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ProductInformation

Protein Tyrosine Kinase Assay Kit, Non-Radioactive

Catalog Number **PTK101**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Protein Tyrosine Kinase (PTK) Assay Kit, Non-Radioactive is intended for the measurement of tyrosine kinase activity from crude samples (column fractions during purification, etc.) or purified preparations, for the characterization of protein tyrosine kinases (natural and recombinant), and the detection of PTK activity in cell and tissue extracts.

Protein tyrosine phosphorylation is a central mechanism that mediates signal transduction events involved in a wide range of cellular processes. Protein tyrosine kinases (PTKs) are considered to play a critical role in signal transduction pathways. They control cell-cycle progression, transcriptional regulation, cell transformation, proliferation, differentiation, and apoptosis. Protein tyrosine kinase activity is often associated with receptor tyrosine kinases (InsulinR, EGFR, PDGFR, TRKs) and soluble non-receptor tyrosine kinases (p60src, lyn, fyn, etc.). Several of the PTKs have been identified as oncogenes.

Measurement of PTK activity is a basic requirement in the following areas of signal transduction research:

- Purification and characterization of protein tyrosine kinases (natural and recombinant)
- Elucidation of PTKs biological function
- Development of specific PTK inhibitors: High-throughput (HTP) screening *in vitro* of PTK inhibitors

Advantages of the PTK Assay Kit

Radioactive methods using ^{32}P-labeled ATP are labor intensive, produce large amounts of hazardous radioactive waste, and require a constant supply of radioactive labeled ATP. In addition, the half-life of the ^{32}P isotope is relatively short (14.3 days).

The Protein Tyrosine Kinase Assay Kit totally avoids the use of radioactive reagents and has several advantages over conventional radioactive techniques:

- High Specificity:
  - No cross-reactivity with tyrosine, phospho-threonine, or phosphoserine. Synthetic polymeric peptide substrate has no Ser or Thr residues.
  - Monoclonal antibody–peroxidase conjugate allows a direct and specific detection of only phosphorylated tyrosine residues.
- High Sensitivity comparable to radioactive methods
- Easy and reliable calibration
- Fast performance (1.5–2.0 hours hands-on assay)
- Convenient component sizes for easy handling

Components

Reagents and materials supplied in the PTK Assay Kit are sufficient for 96 reactions.

Protein Tyrosine Kinase (PTK) Substrate, poly-Glu-Tyr (PGT), Catalog Number P4476 — 5 mg

96-Well Multiwell Plate with a strip holder and plate, 8 wells × 12 strips in a strip holder and plate cover. Packaged in zip lock plastic bag with desiccant, Catalog Number M7552 — 1 each

Adenosine-5′-Triphosphate (ATP), Disodium salt, MW = 551.1, Catalog Number A6714 — 5 mg

Tyrosine Kinase Buffer (10×), 500 mM HEPES buffer, pH 7.4, 200 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM Na_3VO_4, Catalog Number B4550 — 15 ml

Epidermal Growth Factor Receptor (EGFR), Positive control for PTK. Lyophilized with trehalose as cryoprotectant, Catalog Number E2645 — 500 units

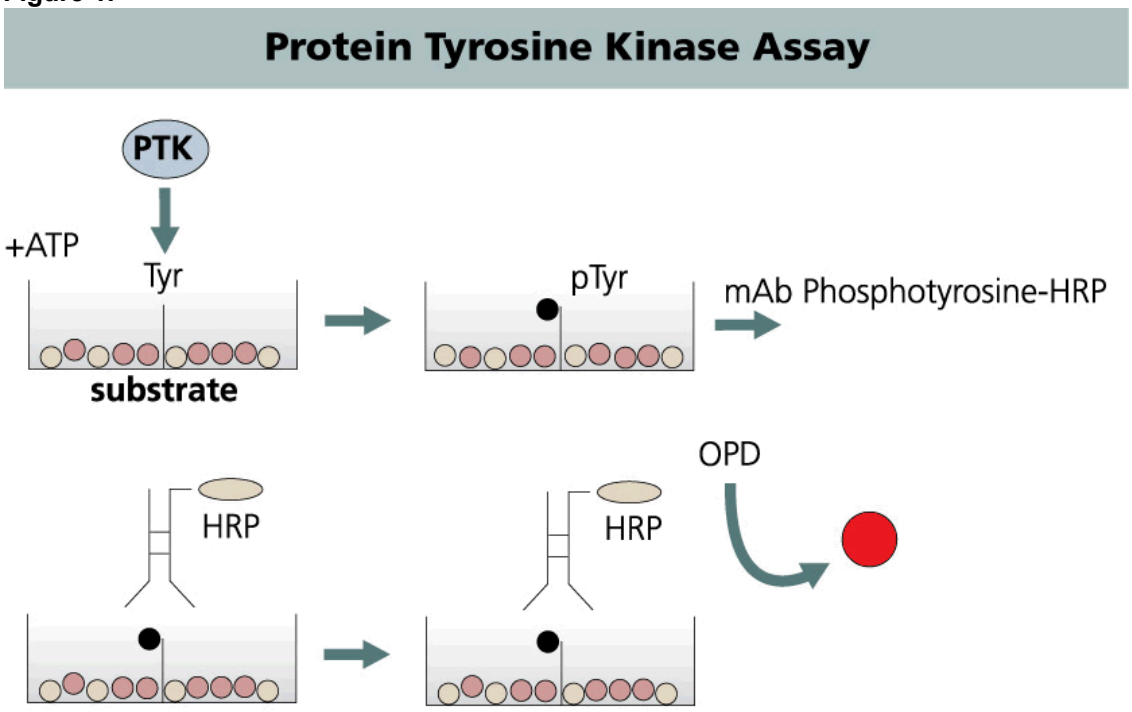
Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate, Clone PT-66, Catalog Number A5964 — 1 vial

Washing Buffer (PBS-TWEEN® 20), 10 mM Phosphate Buffered Saline, pH 7.4, and 0.05% TWEEN, Catalog Number P3563 — 1 packet

SIGMA*FAST*™ (OPD) Tablets, *o*-Phenylenediamine Dihydrochloride, Packaged in zip lock plastic bag, Catalog Number P8936 — 5 tablets in silver foil

SIGMA*FAST* Tablets, Phosphate-Citrate Buffer with Urea Hydrogen Peroxide, Packaged in zip lock plastic bag, Catalog Number P8812 — 5 tablets in gold foil

Figure 1.



Principle of the Assay

The PTK kit assay system for the *in vitro* determination of protein tyrosine kinase activity is based on an ELISA assay using a PTK-specific polymer substrate-coated multiwell plate:

- Multiwell plates are precoated with a synthetic random polymer substrate poly-Glu-Tyr (PGT) containing multiple tyrosine residues. PGT can be phosphorylated by a wide range of PTKs.
- The phosphorylation reaction is initiated by the addition of a protein tyrosine kinase (tissue and cell extracts or a purified tyrosine kinase control such as EGFR) in the tyrosine kinase reaction buffer that contains Mg^{2+}, Mn^{2+}, and ATP.
- The phosphorylated polymer substrate is probed with a purified phosphotyrosine specific monoclonal antibody conjugated to horseradish peroxidase (HRP).
- Color is developed with HRP chromogenic substrate (OPD).
- The color is quantitated by spectrophotometry (ELISA reader) and reflects the relative amount of tyrosine kinase activity in the sample (qualitative).
- Protein Tyrosine kinase activity in the sample (quantitative) is derived from the EGFR control or extrapolated from the EGFR standard curve (absorbance at 492 nm vs. units of EGFR activity).

Assay time:

Protein tyrosine kinase reaction and ELISA, 1.5–2.0 hours.

Reagents and Equipment Required but Not Provided:

1. PBS - 10 mM Phosphate Buffered Saline, pH 7.2–7.4, (Catalog Number P3813, not provided in kit)
2. Protein assay reagent and BSA standards (optional) for determination of protein content in samples (e.g., cell lysates) (Catalog Number BCA1, QPBCA, or TP0300)
3. Microcentrifuge and tubes
4. Graduated cylinders
5. Glycerol (Catalog Number G5516)
6. 0.1 M "Activated" sodium vanadate solution (see Preparation Instructions)
7. Lysis Buffer - 50 mM HEPES buffer, pH 7.4, containing 0.1–1% TRITON® X-100, 10% glycerol, 1 mM dithiothreitol (DTT), and 1 mM "activated" sodium vanadate (see Preparation Instructions). Add protease inhibitors: 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A, or protease inhibitor cocktail (Catalog Number P8340), 10 μl/ml cell suspension.
8. Homogenization Buffer - 20 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, and 2 mM activated sodium vanadate. Add protease inhibitors: 1 mM PMSF, 50 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin A, and 2 mM dithiothreitol (DTT).
9. ELISA Reader capable of measuring absorbance of 96-well plates at wavelength of 492 nm.
10. Ultrapure water
11. 2.5 N H_2SO_4 solution in ultrapure water
12. Precision pipettors (2–20, 20–100, and 200–1000 μl) with disposable tips
13. 37 °C Incubator
14. PTK Reaction (a Positive Control, Catalog Number E2645, provided in kit and Test Sample)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

A. Sample Preparation: Cell Lysates and Tissue Extracts

Important Notes:

- Different cells, tissues, and tyrosine kinases (membrane type, cytosolic, etc.) may require different lysis buffers compositions (with detergents, protease inhibitors, etc.) and conditions (e.g., lysis time, etc.). The lysis/homogenization buffers and conditions described are provided only as guidelines. For a particular application or assay, the buffer and lysis conditions must be optimized.
- All following procedure steps are carried out rapidly on ice, using the appropriate **ice-cold** lysis buffer unless noted otherwise.
- Reagents added during the procedure (buffers, detergents, proteins) may interfere with the assay. For example, **buffers containing azides are not compatible with this assay** and detergents concentrations above 0.1% (e.g., SDS, TRITON X-100) may interfere with the PTK activity in this assay. Perform the PTK assay to find the optimal concentration.
- Endogenous tyrosine phosphatases may interfere with the PTK assay by reversing the tyrosine phosphorylation reaction, thus resulting in lower levels of phosphorylation. Sodium vanadate is an effective inhibitor of endogenous phosphatase. It should be activated (see Preparation of 0.1 M "Activated" sodium vanadate solution), prior to its addition to homogenization/lysis buffers.

Preparation of 0.1 M "Activated" sodium vanadate solution

a. Weigh out 0.64 g of Sodium orthovanadate (Na_3VO_4), Catalog Number S6508.
b. Add 35 ml of ultrapure water to an appropriate container equipped with a stirring bar. Place on magnetic stirrer and begin stirring.
c. Add sodium orthovanadate.
d. Mix until completely dissolved. Mark vessel "Vanadate Solution".
e. Adjust pH of vanadate solution to 10 with 1 N HCl or 1 N NaOH. (The pH of vanadate solution may vary from batch to batch).
f. If the solution turns yellow, boil it (~5 minutes) until it becomes colorless.
g. If required, repeat the last two steps until the vanadate solution remains colorless.

Preparation of Cell Lysates

1. Grow cells in appropriate culture medium.
2. Wash cells or rinse plates with ice-cold PBS (2 × 10 ml). For cells in suspension, centrifuge at 800 × *g* for 5 minutes at room temperature. Remove supernatant.
3. For adherent cells, add freshly prepared Lysis Buffer at 4 °C. For cells in suspension, add Lysis Buffer to washed cell pellet in a 15 ml conical tube.
4. Transfer cell lysate to microcentrifuge tube and leave for 10–20 minutes at 4 °C.
5. Centrifuge lysate at 10,000 × *g* for 15 minutes at 4 °C.
6. Collect supernatant containing lysate fraction. Discard pellet containing nuclear and cell debris.
7. Remove 0.2 ml aliquot of lysate for protein determination using protein standard (e.g., BSA).
8. Sample of the lysate may be used in the PTK assay. It is recommended to test different lysate dilutions to obtain an optimal signal.
9. Aliquot 0.1 ml samples of the cell lysate and store at –70 °C. Protein tyrosine kinases from cell lysates or tissue homogenates may not be recovered after freezing and thawing. To enhance stability of PTKs, glycerol may be added to the lysate up to a final concentration 50% (w/v) and the samples should be stored at –70 °C.

Preparation of Tissue Extracts

1. Rapidly dissect required tissue (e.g., brain, spleen, etc.) and collect into ice-cold homogenization buffer. Weigh tissue.
2. Homogenize tissue in 2–5 volumes of ice-cold homogenization buffer using a PTFE/glass or mechanical homogenizer at maximum speed (3 times with 1 minute rest in between).
3. Centrifuge homogenate at 1,000 × *g* for 10 minutes at 4 °C to remove nuclei and cell debris. Collect supernatant (S1).
4. Centrifuge supernatant at 10,000 × *g* for 20 minutes at 4 °C. Collect clear supernatant (S2). Retain the pellet (P2), representing the crude membrane fraction. The S2 fraction contains cytosolic proteins and may be used for assay of PTK activity or further purification by chromatography.
5. If the activity is presumed to be present in the membrane fraction (P2), solubilize the pellet in 1–2 volumes of lysis buffer for 10 minutes on ice.
6. Centrifuge lysate at 10,000 × *g* for 10 minutes at 4 °C. Collect the clear supernatant representing the lysate of the crude membrane fraction. This fraction contains the solubilized membrane proteins and may be used for assay of PTK activity or further purification by chromatography.
7. Remove an aliquot of the cytosolic fraction or membrane extract for protein determination using a protein assay kit.
8. Aliquot samples of the tissue extract and store at –70 °C. Protein tyrosine kinases from tissue homogenates may not be recovered after freezing and thawing. To enhance stability of PTKs, glycerol may be added to the lysate up to a final concentration 50% (w/v) and the samples should be stored at –70 °C.

B. Assay Reagents

Prepare the reagent solutions as close as possible to the time of use. Store reagent solutions (where applicable) at the appropriate temperature until used.

1. PBS - 10 mM Phosphate Buffered Saline, pH 7.2–7.4, (Catalog Number P3813, not provided in kit).
2. 10% Glycerol in ultrapure water (not provided in kit).
3. Protein Tyrosine Kinase (PTK) Substrate - Reconstitute contents of P4476, PTK substrate, with 1 ml PBS (PTK Substrate Stock Solution). Add 1 ml of the PTK Substrate Stock Solution to 19 ml of PBS in an appropriate tube (50 ml). Mix well. The PTK Substrate Diluted Solution can be stored in aliquoted samples (e.g., 1 ml) at –20 °C. Avoid repeated freeze and thaw of this reagent.
4. Epidermal Growth Factor Receptor (EGFR) - Reconstitute contents of E2645, EGFR, with 125 µl of 10% glycerol in ultrapure water. Mix gently until dissolved. The EGFR Stock Solution can be stored in small aliquots (e.g., 5 µl, 20 units) at –70 °C. Avoid repeated freeze and thaw of this reagent.
5. Adenosine-5′-Triphosphate (ATP) - Reconstitute contents of A6714, ATP, with 1 ml of ultrapure water. Mix gently until dissolved. Final concentration of stock solution is ~5 mg/ml (9 mM). The ATP solution can be stored in small aliquots (e.g., 50 µl) at –20 °C. Avoid repeated freeze and thaw of this reagent.
6. Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate - Reconstitute contents of A5964, Anti-Phosphotyrosine–Peroxidase, in 0.2 ml of ultrapure water. Mix gently until dissolved. The antibody-peroxidase conjugate solution can be stored frozen in aliquots at –20 °C. Dilute reconstituted A5964, Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate in P3563, Washing Buffer. Refer to Certificate of Analysis for lot specific dilution factor.

7. Washing Buffer (PBS-TWEEN 20) - Dissolve contents of P3563, PBS-TWEEN 20, powder package in 1 liter of ultrapure water to give 10 mM sodium phosphate buffer, 138 mM NaCl, 27 mM KCl and 0.05% TWEEN 20, pH 7.4. Store at 2–8 °C. For prolonged storage, keep solution frozen at –20 °C.

Storage/Stability
Store Kit at –20 °C. Avoid repeated freeze and thaw. Avoid "frost-free" freezers.

Protein Tyrosine Kinase (PTK) Substrate, Catalog Number P4476 - Store reconstituted solution at –20 °C.

96-Well Multiwell Plate with a strips holder and plate, Catalog Number M7552 - Store at room temperature.

Adenosine-5′-Triphosphate (ATP), Catalog Number A6714 - Store reconstituted stock solution in aliquots at –20 °C.

Tyrosine Kinase Buffer (10×), Catalog Number B4550 - Store solution at –20 °C. If required, the diluted Tyrosine Kinase Buffer can be kept in frozen aliquots at –20 °C, for a prolonged period.

Epidermal Growth Factor Receptor (EGFR), Catalog Number E2645 - Store reconstituted stock solution in aliquots at –70 °C.

Monoclonal Anti-Phosphotyrosine–Peroxidase Conjugate, Catalog Number A5964 - Store reconstituted solution in aliquots at –20 °C.

Washing Buffer (PBS-TWEEN 20), Catalog Number P3563 - Store solution at 2–8 °C.

SIGMA*FAST* (OPD) Tablets, *o*-Phenylenediamine Dihydrochloride. Catalog Number P8936 - Store at 2–8 °C. Do not store the prepared solution.

SIGMA*FAST* Tablets, Phosphate-Citrate Buffer with Urea Hydrogen Peroxide. Catalog Number P8812 - Store at 2–8 °C. Do not store the prepared solution.

Assay Procedure
A. Coating of 96-Well Plate
Note: The PTK Assay Kit is supplied with removable wells of strips so that the assay can be performed only with the required number of wells in a particular experiment. It is recommended to coat only the number of wells required for a particular assay.

1. Remove the plate from the plastic bag. Place the required number of wells or strips in the well holder. Return any unused strips to the plastic bag, seal the zip lock and store at room temperature.
2. Thaw a sample of the PTK Substrate Diluted Solution.
3. Add 125 µl of PTK Substrate Diluted Solution to each well. Cover multiwell plate.
4. Incubate plate overnight at 37 °C. If required, the coating time may be shortened to 4 hours. However, this may result in a lower signal.
5. Remove coating solution and wash each well with 200 µl of Washing Buffer (PBS-TWEEN 20).
6. Remove Washing Buffer and dry wells for 2 hours at 37 °C.

B. PTK Assay
Note: Perform this assay in duplicates for EGFR (control PTK) and test samples. The EGFR and ATP dilutions **must be** freshly prepared and kept on ice while used. Do not store these dilutions.

Prepare your PTK test sample (purified PTK or cell lysate, tissue extract etc.). For guidelines on the preparation of cell or tissue samples, refer to Preparation Instructions.

1. Prepare 1× Tyrosine Kinase Buffer by adding 1 ml of B4550, the supplied 10× concentrate, to 9 ml of ultrapure water. Mix well.
2. Thaw a 5 µl sample (20 units) of the EGFR (see Assay Reagents). Add 95 µl of 1× Tyrosine Kinase Buffer. Mix gently and place on ice.
3. Thaw a sample of the ATP stock solution (see Assay Reagents). Add 40 µl of ATP solution to 1 ml of 1× Tyrosine Kinase Buffer. Mix well and place on ice.
4. Add 90 µl of 1× Tyrosine Kinase Buffer containing ATP to each well.
5. **Blank** (control without EGFR): Add 20 µl of 1× Tyrosine Kinase Buffer.
6. Add to the appropriate wells, 20 µl (4.0 units) of EGFR or test samples (cell or tissue lysates), diluted 1:1 or 1:2, etc. in 1× Tyrosine Kinase Buffer. Final concentration of ATP in the PTK reaction is 0.3 mM.
7. Cover wells with plate cover. Incubate wells at room temperature for 30 minutes

8. Remove reaction mixture from each well.
9. Wash each well with 200 µl of Washing Buffer. After each wash, blot the plate on a paper towel until all residual moisture is removed. Repeat this step 5 times.
10. Add to each well 100 µl of the appropriate dilution of antibody conjugate.
11. Cover wells with plate cover. Incubate wells for 30 minutes at room temperature.
12. Freshly prepare peroxidase substrate solution by dissolving one P8936, OPD tablet (silver foil)) and one P8812, Urea Hydrogen Peroxide tablet (gold foil) in 20 ml of ultrapure water. Mix well until dissolved. Protect from light until used. Do not store this solution.
13. Remove antibody solution from wells.
14. Wash each well with 200 µl of Washing Buffer. After each wash, blot the plate on a paper towel until all residual moisture is removed. Repeat this step 5 times.
15. Add 100 µl of freshly prepared OPD substrate solution to each well and incubate for **exactly** 7 minutes, in the dark, at room temperature. An orange-yellow color should develop in positive wells.
16. Stop reaction by adding 100 µl of 2.5 N H_2SO_4 to each well.
17. Read wells in a multiwell plate ELISA reader set at 492 nm. The wells must be read within 30 minutes of addition of stop solution.

PTK Standard Curve (EGFR control)
If required, a PTK standard curve can be prepared using the EGFR control. Proceed as previously described with the following modifications:

Step 2. Thaw a 5 µl sample (20 units) of the EGFR (control PTK). Add 45 µl of 1× Tyrosine Kinase Buffer. Mix gently. This corresponds to 8 units EGFR/20 µl. Make (1:1) serial dilutions of the EGFR with of 1× Tyrosine Kinase Buffer (e.g., 0.25, 0.5, 1, 2, and 4 units EGFR).

The PTK standard curve with EGFR is not linear at all concentrations. It is advised to use only the recommended concentration range (resulting in up to 2–4 units EGFR /well) for a PTK standard curve and for PTK activity kinetic studies.

Results

PTK Standard Curve (EGFR control)

Prepare a PTK standard curve by plotting the absorbance of EGFR control serial dilutions at 492 nm (A_{492}) versus the EGFR activity (units). A typical PTK standard curve using EGFR is shown in Figure 2. Determine the activity of the test sample (e.g., cell lysate) using the linear part of the PTK standard curve and by extrapolating the units of PTK activity in the sample from the absorbance obtained at 492 nm.

It is always recommended to run a PTK standard curve. However, after running the standard curve another sample's activity may be determined from the absorbances of a single EGFR test and the test sample obtained at 492 nm. For example, if the absorbance for 4 units EGFR is 1.4 and therefore, similar to the previously run standard curve, the activity of the test sample with absorbance 0.6 is ~1.1 units.

References

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3. Levitzki, A., and Gazit, A., *Science*, **267,** 1782 (1995).
4. Rijksen, G., *et al.*, *Methods Enzymol.*, **200,** 98 (1991).
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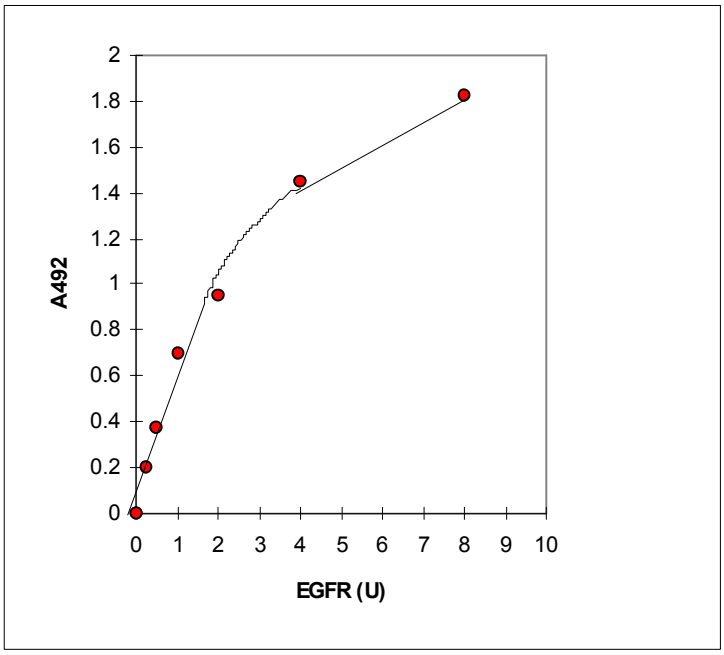
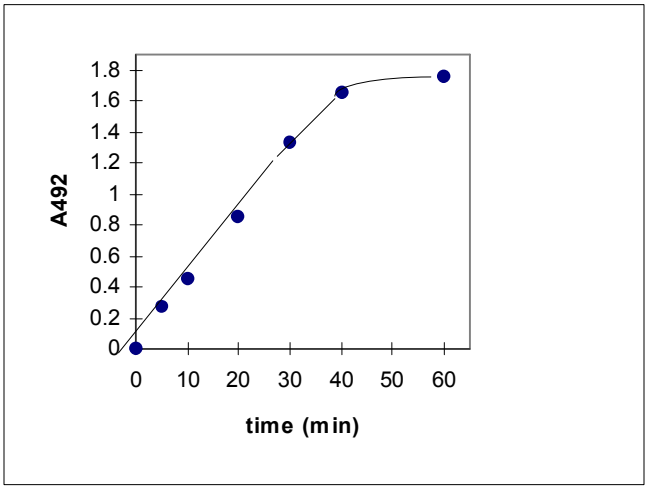
KAA,LPG,AH,KTA,MAM 07/07-1

Figure 2.
Protein Tyrosine Kinase (EGFR) Assay



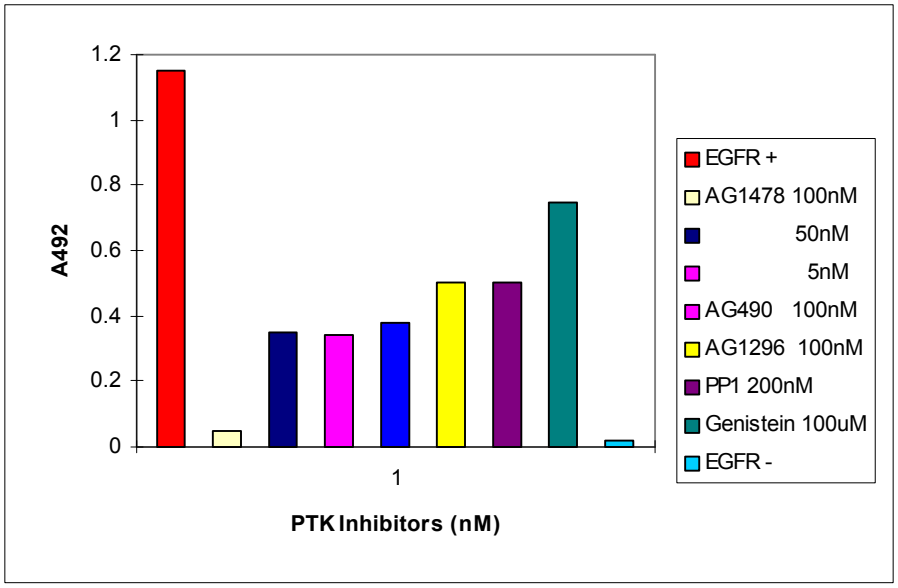
Epidermal Growth Factor Receptor (EGFR) (0.25–8.0 units) was incubated with 0.3 mM ATP on PGT-coated wells for 30 minutes at room temperature. Phosphorylated PGT was detected by ELISA using monoclonal anti-phosphotyrosine–HRP (clone PT-66) and OPD substrate. Optical density of each well was determined at 492 nm in a multiwell plate reader.

Figure 3.
Time course of Tyrosine Kinase Activity



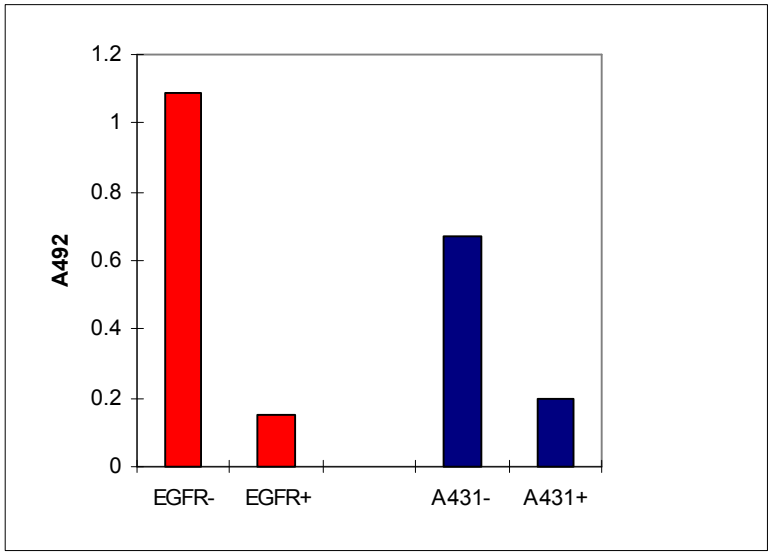
Time dependence of PTK assay. EGFR (4 units) was incubated on PGT-coated wells for different time periods at room temperature and activity was detected as described under Figure 2.

Figure 4.
Inhibition of Tyrosine Kinase Activity



Inhibition of EGFR activity. EGFR (4 units) was preincubated with different concentrations of several PTK inhibitors (see Figure insert) for 10 minutes on ice, and then assayed on PGT-coated wells for as described under Figure 2.

Figure 5.
Inhibition of Tyrosine Kinase Activity in Cell Lysate



Inhibition of purified EGFR activity and PTK activity of an A431 cell lysate. EGFR (4 units) and A431 cell lysate were preincubated with and without 50 nM tyrphostin analog AG1478 (Catalog Number T4182), for 10 minutes on ice, then assayed on PGT-coated wells as described under Figure 2.