Promega

Technical Bulletin

SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase Assay System

INSTRUCTIONS FOR USE OF PRODUCT V8161.

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SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System

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1. Description

The SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System[(a)] provides an improved method to quantitate CaM KII protein kinase activity, both in purified enzyme preparations and in cell or tissue extracts. The SignaTECT® System uses the unique SAM2® Biotin Capture Membrane[(a)], which is 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^{-15}M). The SAM2® Membrane binds biotinylated substrate at a minimum of 1.1nmol/cm^2. In addition, the membrane is optimized for low nonspecific binding.

The SignaTECT® CaM KII Assay System overcomes the problem of nonspecific substrate binding by using a biotinylated peptide that is selective for CaM KII in conjunction with the SAM2® Biotin Capture Membrane (1,2). The high binding capacity of the SAM2® Membrane for the CaM KII biotinylated peptide

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1. Description (continued)

substrate and the low backgrounds observed with this system maximize the signal-to-noise ratio. Following phosphorylation of the CaM KII biotinylated peptide substrate and binding to the SAM2® Membrane, the excess free [γ-^{32}P]ATP and nonbiotinylated proteins are removed via a simple washing procedure. The binding of biotin to streptavidin is rapid and strong. Once formed, the biotin-streptavidin association is unaffected by extremes in pH, temperature, salt concentration and denaturing agents. Due to the strength of this binding interaction, the ^{32}P-labeled CaM KII biotinylated peptide substrate is unlikely to be removed during the washing procedure.

Figure 1 depicts the sensitivity and linearity of CaM KII detection using the SignaTECT® CaM KII Assay System. The linear range of CaM KII detection in Figure 1 is 0–10ng. The slope of the line will depend on the specific activity of the enzyme preparation being analyzed.

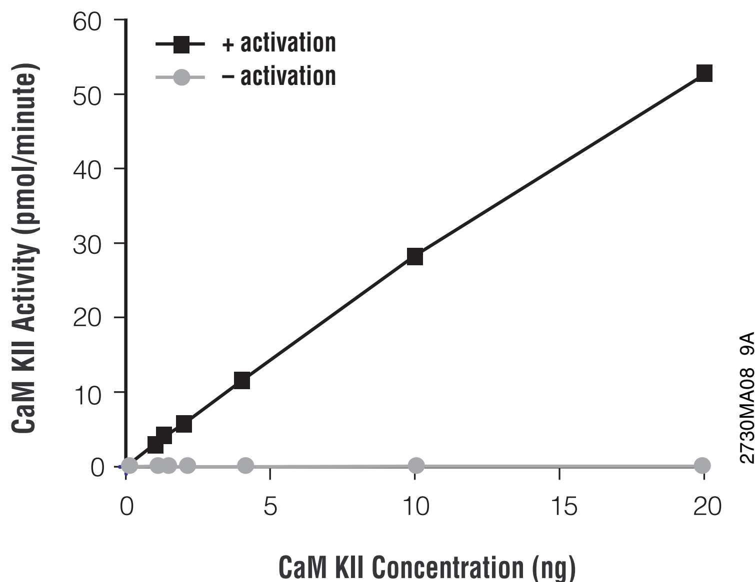

Figure 1. Sensitivity and linearity of CaM KII detection using a commercially available, purified CaM KII enzyme and the SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System. The slope of the line depends on the specific activity of the enzyme preparation being tested.

The multifunctional Ca^{2+}/calmodulin-dependent protein kinase, also known as CaM kinase II (CaM KII) is a well known effector of calcium- and calmodulin-mediated functions. It is present in many tissues but is most abundant in brain. The enzyme has broad substrate specificity, suggesting that it may play a role in many cellular functions (3,4). It is a major neuronal mediator of calcium signaling that integrates multiple related functions, ranging from neurotransmitter synthesis and release to modulation of neurotransmitter receptors and ion channels, gene expression and neurite outgrowth. CaM KII is also required for synaptic plasticity such as long-term potentiation (LTP), a cellular model of learning and memory (3–5). α-CaM KII knockout mice display behavioral abnormalities that include decreased fear response, decreased serotonin release in putative serotonergic neurons of the dorsal raphe (6,7) and increased defensive aggression. The enzyme was recently shown to be involved in myocardial hypertrophy, which is characterized by increased cell size due to changes in myofilament organization and re-expression of the embryonic gene for atrial natriuretic factor (ANF; 8,9).

In addition to phosphorylation of neuronal proteins, the enzyme phosphorylates Ca^{2+}-ATPase and phospholamban. It also phosphorylates transcription factors such as cAMP responsive element binding protein (CREB) (10), and its activation is required for adipogenesis (11). The enzyme is composed of 8–12 isoforms (α, β, γ and δ), ranging in molecular weight from 51kDa (α) to 58–61kDa (β, γ and δ). The α and β isoforms are the predominant forms in the nervous system where they are expressed exclusively. The γ and δ isoforms are expressed in all tissues including brain (12,13). Autophosphorylation of the enzyme has a profound effect on its activity. The phosphorylation of threonine 286 (T^{286}) in the α isoform (or T^{287} of the β isoform) results in a calcium-independent enzyme (12,13). The generation of this autonomous kinase may underlie some long-term enhancement of transient calcium signals (3–5).

Selected Citations Using the SignaTECT® CaM KII Protein Kinase Assay System

- Fasanaro, P. *et al.* (2006) Cyclin D1 degradation enhances endothelial cell survival upon oxidative stress. *FASEB J.* **20**, 1242–4.

  Human umbilical vein endothelial cells (HUVEC) were resuspended in CaMK extraction buffer and lysed by Dounce homogenization. CaMK activity was measured using the SignaTECT® CaM KII Protein Kinase Assay System.

- Dateki, M. *et al.* (2005) Neurochondrin negatively regulates CaM KII phosphorylation, and nervous system-specific gene disruption results in epileptic seizure. *J. Biol. Chem.* **280** , 20503-8.

  SignaTECT® CaM KII Protein Kinase Assay System was used to determine CaM KII activity in mouse hippocampus lysates.

For additional peer-reviewed articles that cite use of the SignaTECT® CaM KII Protein Kinase Assay System, visit: **www.promega.com/citations/**

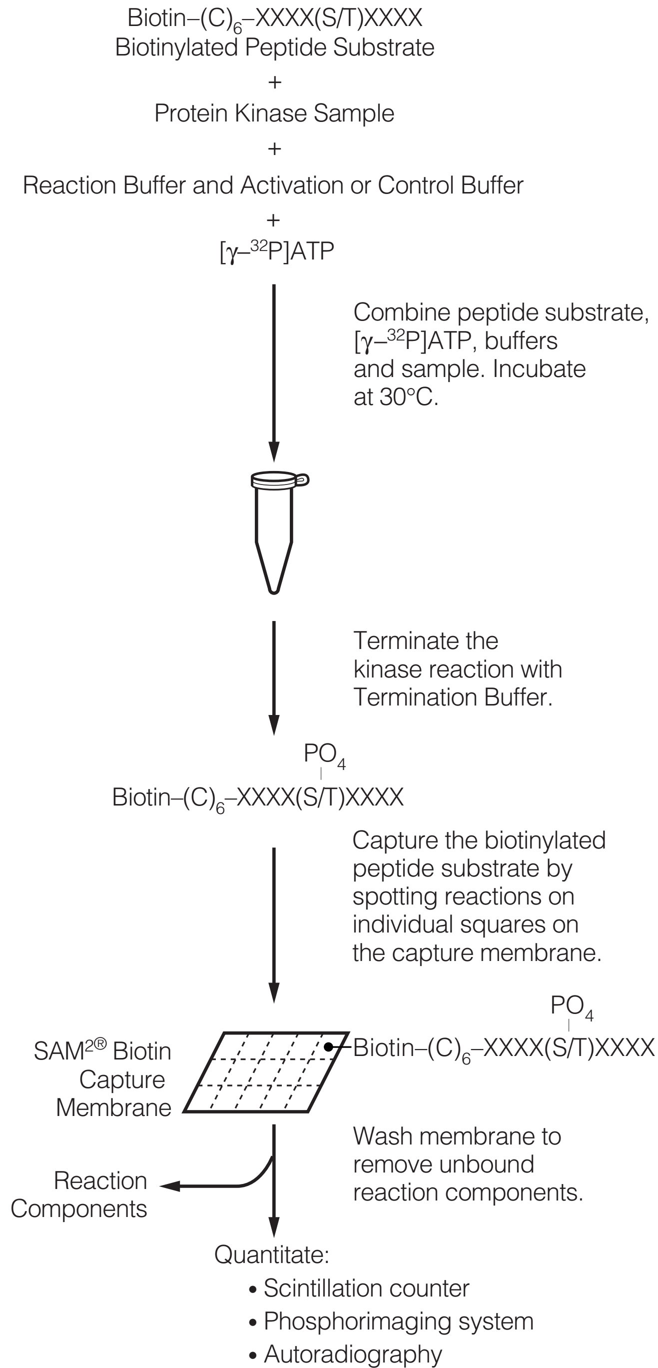

Figure 2. Overview of the SignaTECT® Assay procedure.

2. Product Components and Storage Conditions

Product	Size	Cat.#
SignaTECT® CaM KII Protein Kinase Assay System	96 reactions	V8161

Each system contains sufficient reagents to perform 96 kinase reactions. Includes:

- 600µl ATP, 0.5mM
- 1,400µl Termination Buffer (2 × 700µl)
- 300µl CaM KII Biotinylated Peptide Substrate, 0.5mM
- 1,000µl CaM KII Reaction 5X Buffer
- 500µl CaM KII Activation 5X Buffer
- 500µl CaM KII Control 5X Buffer
- 200µl Bovine Serum Albumin (BSA, 10mg/ml)
- 1 SAM2® Biotin Capture Membrane

Storage Conditions: The system components can be stored at –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 one month, the SAM2® Membrane can be stored at 4°C (return the unused portion of the membrane to the resealable plastic bag). The Termination Buffer can be stored at room temperature (20–25°C), where it is stable for at least one year from the date of purchase.

3. Quantitation of CaM KII Kinase Activity

CaM KII kinase activity can be quantitated in purified enzyme samples (Figure 1) as well as in cell or tissue extracts. Figure 2 is an overview of the SignaTECT® Assay. Figure 3 (Section 3.C) depicts CaM KII activity detected in rat brain extract—see the Appendix, Section 6.A, for a suggested protocol to prepare rat brain extract for use in the CaM KII assay. The following protocol describes the assay developed by Promega to quantify CaM KII kinase activity.

3.A. CaM KII Kinase Assay Protocol

Note: For best results kinase assays should be run with the following controls: (i) assay without substrate, and (ii) assay with and without activator.

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

- [γ-^{32}P]ATP (at 3,000Ci/mmol, 10mCi/ml)
- 2M NaCl
- 2M NaCl in 1% H_3PO_4
- 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
- **optional:** orbital platform shaker
- **optional:** heat lamp
- **optional:** Whatman® 3MM filter discs

3.A. CaM KII Kinase Assay Protocol (continued)

1. Thaw the Termination Buffer at 20–30°C, then vortex well. Thaw the rest of the frozen components on ice and vortex gently.
2. Wearing gloves, cut (using scissors or a razor blade) the required number of squares from the SAM2® Biotin Capture Membrane. The squares may remain connected as a partial sheet to minimize handling. Return the unused SAM2® Membrane to the resealable plastic bag at 4°C for storage of less than one month or at –20°C.
3. Prepare the ATP mix as follows:

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

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

Reaction in the Presence of Activation Buffer:

Component	Final Volume per Reaction	Volume per 20 Reactions
CaM KII Biotinylated Peptide Substrate	2.5µl	50µl
CaM KII Reaction 5X Buffer	5µl	100µl
CaM KII Activation 5X Buffer	5µl	100µl
[γ-^{32}P]ATP mix (Step 3)	5µl	100µl
deionized water	2.5µl	50µl

Reaction in the Presence of Control Buffer:

Component	Final Volume per Reaction	Volume per 20 Reactions
CaM KII Biotinylated Peptide Substrate	2.5µl	50µl
CaM KII Reaction 5X Buffer	5µl	100µl
CaM KII Control 5X Buffer	5µl	100µl
[γ-^{32}P]ATP mix (Step 3)	5µl	100µl
deionized water	2.5µl	50µl

Note: The final peptide concentration is 50µM. Other concentrations may be used but should not exceed 200µM.

5. Mix gently, and preincubate the reaction mix (Step 4) at 30°C for 3 minutes.
6. Prepare appropriate dilutions of the enzyme samples in enzyme dilution buffer. Prepare enzyme dilution buffer as follows: Dilute the 5X Reaction Buffer to 1X with deionized water. Add BSA to a final concentration of 0.1mg/ml.

   Note: We recommend preparing and testing crude samples undiluted and serially diluted twofold from 1:2 to 1:16. Purified enzyme preparations may require greater dilution.

7. Initiate the reaction by adding 5μl of the enzyme sample (Step 6) to the reactants in Step 5. The total reaction volume will be 25μl. Incubate at 30°C for 2 minutes. For low enzyme concentrations it may be desirable to incubate for up to 5 minutes. However, for incubations longer than 2 minutes we recommend monitoring enzyme activity at both 2- and 5-minute time points.

   Note: Incubation times >2 minutes are not recommended, as there may be a loss of linearity in the reaction after approximately 2 minutes.

8. Terminate the reaction by adding 12.5μl of Termination Buffer to each reaction; mix well. The terminated reaction can be kept at room temperature during processing.

9. Spot 10μl of each terminated reaction onto a prenumbered square of the SAM2® 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. (Minor seepage of liquid onto adjacent squares would cause very minimal contamination as the biotinylated peptide is rapidly immobilized to the SAM2® Membrane before liquid migration is complete.)

   ! **Do not** exceed 30μl per membrane square.

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 1 time 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.

    Step 10 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; the number of washes 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.

3.A. CaM KII Kinase Assay Protocol (continued)

11. Dry the SAM2® Membrane 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 has been 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 from any two reaction tubes remaining from Step 9, and spot onto individual SAM2® Membrane squares or Whatman® 3MM filter discs. For this step, dry SAM2® Membrane squares or discs without washing. After analysis, use these results to calculate the specific activity of [γ-^{32}P]ATP in Section 3.C.

    Note: If 5µl is not available from a single tube you may combine contents of several tubes.
13. **Analysis by scintillation counting:** If SAM2® Membrane squares are still connected, separate them (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.

    Analysis by phosphorimaging: Alternatively, the SAM2® Membrane squares may remain intact, and the intact squares or 3MM filter discs may be analyzed using a phosphorimaging system.

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

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

where:

37.5 is the total reaction volume (25µl) + Termination Buffer volume (12.5µl)

5 is the volume (microliters) of the samples from Section 3.A, Step 12

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

2,500 is the amount (pmol) of ATP in the reaction

3.C. Calculation of CaM KII Enzyme Activity

The enzymatic activity of CaM KII can be determined by subtracting the activity of the enzyme without calcium/calmodulin (in Control Buffer) from that of the enzyme with calcium/calmodulin (in Activation Buffer).

Enzyme activity in pmol/min/µg of protein =

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

where:

37.5 is the total reaction volume (25µl) + Termination Buffer volume (12.5µl)

10 is the volume in microliters of the samples from Section 3.A, Step 9

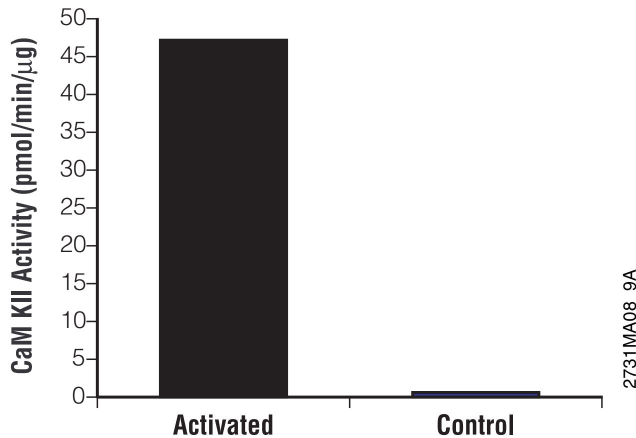

Figure 3. Detection of CaM KII activity using the SAM2® Biotin Capture Membrane. Extract was isolated from rat brain, as described in Section 6, and CaM KII activity measured using the SignaTECT® CaM KII Protein Kinase Assay System.

4. Composition of Buffers and Solutions

2M NaCl

116.9g/L	NaCl

2M NaCl in 1% H_3PO_4

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

CaM KII Activation 5X Buffer

5mM	$CaCl_2$
5μM	calmodulin
0.1mg/ml	BSA

CaM KII Control 5X Buffer

5mM	EGTA (pH 7.2)

CaM KII Reaction 5X Buffer

250mM	Tris-HCl (pH 7.5)
50mM	$MgCl_2$
2.5mM	DTT

enzyme dilution buffer

1X	CaM KII Reaction Buffer
0.1mg/ml	BSA

extraction buffer

20mM	Tris-HCl (pH 8.0)
2mM	EDTA
2mM	EGTA
20μg/ml	soybean trypsin inhibitor
10μg/ml	aprotinin
5μg/ml	leupeptin
2mM	DTT
25mM	benzamidin
1mM	PMSF (in 100% ethanol)*

*Add PMSF stock solution to the extraction buffer just before use (modified from reference 14).

Termination Buffer

7.5M	guanidine hydrochloride

5. References

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6. Appendix

6.A. Preparation of Rat Brain Extract for CaM KII Assay

This protocol is a suggested technique to prepare rat brain extract for use in the CaM KII Assay. The protocol is a modification of Carlin *et al.* (14) with protease inhibitor added to the extraction buffer (Section 4).

Note: For Step 4, cool the centrifuge to 4°C at least 30 minutes before use.

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

- extraction buffer
- fresh rat brain (store at –80°C until use)
- homogenizer (e.g., Tissue-Tearor™, Fisher Cat.# 15-338-55; chill on ice before use)

1. Harvest rat brain tissue immediately following euthanasia. Quick-freeze the brain tissue in liquid nitrogen within 30 seconds of harvest.
2. Immediately before use, place the brain tissue on ice and cut it into small pieces (approximately 0.5–1.0cm^2/piece). Weigh the tissue.
3. Add PMSF to the extraction buffer immediately prior to use, as described in Section 4. Add 3ml/g of extraction buffer (plus PMSF) to the brain tissue. Homogenize on ice.
4. Centrifuge at 350 × *g* for 5 minutes at 4°C. Dispense the supernatant into aliquots (50–100µl/vial), and freeze at –80°C or proceed immediately to Step 6, Section 3.A.
5. **Optional:** Determine the protein concentration of the supernatant using a modified Lowry protein assay (Sigma-Aldrich Cat.# L1013). The final concentration of rat brain extract must be ≥8mg/ml.

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6.B. SAM$^{2®}$ Biotin Capture Membrane Template

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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

7. Related Products

Product	Size	Cat.#
Anti-ACTIVE® CaM KII pAb, Rabbit, (pT^{286})	40µl	V1111
SignaTECT® PKA Assay System	96 reactions	V7480
SignaTECT® PKC Assay System	96 reactions	V7470
SignaTECT® DNA-PK Assay System	96 reactions	V7870
SignaTECT® cdc2 PK Assay System	96 reactions	V6430
SignaTECT® PTK Assay System	96 reactions	V6480
PepTag® Non-Radioactive PKC Assay	120 reactions	V5330
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay	120 reactions	V5340
Kinase-Glo® Luminescent Kinase Assay	10ml*	V6711
Kinase-Glo® Plus Luminescent Kinase Assay	10ml*	V3771
Pro-Fluor® PKA Assay	4 plate	V1240
	8 plate	V1241

*Available in additional sizes.

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

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