3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone (800) 325-5832 (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Tyrosine Kinase Assay Kit

Product Code **CS0730**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Protein tyrosine kinases (PTKs) consist of a large and diverse multigene family. They contain highly conserved catalytic domains with unique subdomain motifs that clearly identify members as tyrosine kinases and differentiate them from protein serine/threonine and dual-specificity kinases. Tyrosine kinases are involved in the regulation of multicellular aspects of the organism. Cell to cell signals concerning growth, differentiation, adhesion, motility, and death are frequently transmitted through tyrosine kinases. Tyrosine kinases have been demonstrated to play significant roles in the development of many disease states, including diabetes and cancer.

The Tyrosine Kinase Assay Kit offers an easy, convenient, and sensitive way to assay tyrosine kinase activity. The kit provides a means to examine the biological role of a particular tyrosine kinase signaling cascade and to explore new tyrosine kinase signaling stimuli, inhibitors, activators, and proteins that are conceivably regulated directly or indirectly by a kinase.

The assay is based on immunoprecipitation of the tyrosine kinase of interest using a specific anti-tyrosine kinase protein antibody. Since the kit is designed to assay any potential tyrosine kinase, the scientist should use a protein specific immuno-precipitating antibody. The immunoprecipitated kinase is incubated with γ–^{32}P-ATP and the ^{32}P incorporation into the substrate is measured. The kit provides a peroxidase conjugated anti-phosphotyrosine antibody, which enables substrate analysis by dot blot assay. This assay serves as an alternative, short, and easy non-radioactive detection method.

Epidermal Growth Factor Receptor has tyrosine kinase activity and is supplied as a positive enzyme control.

Reagents

The kit is sufficient for 50 assays.

- Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate — 1 vial
  Clone PY-66
  Product Code A 5964
- Assay Buffer For Tyrosine Kinase Activity — 1 ml
  Product Code A 7354
- Wash Buffer 10X — 8 ml
  Product Code W 3264
- EZview™ Red Protein A Affinity Gel — 1 ml
  Product Code P 6486.
- Epidermal Growth Factor Receptor — 500 units
  Product Code E 2645
- P81 Cellulose Phosphate — 10 each
  Product Code P 5497

Reagents and Equipment Required but Not Provided

- Antibody for the immunoprecipitation of the tyrosine kinase of interest
- Microcentrifuge e.g. Eppendorf® microcentrifuge 5415 Series (Product Code Z60,406-2) or equivalent.
- Dulbecco's Phosphate Buffered Saline (PBS, Product Code D 8537).
- Phosphoric acid 85% (Product Code 79617)
- Ethanol (Product Code 27,074-1)
- Acetone (Product Code 17,912-4)
- γ–^{32}P-ATP – ~3,000 Ci/mmole, 10 mCi/ml
- 10% (w/v) Glycerol Solution. Prepared from glycerol (Product Code G 7757)
- Microcentrifuge tubes (Product Code T 6649 or T 9661, or equivalent)

For Dot Blot Analysis
- 0.5 M EDTA Solution (Product Code E 7889 or 03690, or equivalent)
- PBS containing 0.2% TWEEN® 20 (PBST, Product Code P 3563)
- Equipment and materials required for immunoblot analysis procedure, such as the ProteoQwest™ Chemiluminescent Western Blotting Kit, CPS Substrate (Product Code PQ0201) or equivalent.
- Nitrocellulose membrane (Product Code N 8267 or equivalent) for protein transfer.

Precautions and Disclaimer
The kit is for R&D use only, not for drug, household or other uses. Please refer to the Material Safety Data Sheet (MSDS) for information regarding hazards and safe handling practices.

Preparation Instructions
Use ultrapure water (17 MΩ·cm or equivalent) for preparation of reagents and throughout the procedure.

Epidermal Growth Factor Receptor (EGFR) Solution – Reconstitute the Epidermal Growth Factor Receptor by adding 0.1 ml of 10% (w/v) Glycerol Solution to the EGFR bottle. Mix well by pipetting. Aliquot the EGFR Solution and store at –70 °C.

Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate - Reconstitute the Anti-Phosphotyrosine–Peroxidase Conjugate by addition of 100 µl of ultrapure water to the vial. Mix well by pipetting. Aliquot the antibody solution and store at –20 °C.

1X Wash Buffer – Prepare a 10-fold dilution of a portion of the Wash Buffer 10X with ultrapure water. For each sample, dilute 160 µl of Wash Buffer 10X. For multiple samples, calculate accordingly.

0.5% Phosphoric Acid Solution – Add 11.8 ml of ~85% phosphoric acid to 2 liters of ultrapure water and mix well.

Storage/Stability
The kit is shipped on dry ice and storage at –20 °C is recommended. Upon reconstitution, the EGFR (Product Code E 2645) Solution should be stored in working aliquots at –70 °C.

Procedure
Principle of the assay
The tyrosine kinase of interest is immunoprecipitated from the sample with an antibody to the kinase and the EZview Red Protein A Affinity Gel. The immunoprecipitated kinase is incubated with the substrate [poly(Glu, Tyr) (4:1), a component of the assay buffer] in the presence of γ–^{32}P-ATP. Subsequently, the ^{32}P incorporation is measured.

An alternative detection method of the level of phosphorylated substrate that does not require radioactively labeled ATP, is a dot blot analysis using the Anti-Phosphotyrosine–Peroxidase Conjugate.

General comments
- The antibody for the immunoprecipitation of the tyrosine kinase of interest is not provided with the kit.
- All steps of the Immunoprecipitation procedure should be performed on ice, unless otherwise stated.
- For each sample, perform a negative control reaction with no immunoprecipitation antibody in order to detect any non-specific protein binding to the agarose beads.
- A positive enzyme control reaction for the detection of the phosphorylated substrate can be performed using the EGFR provided with the kit (see Table 1). Epidermal Growth Factor Receptor has tyrosine kinase activity.

Table 1.
Reaction Scheme for Radioactive Detection

	Kinase Immunoprecipitation			Substrate Phosphorylation		
	Cell lysate	**Anti-tyrosine kinase (of target enzyme)**	**EZview Protein A**	**Assay Buffer**	**EGFR Solution**	**Immuno-precipitate**
Negative Control	250-1000 µl	–	30 µl	15 µl	–	–
Positive Control for substrate phosphorylation	–	–	–	15 µl	2 µl + 15 µl of 10% Glycerol	–
Reaction	250-1000 µl	4 µl*	30 µl	15 µl	–	+

* Suggested volume of an antibody

The procedure described is for one assay. Adjust the procedure according to your experiment. It is highly recommended to perform assays in duplicates.

A. Kinase Immunoprecipitation

1. Transfer up to 1 ml of cell lysate/sample (200-500 µg of protein) into a microcentrifuge tube. The optimal cell lysate volume is 250 µl.
2. Add the appropriate amount of anti-tyrosine kinase antibody (usually 2-4 µl). Mix gently and set the tube on ice while equilibrating the EZview Red Protein A Affinity Gel beads.
3. Equilibration of the EZview Red Protein A Affinity Gel beads:
   a. Carefully mix the gel beads until uniformly suspended. Aliquot 30 µl of the 50% slurry into a clean 1.5 ml microcentrifuge tube. For dispensing of the beads, use a wide orifice pipette tip or cut off about 1 mm a regular tip to enlarge the opening and allow unrestricted flow of the bead suspension.
   b. Wash/equilibrate the beads with ice cold PBS. Add 750 µl of PBS to the tube, vortex, and centrifuge in a microcentrifuge for 30 seconds at ~8,000 x *g*. Carefully remove the supernatant with a micropipette or carefully aspirate the supernatant.
   c. Repeat the wash/equilibrate step (step 3b) once. After removing the supernatant, set the washed bead pellet on ice.

   Note: For assays of multiple samples, it is possible to equilibrate the total volume of resin required for all the samples at once, according to this procedure (Kinase Immunoprecipitation, step 3). At the last wash, dispense the resin into clean 1.5 ml microcentrifuge tubes according to the number of assays.
4. Briefly centrifuge the tube containing the sample and the antibody (Kinase Immunoprecipitation, step 2) for several seconds at ~8,000 x *g* and 4 °C to collect all of the liquid to the bottom part of the microcentrifuge tube. Carefully transfer all of the liquid into the tube containing the washed EZview Red Protein A Affinity Gel beads from step 3c.
5. Vortex briefly and incubate with thorough, gentle rocking for 4 hours at 2–8 °C to allow the antibody-antigen complexes to bind the Protein A on the EZview Red Protein A Affinity Gel beads.
   Note: 2 to 4 hours are usually enough for immmunoprecipitation; however, in some cases overnight incubation would enhance the detected signal.
6. Centrifuge the tube in a microcentrifuge for 30 seconds at ~8,000 x *g* and 4 °C. Set the tube on ice.
7. Aspirate the supernatant carefully or remove with a micropipette and set the tube containing the bead pellet on ice.
   Note: Because of the enhanced visibility of the red affinity resin beads, it is easy to see if beads were accidentally removed during the washing steps. If this happens, transfer the washed supernatant back into the tube and repeat the centrifugation step to pellet the resin once again.

8. Wash the bead pellet by adding 400 µl of ice cold 1X Wash Buffer. Vortex briefly and incubate with a thorough and gentle rocking at 2–8 °C for 1 minute.
9. Centrifuge the tube in a microcentrifuge for 30 seconds at ~8,000 x *g* and 4 °C. Aspirate the supernatant carefully or remove with a micropipette and set the tube with the bead pellet on ice.
10. Perform 2-3 additional washes (steps 8-9).

B. Substrate Phosphorylation and Detection
Radioactive Detection

1. Add 1 µl of γ–^{32}P-ATP with a specific activity of 10 mCi/ml to 100 µl of Assay Buffer. This volume is sufficient for 6 assays.
2. Suspend the immunoprecipitated pellet by gentle pipetting in 15 µl of Assay Buffer containing the radioactive ATP.
3. Perform a positive control assay. Add 15 µl of Assay Buffer containing the radioactive ATP to the positive control tube containing 15 µl of 10% Glycerol Solution and 2 µl of EGFR Solution.
4. Perform a negative control assay. Add 15 µl of Assay Buffer containing the radioactive ATP to a tube containing 15 µl of 10% Glycerol Solution.
5. Incubate the tubes for 30 minutes at 30 °C. From this step on, work at room temperature.
6. Terminate the reactions by spotting 10 µl of the liquid phase of the assay mixture on 2 cm x 2 cm phosphocellulose P81 squares.
7. Soak the phosphocellulose squares in 0.5% Phosphoric Acid Solution.
8. Wash the phosphocellulose squares 4 times with 0.5% Phosphoric Acid Solution. For each wash, agitate gently for 5-6 minutes.
9. Wash once with ethanol for 1 minute.
10. Wash once with acetone for 1 minute.
11. Dry the phosphocellulose squares at room temperature or under a heat lamp and count the incorporated radioactivity using Cerenkov mode (i.e. count the emission without scintillation liquid, using tritium channel).

Dot Blot Analysis

1. Add 15 µl of Assay Buffer to the bead pellet (Kinase Immunoprecipitation, step 10). Suspend the pellet by gentle pipetting.
2. Incubate for 30 minutes at 30 °C.
3. Terminate the reaction with 1 µl of 0.5 M EDTA Solution. Mix well and spin down for 30 seconds. Save the supernatant.
4. Cut nitrocellulose membrane into oblong strips of 5 cm x 2 cm (total of 10 cm^2). This is sufficient for 10 samples. Each additional sample (spot) requires an additional membrane area of 1 cm^2.
5. Spot 1 µl of the reaction mixture (step 3) on the nitrocellulose membrane. Let it dry for a few minutes.
6. Rinse briefly with PBS (or an equivalent buffer such as Tris buffered saline, TBS, Product Code T 6664).
7. Incubate the membrane in a blocking solution for 15 minutes.
8. Incubate the membrane with a 1:2000 dilution of the reconstituted detection antibody (Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate, Product Code A 5964) in blocking buffer. Incubate with gentle agitation for 20 minutes at room temperature.
9. Wash the membrane five times with PBST (or an equivalent buffer such as Tris buffered saline containing TWEEN 20, Product Code T 9039), for 4 minutes each time.
10. Briefly rinse in PBS or equivalent (with no detergent).
11. Prepare a Chemiluminescent Peroxidase Substrate solution, enough to cover the membrane (based on 0.125 ml/cm^2 membrane). Incubate the membrane in the reagent, with gentle agitation, for 1 minute at room temperature.
12. Drain the membrane of excess reagent solution, wrap in plastic wrap and expose to X-ray film. An initial 10 seconds exposure will indicate the need for different exposure times.

References

1. Schlessinger, J., Cell signaling by receptor tyrosine kinases. Cell, **103**, 211–225 (2000).
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3. Hubbard, S.R., *et al*., Autoregulatory mechanisms in protein-tyrosine kinases. J. Biol. Chem., **273**, 11987–11990 (1998).
4. Marshall, C.J., Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell, **80**, 179–185 (1995).
5. Ullrich, A., and Schlessinger, J., Signal transduction by receptors with tyrosine kinase activity. Cell, **61**, 203–212 (1990).

Troubleshooting Guide

Problem	Possible cause	Solution
The signal is very poor or no signal is observed.	The amount of the tyrosine kinase of interest in the sample is very low.	• Add more than 4 µl of anti-tyrosine kinase antibody in step 2 of the immunoprecipitation procedure. • Increase the sample volume - increasing the sample volume up to 1 ml usually does not affect the interaction between the antibody and the tyrosine kinase. • Increase the reaction incubation time from 30 minutes up to 90 minutes (Radioactive Detection, step 3 or Dot Blot Analysis, step 2). For Dot Blot: • Use a higher concentration of anti-phosphotyrosine-peroxidase conjugate.
	There is no activated tyrosine kinase in the sample.	• Prepare a fresh lysate. • Add the appropriate phosphatase inhibitors to the sample (Product Codes P 2850 and P 5726) or increase their concentration to prevent dephosphorylation of activated tyrosine kinase. • Add the appropriate protease inhibitors to the sample (Product Code P 8340) or increase their concentration to prevent degradation of activated tyrosine kinase. • Verify that the sample is appropriate or that the induction procedure for tyrosine kinase activation is appropriate. Determine the presence of the tyrosine kinase of interest in the sample by immunoblotting of the sample using anti-tyrosine kinase antibodies of interest prior to the performance of the immunoprecipitation.
	Incubation time is inadequate.	• Prolong the incubation duration of the anti-tyrosine kinase antibody with the EZview Red Protein A and cell lysate from several hours to overnight.
	Interfering substance is present in sample.	• Excessive detergent concentration may interfere with the interaction between the antibody and the tyrosine kinase of interest. • Make sure the extraction buffer is not interfering with the kinase activity. Some extraction buffers that could be used for immunoprecipitation are not suitable for activity assays. We highly recommend using the CelLytic™-M Cell Lysis Reagent (Product Code C 2978) for cell extract preparation.

Troubleshooting Guide (continued)

Background is too high.	Proteins bind non-specifically to Protein A, the resin beads, or the microcentrifuge tube.	• Pre-clear the sample once or several times by pre-incubation with EZview Red Protein A Affinity Gel (without the anti-tyrosine kinase antibody of interest) to remove proteins that may bind non-specifically. • During the final wash (Kinase Immunoprecipitation, step 10), after suspending the resin, transfer the entire sample to a clean microcentrifuge tube before centrifuging the sample.
	Insufficient washes in the immunoprecipitation step	• Increase the number of washes. • Prolong the duration of the washes to at least 15 minutes incubation. • Centrifuge at a lower speed to avoid non-specific trapping of lysate proteins during the initial centrifugation of Protein A/antigen complexes.
	For Dot Blot: Detection antibody is in excess.	• Use a more dilute anti-phosphotyrosine-peroxidase conjugated antibody.

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