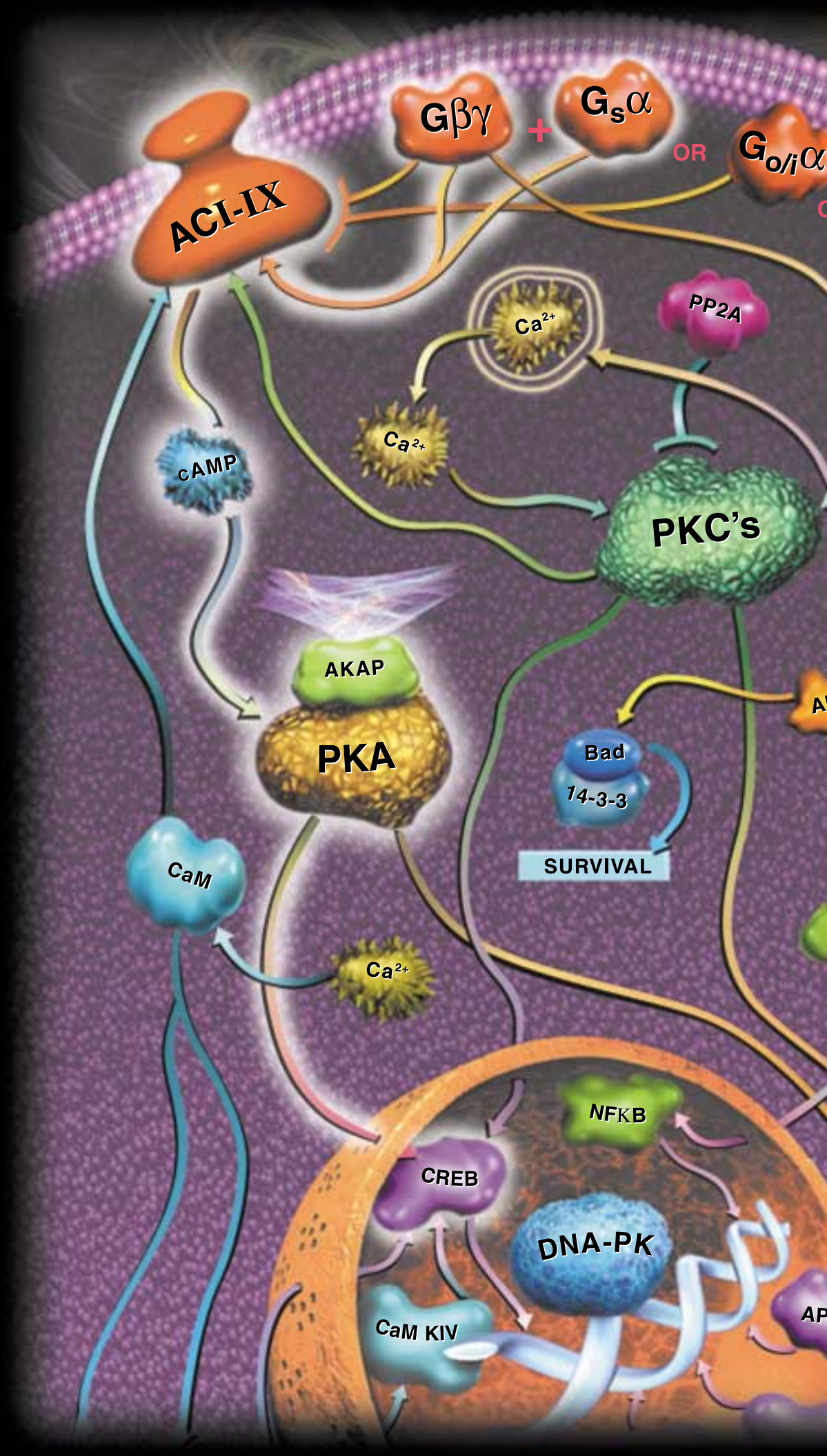


# 2

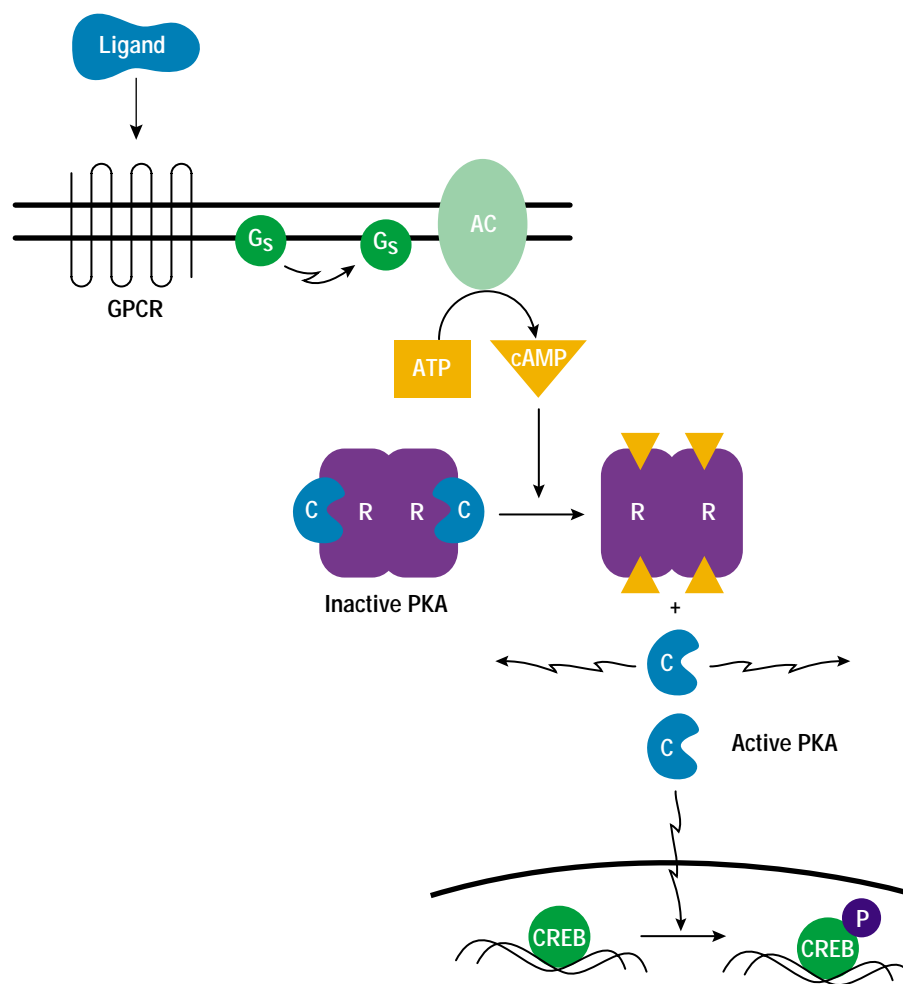
## CHAPTER

### PROTEIN KINASE A

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## SIGNAL TRANSDUCTION RESOURCE



3024IMAGE\_OA

**Figure 2.1. Protein kinase A cascade.** Ligand binding to the G-Protein-Coupled Receptor (GPCR), activates a stimulatory G-protein ( $G_s$ ). The activated G-protein interacts with adenylate cyclase (AC) causing accumulation of cAMP. Active PKA catalytic subunits (C) are released following cAMP binding to the PKA regulatory subunits. The active catalytic subunits phosphorylate targets in both the cytoplasm and the nucleus. One target of phosphorylation by PKA in the nucleus is the cAMP Response Element Binding Protein (CREB). Phosphorylated CREB increases activation of transcription.

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### cAMP-Dependent Protein Kinase (PKA)

cAMP-dependent protein kinase, commonly known as protein kinase A (PKA), is a second messenger-dependent enzyme (1) that has been implicated in a wide range of cellular processes, including transcription (2), metabolism (3), cell cycle progression (4) and apoptosis (5). Known modulators of PKA activity include factors that either activate or inhibit adenylate cyclase, resulting in an increase or decrease in cAMP levels. The enzyme occurs naturally as a four-membered structure with two regulatory (R) and two catalytic (C) subunits.

The R subunit exists in two forms, RI and RII, while just one form of the C subunit is present in eukaryotic cells. Both R subunits (RI and RII) and the single C subunit exist as multiple isoforms (see Table 2.1). 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 (1,6). Regulation of PKA in the cell is related primarily to modulation of its phosphotransferase activity. The holoenzyme contains two C subunits bound to homo- or heterodimers of either RI or RII subunits (7,8). The C subunits do not interact with one another. Activation proceeds by the cooperative binding of two molecules of cAMP to each R subunit, which causes the dissociation of each active C subunit from the R subunit dimer. The consensus sequence for phosphorylation by the C subunit is, most stringently, K/R-R-X-S/TY and, less stringently, R-X-X-S/TY where Y tends to be a hydrophobic residue (9,10). A phenylalanine in the nearby sequence tends to be a negative determinant for phosphorylation by the enzyme (9).

**Table 2.1. Subunit Structure and Isoforms of the Catalytic (C) and Regulatory (R) Subunits of cAMP-Dependent Protein Kinase (9).**

Subunit	Amino Acids	Predicted Molecular Weight	Apparent Molecular Weight by SDS-PAGE
C $\alpha$	351	40,589	40kDa
C $\beta$	351	40,622	41kDa
C $\gamma$	351	40,412	39kDa
RI $\alpha$	381	42,981	49kDa
RI $\beta$	380	43,027	53.5kDa
RII $\alpha$	404	45,518	51kDa*
RII $\beta$	418	46,346	53kDa

\*The apparent molecular weight of the phosphorylated form is 54kDa.

PKA is classified as a Type I or Type II enzyme depending upon the associated R subunit (i.e., RI or RII). Type I PKA is predominantly located in the cytoplasm, while Type II associates with cellular structures and organelles (7). Type II PKA is not a "free floating" enzyme but is anchored to specific locations within the cell by specific proteins called **A** **K**inase-**A**nchoring **P**roteins (AKAPs). The AKAPs keep PKA localized and limit the targets that can be phosphorylated, preventing the indiscriminate phosphorylation expected from free PKA in the cytoplasm (11). Most AKAPs described thus far bind the RII subunits with nanomolar affinities (12) while binding RI subunits in the micromolar range (13). RII-binding AKAPs range in size from 15–300kDa and are capable of binding other kinases as well as phosphatases (14). Anchored protein kinase A modulates the activity of various cellular proteins, including AMPA/kainate channels, glutamate receptor-gated ion channels, L-type Ca<sup>2+</sup> channels in skeletal muscle (14), hormone-mediated insulin secretion in clonal beta cells (15), vasopressin-mediated translocation of aquaporin-2 into the cell membrane of renal principal cells (16), motility of mammalian sperm (17,18) and the sperm acrosome reaction (19). Peptides derived from AKAPs can be used to inhibit PKA activity by interrupting the specific localization of the kinase (16). Recent studies are defining AKAPs that interact with RI domains and tether Type I PKA to specific locations in the cell (20). Further evidence of RI-specific or RI/RII dual specificity AKAPs has been provided by RI localization in RII knockout mice (13) and two-hybrid screens (21,22). An AKAP for an RI-like regulatory subunit of PKA has been described in *Caenorhabditis elegans* that has absolute specificity over the RII-like regulatory subunit (23). Thus, AKAPs allow PKA to be localized to specific areas of the cell where PKA activity is needed, rather than a random diffuse burst of activity.

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## Promega Resources

### Protocol

SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System.....**TB241**

### Publications

Goueli, S. *et al.* (1996) SAM<sup>2</sup>® 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

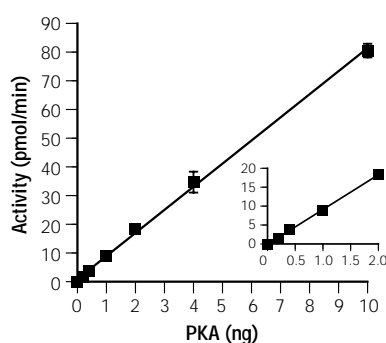
Product	Size	Catalog #
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480

**Description:** The SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System<sup>(a)</sup> provides an improved method for assaying PKA activity. The SignaTECT® PKA Assay System uses biotinylated Kemptide (LRRASLG), a peptide substrate derived from the *in vivo* substrate pyruvate kinase. This peptide substrate is highly specific for PKA. Use of this substrate provides increased specificity for PKA activity in samples that contain multiple kinases. The <sup>32</sup>P-labeled, biotinylated substrate is recovered from the reaction mix with the SAM<sup>2</sup>® Biotin Capture Membrane<sup>(a)</sup>, which 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. In addition, the membrane is optimized for low nonspecific binding.

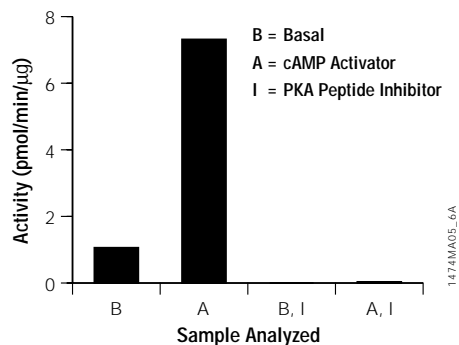
<sup>(a)</sup>U.S. Pat. No. 6,066,462 has been issued to Promega Corporation for quantitation of protein kinase activity.

### Features

- **High Signal-to-Noise Ratios:** The high specificity and strong affinity of the SAM<sup>2</sup>® 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/cm<sup>2</sup> range—allows for kinetic studies.
- **Convenient:** SignaTECT® Systems require less “hands-on” manipulation than other assay methods.
- **Versatile:** The SAM<sup>2</sup>® Membrane can be used in a variety of buffer and reaction conditions.



**Figure 2.2. Sensitivity and linearity of assay.** Promega's cAMP-Dependent Protein Kinase (PKA) Catalytic Subunit (Cat.# V5161) was diluted in 0.1mg/ml BSA, 1,000- to 50,000-fold, and assayed as described in Technical Bulletin #TB241. The inset shows data below 2ng.



**Figure 2.3. PKA detection in tissue extracts.** Crude rat brain extract was prepared and analyzed for PKA activity as in Technical Bulletin #TB241. Activity was increased 7-fold by the addition of the activator, cAMP, and was completely inhibited by Promega's PKA inhibitor, cAMP-Dependent Protein Kinase (PKA) Peptide Inhibitor (Cat.# V5681).

**PepTag® Non-Radioactive Protein Kinase Assay**

Product	Size	Catalog #
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay	120 reactions	V5340

**Description:** The PepTag® Non-Radioactive cAMP-Dependent Protein Kinase (PKA) Assay<sup>(a)</sup> provides a rapid, sensitive and non-radioactive method to detect PKA activity. The PepTag® PKA Assay uses a brightly colored, fluorescent peptide substrate that is highly specific for PKA, the PepTag® A1 peptide (L R R A S L G) (Kemptide). The bright pink color is imparted by the addition of a dye molecule to the PepTag® A1 peptide substrate. Phosphorylation by PKA 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® PKA 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 column fractionations. The PepTag® PKA 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 bovine heart PKA purified by the method of Flockhart and Corbin (1) as a positive control.

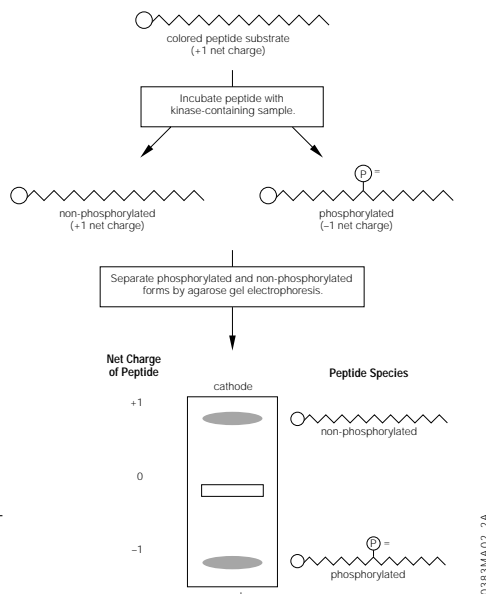
<sup>(a)</sup>U.S. Pat. No. 5,580,747 has been issued to Promega Corporation for a non-radioactive enzyme assay.

**Reference**

1. Flockhart, D.A. and Corbin, J.D. (1984) In: *Brain Receptor Methodologies*, Part A, Marangos, P.J. et al., eds., Academic Press, Orlando, FL, 209.

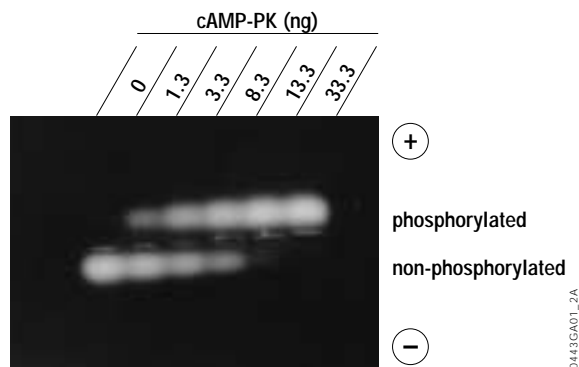
**Features**

- **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 success 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 2.4. Schematic diagram of the PepTag® Non-Radioactive Protein Kinase Assay procedure.**

**Figure 2.5. Detection of cAMP-dependent protein kinase.** Two micrograms of PepTag® A1 Peptide were incubated in the standard PKA assay with varying amounts (1.3–33.3ng) of PKA 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.



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**Protocol**

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

**Publications**

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**Catalytic Subunit**

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**Regulatory Subunit**

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Promega Resources

**Protocol**

cAMP-Dependent Protein Kinase, Catalytic and Regulatory Subunits .....**TB515**

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Catalytic and Regulatory Subunits

**cAMP-Dependent Protein Kinase, Catalytic Subunit**

Product	Size	Catalog #
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500u	V5161

**Description:** The purified 40kDa PKA Catalytic Subunit supplied by Promega may be used to phosphorylate target proteins in vivo via microinjection, or for in vitro enzymological studies of neural and hormonal signal transduction (1-3). Intracellular targets include ion channels (4), transcriptional activator proteins (5), and regulatory enzymes of glycogen metabolism (1). The PKA Catalytic Subunit has been purified from bovine heart by the method of Flockhart and Corbin (6).

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**cAMP-Dependent Protein Kinase, Regulatory Subunit (Type II)**

Product	Size	Catalog #
cAMP-Dependent Protein Kinase, Regulatory Subunit (Type II)	2,500u	V5221

**Description:** The purified PKA Regulatory Subunit ( $R_2$ ) is useful for confirming phosphorylation arising from PKA. Inhibition of phosphorylation by addition of  $R_2$ , and reversal of inhibition by subsequent addition of cAMP, provides definitive evidence that phosphorylation is catalyzed by PKA. The Regulatory Subunit has been purified from bovine heart by the method of Rannels, Beasley and Corbin (1).

**Reference**

- Rannels, S.R., Beasley, A. and Corbin, J.D. (1983) Regulatory subunits of bovine heart and rabbit skeletal muscle cAMP-dependent protein kinase isozymes. *Meth. Enzymol.* **99**, 55.

## Substrates and Inhibitors

## Kemptide (PKA) Peptide Substrate

Product	Size	Catalog #
Kemptide (PKA) Peptide Substrate	1mg	V5601

**Description:** Kemptide (PKA) Peptide Substrate is a specific peptide substrate for cAMP-dependent protein kinases (PKA). It is supplied ready for use in kinase reactions. The sequence of Kemptide is: LRRASLG (1,2). Its molecular weight is 772Da.

## References

1. Kemp, B.E. *et al.* (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **252**, 4888.
2. Casnellie, J.E. (1991) Assay of protein kinases using peptides with basic residues for phosphocellulose binding. *Meth. Enzymol.* **200**, 115.

## cAMP-Dependent Protein Kinase Peptide Inhibitor

Product	Size	Catalog #
cAMP-Dependent Protein Kinase Peptide Inhibitor	1mg	V5681

**Description:** The cAMP-Dependent Protein Kinase (PKA) Inhibitor (also known as PKI), TTYAD-FIASGRRNAIHD, inhibits phosphorylation of target proteins by binding to the protein-substrate site of the catalytic subunit of PKA. It corresponds to the region 5–24 of the naturally occurring protein kinase A inhibitor (1).

## Reference

1. Kemp, B.E., Pearson, R.B. and House, C.M. (1991) Pseudosubstrate-based peptide inhibitors. *Meth. Enzymol.* **20**, 287.

## cAMP

Product	Size	Catalog #
cAMP, 1mM	500µl	V6421

**Description:** cAMP (adenosine-3',5'-cyclic monophosphate) is an activator of cAMP-Dependent Protein Kinase (Cat.# V5161).

## Promega Product Citations

## Kemptide

Chang, B.Y. *et al.* (1998) RACK1, a receptor for activated C kinase and a homolog of the  $\beta$  subunit of G proteins, inhibits activity of Src tyrosine kinases and growth of NIH3T3 cells. *Mol. Cell. Biol.* **18**, 3245.

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## Promega Product Citations

This is a new product offered by Promega and no citations currently exist. The following references are excellent background articles on the use of these peptides.

Harrison, D.A., Carr, D.W. and Meizel, S. (2000) Involvement of protein kinase A and A kinase anchoring protein in the progesterone-initiated human sperm acrosome reaction. *Biol. Reprod.* **62**, 811.

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Vijayaraghavan, S. *et al.* (1997) Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility. *J. Biol. Chem.* **272**, 4747.

## Promega Resources

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Goueli, S. and Hsaio, K. (2000) Using InCELLect™ Cell-Permeable, Stearated Peptides to probe cAMP-dependent protein kinase-mediated cellular signaling reactions in vivo. *Promega Notes* **75**, 29.

## InCELLect™ Peptides

## InCELLect™ AKAP St-Ht31 Inhibitor Peptide

Product	Size	Catalog #
InCELLect™ AKAP St-Ht31 Inhibitor Peptide	150µl	V8211

**Description:** InCELLect™ AKAP St-Ht31 Inhibitor Peptide<sup>(a)</sup> is a stearated (St) form of the peptide Ht31 (derived from human thyroid AKAP or A Kinase-Anchoring Protein). This peptide has been shown to inhibit the interaction between RII subunits of cAMP-Dependent Protein Kinases (PKA) and the A Kinase-Anchoring Protein (AKAP) in cell extracts (1–3). The presence of the hydrophobic stearated moiety enhances the cellular uptake of the peptide through the lipophilic microenvironment of the plasma membrane. InCELLect™ St-Ht31P Control Peptide (Promega Cat.# V8221), which has a very similar sequence but does not inhibit the interaction between protein kinase A and AKAP, has been used as a control peptide for studies with InCELLect™ AKAP St-Ht31 Inhibitor Peptide.

<sup>(a)</sup>Patent Pending.

## References

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## Feature

- **Cell Permeable:** The InCELLect™ AKAP St-Ht31 Inhibitor Peptide is taken up by intact cells. Allows for in vivo studies on PKA localization.

## InCELLect™ St-Ht31P Control Peptide

Product	Size	Catalog #
InCELLect™ St-Ht31P Control Peptide	150µl	V8221

**Description:** InCELLect™ St-Ht31P Control Peptide<sup>(a)</sup> is a stearated form (St) of the peptide Ht31P. This peptide has been used as a control peptide for the study of InCELLect™ AKAP St-Ht31 Inhibitor Peptide (Promega Cat.# V8211). References 1–3 describe experimental systems using the stearated forms of the Ht31 and Ht31P peptides. InCELLect™ St-Ht31P Control Peptide has a very similar sequence to InCELLect™ AKAP St-Ht31 Inhibitor Peptide; the replacement of two isoleucine residues with prolines renders it unable to inhibit the interaction between the RII subunit of cAMP Dependent Protein Kinase (PKA) and the A Kinase-Anchoring Protein (AKAP). The presence of the hydrophobic stearated moiety enhances the cellular uptake of the peptide through the lipophilic microenvironment of the plasma membrane.

<sup>(a)</sup>Patent Pending.

## References

1. Vijayaraghavan, S. *et al.* (1997) Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility *J. Biol. Chem.* **272**, 4747.
2. Vijayaraghavan, S. *et al.* (1997) A tyrosine-phosphorylated 55-kilodalton motility-associated bovine sperm protein is regulated by cyclic adenosine 3', 5'-monophosphates and calcium. *Biol. Reprod.* **56**, 1450.
3. Klussmann, E. *et al.* (1999) Protein kinase A anchoring proteins are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. *J. Biol. Chem.* **274**, 4934.

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