Technical Bulletin

SignaTECT® Protein Tyrosine Kinase Assay System

INSTRUCTIONS FOR USE OF PRODUCTS V6480.

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1. Description

Protein tyrosine kinases (PTKs) play a critical role in the modulation of many cellular events, including differentiation, growth and metabolism (1,2). Phosphorylation of tyrosine residues is essential for maintaining cellular homeostasis, yet this post-translational modification also provides the means by which a number of cellular oncogenes deregulate various signaling pathways and induce transformation. This class of enzymes is therefore an important target for both basic research and drug development (3).

PTKs represent a diverse group of protein kinases, comprising both transmembrane and cytoplasmic enzymes. Although protein phosphorylation is a common modification (approximately 30% of all proteins are phosphorylated), tyrosine phosphorylations make up only 0.01% of these events (4). Widespread appreciation of the complexity of signaling pathways involving this diverse class of enzymes has resulted in the need for more sophisticated reagents to dissect the role of PTKs in normal and disease states (3,5). To meet this need, Promega has developed a novel, broad-specificity SignaTECT® Protein Tyrosine Kinase Assay System[a] to measure

rapidly and specifically the enzymatic activities of a variety of PTKs, including both transmembrane receptor PTK enzymes (epidermal growth factor receptor, insulin receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor) and cytosolic PTK enzymes (p60src, p56lck, p93fes and p43abl).

The most common method for assaying PTK activity involves measuring the transfer of ^{32}P to a protein or peptide substrate. Although phosphocellulose filter assays are commonly used to quantitate peptide substrate phosphorylation, this method has a number of disadvantages. The positively charged, ^{32}P-labeled kinase substrate is typically bound to these filters via weak electrostatic interactions. Therefore, some of the substrate can be lost from the filters during the washing procedure. In addition, peptide substrates often exhibit wide variability in binding to phosphocellulose filters (6). In the presence of multiple kinases (e.g., in a cell or tissue extract), the ^{32}P-labeled peptides or proteins bound to the phosphocellulose filter may reflect kinase activity other than that due to PTKs. Additionally, [γ-^{32}P]ATP preparations can contain ^{32}P-labeled contaminants that possess a positive charge at low pH, allowing them to bind to phosphocellulose filters. This nonspecific binding results in high backgrounds and low signal-to-noise ratios (7).

The SignaTECT® Protein Tyrosine Kinase Assay System overcomes the problems of nonspecific binding by using PTK Biotinylated Peptide Substrates in conjunction with the unique SAM^{2}® Biotin Capture Membrane (SAM^{2}® Membrane), which is a streptavidin matrix. The high binding capacity of the SAM^{2}® Membrane for the PTK Biotinylated Peptide Substrates and the low backgrounds observed with this system maximize the signal-to-noise ratio. Following phosphorylation of the PTK Biotinylated Peptide Substrates and binding to the SAM^{2}® Membrane, the excess free [γ-^{32}P]ATP and nonbiotinylated proteins are removed by a simple washing procedure. The binding of biotin to streptavidin is rapid and strong. Once formed, the biotin-streptavidin association is unaffected by wide extremes of pH, temperature, salt concentrations and denaturing agents. Due to the strength of this binding interaction, the ^{32}P-labeled PTK Biotinylated Peptide Substrate is unlikely to be removed by the washing procedure.

The amount of ^{32}P incorporated into the PTK Biotinylated Peptide Substrate is determined by liquid scintillation counting (Figure 1, Section 3.F), phosphorimaging analysis or by conventional autoradiography.

2. Product Components and Storage Conditions

Product	Size	Cat.#
SignaTECT® Protein Tyrosine Kinase Assay System	96 reactions	V6480

Each system contains sufficient reagents to perform 96 kinase reactions. Includes:

- 1 SAM²® Biotin Capture Membrane
- 600µl ATP, 0.5mM
- 200µl BSA, 10mg/ml
- 1.4ml Termination Buffer (2 × 700µl)
- 240µl PTK Biotinylated Peptide Substrate 1, 2.5mM
- 240µl PTK Biotinylated Peptide Substrate 2, 2.5mM
- 500µl PTK Assay 5X Buffer
- 200µl PTK Enzyme Dilution 5X Buffer
- 400µl Trichloroacetic Acid (TCA), 43%
- 600µl TCA Neutralization 5X Buffer
- 100µl DTT, 100mM
- 250µl Sodium Vanadate, 1mM

Storage Conditions: Store all components of the SignaTECT® Protein Tyrosine Kinase Assay System at –20°C, where they are stable for at least six months from the date of purchase. Avoid multiple freeze-thaw cycles. For storage for less than 1 month, the SAM²® Biotin Capture Membrane and TCA (43%) can be stored at 4°C (return the unused portion of the SAM²® Membrane to the resealable plastic bag). The Termination Buffer and TCA Neutralization Buffer may be stored at room temperature (20–25°C).

Note: The SAM²® Biotin Capture Membrane is prenumbered and perforated into 96 1.25 × 1.15cm squares.

3. Quantitation of Protein Tyrosine Kinase Activity

Due to the heterogeneity of PTK enzymes and the samples assayed for their activity, optimization, including the addition of components such as denaturing agent, glycerol, or detergent to the enzyme dilution buffer, may be necessary. For information concerning the substrates and general reaction conditions, refer to Section 4.

3.A. Preparation of Samples for PTK Assay

Materials to Be Supplied by the User
(Solution compositions are provided in Section 4.C.)

- PTK enzyme sample (see Sections 4.A and 4.B)
- [γ-^{32}P]ATP (3,000Ci/mmol; 10µCi/µl)
- 2M NaCl
- 2M NaCl in 1% H_3PO_4
- washing container (e.g., Nalgene® plastic utility box, 7.5 × 6 × 4 inches)
- scintillation counter or phosphorimaging device
- 30°C heating block or water bath
- deionized water
- orbital platform shaker (optional but recommended)
- heat lamp (optional)

Note: The optimal Biotinylated Peptide Substrate for each PTK sample must be determined empirically (see Section 4.B and Figure 1).

1. Assemble reagents and materials for the SignaTECT® Protein Tyrosine Kinase Assay System as follows:

a. Thaw the PTK Biotinylated Peptide Substrate 1 or the PTK Biotinylated Peptide Substrate 2, 1mM Sodium Vanadate, PTK Assay 5X Buffer, PTK Enzyme Dilution 5X Buffer, 100mM DTT (optional) and 0.5mM ATP on ice.

b. Thaw the Termination Buffer (Section 4.C) or 43% TCA and TCA Neutralization 5X Buffer at 20–30°C. The TCA and TCA Neutralization 5X Buffer will only be needed if Termination Protocol B is used (see Section 3.C).

c. Wearing gloves, cut (using scissors or a razor blade) the required number of squares from the SAM$^{2®}$ Biotin Capture Membrane. The squares may remain connected as a partial sheet to minimize handling. Return the unused SAM$^{2®}$ Membrane to the resealable plastic bag at 4°C or –20°C. The template provided in Section 4.D will allow you to identify samples using the SAM$^{2®}$ Membrane square numbers.

2. Dilute the PTK Enzyme Dilution 5X Buffer fivefold in deionized water (e.g., add 20µl of the 5X stock to 80µl of water). The PTK samples can be diluted using the resulting PTK Enzyme Dilution 1X Buffer or an alternative buffer. (Section 4 provides important information on choice of buffer for enzyme dilution and additives with which to optimize the buffer.) The diluted enzyme should be stored on ice.

3.B. PTK Assay Protocol

1. Prepare the ATP mix as follows:

Component	Final per Reaction	20 Reactions
0.5mM ATP	1.00µl	20µl
[γ-^{32}P]ATP (3,000Ci/mmol, 10µCi/µl)	0.05µl	1.0µl
water	1.45µl	29µl
Final Volume of:	2.5µl	50µl

Note: Adding 2.5µl of this [γ-^{32}P]ATP mixture per reaction will deliver 0.5µCi or 1.11 × 10^6dpm, depending on the reference date of the isotope. However, greater amounts of ^{32}P can be added to the reaction to increase the specific activity of the ATP solution.

2. Prepare the following reaction in 0.5ml or 1.5ml microcentrifuge tubes (see Note 1):

Component	Final per Reaction	20 Reactions
PTK Assay 5X Buffer	5.0µl	100µl
PTK Biotinylated Peptide Substrate, 2.5mM (see Note 2)	2.5µl	50µl
Sodium vanadate, 1mM (see Note 3)	2.5µl	50µl
ATP, 200µM with 0.2µCi/µl [γ-^{32}P]ATP	2.5µl	50µl
deionized water or activator/inihibitor as appropriate	7.5µl	150µl
Final volume of:	20µl	400µl

Note: Perform initial assays without substrate to calculate specific activity (see Section 3.E). In addition, initial substrate dilutions should be assayed to test the substrate dependency of the PTK reaction (see Note 2).

3. Mix gently and preincubate the reaction mix from Section 3.B, Step 2 at 30°C for 1–5 minutes.

4. Initiate the reaction by adding 5µl of the enzyme from Section 3.A, Step 2 to the reaction mix in Section 3.B, Step 3. Total reaction volume will be 25µl.

   For multiple enzyme samples, make additions to the reactions approximately 20 seconds apart to ensure that incubations proceed for the same length of time. For negative control reactions (no enzyme), add 5µl of PTK Dilution 1X Buffer (see Note 4).

5. Incubate the reactions at 30°C for 15 minutes (other time points and temperatures may be tested if desired). Proceed to the termination protocol, Section 3.C.

3.B. PTK Assay Protocol (continued)

Notes:

1. Typically, it is convenient to make up a large master mix of the standard reaction mixture (without the PTK), sufficient for the number of samples being tested. Prewarm the mixture and then dispense 20µl to each reaction tube. Initiate the reaction by adding the appropriate amount of PTK.
2. In initial experiments, serial dilutions (0–1mM final concentration) of the PTK Biotinylated Peptide Substrates 1 and 2 should be made, in water, to test the substrate dependency of the PTK reaction.
3. Addition of Sodium Vanadate, an inhibitor of PTPases, or the reducing agent, DTT, is optional and depends on the nature of the sample (see Section 4.B).
4. Initially, it may be useful to titrate the enzyme in the sample dilution buffer to assess the linear range of the enzyme. Epidermal Growth Factor (EGF) Receptor (Cat.# V5551) can be used as a positive control (use 0.025–0.25 units of undiluted EGF Receptor per 25µl reaction).

3.C. Termination of Reactions and Sample Processing

1. Terminate the reaction using either Termination Protocol A or B, depending on the extent of nonspecific sample binding to the SAM²® Membrane (see Section 4.B). The degree of nonspecific binding must be determined empirically.

Termination Protocol A

Use this protocol for purified or partically purified enzymes and crude cell or tissue extracts.

1. Add 12.5µl of Termination Buffer (7.5M guanidine hydrochloride) to each reaction and gently mix the contents of each tube.

2. Centrifuge each tube in a microcentrifuge at 14,000 × *g* for 10 seconds (optional) and proceed directly to Step 2 below.

Termination Protocol B

Use this protocol for samples that have high backgrounds with protocol A.

1. Terminate reactions by adding 3.5µl of 43% TCA. Mix and place on ice.

2. Add 1.5µl of 10mg/ml BSA. Keep samples on ice for 5 minutes.
Note: Current volume is 30µl.

3. Centrifuge each tube in a microcentrifuge at 14,000 × *g* for 5 minutes and remove 24µl of the supernatant without disturbing the white protein pellet at the bottom of the tube.

4. Transfer this aliquot to another microcentrifuge tube containing 6µl of TCA Neutrialization 5X Buffer. Mix thoroughly and keep the tubes on ice (final volume is 30µl).

2. Spot 12.5µl of each terminated reaction onto a prenumbered SAM$^{2®}$ Membrane square. After all samples have been spotted, follow the wash and rinse steps as described below. Save the reaction tubes for Section 3.C, Step 5.

   Note: The 12.5µl sample contains 2.1nmol of the PTK Biotinylated Peptide Substrate. This substrate concentration has been optimized for the assay and does not exceed the linear binding capacity of the SAM$^{2®}$ Membrane square. Larger volumes can be spotted; however, the maximum binding capacity per membrane square for this substrate is 2.6nmol.

3. Place the SAM$^{2®}$ Membrane squares containing samples from Section 3.C, Step 2 into a washing container. Wash using an orbital platform shaker set on low or by manual shaking, as follows:

   a. Wash 1 time for 30 seconds with 200ml of 2M NaCl.
   b. Wash 3 times for 2 minutes each with 200ml of 2M NaCl.
   c. Wash 4 times for 2 minutes each with 200ml of 2M NaCl in 1% H_3PO_4.
   d. Wash 2 times for 30 seconds each with 100ml of deionized water.
   Total wash time <20 minutes.

   Notes:

   Dispose of the radioactive wash solution in accordance with the regulations of your institution.

   More or less washing may be appropriate to achieve acceptably low background counts; this should be determined empirically.

   For rapid drying, a final 15-second, 95% ethanol wash (100ml) can be used. Longer washes with ethanol may cause the ink to smear but will not affect the assay.

4. Dry the SAM$^{2®}$ Biotin Capture Membrane squares on a piece of aluminum foil under a heat lamp for 5–10 minutes or air-dry at room temperature for 30–60 minutes. (If the SAM$^{2®}$ Membrane has been washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)

5. Determine total counts for calculation of the specific activity of [γ-^{32}P]ATP as follows: Remove 5µl aliquots from any 2 reaction tubes from Section 3.C, Step 2 and spot onto individual SAM$^{2®}$ Membrane squares or Whatman® 3mm filter discs. For this step dry the SAM$^{2®}$ Membrane squares or filter discs without washing. After analysis use these results to calculate the specific activity of [γ-^{32}P]ATP in Section 3.D.

   Note: If 5µl is not available from a single tube, combine the contents of several tubes.

3.C. Termination of Reactions and Sample Processing (continued)

6. **Analysis by scintillation counting:** If still connected, separate the SAM²® Membrane squares (from Section 3.C, Steps 4 and 5) using forceps, scissors or a razor blade and place the SAM²® Membrane squares or 3mm filter discs into individual scintillation vials. Add scintillation fluid to the vials and count.

   Analysis by phosphorimaging: Alternatively, the SAM²® Membrane may remain intact and the intact SAM²® Membrane or 3mm filter discs may be analyzed by phosphorimaging.

3.D. Calculation of the Specific Activity of [γ–^{32}P]ATP

Specific activity of the [γ-^{32}P]ATP in cpm/pmol of ATP = $\frac{(37.5/5)(X)}{500} = 0.015X$

where:

37.5 is the sum of the reaction volume (25µl) + the Termination Buffer (12.5µl)
5 is the volume in microliters of the samples from Section 3.C, Step 5
X is the average counts/minute of the 5µl samples from Section 3.C, Step 5
500 is the pmol of ATP in the reaction (25µl at 20µM)

Note: The final volume of neutralized TCA supernatant is 30µl instead of 37.5µl because not all of the TCA supernatant is recovered. However the dilution of samples is the same whether using Termination Protocol A or B, thus the above formula applies to both protocols.

3.E. Calculation of the Specific Activity of the PTK Enzyme

Enzyme specific activity in pmol of phosphate/minute/µg or µl of sample =

$$\frac{[(\text{cpm}_{\text{reaction with enzyme and substrate}}) - (\text{cpm}_{\text{reaction with enzyme alone}})]\ (37.5)}{(12.5)(15)(\text{amount of PTK per reaction})(\text{specific activity of ATP})}$$

where:

37.5 is the sum of the reaction volume (25µl) + the Termination Buffer (12.5µl)
12.5 is the volume in microliters of sample in Section 3.C, Step 2
15 is the incubation time in minutes

Sample Calculation:

This sample calculation was formulated using 5µl of EGF Receptor (Cat.# V5551), 250µM PTK Biotinylated Peptide Substrate 1 or 2 and 0.92µCi of [γ-^{32}P]ATP per reaction (680,055cpm spotted per square in 12.5µl). The average cpm for standards as prepared in Section 3.C, Step 5 was 272,022. Note that backgrounds are <0.06% of input radioactivity.

Condition	Average cpm ± S.D.*	% of Input
5µl of EGFR enzyme alone (no peptide substrate)	353 ± 59	0.052
PTK Biotinylated Peptide Substrate 1 alone (no EGFR)	322 ± 112	0.047
PTK Biotinylated Peptide Substrate 2 alone (no EGFR)	120 ± 11	0.018
EGFR + PTK Biotinylated Peptide Substrate 1	24,264 ± 163	
EGFR + PTK Biotinylated Peptide Substrate 2	26,909 ± 707	

*Intra-assay deviation with n = 3.

Specific activity of the [g-32P]ATP = $\frac{(37.5/5)(272,022)}{500}$ = 4,080

Enzyme specific activity in pmol of phosphate/minute/ml of sample:

PTK Peptide 1:

$\frac{(24,264\text{cpm} - 353\text{cpm})(37.5)}{(12.5)(15)(5)(4,080)}$ = 0.234pmol/minute/microliter or 234units/ml

PTK Peptide 2:

$\frac{(26,909\text{cpm} - 353\text{cpm})(37.5)}{(12.5)(15)(5)(4,080)}$ = .026pmol/minute/microliter or 260units/ml

Note: 1 unit = 1pmol phosphate transferred/minute

3.F. Expected Results

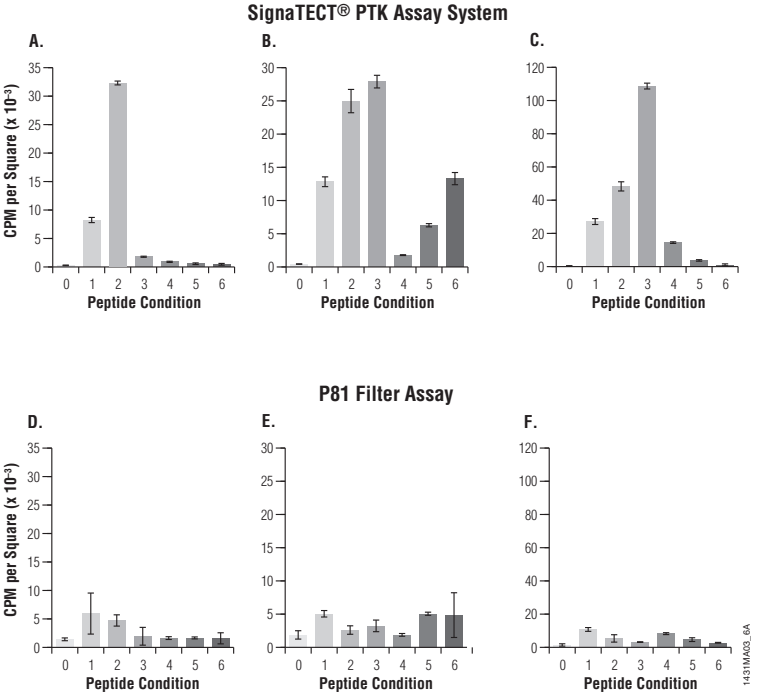
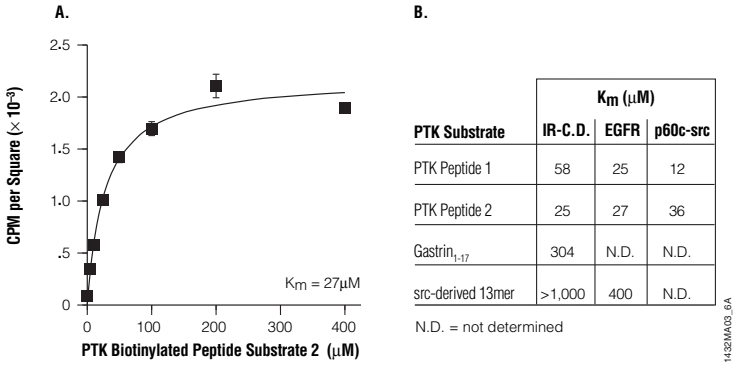


Figure 1. Comparison of PTK activity measurements: SignaTECT® PTK Assay System vs. standard phosphocellulose filter P81 assay. Activities were assessed for a panel of PTK biotinylated peptide substrates with three distinct PTK enzymes. Assays were performed as described in Section 3 using Termination Protocol A for the SignaTECT® PTK Assay System (**Panels A, B and C**) or a P81 filter assay (**Panels D, E and F**). All peptides were biotinylated and were used at a final concentration of 250µM. PTKs included: (**Panels A and D**) IR-C.D. (insulin receptor, cytosolic domain), a soluble 41kDa derivative of the PTK domain of the human insulin receptor, used at 5ng or 125fmol; (**Panels B and E**) EGFR (Epidermal Growth Factor Receptor, Cat.# V5551) purified from A431 cells, at 185ng or 1pmol; (**Panels C and F**) p60c-src PTK at approximately 10ng or 167fmol (Oncogene Science Cat.# PK02).

Peptide conditions:

0 no peptide
1 $gastrin_{1-17}$
2 PTK Peptide Substrate 1
3 PTK Peptide Substrate 2
4 p34cdc2-derived peptide
5 p60c-src derived peptide
6 EGFR-selective peptide



PTK Substrate	Km (µM) IR-C.D.	EGFR	p60c-src
PTK Peptide 1	58	25	12
PTK Peptide 2	25	27	36
$Gastrin_{1-17}$	304	N.D.	N.D.
src-derived 13mer	>1,000	400	N.D.

N.D. = not determined

Figure 2. Kinetic analysis of PTK activity using the SignaTECT® PTK Assay System. **Panel A.** Assays were performed as described in Section 3 using Termination Protocol A (except that the reactions were conducted at 0°C). EGFR (Cat.# V5551) was used at 185ng or 1pmol per reaction. K_m values were determined by nonlinear curve fitting using a hyperbolic function. The K_m value for EGFR and PTK Peptide 2 is 27µM. **Panel B.** K_m values obtained with PTK Biotinylated Peptide Substrates 1 and 2 were analyzed using the SignaTECT® PTK Assay System and Termination Protocol A. K_m values are strikingly low compared to other peptide substrates commonly used to measure PTK activity (8). (IR-C.D. is insulin receptor, cytosolic domain.)

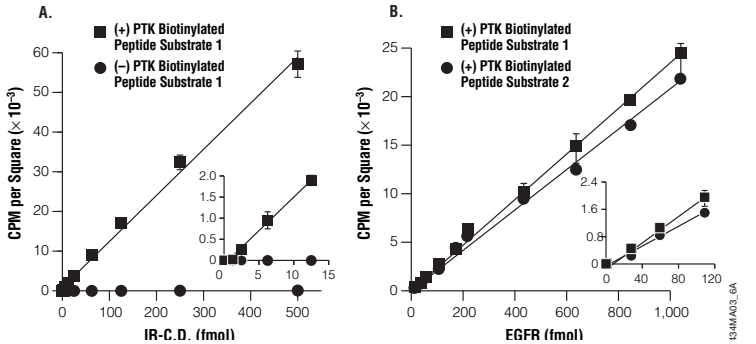


Figure 3. Linear detection of PTK activity using the SignaTECT® PTK Assay System. Assays were performed as described in Section 3 using Termination Protocol A. All peptides were biotinylated and were used at a final concentration of 250µM. Insets show expanded view of low enzyme concentrations. **Panel A.** IR-C.D. activity was measured in the absence (circles) or presence (squares) of PTK Biotinylated Peptide Substrate 1. **Panel B.** EGFR activity (Cat.# V5551) was measured in the presence of either PTK Biotinylated Peptide Substrate 1 (squares) or PTK Biotinylated Peptide Substrate 2 (circles). Summary: The high V_{max} and low K_m values observed with PTK Biotinylated Peptide Substrates 1 and 2, combined with the low backgrounds obtained with the SignaTECT® Protein Tyrosine Kinase Assay System, provide increased sensitivity (IR-C.D. and EGFR detection limits of approximately 2.5fmol and 25fmol, respectively).

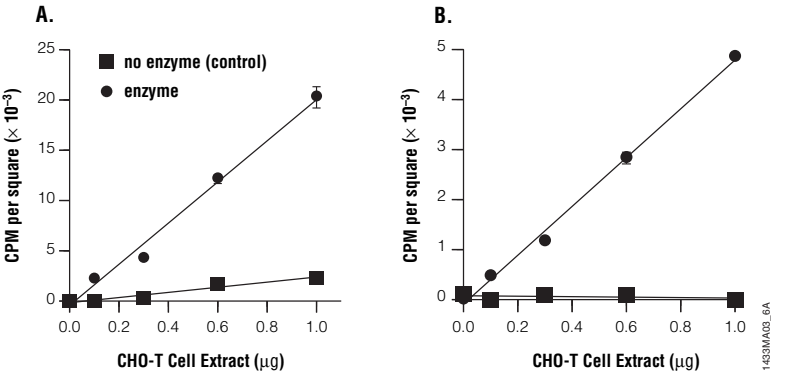


Figure 4. Detection of PTK activity in crude cell extracts using the SignaTECT® PTK Assay System. Crude cell extracts were prepared from CHO-T cells (approximately 1 × 10^6 insulin receptors per cell). The PTK sample was exchanged into a PTK sample buffer as described in Section 6 and 0–1µg was used per 25µl final reaction volume. Assays were performed as described in Section 3 and were terminated with either (**Panel A**) 7.5M guanidine hydrochloride (Termination Protocol A) or (**Panel B**) 43% TCA (Termination Protocol B). The PTK Biotinylated Peptide Substrate 1 was used at a final concentration of 250µM.

4. Appendix

4.A. Preparation of Cell Extract Samples for the SignaTECT® Protein Tyrosine Kinase Assay System

The following protocol is based on the method of Ellis *et al.* (9) for detection of insulin receptor (a transmembrane PTK) activity in crude cell extracts. This protocol was used to generate the data presented in Figure 4 (see Section 3.F).

Materials to Be Supplied by the User

(Solution compositions are provided in Section 4.C.)

- cell extraction buffer(s) including protease inhibitors, if required
- phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}
- (Invitrogen Cat.# D-PBS)
- rocker platform at 4°C

PTK Extraction from Cultured Cells

Culture cells in 10cm tissue culture plates in the appropriate growth medium supplemented with 5–10% fetal bovine serum until approximately 75% confluent (approximately 7 × 10^6 cells). Wash cells twice with phosphate buffered saline (PBS) and then incubate for 12–18 hours in growth medium without serum (serum starvation). Replace the medium with the identical medium but containing an appropriate inducer of PTK activity (e.g., insulin for cells expressing insulin receptors or 10% serum as a general inducer) and incubate for 30 minutes at 37°C. Wash the cells with PBS (5ml per 10cm dish) and remove the buffer completely. Add the appropriate extraction buffer and process as described below:

Solubilize the cells by adding 2ml of membrane-bound PTK extraction buffer (containing 1% Triton® X-100) per 10cm plate for 10 minutes on ice. Detach any residual cells using a cell scraper. Gently mix the cell suspension several times with a pipet tip or syringe and 20-gauge needle. Collect the suspension into an appropriately sized tube and rock for 15 minutes at 4°C. Centrifuge the suspension at 100,000 × g at 4°C for 1 hour and save the supernatant. Proceed to Step 3.A of the PTK Assay Protocol. **Note:** Each of these preparations can be dispensed into small volumes and stored at –80°C, where they are stable for at least 3 months.

4.B. Factors Influencing Quantitation of Protein Tyrosine Kinase Activity

The PTK Biotinylated Peptide Substrate

The supplied PTK Biotinylated Peptide Substrates ensure broad applicability of the SignaTECT® Protein Tyrosine Kinase Assay System, allowing analysis of multiple PTKs, including membrane-bound receptors (of which there are at least 6 different classes; see reference 9) and cytoplasmic enzymes (e.g., p60src; 2). The Peptide Substrates are novel and proprietary sequences, which contain a single tyrosine phosphorylation site. These peptides are readily phosphorylated by a variety of PTKs, including both transmembrane receptor PTKs (epidermal growth factor receptor, insulin receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor) and cytosolic PTK enzymes (p60src, p56lck, p93fes and p43abl). **Empirically determine which of the supplied PTK Biotinylated Peptide Substrates works best for each application.**

Note: The supplied reaction buffer provides 20mM $MgCl_2$ and 1mM $MnCl_2$ as final concentrations.

The PTK Assay Buffer

The PTK Assay 5X Buffer provided has been optimized for measuring the PTK activity of several distinct classes of PTK enzymes (see Figure 1, Section 3.F). The optimum levels of divalent cations and detergents must be determined empirically for each enzyme assayed.

Protein Tyrosine Phosphatase Inhibitors

In the SignaTECT® Protein Tyrosine Kinase Assay System, we include 8mM β-glycerophosphate (supplied in the assay buffer) and 0.1mM Sodium Vanadate as inhibitors of protein tyrosine phosphatases (PTPases). PTPases present in the sample could cause dephosphorylation of the PTK Biotinylated Peptide Substrate. Addition of Sodium Vanadate is optional, since not all samples will contain PTPases. The presence of PTPases and the effectiveness of PTPase inhibitors can rapidly be determined using the non-radioactive Tyrosine Phosphatase Assay System (Cat.# V2471).

Reducing Agent

The SignaTECT® PTK Assay System includes a 100mM stock of Dithiothreitol (DTT) that can be used as a reducing agent in the PTK reaction (typical final concentration: 1–2mM). The presence of a reducing agent may aid in

4.B. Factors Influencing Quantitation of Protein Tyrosine Kinase Activity (continued)

determining the activity of a variety of soluble PTK enzymes, including thenaturally soluble p60^{src}-like PTKs and the soluble, truncated derivatives that contain the catalytic domain of transmembrane PTKs (such as those generated from the insulin receptor and EGFR). Reducing agents prevent disulfide bond formation between free thiol groups. They can also inhibit certain proteases that might degrade the PTK or the Peptide Substrate.

In contrast to their benefits for soluble PTKs, reducing agents may adversely affect the full-length transmembrane receptor PTKs due to the presence of critical disulfide bonds within their extracellular domains. However, we recommend testing the sensitivity of full-length transmembrane PTK enzymes to reducing agents because low concentrations of a reducing agent (e.g., less than 1mM DTT) may result in higher PTK activity.

Sample Buffer

PTK enzyme preparations can be suspended in a variety of sample buffers. Individual buffer components should be tested separately to determine the optimal sample buffer for the particular PTK under investigation. This PTK buffer then can be incorporated into the final step of a purification protocol. Certain PTK inhibitors can be present in buffers, causing low PTK activity. To optimize a sample that has low PTK activity, the final preparation can be rapidly exchanged into an optimized sample buffer using a commercially available spin column (e.g., Sephadex® G-25 Resin; GE Healthcare Biosciences) prior to assaying PTK activity (Section 3.B).

Compatibility with a Variety of Biological Samples

The SignaTECT® Protein Tyrosine Kinase Assay System measures PTK activity in a variety of biological samples. Samples generally vary in the levels and the specific activity of these enzymes. Some samples may contain high levels of proteins that are readily phosphorylated and can bind to various membranes. The SAM^{2}® Membrane reduces this nonspecific signal while remaining compatible with a variety of samples. However, some extracts may require additional optimization to obtain ideal signal-to-noise ratios. To accommodate this potential need, we have developed two termination protocols, which are described in Section 3.C. Using Termination Protocol A, the reaction is simply terminated by adding guanidine hydrochloride to a final concentration of 2.5M. Using Termination Protocol B, the reactions are terminated by a modified TCA precipitation step.

Note: If backgrounds in the absence of substrate and using Termination Protocol A are higher than desired, use Protocol B to reduce background further (e.g., if the signal-to-noise ratio is less than 3 with Protocol A, use of Protocol B may be appropriate, depending on the nature of the sample). When samples are matched with the appropriate termination protocol, both procedures produce low background values (e.g., 100–500cpm using 0.5µCi of [γ-^{32}P]ATP [approximately 1.11 × 10^{6}dpm] per reaction).

4.C. Composition of Buffers and Solutions

PTK Assay 5X Buffer

40mM	imidazole hydrochloride (pH 7.3)
40mM	β-glycerophosphate
1mM	EGTA
100mM	$MgCl_2$
5mM	$MnCl_2$
0.5mg/ml	bovine serum albumin (BSA)

PTK Enzyme Dilution 5X Buffer

40mM	imidazole hydrochloride (pH 7.3)
40mM	β-glycerophosphate
0.5mg/ml	bovine serum albumin (BSA)

Termination Buffer

7.5M	guanidine hydrochloride

Trichloroacetic Acid

43%	Trichloroacetic Acid (TCA)

TCA Neutralization 5X Buffer

A proprietary mixture that facilitates neutralization of the TCA supernatant and reduces non-specific binding to the SAM²® Membrane.

membrane-bound PTK extraction buffer

20mM	HEPES (pH 7.4)
1%	Triton® X-100
5mM	EDTA
50mM	NaCl
30µM	β-glycerophosphate
50mM	sodium fluoride
50µg/ml	aprotinin
10µM	leupeptin
1mM	AEBSF

10X protease inhibitor stock (100µl)

15µl	aprotinin, 6.6mg/ml
1µl	leupeptin, 10mM
5µl	AEBSF, 200mM
79µl	deionized water

optimized PTK sample buffer

Listed below is an example of a sample buffer that has been optimized for a variety of PTKs in crude cell extracts. This buffer can be used to exchange final PTK samples as described in Section 4.B.

8mM	imidazole hydrochloride (pH 7.3)
8mM	β-glycerophosphate (pH 7.3)
50mM	NaCl
50µg/ml	aprotinin
10µM	leupeptin
1mM	AEBSF
100µM	sodium vanadate
5.0µM	zinc chloride
0.05%	Triton® X-100

2M NaCl

116.9g/L	NaCl

2M NaCl in 1% H_3PO_4

116.9g/L	NaCl
11.8ml/L	85% H_3PO_4

PBS with Ca^{2+}, Mg^{2+}

0.1g/L	$CaCl_2$
0.2g/L	KCl
0.2g/L	KH_2PO_4
0.1g/L	$MgCl_2 \cdot 6H_2O$
8.0g/L	NaCl
1.15g/L	Na_2HPO_4
2.16g/L	$Na_2HPO_4 \cdot 7H_2O$

4.D. SAM2® Biotin Capture Membrane Template

Promega				SAM2® Biotin Capture Membrane							
1	9	17	25	33	41	49	57	65	73	81	89
2	10	18	26	34	42	50	58	66	74	82	90
3	11	19	27	35	43	51	59	67	75	83	91
4	12	20	28	36	44	52	60	68	76	84	92
5	13	21	29	37	45	53	61	69	77	85	93
6	14	22	30	38	46	54	62	70	78	86	94
7	15	23	31	39	47	55	63	71	79	87	95
8	16	24	32	40	48	56	64	72	80	88	96

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4.E. References

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4.F. Related Products

Product	Size	Cat.#
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase Assay System	96 reactions	V8161
ProFluor® Src-Family Kinase Assay	4 plate	V1270
	8 plate	V1271
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	100ml	V6713
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	100ml	V3773
Kinase-Glo® Max Luminescent Kinase Assay	10ml	V6071
	100ml	V6073
Tyrosine Phosphatase Assay System	96 reactions	V2471
Serine/Threonine Phosphatase Assay System	96 reactions	V2460
Epidermal Growth Factor, Human, Recombinant	100µg	G5021
Epidermal Growth Factor Receptor (EGFR)	10 units	V5551

[a]U.S. Pat. Nos. 6,066,462, 6,348,310 and 6,753,157 and European Pat. No. 0 760 678 B9 have been issued to Promega Corporation for quantitation of protein kinase activity.