluent culture (2×10^7 cells) into centrifuge tubes. Spin at 1,500 rpm and collect the cells as a pellet. PTP fraction is prepared.

2) Add 400 μl of Protein A-streamline (Amersham Pharmacia Biotech) pre-washed with 5 mM PIPES buffer (pH7.0), and shake completely. Spin at 14,500 rpm (1,000 x g) to precipitate insoluble substances from the cell and Protein A slurry. Use the spernatant as a sample.

3) Dispense the supernatant equally into three tubes (the final volume per tube is around 700 μl) . Into the tubes, add the antibodies which are specific to the target PTP, 10 μg of SH-PTP-1 rabbit IgG (Santa Cruz Biotechnology, Inc., Cat.#sc-287), 10 μg of SH-PTP-2 rabbit IgG (Santa Cruz Biotechnology, Inc., Cat.#sc-280), and 10 μg of blank rabbit IgG antibody (in-house preparation at Takara). Incubate at room temperature for 30 min.

4) After incubation, add 100 μl slurry of Protein A-streamline washed with 50 mM PIPES buffer (pH7.0), and leave for 15 min.

5) Spin at 10,000 rpm to recover the slurry containing the complex of the antibody and PTP. Wash the slurry in each microtube with 1 ml of 50 mM PIPES buffer (pH7.0) containing 0.5% Tween.

6) Suspend the each slurry in 200 μl of Phosphate reacting solution including 2-mercaptoethanol. Add this suspension onto four wells in 50 μl/well and perform dephosphorylation. The PTP activity in each specific antibody is calculated by deducting the value of PTP activity in the blank antibody.

Cell	Specific antibody	Specific PTP activity (units/μl)
K562 cells (human leukocyte cell) $2 \times 10^7 \times 1/3$	SH-PTP-1 rabbit IgG 10 μg (Santa Cruz Biotechnology, Inc., Cat.#sc-287)	21.0×10^{-5}
	SH-PTP-2 rabbit IgG 10 μg (Santa Cruz Biotechnology, Inc., Cat.#sc-280)	11.7×10^{-5}

11. Note

For research use only. Not for use in diagnostic or therapeutic procedures.