



Promega

Technical Bulletin

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

INSTRUCTIONS FOR USE OF PRODUCT V7480.



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SignaTECT[®] cAMP-Dependent Protein Kinase (PKA) Assay System

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1. Description	1
2. Product Components and Storage Conditions	2
3. Quantitation of cAMP-Dependent Protein Kinase (PKA) Activity	3
A. Preparation of Tissue or Cell Samples for PKA Assay	3
B. PKA Assay Protocol	3
C. Calculation of the Specific Activity of [γ - ³² P] ATP	6
D. Calculation of Protein Kinase A Enzyme Activity	6
E. Sample Data	7
4. Composition of Buffers and Solutions	7
5. Related Products	8
6. References	9
7. SAM ^{2®} Biotin Capture Membrane Template	9

1. Description

The cAMP-dependent protein kinase (PKA) is a second messenger-dependent enzyme that plays a pivotal role in mediating cellular responses to a variety of extracellular stimuli (1). Factors that either activate or inactivate adenylate cyclases, thereby resulting in an increase or decrease in cAMP levels, are known modulators of PKA activity. This enzyme is a tetramer with two catalytic subunits and two regulatory subunits. Binding of cAMP to regulatory subunits causes the regulatory subunits to dissociate from the catalytic subunits, releasing an active catalytic subunit. PKA-mediated phosphorylation is implicated in a variety of cellular processes (1-3). Thus, determining its activity in cellular or tissue extracts is an important step toward understanding its diverse functions in the cell.

The most common method to assay PKA activity involves measuring the transfer of ³²P-labeled phosphate to a protein or peptide substrate that is captured on phosphocellulose filters via weak electrostatic interactions. However, in the presence of multiple kinases (e.g., in a tissue extract) the ³²P-labeled peptides/proteins bound to the phosphocellulose filter may reflect kinase activity other than that due to PKA. To increase the specificity of the

1. Description (continued)

PKA assay, the SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System^(a) uses biotinylated Kemptide (LRRASLG), a peptide substrate derived from the in vivo substrate pyruvate kinase (4). This substrate is highly specific for PKA. The high affinity of Kemptide for PKA ($K_m = 5\text{--}10\mu\text{M}$) provides sufficient sensitivity to detect the enzyme at levels typically found within most biological samples.

The ³²P-labeled, biotinylated substrate is recovered from the reaction mix with the SAM²® Biotin Capture Membrane, which is a novel streptavidin matrix (5). The SAM²® Membrane is prenumbered and partially cut so that individual squares can be easily identified, separated and placed into scintillation vials. Alternatively, the intact SAM²® Membrane can be analyzed using a phosphoimaging device or by conventional autoradiography. This convenient and rapid technique provides low backgrounds and high signal-to-noise ratios, even with complex samples such as crude cell extracts, while retaining the high substrate capacity necessary to maintain optimum reaction kinetics. Sample data obtained with this system are illustrated in Section 3.E. Specificity of this assay system is further demonstrated through the use of the cAMP-Dependent Protein Kinase Peptide Inhibitor (Cat.# V5681).

2. Product Components and Storage Conditions

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

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)
- 600µl PKA Biotinylated Peptide Substrate, 0.5mM
- 600µl PKA Assay 5X Buffer
- 600µl cAMP, 0.025mM

Storage Conditions: Store all components of the SignaTECT® cAMP-Dependent Protein Kinase (PKA) 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 can be stored at 4°C (return the unused portion of the SAM²® Biotin Capture Membrane to the resealable plastic bag). The Termination Buffer can be stored at room temperature (20–25°C).

3. Quantitation of cAMP-Dependent Protein Kinase (PKA) Activity

PKA activity can be quantitated in a variety of biological samples from purified enzyme preparations to tissue or cellular extracts. The following protocols describe methods used at Promega to accurately measure this activity. Specificity can be demonstrated by using 10 μ M cAMP-Dependent Protein Kinase Peptide Inhibitor (Cat.# V5681).

3.A. Preparation of Tissue or Cell Samples for PKA Assay

Materials to Be Supplied by the User

(Solution compositions are provided in Section 4.)

- extraction buffer
- phosphate buffered saline (PBS)
- homogenizer: (e.g., a Polytron® homogenizer or similar homogenizer for tissue samples or a Dounce homogenizer or similar homogenizer for cultured cells)
- microcentrifuge capable of 14,000 \times g

Note: Crude extracts should be assayed the same day they are prepared to retain maximal activity and obtain optimal results.

1. Cool the appropriate homogenizer and extraction buffer to 0–4°C.
2. **Tissue samples:** Homogenize 1g of tissue in 5ml of cold extraction buffer with a cold homogenizer (e.g., a Polytron® homogenizer).

Cultured cells: Wash 5 \times 10⁶ to 1 \times 10⁷ cells with phosphate buffered saline (PBS) (5ml per 100mm dish), and remove the buffer completely. Suspend the cells in 0.5ml of cold extraction buffer, and homogenize using a cold homogenizer (e.g., Dounce homogenizer).

3. Centrifuge the lysate for 5 minutes at 4°C at 14,000 \times g in a microcentrifuge, and save the supernatant. Proceed to Section 3.B.

3.B. PKA Assay Protocol

Materials to Be Supplied by the User

(Solution compositions are provided in Section 4.)

- [γ -³²P]ATP (3,000Ci/mmol) 10 μ Ci/ μ l
- 2M NaCl
- 2M NaCl in 1% H₃PO₄
- deionized water
- 30°C heating block or water bath
- scintillation counter or phosphoimaging device
- washing container (e.g., Nalgene® plastic utility box, 19 \times 15 \times 10cm)
- **optional:** orbital platform shaker
- **optional:** heat lamp

3.B. PKA Assay Protocol (continued)

1. If the Termination Buffer is frozen, thaw at 20–30°C, then vortex well. Thaw the rest of the frozen components on ice.
2. 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 7 can be used to identify samples using SAM^{2®} Membrane square numbers.

Note: The SAM^{2®} Biotin Capture Membrane is prenumbered and perforated into 96 1.25cm × 1.15cm sections.

3. Prepare the ATP mix as follows:

Component	Final per Reaction	20 Reactions
0.5mM ATP	5.00µl	100µl
[γ- ³² P]ATP (3,000Ci/mmol) 10µCi/µl	0.05µl	1µl

4. Prepare the following reaction in 0.5–1.5ml microcentrifuge tubes:

Component	Per Reaction	20 Reactions
PKA Assay Buffer 5X	5µl	100µl
cAMP, 0.025mM ¹	5µl	100µl
PKA Biotinylated Peptide Substrate ²	5µl	100µl
[γ- ³² P]ATP mix (see Section 3.B)	5µl	100µl

¹This component is not required if the purified PKA catalytic subunit is the source of activity. Replace with 5µl of deionized water.

²Final concentration is 100µM. Other concentrations may be used but should not exceed 200µM.


Note: A control reaction without substrate should also be performed to determine background counts.

5. Prepare appropriate dilutions of the enzyme samples in 0.1mg/ml BSA. Place on ice. We recommend preparing and testing crude lysate samples undiluted and serially diluted 2- to 16-fold. Purified enzyme preparations may require greater dilution.
6. Mix gently, and preincubate the reaction mix (see Step 4) at 30°C for 1–5 minutes.
7. Initiate the reaction by adding 5µl of the enzyme sample (see Step 5) to the reactants in Step 4. Total reaction volume will be 25µl.

Incubate the reaction at 30°C for 5 minutes. (Other time points and temperatures may be tested if desired.)

8. Terminate the reaction by adding 12.5µl of Termination Buffer to each reaction; mix well.
9. Spot 10µl of each terminated reaction onto a prenumbered SAM²[®] Membrane square. After all samples are spotted, follow the wash and rinse steps as described below. Save the reaction tubes for Step 12.

Note: Larger volumes may be spotted; however, if more than 15µl is to be spotted, separate the squares first to prevent cross contamination.

 **Do not** exceed 30µl per square. (Minor seepage of liquid onto adjacent squares does not cause contamination, as the biotinylated peptide is rapidly immobilized to the SAM²[®] Membrane before liquid migration is complete.) The linear capacity of the Membrane is 1.3nmol biotinylated substrate/10µl of terminated reaction volume.

10. Place the SAM²[®] Membrane squares containing samples from Step 9 into a washing container. Wash using an orbital platform shaker set on low or by occasional manual shaking as follows:

Wash once for 30 seconds with 200ml of 2M NaCl.



Wash 3 times for 2 minutes each with 200ml of 2M NaCl.



Wash 4 times for 2 minutes each with 200ml of 2M NaCl in 1% H₃PO₄.



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 run slightly.
 - Specificity of activity can be assessed by performing the assay in the presence of PKA activators and inhibitors.
11. Dry the SAM²[®] 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 SAM²[®] Membrane was washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)

3.B. PKA Assay Protocol (continued)

12. Determine total counts to calculate the specific activity of [γ - 32 P]ATP as follows: Remove 5 μ l aliquots from any two reaction tubes from Step 9, and spot onto individual SAM²[®] Membrane squares or Whatman[®] 3mm filter discs. For this step, dry **without** washing. After analysis, use these results to calculate the specific activity of [γ - 32 P]ATP in Section 3.C.

Note: If 5 μ l is not available from a single tube you may combine the contents of several tubes for this step.

13. **Analysis by scintillation counting:** If still connected, separate the SAM²[®] Membrane squares with samples (from Steps 11 and 12) using forceps, scissors or a razor blade, and place the squares or 3mm filter discs into individual scintillation vials. Add scintillation fluid to the vials and count.

Phosphoimaging analysis: Alternatively, the SAM²[®] Membrane may remain intact, and the intact SAM²[®] Membrane or 3mm filter discs may be analyzed using a phosphoimaging device.

3.C. Calculation of the Specific Activity of [γ - 32 P]ATP

$$\text{The specific activity of } [\gamma\text{-}^{32}\text{P}]\text{ATP in cpm/pmol of ATP} = \frac{(37.5/5) (x)}{2,500}$$

where:

- 37.5 is the sum of the reaction volume (25 μ l) and the Termination Buffer volume (12.5 μ l).
- 5 is the volume in microliters of the samples from Section 3.B, Step 12.
- x is the average cpm of the 5 μ l samples from Section 3.B, Step 12.
- 2,500 is the number of pmol of ATP in the reaction.

3.D. Calculation of Protein Kinase A Enzyme Activity

Enzyme specific activity in pmol ATP/minute/ μ g of protein =

$$\frac{(\text{cpm}_{\text{reaction with substrate}} - \text{cpm}_{\text{reaction without substrate}}) \times (37.5)}{(10) \times (\text{incubation time}^*_{\text{minutes}}) \times (\text{amount of protein in reaction}_{\mu\text{g}}) \times (\text{specific activity of } [\gamma\text{-}^{32}\text{P}]\text{ ATP})}$$

where:

- 37.5 is the sum of the reaction volume (25 μ l) and Termination Buffer volume (12.5 μ l)
- 10 is the volume in microliters of the sample from Section 3.B, Step 9.

***Note:** In this protocol, recommended incubation time is 5 minutes (see Section 3.B, Step 7).

3.E. Sample Data

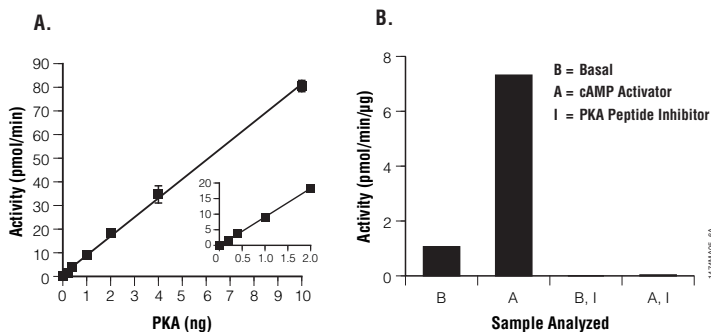


Figure 1. Panel A. Sensitivity and linearity of assay. cAMP-Dependent Protein Kinase (PKA) Catalytic Subunit (Cat.# V5161) was diluted in 0.1mg/ml BSA, 1,000- to 50,000-fold, and assayed as described in Section 3. The inset shows data below 2ng. **Panel B. PKA detection in tissue extracts.** Crude rat brain extract was prepared and analyzed for PKA activity as in Section 3. Activity was increased sevenfold by adding the activator, cAMP, and was completely inhibited by cAMP-Dependent Protein Kinase (PKA) Peptide Inhibitor (Cat.# V5681).

4. Composition of Buffers and Solutions

extraction buffer

(see Reference 5)

25mM	Tris-HCl (pH 7.4)
0.5mM	EDTA
0.5mM	EGTA
10mM	β -mercaptoethanol
1 μ g/ml	leupeptin
1 μ g/ml	aprotinin

Store at 4°C or, for up to six months, at -20°C. Just before use, add 0.5ml of PMSF stock solution (100mM PMSF in 100% ethanol) per 100ml of extraction buffer.

2M NaCl

116.9g/L NaCl

phosphate buffered saline (PBS)

0.2g/L	KCl
8.0g/L	NaCl
0.2g/L	KH_2PO_4
1.15g/L	Na_2HPO_4

PKA Assay Buffer 5X

200mM	Tris-HCl (pH 7.4)
100mM	MgCl_2
0.5mg/ml	bovine serum albumin (BSA)

Termination Buffer

7.5M guanidine hydrochloride

2M NaCl in 1% H_3PO_4

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

5. Related Products

Product	Size	Cat.#
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500u	V5161
cAMP-Dependent Protein Kinase Peptide Inhibitor	1mg	V5681
PepTag® Non-Radioactive		
cAMP-Dependent Protein Kinase Assay	120 reactions	V5340
cAMP, 1mM*	500µl	V6421
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT® Protein Tyrosine Kinase (PTK) Assay System	96 reactions	V6480
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System	96 reactions	V8161
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430
InCELLect® AKAP St-Ht31 Inhibitor Peptide	150µl	V8211
InCELLect® St-Ht31P Control Peptide	150µl	V8221
ProFluor® Src-Family Kinase Assay	4 plate	V1270
	8 plate	V1271
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241
ProFluor® Ser/Thr PPase Assay	4 plate	V1260
	8 plate	V1261
ProFluor® Tyrosine Phosphatase Assay	4 plate	V1280
	8 plate	V1281
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10 ml	V3772
	100ml	V3773
	10× 100ml	V3774

*For Laboratory Use.

6. References

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4. Pilkis, S.J. *et al.* (1980) Phosphorylation of rat hepatic fructose-1,6-bisphosphate and pyruvate kinase. *J. Biol. Chem.* **255**, 2770-5.
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7. SAM²® Biotin Capture Membrane Template

Promega		SAM ² ® 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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