Promega Notes Magazine **Number 58, 1996, p. 22**

SAM^{2TM} Biotin Capture Membrane and SignaTECT^{TM} Protein Kinase Assay Systems

By Said Goueli, Erik Schaefer and Allan Tereba
Promega Corporation

Promega's SignaTECT^{TM} Protein Kinase Assay Systems combine the use of biotinylated target peptide substrates with a unique Streptavidin Matrix Membrane to specifically and accurately quantitate the activity of protein kinases in a variety of samples. This technique provides low backgrounds and high signal-to-noise ratios even with complex samples such as crude cell extracts, while retaining the high capacity necessary to maintain optimum reaction kinetics.

Introduction

Recently, substantial progress has been made toward identifying and understanding the role of biologically active molecules responsible for signal transduction in a variety of organisms (1). The signal transduction cascade is generally triggered by the binding of growth factors, cytokines or hormones to specific cell surface receptors. These receptors transmit the stimulus to the interior of the cell, where the signal becomes amplified and directed in a specific manner.

The propagation and amplification of the primary signal involves a wide array of enzymes with very specialized functions. Some of these signaling enzymes are responsible for post-translational modifications of cellular proteins. One of the most common modifications, phosphorylation, plays a dominant role in almost all events in cellular regulation, including cell growth, development, homeostasis and cell death (1). The state of phosphorylation of the intracellular targets is determined by the interplay between protein kinases and protein phosphatases -- some enzymes require phosphorylation for activity, while others are inactivated by phosphorylation (2,3).

Promega recently introduced the SignaTECT^{TM} Protein Kinase Assay Systems. These systems, which feature the SAM^{2TM} Biotin Capture Membrane*, are designed for the sensitive and specific detection of protein tyrosine kinases (PTKs), cAMP-dependent protein kinases (PKA), Ca^{2+} or phospholipid-dependent protein kinases (PKCs) and cdc2 protein kinase (Table 1).

**Patent Pending*

Table 1. Summary of the SignaTECT™ Protein Kinase Assay Systems.

Protein Kinase Assay System	Target Substrate[a] (* - site of PO_4)	Sensitivity	Comments
SignaTECT™ cAMP-Dependent Protein Kinase (PKA) Assay System	Kemptide: LRRAS*LG	≈0.2ng or 5fmol	Directly assay crude extracts
SignaTECT™ Protein Kinase C (PKC) Assay System	Neurogranin$_{(28-43)}$: AAKIQAS*FRGHMARKK	≈0.4ng or 10fmol	Directly assay crude extracts
SignaTECT™ cdc2 Protein Kinase Assay System	Histone H1-derived peptide: PKT*PKKAKKL	0.2µg of mitotic HeLa cell extract	Directly assay crude extracts
SignaTECT™ Protein Tyrosine Kinase (PTK) Assay System	Two novel, proprietary peptides, each with a single tyrosine residue	PTK dependent: e.g., EGFR, 25fmol; IR-CD, 2.5 fmol	Directly assay crude extracts or use a modified TCA precipitation step; shown to detect eight different enzymes.

[a]All peptide substrates are biotinylated at the amino terminus using long-chain biotin.
Abbreviations used: EGFR, epidermal growth factor receptor; IR, insulin receptor.

SAM^{2TM} Biotin Capture Membrane

Promega's proprietary SAM^{2TM} Biotin Capture Membrane features streptavidin covalently attached to a solid support (4). This membrane exhibits a high linear binding capacity for biotinylated target substrates (>2nmol/cm^2) and very low nonspecific binding. These advantages allow for detection of low femtomole levels of protein kinases. Binding of the biotinylated peptide substrate to the membrane is independent of its sequence, allowing comparison of multiple target peptides. In contrast to other protein kinase assay techniques which often require alteration of the amino acid sequence of the target peptide (potentially resulting in reduced specificity and poor binding kinetics), tagging of the target peptide with biotin via a six carbon spacer minimizes alteration of the intrinsic properties of the peptide. The selective capture of the biotinylated peptide substrate by the SAM^{2TM} Biotin Capture Membrane makes it possible to quantitate a particular protein kinase even in the presence of other kinases.

Protein Kinase Assay Systems

The most commonly used method to quantitate peptide substrate phosphorylation, the P-81 phosphocellulose filter assay (5), suffers from a number of distinct disadvantages. First, because the positively charged ^{32}P-labeled kinase substrate is bound to P-81 filters by weak electrostatic interactions, the labeled substrate can be lost during the washing procedure. Second, peptide substrates of equal positive charge often exhibit wide variability in binding to phosphocellulose filters (5). Third, in the presence of other kinases, the amount of ^{32}P bound to the phosphocellulose filter may not accurately reflect the amount of ^{32}P incorporated into the peptide substrate, since any positively charged, phosphorylated protein will bind to the filter. Finally, [gamma-^{32}P]ATP preparations can contain radiolabeled contaminants that possess a positive charge at low pH. These contaminants may bind to phosphocellulose filters, resulting in higher backgrounds and lower signal-to-noise ratios (6).

Promega's SignaTECT™ Protein Kinase Assay Systems overcome the problem of nonspecific binding by using a biotinylated peptide substrate in conjunction with the SAM2™ Biotin Capture Membrane. The binding of biotin to streptavidin is rapid and strong, and the association is unaffected by rigorous washing procedures, denaturing agents, and wide extremes in pH, temperature and salt concentration. High signal-to-noise ratios are generated even with complex samples, while the high substrate capacity allows for optimum reaction kinetics. The systems can be used to measure protein kinase activities using low femtomole levels of purified enzyme or crude cellular extracts (see Table 1).

As outlined in Figure 1, the assay steps and analysis of results are straightforward and require only common laboratory equipment. Following phosphorylation and binding of the biotinylated substrate to the numbered and partially cut squares of the SAM2™ Biotin Capture Membrane, unincorporated [gamma-^{32}P]ATP is removed by a simple washing procedure. This procedure also removes nonbiotinylated proteins that have been phosphorylated by other kinases in the sample. The bound, labeled substrate is then quantitated by scintillation counting or PhosphorImager™ analysis.

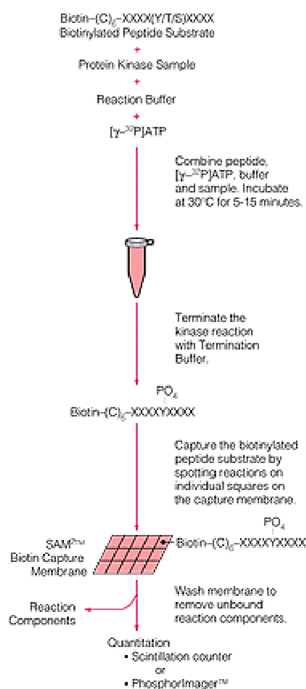

Figure 1. Schematic diagram of the SignaTECT™ Protein Kinase Assay protocol. The illustration shows an overview of the protocol steps to prepare, run and analyze a specific protein kinase activity using any of the SAM2™ Protein Kinase Assay Systems.

SignaTECT™ Protein Tyrosine Kinase Assay System

Protein tyrosine kinases (PTKs) play critical roles in differentiation, growth, metabolism and programmed cell death (1,7). Phosphorylation of tyrosine residues is essential for maintaining cellular homeostasis, yet this post-translational modification is also implicated in the deregulation of various signaling pathways that results in oncogenic transformation (7,8). PTKs represent a diverse group of protein kinases, consisting of both transmembrane and cytoplasmic enzymes. Although protein phosphorylation is a common modification, tyrosine is the target of only 0.01% of all protein phosphorylation events.

The complexity of the signaling pathways (8,9) involving this varied class of enzymes points to the need for more sophisticated experimental reagents. The SignaTECT™ Protein Tyrosine Kinase Assay System rapidly and specifically measures the enzymatic

activities of a variety of transmembrane receptor (e.g., epidermal growth factor receptor, insulin receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor) and cytosolic (e.g., $p43^{abl}$, $p56^{lck}$, $p60^{src}$, $p93^{fes}$) PTK enzymes. The system uses two novel biotinylated peptides that have very low K_m (1060µM) and high V_{max} values for each of the PTKs tested. These peptides, combined with the high binding capacity and low nonspecific binding of the SAM^{2TM} Biotin Capture Membrane, result in high signal-to-noise ratios and the ability to compare signals obtained with multiple PTKs and PTK peptide substrates (Figure 2). This assay can be used to measure enzyme activity of femtomole levels of PTKs using purified or partially purified enzyme preparations or crude cellular extracts.

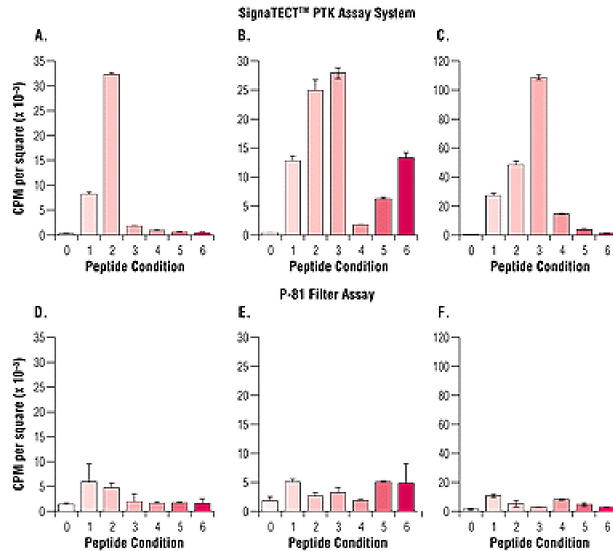

Figure 2. Comparison of PTK activity measurements: Promega's SignaTECT^{TM} PTK Assay System vs. standard P-81 phosphocellulose filter assay. Activities were assessed for a panel of PTK biotinylated peptide substrates with three distinct PTK enzymes. Assays were performed as described for Promega's SignaTECT^{TM} PTK Assay System (Panels A, B and C; 31) or a P-81 filter assay (Panels D, E and F; 5). All peptides were biotinylated and were used at a final concentration of 250µM. PTKs included: **(Panels A and D)** IR-C.D., a soluble 41kDa derivative of the PTK domain of the human insulin receptor, used at 5ng (125fmol); **(Panels B and E)** EGFR (Epidermal Growth Factor Receptor) purified from A431 cells, at 185ng (1pmol) (Promega Cat.# V5551); **(Panels C and F)** p60c-src PTK at ~10ng (167fmol) (Oncogene Science Cat.# PK02). Peptide Condition: 0, no peptide; 1, $gastrin_{1\text{-}17}$; 2, PTK Peptide Substrate 1; 3, PTK Peptide Substrate 2; 4, $p34^{cdc2}$-derived peptide; 5, $p60^{c\text{-}src}$-derived peptide; 6, EGFR-selective peptide.

SignaTECT^{TM} cAMP-Dependent Protein Kinase (PKA) Assay System

cAMP-dependent protein kinase (PKA) is a second messenger-dependent enzyme (10) that has been implicated in a wide range of cellular processes, such as transcription (11), metabolism (12), cell cycle progression (13), and apoptosis (14). Known modulators of PKA activity include factors that either activate or deactivate adenylate cyclase, resulting in a corresponding increase or decrease in cAMP levels. PKA is composed of two regulatory (R) and two catalytic (C) subunits. There are two forms of the R subunit, and both the R subunits and the C subunit exist as multiple isotypes. Although there are major differences in the tissue distribution, biochemical and physical properties of the R subunit isoforms, differences between the various isoforms of the C subunit are more subtle (10,15). Regulation of PKA in the cell is related primarily to modulation of its phosphotransferase activity.

Promega's SignaTECT^{TM} cAMP-Dependent Protein Kinase (PKA) Assay System uses biotinylated Kemptide, a seven-residue peptide derived from pyruvate kinase (16), as a substrate. This substrate is specific and exhibits high affinity for PKA. Because Kemptide has a very low K_m (510µM) for PKA, the sensitivity of the assay readily allows detection of this kinase in biological samples. The assay measures the enzyme activity of as little as 0.2ng of purified PKA (Figure 3A), and can quantitate PKA activity in a variety of cellular or tissue extracts. Even in crude extracts, the phosphorylation of the target peptide is due specifically to the activity of PKA as demonstrated by the fact that addition of the PKA-specific inhibitor (PKI) to the reaction results in no detectable phosphorylation of the substrate (Figure 3B).

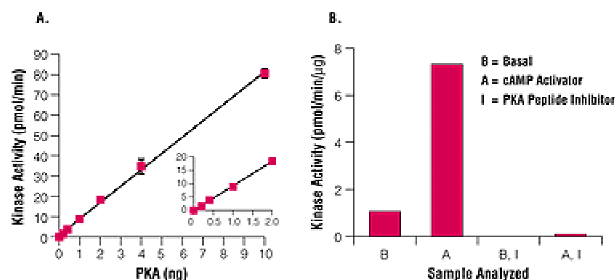

Figure 3. Detection of PKA activity using the SignaTECT™ PKA Assay System. Panel A: Sensitivity and linearity of the PKA Assay System. Promega's cAMP-Dependent Protein Kinase (PKA) Catalytic Subunit (Cat.# V5161) was diluted 1,000- to 50,000-fold in 0.1mg/ml BSA, and assayed as described (4,32). The inset shows data below 2ng. **Panel B:** Crude rat brain extract was prepared and analyzed for PKA activity as described (4,32). Activity was increased 7-fold by the addition of 5µM cAMP, and was completely inhibited by 1mM PKA Peptide Inhibitor (Cat.# V5681).

SignaTECT™ Protein Kinase C (PKC) Assay System

Ca^{2+}/phospholipid-dependent protein kinase (PKC) enzymes are crucial to cellular proliferation, differentiation, apoptosis and exocytotic release in a number of non-neuronal systems (17). The enzymes also phosphorylate several neuronal proteins, where they presumably regulate neurotransmitter release and alter long-term potentiation in memory formation (18,19). PKCs are stimulated by Ca^{2+} and phospholipids, thus factors that increase or decrease the turnover or availability of phospholipids or calcium also alter the enzymatic activity of PKC (20).

PKC enzymes are composed of a regulatory and a catalytic domain. The mammalian PKC enzyme family consists of at least twelve different isozymes that confer unique functional properties (17). These isozymes respond differently to Ca^{2+}, diacylglycerol and phospholipids. Therefore, the various isozymes are differentiated on the basis of their cofactor requirements (17,20).

The SignaTECT™ Protein Kinase C (PKC) Assay System exploits the properties of $Neurogranin_{(28-43)}$ as a specific substrate for PKC isozymes. This substrate has greater selectivity for PKC than any other available substrate; over 95% of the phosphate incorporated into $Neurogranin_{(28-43)}$ is inhibited by the selective PKC inhibitor, $PKC_{(19-36)}$ (21). The K_m for $Neurogranin_{(28-43)}$, using PKC in hippocampal tissue homogenates, is similar to that obtained with purified enzyme (K_m = 147nM) (22). The use of a biotin-tagged neurogranin peptide substrate in the SignaTECT™ PKC Assay System allows for increased selectivity as compared to other available substrates. The system is capable of measuring the activity of 0.4ng of purified enzyme (Figure 4A), and can be used to measure the kinase activity in tissue and cellular extracts (Figure 4B).

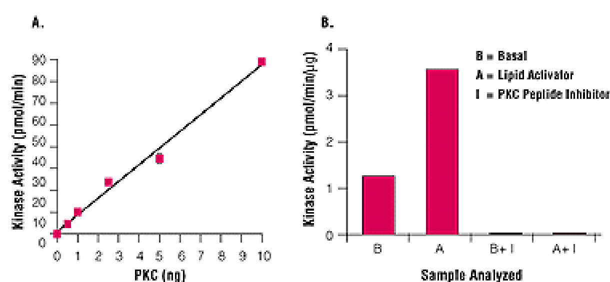

Figure 4. Detection of PKC activity using the SignaTECT™ PKC Assay System. Panel A: Sensitivity and linearity of the PKC Assay System. Promega's Protein Kinase C (PKC) (Cat.# V5261) was diluted 12.5- to 250-fold in 0.1mg/ml BSA and 0.05% Triton® X-100, and assayed as described (32). **Panel B:** Crude rat brain extract was prepared and assayed as described (4,32). Activity was increased 3-fold by the addition of phospholipid activator and was completely inhibited by 50µM Myristoylated Protein Kinase (PKC) Peptide Inhibitor (Cat.# V5691).

SignaTECT™ cdc2 Protein Kinase Assay System

Cyclin-dependent protein kinases (cdks) are a family of $p34^{cdc2}$-related serine/threonine kinases that control cell cycle progression, entry into apoptosis and oncogenic transformation (23,24). The cdks require both a kinase catalytic subunit and a cyclin regulatory subunit for activity, and they interact with a variety of positive and negative protein regulators (25-27).

The complex phosphorylation dependent regulation of the cdks (29), the cell cycle-specific destruction of the cyclins, and the interaction of multiple regulators, make it difficult to determine cdk activity using immunoprecipitation-based kinase assays. The SignaTECT™ cdc2 Protein Kinase Assay System provides a convenient method to determine this activity. The system uses a biotinylated peptide that is derived from histone H 1, an endogenous substrate of cdc2 kinase (30), to assay this enzyme (Figure 5D). The low K_m (2µM) allows for optimal reaction conditions. cdc2 kinase activity can be analyzed in the presence of regulatory proteins which do not co-purify with cdc2 kinase by assaying the activity in crude extracts prepared from fractionated cell populations. Using the SignaTECT™ cdc2 Protein Kinase Assay System, as little as 100ng of mitotic extract protein can be analyzed (Figure 5A), with kinetics that are identical to those of purified cdc2 kinase (Figure 5C).

In extracts containing more than one active cyclin-dependent kinase (e.g., activated starfish oocytes), multiple cyclin-dependent kinases can be identified using this system. Figure 5B shows that, at low substrate concentrations, a kinase activity with a K_m roughly equal to that of purified cdc2 kinase can be detected. However, at higher substrate concentrations, at least one other kinase is detected (apparent K_m = 24µM). The simple inhibition kinetics of this sample with Olomoucine (Figure 5F), a selective inhibitor of a subset of cdks, suggests that the second activity is due to cdk2 activity (28).

To confirm the specificity, immunoprecipitates can be analyzed for kinase activity. Figure 5E compares the kinase activity from mitotic HeLa cell extract to that obtained from the same volume of HeLa extract that had been precipitated with a carboxy-terminus cdc2 kinase specific antibody. The recovery of one-third of the activity in the immune complex, the absence of activity in asynchronous cultures and the inhibition of activity by Olomoucine all support the specificity of this assay system, but the data also illustrate the inherent difficulties in quantitating cell activities by this method.

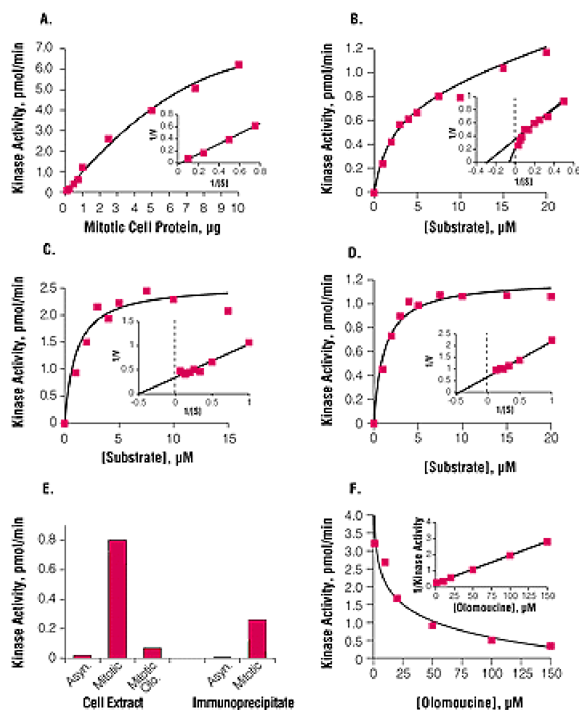

Figure 5. Analysis of cdc2 activities using the SignaTECT™ cdc2 Kinase Assay System. Except where indicated, the cdc2 kinase assays were performed as described (34). **Panel A:** The indicated amounts of mitotic HeLa cell extract protein were assayed for 15 minutes. The inset shows the results obtained with less than 1µg of extract protein. **Panels B and C:** Kinetic analysis of extracts. Activated starfish oocyte extract (10µg) (Panel B) or mitotic HeLa cell extract (2.5µg) (Panel C) were incubated for 10 minutes with increasing concentrations of the peptide substrate. The inset figures show double reciprocal plots. **Panel D:** Kinetic analysis of purified cdc2 Kinase. cdc2 Kinase (Cat.# V6341) purified from starfish was incubated with increasing concentrations of the peptide substrate. The inset figure shows a double reciprocal plot. **Panel E:** Extracts prepared from actively growing HeLa cells or HeLa cells blocked in mitosis (34) were analyzed for cdc2 kinase activity either directly or after immunoprecipitation with 1µg of Anti-pp34^{cdc2} Kinase C-Terminus Polyclonal Antibody (Cat.# V6291) and Protein A Agarose, either in the presence or absence of the cdc2 kinase inhibitor Olomoucine. Approximately one-third of the cdc2 kinase activity was recovered in the immunoprecipitates. **Panel F:** Activated starfish oocyte extract was incubated with 50µM ATP and the indicated concentrations of Olomoucine. The inset, showing a single reciprocal plot, indicates simple inhibition kinetics in the range reported for cdc2 kinase.

Summary

Promega has developed a proprietary streptavidin-coated matrix technology, the SAM2TM Biotin Capture Membrane, that is designed to rapidly and specifically capture biotinylated molecules under a variety of assay conditions. This technology, in combination with highly specific biotinylated peptide substrates, has been applied to develop the SignaTECT™ Protein Kinase Assay Systems. These systems, designed for the specific and sensitive detection of PKA, PKC, cdc2 and PTKs, provide significant advantages over other methods commonly used for the quantitation of protein kinase activity.

References

1. van der Geer, P., Hunter, T. and Lindberg, R.A. (1994) *Annu. Rev. Cell Biol.* **10,** 251.
2. De Meyts, P. *et al.* (1995) *Metabolism* **44,** 2.
3. Denton, R.M. and Tavare, J.M. (1995) *Eur. J. Biochem.* **227,** 597.
4. Goueli, S.A. *et al.* (1995) *Anal. Biochem.* **225,** 10.
5. Toomik, R., Ekman, P. and Engstrom, L. (1992) *Anal. Biochem.* **204,** 311.
6. Casnellie, J.E. (1991) *Meth. Enzymol.* **200,** 115.
7. Liu, X. and Pawson, T. (1994) *Recent Prog. Horm. Res.* **49,** 149.
8. Levitzki, A. and Gazit, A. (1995) *Science* **267,** 1782.
9. Hubbard, S.R. *et al.* (1994) *Nature* **372,** 746.
10. Smith, J.A., Francis, S.H and Corbin, J.D. (1993) *Mol. Cell. Biochem.* **127/128,** 51.
11. Huggenvik, J.I. *et al.* (1991) *Mol. Endocrinol.* **5,** 921.
12. Hubbard, M.J. and Cohen, P. (1993) *Trends Biochem. Sci.* **18,** 172.
13. Matten, W. *et al.* (1994) *Mol. Cell. Biol.* **14,** 4419.
14. Gjertsen, B.T. and Doskeland, A. (1995) *Biochim. Biophys. Acta* **1269,** 187.
15. Gamm, D.M. *et al.* (1996) *J. Biol. Chem.* **271,** 15736.
16. Pilkis, S.J. *et al.* (1980) *J. Biol. Chem.* **255,** 2770.
17. Newton, A.C. (1995) *J. Biol. Chem.* **270,** 28495.
18. Tanaka, C. and Nishizuka, Y. (1994) *Annu. Rev. Neurosci.* **17,** 551.
19. Hvalby, O. *et al.*(1994) *Proc. Natl. Acad. Sci. USA* **91,** 4761.
20. Nishizuka, Y. (1995) *FASEB J.* **9,** 484.
21. Chen-S.J. *et al.* (1993) *Biochemistry* **32,** 1032.
22. Gonzales, A. *et al.* (1993) *Anal. Biochem*. **215,** 184.
23. Ahmed, K. and Goueli, S.A. (1987) In: *Biochemical Actions of Hormones, XIV,* Litwack, G., ed., Academic Press, NY.
24. Karin, M. (1994) *Curr. Opin. Cell Biol.* **6,** 415.
25. Grana, X. and Reddy, E.P. (1995) *Oncogene* **11,** 211.
26. Correa-Bordes, J. and Nurse, P. (1995) *Cell* **83,** 1001.
27. Yee, A. *et al.* (1995) *Cancer Res.* **55,** 6058.
28. Vesely, J. *et al.* (1994) *Eur. J. Biochem.* **224,** 771.
29. Morgan, D.O. (1994) *Nature* **374,** 131.
30. Beaudette, K.N. *et al.* (1993) *J. Biol. Chem.* **268,** 20825.
31. *SignaTECT™ Protein Tyrosine Kinase (PTK) Assay System Technical Bulletin* #TB211, Promega Corporation.
32. *SignaTECT™ cAMP-Dependent Protein Kinase (PKA) Assay System Technical Bulletin* #TB241, Promega Corporation.
33. *SignaTECT™ Protein Kinase C (PKC) Assay System Technical Bulletin* #TB242, Promega Corporation.
34. *SignaTECT™ cdc2 Protein Kinase Assay System Technical Bulletin* #TB227, Promega Corporation.

Ordering Information

Product	Size	Cat.#
SignaTECT™ Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT™ cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT™ cdc2 Protein Kinase Assay System	96 reactions	V6430
SignaTECT™ Protein Tyrosine Kinase (PTK) Assay System	96 reactions	V6480

© 1996 Promega Corporation. All Rights Reserved.

SAM2 and SignaTECT are trademarks of Promega Corporation.

PhosphorImager is a trademark of Molecular Dynamics. Triton is a registered trademark of Union Carbide Chemicals and Plastics Co., Inc.