Cat. # MK410

For Research Use

TaKaRa

Universal Tyrosine Kinase Assay Kit

Product Manual

v201608Da

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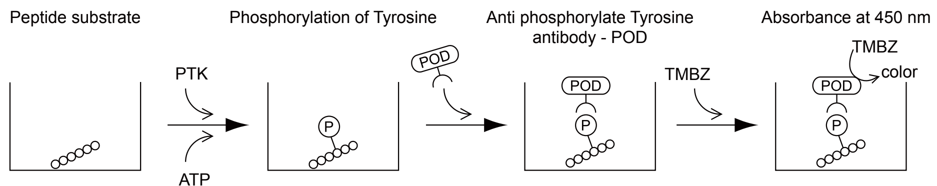
I. Description

Protein Tyrosine Kinase (PTK) is an important enzyme and concerned in the signal transduction pathway which controls cell proliferation, differentiation and carcinogenesis. PTK is classified into two groups; 1) membrane receptor PTK such as Insulin receptor, EGF receptor and 2) non-receptor linked PTK such as Src, FAK. The receptor PTKs consist of three domains, the extracellular domain, the membrane intrinsic domain and the intracellular domain. The extracellular and membrane intrinsic ones bind to ligands such as hormones and growth factors, the intracellular one carries the active site of PTK. The receptor PTKs transduce signals directly into the cell by phosphorylation of tyrosine when ligands bind to its domain. The non-receptor linked PTKs do not possess the membrane intrinsic domain and are divided into three types by the part in which this enzyme exists such as the surface of membrane, cytoplasm and nucleus. This type functions variously, some work as transducers, some response cell-cell contact.

The activity of PTK has been measured by a very complicated protocol which employs RI-labeled chemical (^{32}P-ATP). Universal Tyrosine Kinase Assay Kit enables to measure the activity of PTK in a wide range quickly and specifically with non-RI chemicals. This kit is useful for analysis of the regulation of PTK activity by using the recombinant PTK and for the *in vitro* screening of PTK inhibitors.

II. Principle



III. Features

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

IV. Components (For 96 tests)

1. PTK substrate immobilized microplate 8 wells x 12 — 1 plate
2. Kinase reacting solution — 11 ml
3. 40 mM ATP-2Na (lyophilized, for 0.55 ml / H_2O) — 2
4. Extraction buffer — 11 ml
5. PTK control (lyophilized) — 1
6. Anti-phosphotyrosine (PY20) - Horse Radish Peroxidase (HRP) (lyophilized, for 5.5 ml / H_2O) — 1
7. Blocking solution — 11 ml
8. HRP substrate solution (TMBZ) — 12 ml

V. Storage 4℃

VI. Materials Required but not Provided

- Stop solution (11 ml for a kit): The Stop Solution in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) is available. 1 N H_2SO_4 can be also used.
- Washing buffer: PBS including 0.05% (v/v) Tween 20
- Incubator (37℃)
- Microtiter plate reader with a filter for the use of a wavelength nearby 450 nm
- Multichannel pipettor (10 - 500 μl)
- 2-mercaptoethanol
- Distiled water

VII. Protocol

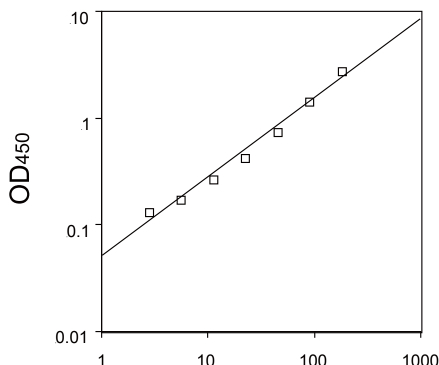
VII-1. Performance

(1) Specificity

Universal Tyrosine Kinase Assay Kit monitors the transfer of γ-phosphate residue from ATP to peptide substrates immobilized on plate.
As employed anti-phosphotyrosine antibody does not cross-react with phosphorylated serine or threonine, this kit enables to measure the PTK activity specifically without the interference with serine / threonine kinases.

[a] Correlation between PTK activity and absorbance in use of rc-Src as sample

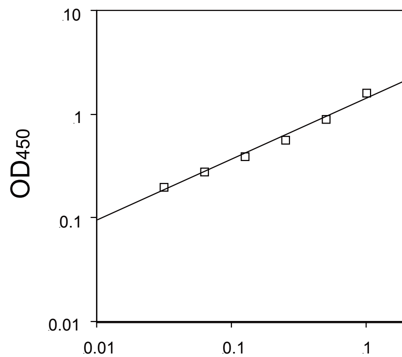
PTK activity ($\times 10^{-5}$ units/μl)	180	90	45	22.5	11.25	5.625	2.813	0
Absorbance (A_{450})	2.753	1.448	0.741	0.422	0.265	0.172	0.133	0.075



r = 0.991
logy =1.30 + 0.741 logx

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

PDilution rate	1/1	1/2	1/4	1/8	1/16	1/32
Absorbance (A_{450})	1.625	0.899	0.565	0.394	0.280	0.198



r = 0.991
logy = 0.156 + 0.593 logx

(2) Sensitivity (as Src kinase)
≧ 2.16×10^{-5} units/μl of sample (which is equal to 32 fmol/well)

(3) Definition of activity
The activity of the enzyme is based on the activity of recombinant c-Src. One unit (U) of enzyme is defined as the amount needed to incorporate 1 pmol of phosphate into the substrate (KVEKIGEGTYGVVYK: 6 - 20 residue of $p34^{cdc2}$) for 1 minute.
The activity of PTK control in this kit is shown as the activity of c-Src.

(4) Assay time
Assay time depends on the time of enzyme reaction. It usually takes 1.5 - 3 hours to perform the whole measurement.

(5) Assay parameters
Since the PTK content in the sample can vary over a wide range depending on its source, the concentration of enzyme and the reaction time should be adapted according to the purpose of experiment.

(6) Substrate
Synthesized peptide (poly (Glu-Tyr)) that possesses a wide spectrum of specificity is immobilized on the well.

(7) Measuring range and phosphopeptide standard curve
The activity of PTK is determined by comparing its absorbance with that of PTK standard supplied in this kit. The measuring range of this kit is from 32 fmol/well to 2 pmol/well (from 2.16×10^{-5} units/sample μl to 135×10^{-5} units/sample μl) .

＊ **The concentration of the standard solution (U/μl) is different in each lot. The standard curve for calculation needs to be established in each assay.**

(8) Interference with phosphoproteins from the sample

Generally, prepared PTK sample contains phosphoproteins and wild type of PTK substrates which are phosphorylated during reaction. But after phosphorylation, the wild type of PTK substrates are removed during the washing step and they do not interfer the result of measurement. If the non-specific phosphoprotein binding to wells is concerned, it is recommended to perform control experiment by using Nunc plate (Nunc, Code. 468607) as a control plate in the same procedure and the background of measurement should be checked.

(9) Interference with endogenous Protein Tyrosine Phosphatase (PTP) from the sample

Most prepared PTK sample contains endogenous PTP. Extraction buffer and Kinase reacting solution include 1 mM of sodium-orthovanadate and 50 mM of NaF for avoiding decomposition of phosphorylated synthesized peptide substrate by PTP.

VII-2. Preparation of solutions

(1) PTK substrate immobilized microplate, Extraction buffer and Blocking solution can be used without any preparations.

(2) Kinase reacting solution

2-mercaptoethanol should be added to Kinase reacting solution to a final concentration of 10 mM just before use.* As 2-mercaptoethanol is unstable, this solution should be prepared freshly in a required amount in each use.

＊: For example, taking the concentration of commercial 2-mercaptoethanol (99%) as 14.4 M, add 2-mercaptoethanol by 1,440 times dilution to the supplied Kinase reacting solution.

(3) 40 mM ATP-2Na

One vial of lyophilized ATP-2Na is dissolved in 0.55 ml of distilled water (for 48 wells/vial).

Note : The prepared ATP-2Na solution must be used up within that day when it isprepared. It cannot be stored.

(4) PTK control

One vial of lyophilized PTK control is dissolved in 100 μl of distilled water. (In case that all volume of solution is not used in one time, the solution can be stored in aliquots at -20℃ for 2 weeks.) Then add 400 μl of Kinase reacting solution including 2-mercaptoethanol (prepared at step (2)) to 100 μl of PTK 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 Kinase reacting solutions prepared at (2). The Kinase reacting solution prepared at step (2) can be used as 0 M standard of PTK. Since the activity (unit/μl) of PTK control could be different in each lot, the standard curve should be taken in each measurement.

(5) 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℃ for 2 weeks.) Do not repeat freeze-thaw cycles.

(6) 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.

VII-3. Sample preparation (extraction of total proteins)

Samples prepared by the following procedure are stable at -80℃ for a few days.

Note : When many samples are handled, Extraction buffer may not be sufficient. In this case, adjust the scale of culture and extraction depending on used samples.

(1) Suspension cells

- Wash 1 - 5 x 10^6 cells with PBS and spin at room temperature for 5 min at 300 X *g*, then recover the cells as pellet.
- Add 1 ml of Extraction buffer, then suspend the pellet by vortex gently.
- Spin at 4℃ for 10 min at 10,000 X *g*, recover the supernatant as sample.

(2) Adherent cells

- Remove the medium from the dish (1 - 5 x 10^6 cells, φ 9 cm dish), and wash the cells with PBS.
- Add 1 ml of Extraction buffer to the dish and recover the cells cautiously from the dish with Cell Scraper. Then spin at 4℃ for 10 min at 10,000 X *g*, and recover the supernatant as sample.

(3) Recovery of cells cultured in 96 wells plate

- Add 50 - 100 μl of Extraction buffer to each well.
- After taking off the cells by pipetting, then spin at 4℃ for 10 min at 10,000 X *g*, and recover the supernatant as sample.

(4) Specific PTK activity assay by immunoprecipitation

1. Add 50 μl Protein A-agarose or Protein G-agarose into 0.5 - 1.0 ml of total protein extracts that are prepared with the above mentioned procedure (1), (2), or (3).
2. Remove non-specific binding proteins to these agaroses by shaking gently at 4℃ for 20 min.
3. Spin at 4℃ for 10 min at 10,000 X *g*, and transfer its supernatant into a fresh sample tube.
4. Add 5 - 30 μg of antibody that is specific to the target PTK into the supernatant and incubate it for 1 hours at room temperature.
5. Add 30 μl Protein A-agarose or Protein G-agarose into the supernatant and incubate it for 20 min at room temperature.
6. Spin at 4℃ for 1 min at 10,000 X *g*, and remove its supernatant.
7. Add 1 ml of PBS to the precipitate, re-suspend by shaking at 4℃ for 10 min. Then spin at 4℃ for 1 min at 10,000 X *g*, and remove its supernatant.
8. Repeat washing procedure with PBS (step 7) three times.
9. Add 150 μl of Kinase reacting solution including 2-mercaptoethanol to the precipitate and re-suspend. This suspension (including gel) is used as a sample for the measurement of PTK activity at 50 μl/well.

VII-4. Assay procedure

(1) Dilution

Dilute the prepared sample (as mentioned in **VII-3. Sample preparation** (1), (2), (3)) with Kinase Reacting Buffer by more than 5 times.

* When extracts of cultured cells are used, dilute by 15 - 100 times.
  Prepared sample is stable at - 80℃ for few days before dilution.
  The diluted sample is unstable and the activity should be measured on the same day.

(2) Phosphorylation

- Add 40 μl of serial dilutions of PTK control or samples into each well with micro pipette in duplicate.
- Add 10 μl of 40 mM ATP-2Na solution into each well and mix well.
- Incubate it for 30 min at 37℃
  Addition of ATP solution starts phosphorylation of tyrosine. If reaction time is over 45 min, non-specific binding of PTK to a plate may occur and may cause high background.

(3) Blocking

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

(4) Addition of antibody

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

(5) Substrate reaction

- Discard the antibody solution and wash each well 4 times with Washing buffer.
- Empty out washing buffer fully on a paper towel.
- Add 100 μl of HRP substrate solution (TMBZ) into each well.
- Incubate it at 37℃. (15 min is recommended as standard reaction time.)

(6) Stop coloring development

- Add 100 μl of stop solution into each well in the same order as HRP substrate solution.

(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 PTK control on the x-axis.
3. Calculate PTK activity of sample on the basis of the prepared standard curve.

Note : The resulting color intensity is stable for 1 hour 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.

VIII. Application

VIII-1. Change in PTK activity with embryo genesis

PTK activities in Mouse embryo 11 days and 15 days of pregnancy (Usually they are born in 20 days of pregnancy.) were measured with Universal Tyrosine Kinase Assay Kit.

Mouse embryo	PTK activity (units/mg protein)
11 days of pregnancy	0.82
15 days of pregnancy	25.6

VIII-2. Influence of INF - γ on U937 cells

PTK activities in U937 cells with treatment of IFM-γ (1,000 U/ml) for 48 hours was compared with ones without treatment.

Treatment	PTK activity (x 10^{-5} units/10^6 cells)
No treatment	1.53
With IFN- γ treatment	2.39

VIII-3. Specific measurements of PTK activities of EGF-R and FAK by combining immunoprecipitation

(1) A431 cells (10^7 cells/ 9 cm dish) were cultured for 48 hours in RPMI - 10% FCS medium including 10 ng/ml of Mouse Epidermal Growth Factor (EGF). Wash cells with PBS once, then add 1 ml of Extraction buffer and collect the cells from the dish by using the Cell Scraper. Recover cell suspension in a fresh 1.5 ml tube.

(2) Add 50 μl Protein A-agarose to the tube, and incubate it for 20 min at room temperature. Spin it at 4℃ for 5 min at 10,000 X *g* and collect its supernatant.

(3) Add the specific antibody into the supernatant and mix well.
(Types and volumes are shown in below.)

(4) Add 30 μl Protein A-agarose or Protein G-agarose into the supernatant and leave it for 20 min at room temperature.

(5) Spin it at 4℃ for 5 min at 10,000 X *g* and remove its supernatant. Add 1 ml of PBS to the precipitate, and mix well by shaking at 4℃ for 10 min Then spin at 4℃ for 1 min at 10,000 X *g* and remove its supernatant.
Repeat this washing procedure with PBS three times.

(6) Add 150 μl of Kinase reacting solution including 2-mercaptoethanol to the precipitate. Take 50 μl of this solution that includes agarose gel into each well, and add 10 μl of 40 mM ATP-2Na solution into each well and mix. Incubate it for 30 min at 37℃ for phosphorylation.

(7) Perform specific detection of various PTK by following the procedure described at **VII-4. Assay procedure**.

Cell	Treatment	Antibody (Qty. /tube)	PTK activity (units/ 10^6 cells)
A431	EGF	Anti-EGFR*1 (24 μg)	763
A431	EGF	Anti-cSrc*2 (10 μl)	100
A431	EGF	Normal mouse Ig (11 μl)	Not detected
WiDr	No treatment	Anti-FAK*3 (10 μg)	79
WiDr	No treatment	Normal rabbit Ig (10 μl)	Not detected

∗ 1 : Mouse monoclonal antibody (Cat. #M059; discontinued)
∗ 2 : Rabbit antiserum (Chemicon, Code. AB1420)
∗ 3 : Rabbit antibody (Cat. #M135; discontinued)

IX. Q & A

Q1 : What kind of principle is applied to this kit ?
A1 : Conventional measurement methods of PTK activity need the use of RI (^{32}P-ATP) and measure the amount of ^{32}P incorporated into synthesized peptide substrates after separating it with electrophoresis or precipitation by trichloroaceticacid or adsorption on paper disk.
In Universal Tyrosine Kinase Assay Kit, ATP and sample that includes PTK are added into 96 well microtiter plate immobilized with synthesized peptide substrates, and after phosphorylation, PTK activity is measured specifically by detecting with the anti-phophorylated Tyrosine antibody labeled with Horse Radish Peroxidase (HRP).

Q2 : Is there a possibility to measure the activities of other Kinases than PTK ?
A2 : There are Ser / Thr kinases other than PTK as Protein kinase.
But antibody that is employed for detection in this kit does not react with phosphorylated Ser / Thr. And the employed synthesized substrate is Poly (Glu-Tyr), so the activities of other protein kinases should not be measured.

Q3 : Is there a possibility that Protein Tyrosine Phosphatase (PTP) could interfere the measurement of PTK activity ?
A3 : PTP and Protein Phosphatase (PP) are included in the cell extraction. For suppressing the interference with these enzymes activities, Sodium Vanadate and NaF (PTP and PP inhibitors) have been added into Extraction buffer and Kinase reacting solution.

Q4 : Is there a possibility that phosphorylated proteins that derive from the extracted sample could interrupt the measurement of PTK activity ?
A4 : In Universal Tyrosine Kinase Assay Kit, the synthesized substrates have been immobilized in advance, so phosphorylated proteins that derive from sample are washed away during the washing procedures. In this point this kit allows more accurate assay than the kits that employ the biotin-avidin method to trap synthesized peptides. But in case that protein concentration of sample is very high, the phosphorylated proteins can be bound to plate non-specifically. In this case, it is recommended that the sample should be diluted or that the non-specific binding should be checked by using a commercial non-treated 96 wells micrtiter plate as a blank.

Q5 : What kind of samples can be available for the assay with this kit ?

A5 : Universal Tyrosine Kinase Assay Kit is basically designed for the measurement of PTK 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, the following procedure is recommended; the tissue is mixed with Extraction buffer, then this mixture is homogenized and spin. The supernatant is applied to the assay.

Q6 : The specificity of synthesized peptide substrates that are used in Universal Tyrosine Kinase Assay Kit

A6 : The employed peptide substrate is Poly (Glu-Tyr) (4 : 1, 20 - 50 kDa). This substrate is used in the measurement of the activities of various PTKs such as FAK, ZAP-70, c-Src, and EGF-R. 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 for measurement. The sensitivity of these kits are not enough either. Universal Tyrosine Kinase Assay Kit allows highly sensitive assay of the activity of a target PTK with a single substrate, without RI, only by combining with a PTK specific antibodies.

Q7 : How is the activity defined ?

A7 : The activity of enzyme is based on the activity of recombinant c-Src. One unit (U) of the enzyme is defined as the amount needed to incorporate 1 pmol of phosphate into the substrate (KVEKIGEGTYGVVYK: 6 - 20 residue of $p34^{cdc2}$) in 1 min

The activity of PTK control in this kit is shown as the activity of c-Src.

Q8 : What is the PTK control ?

A8 : PTK control is a crude extract of Kinase derived from cultured cell and supplied in lyophilized form. It is controled by comparing the unit with that of recombinant c-Src.

Q9 : The sensitivity and the assay range of this kit

A9 : The detection sensitivity is 2.16×10^{-5} units / μl and the measurement range is from 2.16×10^{-5} -135×10^{-5} units/μl (86.4×10^{-5} - $5,400 \times 10^{-5}$ units/well).

The comparative data between Takara's kit and other company's kit is shown in below.

Sample : A431 cell or WiDr cells (1×10^7 cells)

Protocol : The samples were extracted from the cells with 1 ml Extraction buffer.

These samples are diluted three times, then PTK activities of each sample are measured with both kits.

Cell	Absorbance (A_{450})	
	Takara bio kit	Other companies kit
A431	2.207	0.748
WiDr	1.015	0.004

Takara's kit gave higher color development.

Q10 : How many cells are needed to measure PTK activity ?
A10 : The number of needed cell depends on a kind of cell and stimulation.
For example, regarding U937 cells, P3U1 cells, and Neuro2A cells that are cultured as usual, 500 - 1,000 cells are available for detection.

Q11 : Can PTK be extracted only by suspending cells with Extraction buffer included in this kit ?
A11 : Yes.
The assay results were compared by using the following samples.
(1) Sample that was extracted with other companies extraction solution (50 mM of Tris-HCl, pH 7.5 including EDTA, 2-ME, PMSF, and Benzamidine) and sonication.
(2) Sample that was extracted with Takara's Extraction buffer.
(3) Sample that was prepared with 8 times of 5 sec sonication.

Extraction procedure	PTK activity (A_{450})
(1)	0.217
(2)	2.316
(3)	2.324

This result shows that extraction buffer of Takara's kit is more efficient than those in other comanies' kit and that only suspending cells in Extraction buffer is available to measure activity of PTK.

X. Trouble Shooting

Situation	Treatment
High background	Shorten Kinase reaction time to about 15 min
Low absorbance (Weak color development)	Prolong Kinase reaction time to about 60 min
Extremely high absorbance (Strong color development)	PTK activity of sample may be high. It is recommended to determine a proper dilution rate empirically by preparing serial dilutions of the sample, such as 5X, 15X, 45X, 135X, and 405X.

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