

# Detection and Quantitation of Protein Tyrosine Kinases



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*Widespread appreciation of the complexity of signaling pathways involving the protein tyrosine kinase (PTK) superfamily of enzymes has resulted in the need for more sophisticated reagents to dissect the role of PTKs in normal and disease states. To meet this need, Promega has developed the SignaTECT™ PTK Assay System, a novel method based on the use of optimized PTK Biotinylated Peptide Substrates in conjunction with the unique SAM<sup>2</sup>™ Biotin Capture Membrane. This system provides selective, accurate and rapid detection of PTK activity, while minimizing handling and radioisotope requirements. The SignaTECT™ PTK Assay System is a broad-specificity assay that measures the activity of a wide variety of PTKs, including both transmembrane receptor and cytosolic enzymes.*

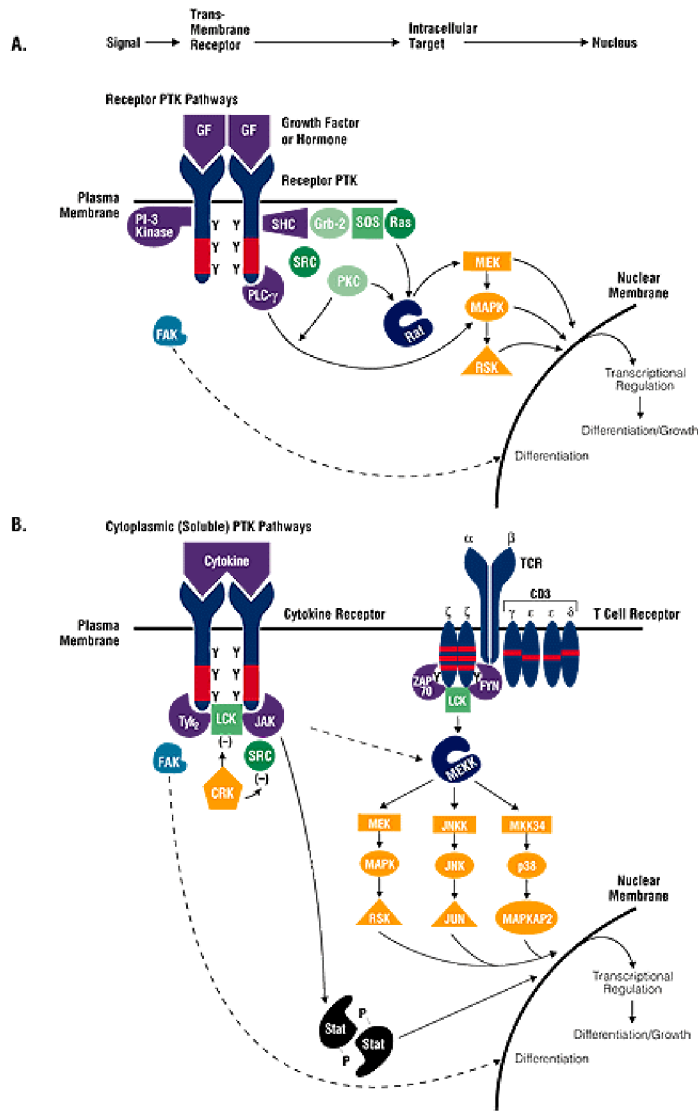
## Introduction

Protein tyrosine kinases (PTKs) modulate a wide variety of cellular events, including differentiation, growth, metabolism and apoptosis (1-4). Phosphorylation of tyrosine residues in target proteins is essential for maintaining cellular homeostasis, yet this posttranslational modification also provides the means by which a number of cellular oncogenes deregulate various signaling pathways and induce transformation. PTKs are therefore important targets for both basic research and drug development efforts (3).

PTKs represent a diverse and rapidly expanding superfamily of protein kinases, including both transmembrane and soluble cytoplasmic enzymes ([Table 1](#)). Activation of the PTK domain of either class of PTK enzymes results in interaction of the protein with other signal transducing molecules and propagation of the signal along a specific signal transduction pathway ([Figure 1](#); 1-9).

**Table 1. Classification and Function of PTKs.**

Transmembrane Receptor PTKs			
PTK Enzyme Family	PTKs	Involvement in Cellular Signaling (Disease States)	Representative References
Axl	Axl, Mer/Nyk, Rse	integrin signaling	Mark <i>et al.</i> (1996) <i>J. Biol. Chem.</i> <b>271</b> , 9785.
Eph	CEK5, CEK8, EBK, ECK, EEK, EHK-1, EHK-2, ELK, EPH, ERK, HEK, MDK2, MDK5, SEK	growth, differentiation, neurobiology (epithelial cell cancer)	Pandey <i>et al.</i> (1996) <i>Curr. Opin. Biol.</i> <b>5</b> , 986.
Epidermal growth factor receptor (EGFR)	EGF-R, HER2/neu, HER3, HER4, ErbB, ErbB2, ErbB3, ErbB4, Xmrk, DER, let23	growth (breast and squamous cell carcinoma, psoriasis)	Carraway and Burden (1996) <i>Curr. Opin. Neurobiol.</i> <b>5</b> , 606.
Fibroblast growth factor receptor (FGFR)	FGF-R1, FGF-R2/BEK/CEK3, FGF-R3/CEK2, FGF-R4/TKF, KGF-R	growth, differentiation (colon and prostate cancer)	Naski <i>et al.</i> (1996) <i>Nature Genetics</i> <b>13</b> , 233.
Hepatocyte growth/scatter factor receptor (HGFR)	HGF-R, MET, RON, SEA, SEX	growth, differentiation (cancer)	Rosen and Goldberg (1996) <i>Adv. Cancer Res.</i> <b>67</b> , 257.
Insulin receptor (IR)	I-R, IGF1-R	differentiation, metabolism (diabetes)	Paz <i>et al.</i> (1996) <i>J. Biol. Chem.</i> <b>271</b> , 6998.
Nerve growth factor receptor (NGFR or Trk)	Trk A, Trk B, Trk C	neuronal differentiation, neurite outgrowth	Carter <i>et al.</i> (1996) <i>Science</i> <b>272</b> , 542.
RET	RET	B cell, kidney and neural crest development (Hirschsprung's disease, multiple endocrine neoplasia, medullary thyroid cancer)	Iwashita <i>et al.</i> (1996) <i>Oncogene</i> <b>12</b> , 481.
Platelet-derived growth factor receptor (PDGFR)	PDGF $\alpha$ -R, PDGF $\beta$ -R, CSF1-R/FMS, SCF-R/KIT, VEGF-R/FLT, NEK/FLK1, FLT3/FLK2/STK-1	growth, differentiation, cytokine and vascular regulation (leukemia, gliomas)	Rousset <i>et al.</i> (1995) <i>J. Mol. Evol.</i> <b>41</b> , 421.
Soluble Cytoplasmic PTKs			
PTK Enzyme Family	PTKs	Involvement in Cellular Signaling (Disease States)	Representative References
ABL	p43 <sup>abl</sup> , ARG	cell cycle regulation, direct coupling to DNA (CML)	Juang <i>et al.</i> (1996) <i>Oncogene</i> <b>12</b> , 1033.
BTK	BTK, ITK/EMT, TEC	B cell activation and development (Bruton's Disease)	Rawlings <i>et al.</i> (1996) <i>Science</i> <b>271</b> , 822.
CSK	p50 <sup>csk</sup> , p56 <sup>lck</sup> , CTK/CRK	T cell activation, negative regulation of src family kinases	Zenner <i>et al.</i> (1996) <i>BioEssays</i> <b>17</b> , 967.
FAK	p112 <sup>FAK</sup> , p125 <sup>hck</sup>	integrin signaling, focal adhesion, differentiation	Plopper <i>et al.</i> (1996) <i>Mol. Cell. Biol.</i> <b>6</b> , 1349.
FPS	p93 <sup>hck/tyk</sup> , p94 <sup>lck</sup>	cytokine signaling, hematopoiesis	Brizzi <i>et al.</i> (1996) <i>J. Biol. Chem.</i> <b>271</b> , 3562.
JAK	p130 <sup>jak1</sup> , p130 <sup>jak2</sup> , p130/p135 <sup>tyk2</sup>	cytokine signaling (STAT), transcriptional activation (ALL)	Schindler and Darnell (1995) <i>Ann. Rev. Biochem.</i> <b>64</b> , 1255.
SRC	p55 <sup>src</sup> , p53/p56 <sup>lck</sup> , p56 <sup>lyn</sup> , p55/p57 <sup>hck</sup> , p59 <sup>hck</sup> , p59 <sup>hck</sup> , p60 <sup>src</sup> , p60 <sup>hck</sup> , p62 <sup>yes</sup>	cell membrane associated via myristate group (cancer)	Marengere <i>et al.</i> (1996) <i>Science</i> <b>272</b> , 1170.
SYK	p70 <sup>syk</sup> , p72 <sup>hck</sup>	B and T cell signaling (autoimmunity)	Kanner <i>et al.</i> (1996) <i>Tissue Antigens</i> <b>46</b> , 145.



**Figure 1. Signaling by protein tyrosine kinases (PTKs).** **Panel A:** Transmembrane receptor PTKs. Autophosphorylation of tyrosine residues on the PTK is triggered by binding of the appropriate extracellular ligand (e.g., growth factor or hormone) to the transmembrane receptor binding site and results in activation of the intrinsic PTK activity of the receptor. This in turn causes additional protein-protein interactions to occur that modulate the activity and location of a variety of intracellular signaling molecules, thereby determining the appropriate physiological response. **Panel B:** Soluble cytoplasmic PTKs. Binding of an extracellular ligand to a non-PTK receptor (e.g., cytokine binding to a cytokine receptor) or activation of the T cell receptor leads to association and activation of a series of intracellular and soluble PTK molecules (e.g., p53/p56<sup>lck</sup>, p60<sup>src</sup>, p70<sup>zap</sup>). Significant overlap exists between the signaling cascades shown in each panel with both positive and negative contributions. Moreover, the balance between protein phosphorylation and dephosphorylation events (protein phosphatases are not shown) determines the final response of the cell to the stimulus. Abbreviations: Y indicates tyrosine residues; dashed line indicates putative involvement; - indicates inhibition.

Activation of transmembrane PTKs is typically initiated by binding of a ligand (e.g., hormone or growth factor) to a specific site within the extracellular domain of the receptor (Figure 1A). Upon ligand binding, these receptors commonly undergo dimerization, resulting in autophosphorylation of tyrosine residues within the cytoplasmic domain. This autophosphorylation event can occur in *trans* (between receptor molecules within the dimer) or in *cis* (within a single receptor molecule in the dimer; 5,6). These phosphorylation events activate the kinase, thereby increasing its intrinsic PTK activity, and produce new binding sites for intracellular adapter molecules. For example, the association of the phosphotyrosine-binding src homology domains (SH-2 or SH-3) of adapter proteins such as Grb2 and Shc with an autophosphorylated receptor provides the molecular interactions that bring signal transduction molecules into close proximity (Figure 1A; 1,3,4). The GTP-bound form of Ras then binds to Raf protein kinase (a MAPK kinase kinase) isoforms, including C-Raf-1, B-Raf and A-Raf. This interaction results in targeting of Raf to the membrane where its protein kinase activity is increased by phosphorylation, thereby allowing it to activate other signaling molecules.

Receptors which lack PTK activity but which harbor sites for tyrosine phosphorylation (often catalyzed by the soluble cytoplasmic PTK

enzymes) activate identical or similar enzyme cascades via the association of their phosphotyrosine residues with adapter molecules (Figure 1B; 2,7,8). For example, phosphorylation of the zeta chain of the T cell receptor by p53/56<sup>lck</sup> or p59<sup>fyn</sup> induces p70<sup>ZAP</sup> association and activation (10). Similarly, phosphorylation of the cytoplasmic domains of cytokine receptors by p53/56<sup>lck</sup> leads to association and activation of the JAK family of soluble cytoplasmic PTKs (8,11).

Significant overlap exists between these general signaling pathways, including the activation of the mitogen-activated/extracellular signal-regulated protein kinase (MAPK or ERK) superfamily (4,9). In general, the ERK1 and ERK2 subfamily is activated by growth factor/hormone signaling, while the JNK and p38 subfamilies are activated by various cytokines and stress (4,9). The resulting cascade involves a series of phosphorylation events mediated by PTKs as well as Ser/Thr protein kinases (e.g., PKA, PKC, MEKs, MEK, etc.) that can either activate or inhibit the proteins involved. The physiological outcome of these signal transduction pathways is determined by the interplay between protein kinases and protein phosphatases.

To facilitate the analysis of PTK activity, Promega recently introduced the SignaTECT™ Protein Tyrosine Kinase (PTK) Assay System. This system combines two unique and optimized peptide substrates with the proprietary SAM<sup>2</sup>™ Biotin Capture Membrane to generate a superior broad-specificity PTK assay system that provides high signal-to-noise ratios.

## Common methods for detecting PTK activity

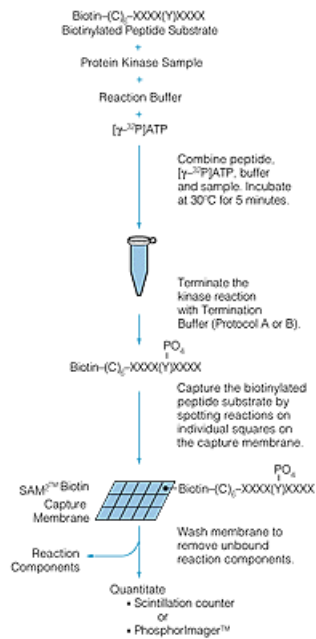
Although protein phosphorylation is a common posttranslational modification (~10% of all proteins are phosphorylated), tyrosine phosphorylation comprises only 0.01% of these events. Detection of PTK phosphorylation is further complicated by the generally low abundance of PTK enzymes in cells and tissues, and the low intrinsic phosphotransferase activity of PTKs compared to other commonly studied Ser/Thr protein kinases (e.g., PKA, PKC, MAP kinases). The most common method for assaying PTK activity involves measuring the transfer of <sup>32</sup>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, <sup>32</sup>P-labeled kinase substrate is typically bound to these filters via weak electrostatic interactions; therefore, substrate can be lost from the filters during the washing procedure. In addition, peptide substrates often exhibit wide variability in binding to phosphocellulose filters (12,13). In the presence of multiple kinases (e.g., in a cell or tissue extract), the <sup>32</sup>P-labeled peptides or proteins bound to the phosphocellulose filter also may reflect kinase activity other than that of PTKs. Additionally, [ $\gamma$ -<sup>32</sup>P]ATP preparations can contain <sup>32</sup>P-labeled contaminants that possess a positive charge at low pH, allowing them to bind to phosphocellulose filters (14). These variables often result in higher backgrounds and lower signal-to-noise ratios.

A variety of other methods are also used to measure PTK activity. Immunoprecipitation-based kinase assays remove the specific kinase of interest from a complex mixture; however, this approach is labor-intensive and the resulting signal depends greatly on the nature of the antibody. Similarly, phosphorylation of proteins with [ $\gamma$ -<sup>32</sup>P]ATP and separation by polyacrylamide gel electrophoresis typically requires extensive handling of large amounts of isotope. The use of [ $\gamma$ -<sup>32</sup>P]ATP to label the protein of interest can be avoided by using a Western blotting approach with an anti-phosphotyrosine antibody to detect the phosphorylated proteins. However, it can be difficult to obtain linear signals for quantitation, and recognition is once again highly dependent on the nature of both the antibody and the reactive epitope within the antigen.

## SAM<sup>2</sup>™ Biotin Capture Membrane and the SignaTECT™ Protein Tyrosine Kinase Assay System

Promega's SignaTECT™ Protein Tyrosine Kinase Assay System (Figure 2) features the SAM<sup>2</sup>™ Biotin Capture Membrane\* (15). This membrane is a unique and proprietary format (14) in which streptavidin is attached covalently to a solid support. The SAM<sup>2</sup>™ Biotin Capture Membrane is provided as a numbered and perforated sheet, allowing for ease of sample application and quantitation of the signal by scintillation counting, autoradiography or PhosphorImager™ analysis. The membrane provides low nonspecific binding and high linear binding capacity (>2nmol/cm<sup>2</sup>). These advantages result in high signal-to-noise ratios and high sensitivity, with enzyme detection limits typically in the low femtomole range. Importantly, capture of the biotinylated target peptide substrates is independent of the amino acid sequence of the substrate. Therefore, one can directly compare multiple target peptides simultaneously as well as measuring the activity of several kinases using different peptide substrates. The low background and high specificity of this system allows protein kinase activity to be assayed from crude cell extracts, even in the presence of contaminating kinases.

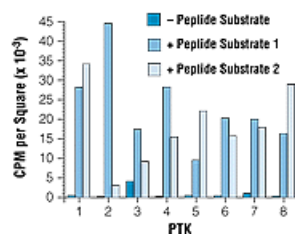
\*Patent pending.



**Figure 2. Flow chart representing the SignaTECT™ PTK Assay Protocol.** The PTK Biotinylated Peptide Substrate is phosphorylated by the enzyme of interest, the reaction is terminated with either Protocol A or B (18) and the peptides are captured onto the SAM<sup>2</sup>™ Biotin Capture Membrane. Following the brief washing step, the signal is quantitated as indicated.

Tagging the peptide substrates with biotin via a long chain (six carbon spacer) minimizes interference from the biotin moiety and eliminates the need to alter the amino acid sequence of the peptide, which is often required in other assay systems based on the phosphocellulose P-81 assay format (13). Such alteration (e.g., addition of multiple basic amino acids on one end of the peptide) may result in decreased binding kinetics or specificity (17). Since the binding of biotin to streptavidin is rapid and strong, the association is unaffected by rigorous washing procedures (unlike standard phosphocellulose filter assays), including wide extremes in pH, temperature, salt concentration and denaturing agents. Following phosphorylation of the peptide substrate, the reaction is terminated with guanidine hydrochloride (final concentration of 2.5M) (Protocol A; 16,18) and the biotinylated substrate is captured onto the membrane. The membrane is then briefly washed in 2M NaCl and 2M NaCl/1% phosphoric acid. The nonbiotinylated components (including free [ $\gamma$ -<sup>32</sup>P]ATP and nonbiotinylated proteins) are removed by this simple 20 minute washing procedure that typically produces background values of 100300cpm (approximately  $1.1 \times 10^6$  dpm) using 0.5 $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP.

The assay buffer supplied with the SignaTECT™ PTK Assay System has been optimized for measuring the PTK activity of several distinct classes of PTK enzymes (i.e., IR, EGFR, p60<sup>src</sup>). The buffer is composed of 8mM imidazole-HCl, 8mM beta-glycerophosphate, 200 $\mu$ M EGTA, 20 $\mu$ M MgCl<sub>2</sub>, 1mM MnCl<sub>2</sub>, 100 $\mu$ M sodium vanadate, 20 $\mu$ M ATP and 100 $\mu$ g/ml bovine serum albumin (final concentrations). The beta-glycerophosphate and sodium vanadate inhibit tyrosine phosphatases, and the bovine serum albumin serves as a carrier to minimize nonspecific interactions. Although this buffer has proven effective with a wide range of PTK enzymes (Figure 3), it may be necessary to determine the specific requirements for the PTK of interest. Reaction parameters that may require optimization include the concentration of divalent cations, reducing agent, phosphatase inhibitor and/or the need for a nonionic detergent (e.g., NP-40 or Triton® X-100) (18).



**Figure 3. Comparison of enzyme activity of multiple PTK enzymes.** Assays containing PTK Biotinylated Substrate 1, PTK Biotinylated Substrate 2 or no substrate were performed as described (18) using termination Protocol A. Following termination of the kinase reactions, 12.5 $\mu$ l of each reaction was spotted onto each square of the streptavidin matrix. This equates to 2.1nmol of biotinylated peptide or 2.5nmoles per cm<sup>2</sup>, which is 69% of the total linear binding capacity of the

SAM<sup>2</sup>™ Biotin Capture Membrane (i.e., 3.6nmol/cm<sup>2</sup> for PTK Biotinylated Substrates 1 and 2). Each biotinylated peptide substrate was used at 250μM. PTK enzymes used were as follows: 1, EGFR (1,000fmol); 2, IR-CD (62.5fmol); 3, PDGFR-CD (2,500fmol); 4, FGFR-CD (125fmol); 5, p60<sup>src</sup> (287fmol); 6, p56<sup>lck</sup> (7,900fmol); 7, p93<sup>fes</sup> (250fmol); 8, p43<sup>abl</sup> (21units).

## Broad spectrum detection of PTK activity using the SignaTECT™ PTK Assay System

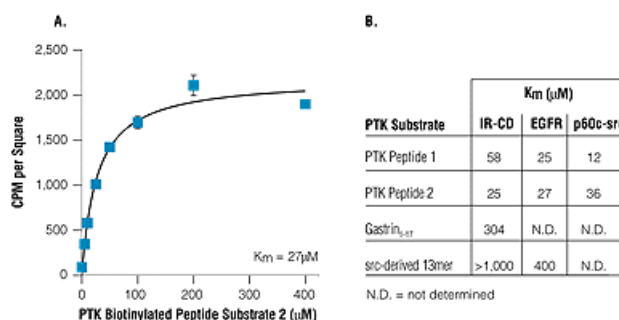
The SignaTECT™ PTK Assay System combines the SAM<sup>2</sup>™ Biotin Capture Membrane with two PTK Biotinylated Substrates\* to overcome the difficulties commonly encountered when measuring PTK activity. The two optimized biotinylated substrates are novel and proprietary sequences that contain a single tyrosine phosphorylation site and are readily phosphorylated by a variety of PTKs. These peptide substrates have been optimized for broad-specificity and ideal kinetic properties (i.e., low  $K_m$  and high  $V_{max}$  values). The ability to efficiently capture the biotinylated peptide substrates onto the streptavidin matrix independent of the amino acid sequence of the peptide allows multiple peptides to be compared. This advantage is illustrated in [Figure 3](#), which compares substrate phosphorylation of the two PTK Biotinylated Peptide Substrates by eight different PTKs, including both transmembrane receptor PTK enzymes (epidermal growth factor receptor [EGFR], insulin receptor [IR], platelet-derived growth factor receptor [PDGFR] and fibroblast growth factor receptor [FGFR]) and cytosolic PTK enzymes (p43<sup>abl</sup>, p53/p56<sup>lck</sup>, p60src and p93<sup>fes</sup>). These results show that PTK Biotinylated Peptide 1 provides the highest signals with the IR, PDGFR, FGFR, p56<sup>lck</sup> and p93<sup>fes</sup> PTKs, while PTK Biotinylated Peptide 2 provides the highest signals with the EGFR, p43<sup>abl</sup> and p60<sup>src</sup> enzymes. With other PTKs, an initial assessment should be made to determine which PTK Biotinylated Peptide Substrate works best for a particular application. While each of the biotinylated substrates results in strong signals with the eight PTKs tested, the corresponding signals obtained with the P-81 based assay method were less than 10% of the signals observed with the SignaTECT™ PTK Assay System (15,18). This finding supports the observation that the acidic nature of most PTK peptide substrates results in poor capture onto phosphocellulose filters even if additional basic amino acids are added to improve capture efficiencies (12,13,17,19).

\*Patent pending.

The use of truncated catalytic domain (CD) derivatives of the IR, FGFR and PDGF PTKs in [Figure 3](#) illustrates a common approach used in characterizing the enzymatic and structural properties of these domains in the transmembrane receptor PTKs. Thus, it is often possible to express recombinant cDNAs that have had the extracellular and transmembrane regions deleted, resulting in soluble and catalytically active forms of the intracellular kinase domain (20,21). These truncated versions can be manipulated without the use of detergents and they are either constitutively active or can be activated with a suitable activation buffer (20).

## Kinetic analysis of PTK activity using the SignaTECT™ PTK Assay System Peptide Substrates

Although a variety of peptide substrates commonly are used to measure PTK enzyme activity (e.g., p34<sup>cdc2</sup>-derived peptide, gastrin<sub>1-17</sub> and src-derived peptide), these peptides typically have  $K_m$  values greater than 300μM and often greater than 1mM ([Figure 4B](#); 22). In contrast, titration of the optimized PTK Biotinylated Peptide Substrates that are supplied with the SignaTECT™ PTK Assay System demonstrate very low  $K_m$  values (10-60μM). In addition, the  $V_{max}$  values observed with these optimized peptide substrates are 2- to 5-fold higher than values obtained with the p34<sup>cdc2</sup>-derived peptide, gastrin<sub>1-17</sub> and the src-derived peptide (15,18).

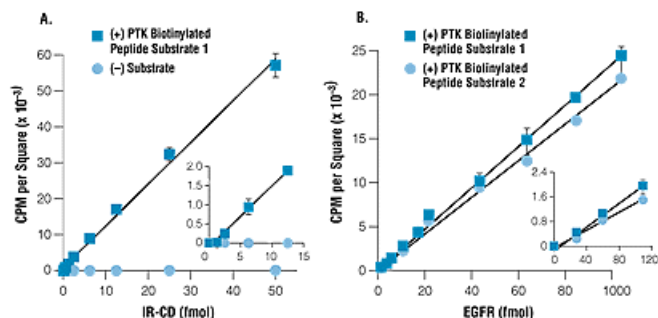


**Figure 4. Kinetic analysis of PTK activity.** Assays were performed as described (18) except that the reactions were incubated at 0°C; termination Protocol A was used.  $K_m$  values were determined by nonlinear curve fitting using a hyperbolic function. **Panel A:** EGFR (Cat.# V5551) was used at 185ng (1pmol) per reaction. The  $K_m$  was 27μM. **Panel B:**

$K_m$  values obtained using PTK Biotinylated Peptide Substrate 1, Peptide Substrate 2 or a biotinylated version of one of the commonly used PTK peptide substrates.

## Linear detection of PTK activity

The high  $V_{max}$  and low  $K_m$  values observed with the PTK Biotinylated Peptide Substrates 1 and 2, combined with the low backgrounds and high linear binding capacity of the SAM<sup>2</sup>™ Biotin Capture Membrane, maximize the linear range of the SignaTECT™ PTK Assay System. Figure 5 illustrates a linear titration of IR-CD and EGFR. Both of these enzymes can be detected in the low femtomole range, with limits of approximately 2.5fmol and 25fmol for IR-CD and EGFR, respectively. This high sensitivity is particularly useful with PTK enzymes that are of low abundance, low intrinsic activity or both.



**Figure 5. Linear detection of PTK activity.** Assays were performed as described (18) using termination Protocol A. All peptides were biotinylated and used at a final concentration of 250 $\mu$ M. Insets show an enlargement of the graph at low enzyme concentrations. **Panel A:** IR-CD activity was measured in the presence of PTK Biotinylated Peptide Substrate 1 or the absence of substrate. **Panel B:** EGFR activity (Cat.# V5551) was measured in the presence of either PTK Biotinylated Peptide Substrate 1 or PTK Biotinylated Peptide Substrate 2.

## Summary

The SignaTECT™ PTK Assay System is well suited for detecting PTK activity in a variety of biological samples, including crude cell extracts and fractions generated during PTK purification and characterization. This system provides a rapid, accurate and highly versatile method to detect and quantitate the enzymatic activity of a variety of PTK enzymes. The current system makes use of two novel and proprietary biotinylated peptide substrates that have been optimized to provide low  $K_m$  values, high  $V_{max}$  values and broad-specificity detection of enzymes in the PTK superfamily. The efficient and uniform capture of the biotinylated peptide substrates onto the SAM<sup>2</sup>™ Biotin Capture Membrane allows for optimum detection and comparison of multiple PTK enzymes and a variety of peptide substrates, thereby offering distinct advantages over other currently available assay technologies.

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### Ordering Information

Product	Size	Cat.#
SignaTECT™ Protein Tyrosine Kinase (PTK) Assay System	96 reactions	V6480

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