Technical Bulletin

SignaTECT® Protein Kinase C (PKC) Assay System

INSTRUCTIONS FOR USE OF PRODUCT V7470.

www.promega.com

PRINTED IN USA.
Revised 1/13

Part# TB242

SignaTECT® Protein Kinase C (PKC) Assay System

All technical literature is available on the Internet at: www.promega.com/protocols/ Please visit the web site to verify that you are using the most current version of this Technical Bulletin. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

1. Description

Ca^{2+}- and phospholipid-dependent protein kinases (PKCs) represent a family of second messenger-dependent protein kinases that are stimulated by Ca^{2+} and phospholipid (1). The PKC enzymes play 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). The enzymes are implicated in phosphorylation of several neuronal proteins that are thought to regulate neurotransmitter release and establish long-term potentiation in memory formation (3–6).

In mammals, the enzymes comprise a highly homologous family of at least ten isozymes, each of which consists of a regulatory domain and a catalytic domain (1,7,8). Members of the first group of isozymes (α, βI, βII and γ) are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), and thus conform to the conventional classification. Members of the second group (δ, ε, η and θ) are stimulated by DAG and PS but do not require calcium for activation, and are thus referred to as unconventional PKC isozymes. Members of the third group (ζ and λ) are stimulated by PS alone and are thus considered atypical PKC isozymes (1). These isozymes can be differentiated based on their cofactor requirements.

1. Description (continued)

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

To increase the specificity of the PKC assay, the SignaTECT® Protein Kinase C (PKC) Assay System[a] uses the biotinylated peptide Neurogranin$_{(28–43)}$ (AAKIQAS*FRGHMARKK), a specific substrate commercially available for PKC activity (9). The biotinylated, ^{32}P-labeled substrate is recovered from the reaction mix with the SAM^{2}® Biotin Capture Membrane (SAM^{2}® Membrane; 10), which is a novel streptavidin matrix. The SAM^{2}® Membrane is prenumbered and partially cut so that individual squares can be easily identified, separated and placed in scintillation vials. Alternatively, the intact SAM^{2}® Membrane can be analyzed using a phosphorimaging system 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.

2. Product Components and Storage Conditions

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

System contains sufficient reagents to perform 96 kinase reactions. Includes:

- 1 SAM^{2}® Biotin Capture Membrane
- 600µl ATP, 0.5mM
- 200µl BSA, 10mg/ml
- 1.4ml Termination Buffer (2 × 700µl)
- 600µl PKC Biotinylated Peptide Substrate, 0.5mM
- 600µl PKC Activation 5X Buffer (3 × 200µl)
- 600µl PKC Coactivation 5X Buffer
- 600µl Control Buffer 5X

Storage Conditions: PKC Activation 5X Buffer must be stored at –70°C. Store the remaining components at –70°C or –20°C, where they are stable for at least six months from the date of purchase. **Avoid multiple freeze-thaw cycles.** For storage of less than 1 month, the SAM^{2}® Biotin Capture Membrane can be stored at 4°C (return the unused portion of the SAM^{2}® Membrane to the resealable plastic bag). The Termination Buffer can be stored at room temperature (20–25°C).

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

3. Quantitation of Protein Kinase C Activity

PKC activity can be quantitated in a variety of biological samples, from purified enzyme preparations to tissue extracts. The following protocols describe methods used at Promega to accurately measure this activity. To demonstrate specificity by phospholipid activation of PKC in crude extracts, endogenous phospholipids should first be removed by a simple and rapid column extraction. Specificity can be further demonstrated using 100µM Myristoylated Protein Kinase C Peptide Inhibitor (Cat.# V5691).

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

Materials to Be Supplied by the User
(Solution compositions are provided in Section 4.)

- extraction buffer
- extraction buffer with 200mM NaCl
- phosphate buffered saline (PBS)
- 1ml column of DEAE cellulose (Whatman® DE52) pre-equilibrated in extraction buffer
- homogenizer (e.g., Polytron® homogenizer or similar homogenizer for tissue samples and a Dounce homogenizer or similar homogenizer for cultured cells)
- microcentrifuge capable of 14,000 × *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. **For tissue:** Homogenize 1g of tissue in 5ml of cold extraction buffer using a cold homogenizer (e.g., a Polytron® homogenizer).

   For cultured cells: Wash 5×10^6 to1×10^7 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, 14,000 × *g* in a microcentrifuge, and save the supernatant.
4. Pass the supernatant over a 1ml column of DEAE cellulose that has been pre-equilibrated in extraction buffer. Wash the column with 5ml of extraction buffer, then elute the PKC-containing fraction using 5ml of extraction buffer containing 200mM NaCl. Proceed to Section 3.B.

3.B. PKC Assay Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 4.)

- [γ-^{32}P]ATP (3,000Ci/mmol) 10µCi/µl
- 2M NaCl
- 2M NaCl in 1% H_3PO_4
- Triton® X-100
- enzyme dilution buffer
- 30°C heating block or water bath
- scintillation counter or phosphorimaging system
- washing container (e.g., Nalgene® plastic utility box, 7.5 × 6 × 4 inches)
- deionized water
- orbital platform shaker (optional)
- heat lamp (optional)

1. Thaw the Termination Buffer at 20–30°C, then vortex well. Thaw the rest of the frozen components on ice. For maximal activation, briefly sonicate the PKC Activation 5X Buffer before use. (Alternatively, vortexing the PKC Activation Buffer for 10 seconds before use often is sufficient.)

2. Wearing gloves, cut (with 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 the SAM$^{2®}$ Membrane square numbers.

3. Prepare the ATP mix as follows:

Component	Final per Reaction	20 Reactions
0.5mM ATP	5µl	100µl
[γ-^{32}P]ATP (3,000Ci/mmol) 10µCi/µl	0.05µl	1µl

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

a. Reaction in the presence of phospholipids (activated PKC reaction):

Component	Final per Reaction	20 Reactions
PKC Coactivation 5X Buffer	5µl	100µl
PKC Activation 5X Buffer	5µl	100µl
PKC Biotinylated Peptide Substrate*	5µl	100µl
[γ-^{32}P]ATP mix (see Step 3)	5µl	100µl
Total volume	**20µl**	**400µl**

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

b. Reaction in the absence of phospholipids (control reaction):

Component	Final per Reaction	20 Reactions
PKC Coactivation 5X Buffer	5µl	100µl
Control Buffer 5X	5µl	100µl
PKC Biotinylated Peptide Substrate	5µl	100µl
[γ-^{32}P]ATP mix (Step 3)	5µl	100µl
Total volume	**20µl**	**400µl**

5. Prepare appropriate dilutions of the enzyme samples to be tested using enzyme dilution buffer (Section 4). We recommend preparing and testing both undiluted and 2- to 16-fold serially diluted crude lysate samples. Purified enzyme preparations may require greater dilution.

6. Mix gently, and preincubate the reaction mix (Step 4) at 30°C for 3 minutes.

7. Initiate the reaction by adding 5µl of enzyme sample (Step 5) to the reactants in Step 4. The 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. This solution can be kept at room temperature during processing.

9. Spot 10µl of each terminated reaction onto a prenumbered square of the SAM$^{2®}$ Membrane. 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 SAM$^{2®}$ Membrane before liquid migration is complete.) The linear capacity of the SAM$^{2®}$ Membrane is 1.1nmol biotinylated substrate (peptide)/10µl of terminated reaction volume.

10. Place the SAM$^{2®}$ 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% H_3PO_4.

↓

Wash 2 times for 30 seconds each with 100ml of deionized water.

Total wash time <20 minutes.

3.B. PKC Assay Protocol (continued)

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.

11. Dry the SAM$^{2®}$ Membrane on a piece of aluminum foil under a heat lamp for 5–10 minutes or air-dry at room temperature 30–60 minutes. (If the SAM$^{2®}$ Membrane was washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)

12. Determine total counts to calculate the specific activity of [γ-^{32}P]ATP as follows: remove 5µl aliquots from any two reaction tubes from Step 8, and spot onto individual SAM$^{2®}$ Membrane squares. For this step, dry SAM$^{2®}$ Membrane squares **without** washing. After analysis, use these results to calculate the specific activity of [γ-^{32}P]ATP in Section 3.C.

(!) You may combine the contents of several tubes if 5µl is not available from a single tube

13. **Analysis by scintillation counting:** If still connected, separate the SAM$^{2®}$ Membrane squares (from Steps 11 and 12) using forceps, scissors or a razor blade, and place the squares into individual scintillation vials. Add scintillation fluid to the vials and count.

    Analysis by phosphorimaging: Alternatively, the SAM$^{2®}$ Membrane squares may remain intact, and the intact SAM$^{2®}$ Membrane or 3mm filter discs may be analyzed using a phosphorimaging system.

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

The specific activity of [γ-^{32}P]ATP in the terminated reaction in cpm/pmol of ATP = $\frac{(37.5/5)(X)}{2{,}500} = 0.003(X)$

where:

37.5 is the sum of the reaction volume (25µl) and Termination Buffer volume (12.5µl).

5 is the volume in microliters of sample used from Step 12, Section 3.B.

X is the average counts/minute of the 5µl samples from Step 12, Section 3.B.

2,500 is the number of pmol of ATP in the reaction.

3.D. Calculation of Protein Kinase C Enzyme Activity

The enzymatic activity of PKC can be determined by subtracting the activity of the enzyme in the absence of phospholipids (Control Buffer) from that of the enzyme in the presence of phospholipids (Activation Buffer).

Enzyme activity in pmol ATP/minute/µg of protein =

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

where:

37.5 is the sum of the reaction volume (25µl) + the Termination Buffer volume (12.5µl).

10 is the volume in microliters of the sample from Step 9, Section 3.B.

3.E. Sample Data

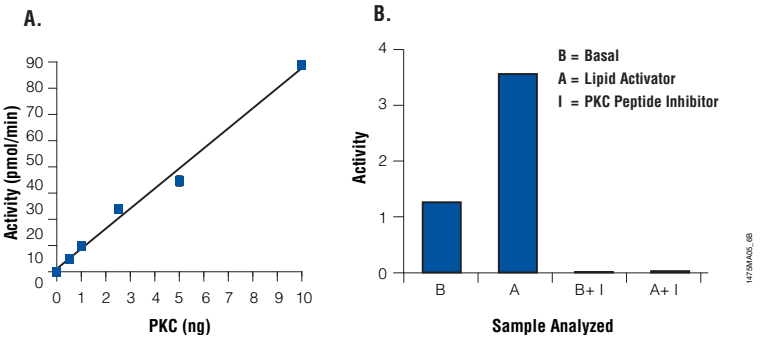

Figure 1. Panel A. Sensitivity and linearity of assay. Protein Kinase C (PKC) (Cat.# V5261) was diluted 12.5- to 250-fold in 0.1mg/ml BSA and 0.05% Triton® X-100 and assayed as described in Section 3. **Panel B. PKC detection in tissue extracts.** Crude rat brain extract was prepared and assayed as described in Section 3. Activity was increased threefold by the addition of activator and was completely inhibited by 100µM of PKC inhibitor, Myristoylated Protein Kinase (PKC) Peptide Inhibitor (Cat.# V5691).

4. Composition of Buffers and Solutions

extraction buffer

25mM	Tris-HCl (pH 7.4)
0.5mM	EDTA
0.5mM	EGTA
0.05%	Triton® X-100
10mM	β-mercaptoethanol
1µg/ml	leupeptin
1µg/ml	aprotinin

Store at 4°C or for up to 6 months at –20°C. Just before use, add 0.5ml of PMSF stock solution (100mM PMSF in 100% ethanol) per 100ml of extraction buffer.

enzyme dilution buffer

0.1mg/ml	bovine serum albumin (BSA)
0.05%	Triton® X-100

Termination Buffer

7.5M	guanidine hydrochloride

PKC Coactivation 5X Buffer

1.25mM	EGTA
2mM	$CaCl_2$
0.5mg/ml	BSA

Control Buffer 5X

100mM	Tris-HCl (pH 7.5)
50mM	$MgCl_2$

PKC Activation 5X Buffer

1.6mg/ml	phosphatidylserine
0.16mg/ml	diacylglycerol
100mM	Tris-HCl (pH 7.5)
50mM	$MgCl_2$

phosphate buffered saline (PBS)

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

2M NaCl

116.9g/L	NaCl

2M NaCl in 1% H_3PO_4

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

5. Related Products

Product	Size	Cat.#
Protein Kinase C (Rat Brain)	1µg	V5261
Myristoylated Protein Kinase C Peptide Inhibitor	1mg	V5691
PMA	5mg	V1171
4α-PMA	1mg	V1181
PepTag® Non-Radioactive Protein Kinase C Assay	120 assays	V5330
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaMKII) Assay System	96 reactions	V8161
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430
SignaTECT® Protein Tyrosine Kinase Assay System	96 reactions	V6480
SAM²® Biotin Capture Membrane*	96 sample	V2861
	7.6 x 10.9cm	V7861
SAM²® 96 Biotin Capture Plate*	96 well plate	V7541
	5 x 96 well plates	V7542
ProFluor® Src-Family Kinase Assay	4-plate	V1270
	8 plate	V1271
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774

*For Laboratory Use.

6. References

1. Newton, A.C. (1995) Protein kinase C: Structure, function, and regulation. *J. Biol. Chem.* **270**, 28495–8.
2. Ohkusu, K. *et al.* (1995) Elucidation of the protein kinase C-dependent apoptosis pathway in distinct subsets of T lymphocytes in MRL-lpr/lpr mice. *Eur. J. Immunol.* **25**, 3180–6.
3. Suzuki, T. (1994) Protein kinases involved in the expression of long-term potentiation. *Int. J. Biochem.* **26**, 735–44.
4. Pasinelli, P. *et al.* (1995) Long-term potentiation and synaptic protein phosphorylation. *Behav. Brain Res.* **66**, 53–9.
5. Seedorf, K., Sherman, M. and Ullrich, A. (1995) Protein kinase C mediates short- and long-term effects on receptor tyrosine kinases. Regulation of tyrosine phosphorylation and degradation. *Ann. NY. Acad. Sci.* **766**, 459–62.
6. Weiler, I.J., Hawrylak, N. and Greenough, W.T. (1995) Morphogenesis in memory formation: Synaptic and cellular mechanisms. *Behav. Brain Res.* **66**, 1–6.
7. Dekker, L.V. and Parker, P.J. (1994) Protein kinase C—a question of specificity. *Trends Biochem. Sci.* **19**, 73–7.
8. Mahoney, C.W., Seki, K. and Huang, K.P. (1995) Phosphorylation of MARCKS, neuromodulin, and neurogranin by protein kinase C exhibits differential responses to diacylglycerols. *Cell. Signal.* **7**, 679–88.
9. 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**, 1032–9.
10. Goueli, B.S. *et al.* (1995) A novel and simple method to assay the activity of individual protein kinases in a crude tissue extract. *Anal. Biochem.* **225**, 10–7.

7. SAM²® Biotin Capture Membrane Template

Promega				SAM²® 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

2732MA08_9A

[a]U.S. Pat. Nos. 6,066,462, 6,348,310 and 6,753,157 and European Pat. No. 0 760 678 B9.

Kinase-Glo, PepTag, ProFluor, SAM² and SignaTECT are registered trademarks of Promega Corporation.

Nalgene is a registered trademark of Nalge Nunc International. Polytron is a registered trademark of Kinematica AG. Triton is a registered trademark of Union Carbide Chemicals & Plastics Technology Corporation. Whatman is a registered trademark of Whatman Paper Company, Ltd.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA
Toll Free in USA 800-356-9526 · Phone 608-274-4330 · Fax 608-277-2516 · **www.promega.com**
Printed in USA. Part# TB242
Revised 1/13