

SIGNAL TRANSDUCTION RESOURCE

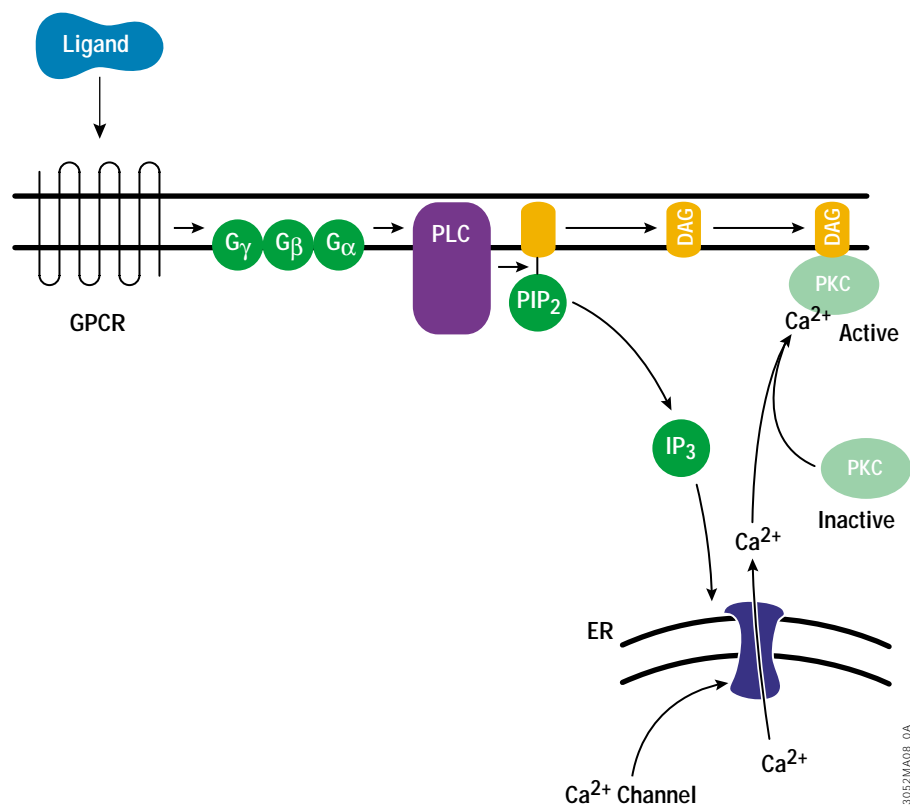


Figure 3.1. Activation of a conventional PKC. External stimulus activates a G-Protein-Coupled Receptor (GPCR), which activates a stimulating G-protein. The G-protein activates phospholipase C (PLC), which cleaves phosphoinositide-4,5-bisphosphate (PIP₂) into 1,2-diaclyglycerol and inositol-1,4,5-trisphosphate (IP₃). The IP₃ interacts with a calcium channel in the endoplasmic reticulum (ER), releasing Ca²⁺ into the cytoplasm. The increase in Ca²⁺ levels activates PKC, which translocates to the membrane, anchoring to diacylglycerol (DAG) and phosphatidylserine.

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Protein Kinase C

Ca²⁺- and phospholipid-dependent protein kinases (PKCs) represent a family of second messenger-dependent protein kinases that are stimulated by Ca²⁺ and/or phospholipid (1). PKC plays a pivotal role in mediating cellular responses to extracellular stimuli involved in proliferation, differentiation, apoptosis, and exocytotic release in a number of non-neuronal systems such as islet cells, chromaffin cells and paramecium (2). PKC has also been implicated in phosphorylation of several neuronal proteins, which are thought to regulate neurotransmitter release and establish long-term potentiation in memory formation (3–6).

PKC is not a single enzyme but a family of serine/threonine kinases. At least 10 isoforms of PKC are known and are classified by their activation requirements (7; Table 3.1). PKC activation occurs when plasma membrane receptors coupled to phospholipase C are activated, releasing diacylglycerol (Figure 3.1). The conventional isoforms, α , β I, β II, and γ , are activated by phosphatidylserine, diacylglycerol and Ca²⁺. The unconventional isoforms, δ , ϵ , η , and θ , require phosphatidylserine and diacylglycerol but do not require Ca²⁺. The ζ and λ isoforms are called atypical and require only phosphatidylserine for activation.

Table 3.1. PKC Isozymes.

Isoform	Amino Acids	Calculated Molecular Weight
PKC α	672	76,704
PKC β I	671	76,663
PKC β II	673	76,806
PKC γ	697	78,268
PKC δ	674	77,458
PKC ϵ	737	83,492
PKC η	683	77,884
PKC θ	707	81,479
PKC ζ	592	67,605
PKC λ	586	67,200

All forms are monomeric. The β I and β II differ in their C-terminal amino acid residues, and the forms are the result of alternative splicing of the 3'-exon (8).

PKC isozymes are synthesized as single polypeptides with an N-terminal regulatory domain (20–40kDa) and a C-terminal catalytic domain (~45kDa). Generally, PKC isozymes contain four conserved regions termed C1–C4. C1 contains a cysteine-rich motif and forms the diacylglycerol binding site (1). The autoinhibitory pseudosubstrate sequence is upstream of the cysteine-rich motif in the same region (9). C2 contains the recognition site for acidic lipids and, in some isozymes, the Ca²⁺ binding site. C3 and C4 form the ATP and substrate binding lobes of the kinase, respectively (1).

In the inactive form, the pseudosubstrate domain is bound to the catalytic domain of PKC (10). Upon stimulation, PKC translocates to the plasma membrane where the C1 and C2 domains interact with DAG and phosphatidylserine, respectively. This interaction causes the pseudosubstrate domain to dissociate from the catalytic domain, which results in activation of PKC. Inactive PKC is not freely distributed throughout the cytoplasm but appears localized to specific sites within the cell. This localization is facilitated by association of PKC with scaffolding proteins, such as AKAP79 (11) and Gravin (12). AKAP79 (A Kinase-Anchoring Protein 79) binds a number of PKC isoforms including α , β II, δ , ϵ , and ζ (13). In the case of β II, the association can be disrupted by Ca²⁺/calmodulin, freeing β II to translocate to the plasma membrane (14). PKC binding proteins have been identified (15–17) that localize PKC. The Receptor Associated with C Kinase (RACK1) binds only activated PKC and directs the PKC β II to its appropriate subcellular site (18).

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Promega Product Citations

Bastianetto, S., Zheng, W.-H. and Quirion, R. (2000) The *Gingko biloba* extract (EGb 761) protects and rescues hippocampal cells against nitric oxide-induced toxicity: Involvement of its flavonoid constituents and protein kinase C. *J. Neurochem.* **74**, 2268.

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Promega Resources

Protocol

SignaTECT® Protein Kinase C (PKC) Assay System.....**TB242**

Publications

Goueli, S. *et al.* (1996) SAM²® Biotin Capture Membrane and SignaTECT® Protein Kinase Assay Systems. *Promega Notes* **58**, 22.

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Enzyme Assay Systems

SignaTECT® Protein Kinase Assay System

Product	Size	Catalog #
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470

Description: The SignaTECT® Protein Kinase C (PKC) Assay System^(a) provides an improved method to quantitate Protein Kinase C activity, both in purified enzyme preparations and in crude cell extracts. Promega's SignaTECT® PKC Assay System overcomes the problem of nonspecific substrate binding by using a biotinylated form of the peptide, Neurogranin₍₂₈₋₄₃₎ (1), the most specific substrate available to assess PKC activity, in conjunction with Promega's SAM²® Biotin Capture Membrane^(a). The SAM²® Membrane is a novel streptavidin matrix produced by a proprietary process that results in a high density of streptavidin on the membrane matrix. This streptavidin matrix provides rapid, quantitative capture of biotinylated substrate molecules, based on the strong affinity of biotin for streptavidin (K_d=10⁻¹⁵M). The SAM²® Membrane can linearly bind biotinylated substrate up to the low nmol/cm² range, allowing kinetic studies to be performed. In addition, the membrane has been optimized for low, non-specific binding.

^(a)U.S. Pat. No. 6,066,462 has been issued to Promega Corporation for quantitation of protein kinase activity.

Reference

- Chen, S.J. *et al.* (1993) Studies with synthetic peptide substrates derived from the neuronal protein neurogranin reveal structural determinants of potency and selectivity for protein kinase C. *Biochemistry* **32**, 1032.

Features

- Specific:** Uses the biotinylated peptide, Neurogranin₍₂₈₋₄₃₎, the most specific commercially available substrate for PKC activity assays.
- High Signal-to-Noise Ratios:** The high specificity and strong affinity of the SAM²® Membrane for biotinylated substrates results in lower background and higher signal-to-noise ratios than traditional capture methods (i.e. P-81 phosphocellulose).
- Linear Binding:** Membrane can linearly bind biotinylated substrates up to the nmol/cm² range—allows for kinetic studies.
- Convenient:** SignaTECT® Systems require less “hands-on” manipulation than other assay methods.
- Versatile:** The SAM²® Membrane can be used in a variety of buffer and reaction conditions.

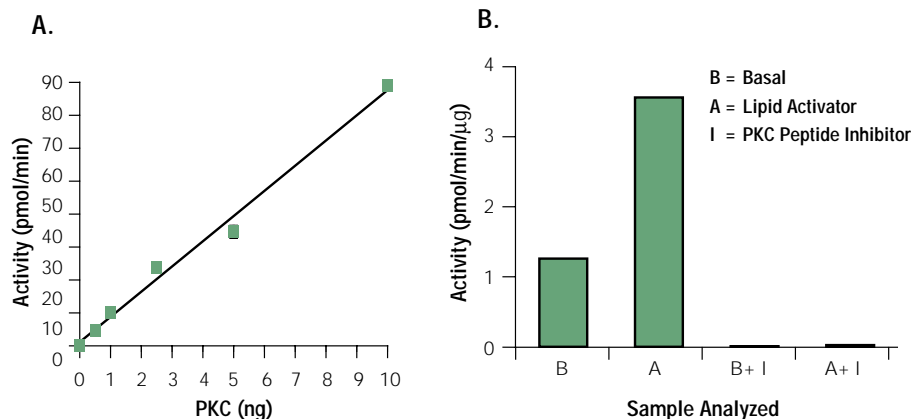


Figure 3.2. Detection of PKC activity using the SignaTECT® PKC Assay System. Panel A: Sensitivity and linearity of the PKC Assay System. Protein Kinase C (Cat.# V5261) was diluted 12.5- to 250-fold in 0.1mg/ml BSA and 0.05% Triton® X-100, and assayed as described in Technical Bulletin #TB242. Panel B: Crude rat brain extract was assayed as described in Technical Bulletin #TB242. Activity was increased 3-fold by the addition of phospholipid activator and was completely inhibited by 50μM Myristoylated Protein Kinase C (PKC) Peptide Inhibitor (Cat.# V5691).

PepTag® Non-Radioactive Protein Kinase Assay

Product	Size	Catalog #
PepTag® Non-Radioactive Protein Kinase C Assay	120 reactions	V5330

Description: The PepTag® Non-Radioactive Protein Kinase C (PKC) Assay^(a) provides a rapid, sensitive and non-radioactive method to detect PKC activity. The PepTag® PKC Assay uses a brightly colored, fluorescent peptide substrate that is highly specific for PKC, the PepTag® C1 peptide (PLSRTL5VAAK). The bright pink color is imparted by the addition of a dye molecule to the PepTag® C1 peptide substrate. Phosphorylation by PKC alters the substrate's net charge from +1 to -1. This change in the net charge of the substrate allows the phosphorylated and nonphosphorylated versions of the substrate to be rapidly separated on an agarose gel at neutral pH. Using the PepTag® PKC Assay, less than 10ng of kinase can be detected in under 2 hours. This allows rapid screening of large numbers of samples such as those produced from the assay of multiple column fractions. The PepTag® PKC Assay can detect kinase in partially purified samples or purified preparations of enzyme, making it a good choice for the rapid screening of column fractions or the screening of kinase activators or inhibitors. In addition to the assay components, the assay includes active rat PKC purified by the method of Walton *et al.* (1) as a positive control.

^(a)U.S. Pat. No. 5,580,747 has been issued to Promega Corporation for a non-radioactive enzyme assay.

Reference

- Walton, G.M. *et al.* (1987) A three-step purification procedure for protein kinase C: Characterization of the purified enzyme. *Anal. Biochem.* **161**, 425.

Features

- Non-Radioactive:** The fluorescent tag on the peptide substrate facilitates quantitation of the phosphorylation reaction without the use of radioactivity.
- Low Background:** Because the phosphorylation of the colored peptide supplied with the system is used to measure kinase activity, phosphorylation of other substrates occurring naturally in the sample does not add to the kinase activity measured.
- Rapid:** The outcome of a phosphorylation reaction can be quickly determined after the electrophoretic separation step.
- Convenient:** Quantitation of the phosphorylated peptide can be accomplished using a densitometer, spectrophotometer, 96 well plate reader or fluorometer.

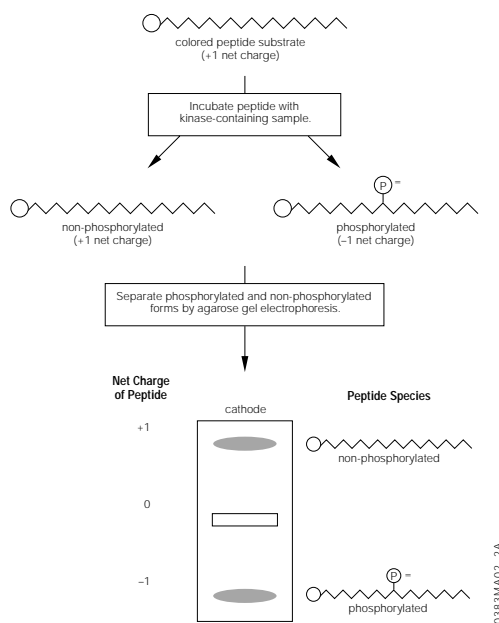


Figure 3.3. Schematic diagram of the PepTag® Non-Radioactive Protein Kinase Assay procedure.

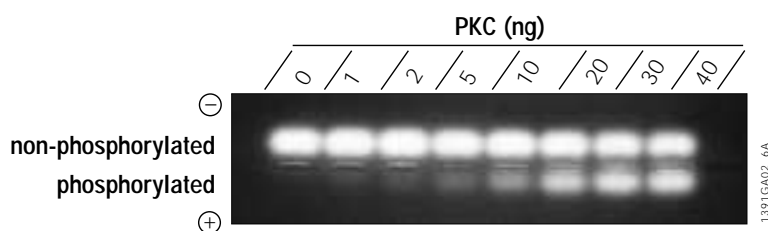


Figure 3.4. Detection of Protein Kinase C. Two micrograms of PepTag® C1 Peptide were incubated as described in Technical Bulletin #TB132 with varying amounts (0–40ng) of PKC in a final volume of 25µl for 30 minutes at room temperature. The reactions were stopped by heating to 95°C for 10 minutes. The samples were electrophoresed on a 0.8% agarose gel at 100V for 15 minutes. Phosphorylated peptide migrated toward the anode (+), while non-phosphorylated peptide migrated toward the cathode (-). The gel was photographed on a transilluminator.

Promega Product Citations

Wagner, A.H. *et al.* (2000) Improvement of nitric oxide-dependent vasodilation by HMG-CoA reductase inhibitors through attenuation of endothelial superoxide anion formation. *Arterioscler. Thromb. Vasc. Biol.* **20**, 61.

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Promega Resources

Protocol

PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase**TB132**

Publications

White, D. and Shultz, J. (1992) A novel method for non-radioactive assays of specific protein kinases. *Promega Notes* **35**, 11.

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Protein Kinase C

Aoki, H. *et al.* (2000) Hepatitis C virus core protein interacts with 14-3-3 protein and activates the kinase Raf-1. *J. Virol.* **74**, 1736.

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Neurogranin

Bush, E.W. *et al.* (2000) Myotonic dystrophy protein kinase domains mediate localization, oligomerization, novel catalytic activity, and autoinhibition. *Biochemistry* **39**, 8480.

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Inhibitor

Bush, E.W. *et al.* (2000) Myotonic dystrophy protein kinase domains mediate localization, oligomerization, novel catalytic activity, and autoinhibition. *Biochemistry* **39**, 8480.

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Li, D., Yang, B. and Mehta, J.L. (1998) Ox-LDL induces apoptosis in human coronary artery endothelial cells: Role of PKC, PTK, Bcl-2 and Fas. *Am. J. Physiol.* **275**, H568.

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Protocol

Protein Kinase C**TB528**

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Enzymes, Substrates and Inhibitors

Protein Kinase C

Product	Size	Catalog #
Protein Kinase C	1µg	V5261

Description: Protein Kinase C is an 82kDa monomeric enzyme consisting of a C-terminal catalytic domain and a cysteine-rich N-terminal regulatory domain. The regulatory domain contains the sites for calcium- and phospholipid-binding and a pseudosubstrate subdomain, the target for PKC autophosphorylation. Many of the PKC target substrates are components of signal transduction pathways and include proteins that regulate ion channels, calcium- and calmodulin-binding proteins, growth factor receptors, structural and regulatory proteins of the cytoskeleton, components of the transcriptional machinery, efflux pumps and many other proteins (1,2). PKC is isolated from rat brain following the procedure of Welton and Colleagues (3). The purified PKC consists primarily of α , β and γ isoforms with lesser amounts of δ and ζ isoforms.

References

1. Azzi, A., Boscoboinik, D. and Hensey, C. (1992) The protein kinase C family. *Eur. J. Biochem.* **208**, 547.
2. Kikkawa, U. and Nishizuka, Y. (1986) The role of protein kinase C in transmembrane signalling. *Ann. Rev. Cell Biol.* **2**, 149.
3. Welton, G.M. *et al.* (1987) A three-step purification procedure for protein kinase C: Characterization of the purified enzyme. *Anal. Biochem.* **161**, 425.

Feature

- **Purity:** PKC is greater than 90% pure as determined by SDS-PAGE.

Neurogranin₍₂₈₋₄₃₎ (PKC) Peptide Substrate

Product	Size	Catalog #
Neurogranin ₍₂₈₋₄₃₎ (PKC) Peptide Substrate	1mg	V5611

Description: Neurogranin₍₂₈₋₄₃₎ (PKC) Peptide Substrate is a specific peptide substrate for calcium- and phospholipid-dependent protein kinases (PKC). Neurogranin₍₂₈₋₄₃₎ (PKC) Peptide Substrate is supplied ready for use in kinase reactions.

The sequence of Neurogranin₍₂₈₋₄₃₎ is AAKIQASFRGHMARKK (1). Its molecular weight is 1,800 daltons (verified by Fast Atomic Bombardment mass spectrometry).

Reference

1. Chen, S.-J. *et al.* (1993) Studies with synthetic peptide substrates derived from the neuronal protein neurogranin reveal structural determinants of potency and selectivity for protein kinase C. *Biochem.* **32**, 1032.

Myristoylated Protein Kinase C Peptide Inhibitor

Product	Size	Catalog #
Myristoylated Protein Kinase C Peptide Inhibitor	1mg	V5691

Description: Myristoylated Protein Kinase C (PKC) Peptide Inhibitor, Myr. RFARKGALRQKNV (MW = 1,754 daltons) specifically inhibits calcium- and phospholipid-dependent protein kinase C (PKC). It is based on the pseudosubstrate region of PKC- α and PKC- β (1). It is supplied ready for use in kinase reactions. Maximum inhibition of PKC activity is usually obtained with 50µM Myristoylated PKC Peptide Inhibitor.

Reference

1. Eichholtz, T. *et al.* (1993) A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. *J. Biol. Chem.* **268**, 1982.

Feature

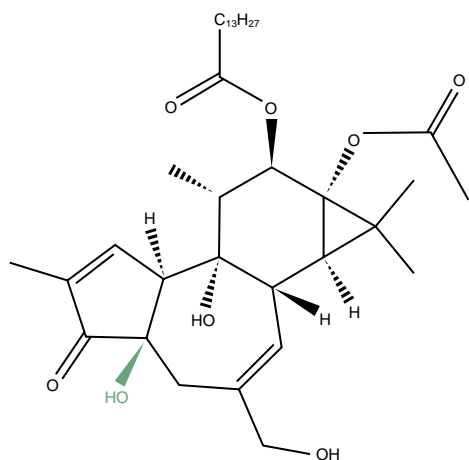
- **Cell Permeable:** The sequence of this peptide should allow it to be taken up by intact cells, permitting in vivo PKC inhibition studies (1).

Activators and Activator Analogs

PMA (Phorbol 12-myristate 13-acetate)

Product	Size	Catalog #
PMA (Phorbol 12-myristate 13-acetate)	5mg	V1171

Description: Phorbol 12-myristate 13-acetate (PMA) is a specific activator of group A (α , β I, β II, γ) and group B (δ , ϵ , η , θ) Protein Kinase Cs (PKCs) (in the 1–100nM range). Phorbol esters, such as PMA, affect PKCs by mimicking diacylglycerol, a natural ligand and activator of PKCs. PMA is an effective skin irritant and potent tumor promoter in mice. A common alternative name for PMA is 12-O-tetradecanoylphorbol 13-acetate (TPA).

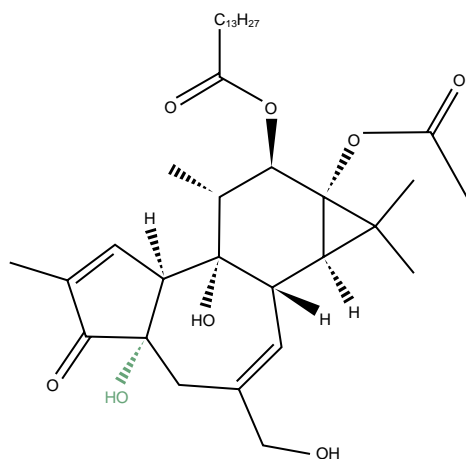


Structure of PMA

4 α -PMA (Inactive PMA Analog)

Product	Size	Catalog #
4 α -PMA	1mg	V1181

Description: 4 α -Phorbol 12-myristate 13-acetate (4 α -PMA) is an inactive analogue of and negative control for phorbol 12-myristate 13-acetate (PMA), which is also known as 12-O-tetradecanoylphorbol 13-acetate (TPA). Effects of PMA in a given system are thought to be specific if 4 α -PMA is not active in the same system.

Structure of 4 α -PMA

Promega Product Citations

The PKC activator, PMA, and its inactive analog, 4 α -PMA, are new to Promega's product line and citations do not currently exist.

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