



Promega

Technical Bulletin

Anti-ACTIVE® CaM KII pAb, (pT286) and Anti-ACTIVE® Qualified Secondary Antibody Conjugates

INSTRUCTIONS FOR USE OF PRODUCTS V1111, V7951 AND V7971.



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Anti-ACTIVE[®] CaM KII pAb, (pT²⁸⁶) and Anti-ACTIVE[®] Qualified Secondary Antibody Conjugates

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1. Description

Promega has developed a polyclonal antibody (pAb) to the multifunctional calcium/calmodulin-dependent protein kinase CaM kinase II (CaM KII) that is phosphorylated on threonine 286 (pT²⁸⁶). The Anti-ACTIVE[®] CaM KII pAb was raised against the phosphothreonine-containing peptide. The recommended dilution of Anti-ACTIVE[®] CaM KII pAb for Western blot analysis is 1:5,000.

Donkey Anti-Rabbit IgG, (H + L), HRP and Donkey Anti-Rabbit IgG, (H + L), AP are affinity purified-horseradish peroxidase (HRP) or alkaline phosphatase (AP) -conjugated secondary antibodies for use with the Anti-ACTIVE[®] pAbs in

1. Description (continued)

Western blot analysis using chemiluminescent and colorimetric detection methods. The antibody conjugates exhibit minimal cross-reactivity to goat, mouse and sheep IgG, bovine serum albumin (BSA) and proteins in mammalian cell extracts. These antibody conjugates provide low backgrounds and highly specific signals when used at the recommended 1:10,000 dilution with Anti-ACTIVE® CaM KII pAb.

2. Product Components and Storage Conditions

| Product | Size | Cat.# |
|---|------|-------|
| Anti-ACTIVE® CaM KII pAb, Rabbit, (pT ²⁸⁶) | 40µl | V1111 |
| Donkey Anti-Rabbit IgG, (H + L), HRP, Anti-ACTIVE® Qualified* | 60µl | V7951 |
| Donkey Anti-Rabbit IgG, (H + L), AP, Anti-ACTIVE® Qualified* | 60µl | V7971 |

*For Laboratory Use.

When used at the recommended dilution, Cat.# V1111 will generate 200ml of blotting solution, while Cat.# V7951 and V7971 will generate 600ml of blotting solution. Each system includes:

- 1 tube Anti-ACTIVE® CaM KII pAb or Anti-ACTIVE® Qualified antibody conjugate

Storage Conditions: Anti-ACTIVE® CaM KII pAb should be stored at -20°C in aliquots, where it is stable for at least six months from the date of purchase. The Donkey Anti-Rabbit IgG antibody conjugates should be stored at -20°C, where they are stable for at least six months from the date of purchase. Avoid multiple freeze-thaw cycles.

3. General Considerations

3.A. Role and Importance of CaM Kinase II

The multifunctional Ca²⁺/calmodulin-dependent protein kinase, also known as CaM kinase II or CaM KII, is a well known effector of calcium- and calmodulin-mediated functions. The enzyme is composed of 8–12 isoforms (α , β , γ and δ), ranging in molecular weight from 52kDa (α) to 58–61kDa (β , γ and δ). The α and β isoforms are abundantly expressed in the nervous system, whereas the γ and δ isoforms are expressed in all tissues, including brain (1,2). In the nonphosphorylated form, CaM KII requires calcium and calmodulin for activity. In the presence of calcium and calmodulin, the enzyme is autophosphorylated on threonine 286 (T²⁸⁶) in the α isoform (T²⁸⁷ of the β isoform) and becomes calcium- and calmodulin-independent. Thus, the generation of this autonomous kinase may underlie some long-term potentiation of transient calcium signals (3–5).

CaM KII is a major neuronal mediator of calcium signaling that integrates multiple related functions, including neurotransmitter synthesis and release, modulation of neurotransmitter receptors and ion channels, gene expression and neurite outgrowth. CaM KII also is 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 and increased defensive aggression, as well as a decrease in serotonin release in putative serotonergic neurons of the dorsal raphe (6,7). The enzyme was recently shown to be involved in myocardial hypertrophy, including increased cell size, myofilament organization and re-expression of the embryonic gene for atrial natriuretic factor (ANF). Several myocardial proteins are involved in calcium transport, such as ryanodine receptor (8) and the sarcoplasmic reticular Ca^{2+} natriuretic factor (9). In addition to neuronal proteins, CaM KII 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).

It is well documented that subcellular localization and compartmentalization of enzymes and their substrates play a significant role in mediating specific cellular responses to external signaling molecules. Consistent with its diverse roles, CaM KII exhibits broad distribution within neurons, and thus discrete localization of the enzyme may play an important role in its physiological functions. The α and β isoforms of CaM KII are abundantly expressed in the brain, with the α isoform making up as much as 2% of total protein in certain brain regions. CaM KII is enriched in postsynaptic densities (PSD), which are cytoskeletal specializations apposed to the postsynaptic membrane of excitatory synapses. PSDs are thought to be scaffolds for neurotransmitter receptors, ion channels and their postsynaptic modulators and effectors, and are involved in the regulation of synaptic functions such as synaptic plasticity, receptor function and structural modification. It was recently shown that autophosphorylation of CaM KII leads to translocation of the enzyme to the PSD fractions (12,13), and upon dephosphorylation it dissociates back to the soluble fraction (14). It is noteworthy that the activation of the enzyme is required for LTP since the phosphorylation of the AMPA-type glutamate receptor, which mediates rapid excitatory synaptic transmission, is correlated with the activation and autophosphorylation of CaM KII on the α isoform (15).

3.B. Detection of Autophosphorylated CaM KII by Western Blot Analysis

The results shown in Figure 1 demonstrate the sensitivity of detection of the autophosphorylated pure enzyme by the Anti-ACTIVE® CaM KII pAb. Figure 2, Panel A, demonstrates the specificity of the Anti-ACTIVE® CaM KII pAb for in vitro-phosphorylated CaM KII in brain homogenate. The band obtained at a molecular weight of 52kDa represents the α subunit of the enzyme, while there also is an indication of the presence of the β subunit, albeit at lower intensity, which corresponds well with the distribution of the two forms in the brain. Figure 2, Panel B, demonstrates similar amounts of CaM KII in both nonphosphorylated and phosphorylated samples of brain extract.

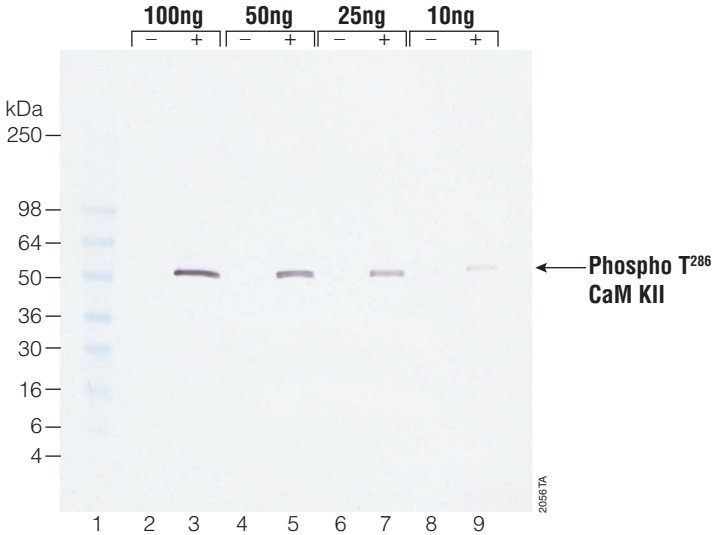


Figure 1. Western blot detection of purified CaM KII by Anti-ACTIVE® CaM KII pAb. Decreasing amounts of nonphosphorylated (-) CaM KII (lanes 2, 4, 6 and 8) and autophosphorylated (+) CaM KII (lanes 3, 5, 7 and 9) were subjected to SDS-PAGE and Western blot analysis using the Anti-ACTIVE® CaM KII pAb at 1:5,000. Detection was performed using a Donkey Anti-Rabbit IgG, AP-conjugated secondary antibody (Cat.# V7971) and Western Blue® Stabilized Substrate (Cat.# S3841). These results demonstrate the high sensitivity and selectivity obtained with Anti-ACTIVE® CaM KII pAb. Lane 1, molecular weight markers (Novex SeeBlue® Pre-Stained Standards, Cat.# LC5625).

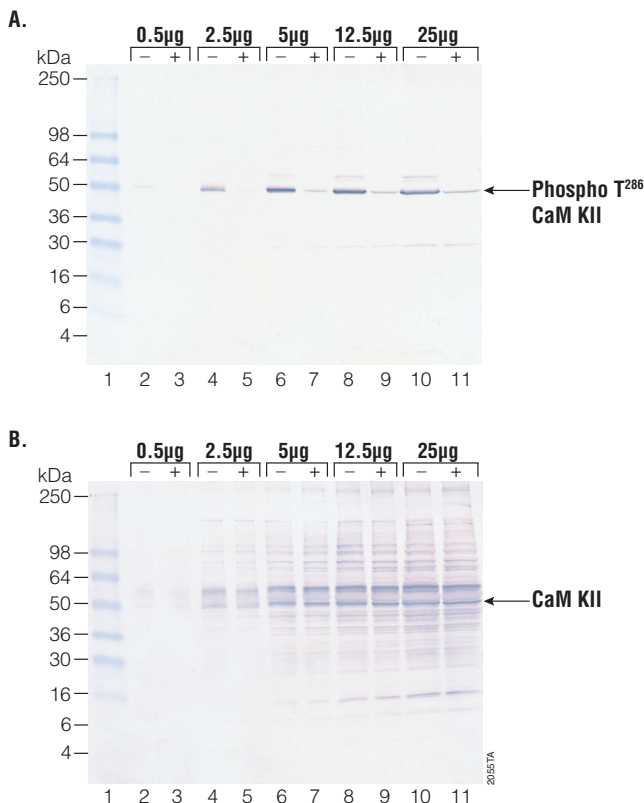


Figure 2. Detection of CaM KII using Anti-ACTIVE® CaM KII pAb and a total CaM KII pAb in Western analysis of brain homogenate. Rat brains were collected immediately following euthanasia and frozen in liquid nitrogen. Brains were homogenized, centrifuged at 350 × g and the cytosolic fraction collected. A sample of this extract was phosphorylated in vitro as described in Section 4 (12), and aliquots of nonphosphorylated and autophosphorylated fractions were analyzed by SDS-PAGE under reducing conditions, followed by immunoblotting onto nitrocellulose membrane (16). **Panels A and B.** Lanes 2, 4, 6, 8 and 10 contain autophosphorylated (+) brain cytosolic protein in the amounts shown; lanes 3, 5, 7, 9 and 11 contain nonphosphorylated (-) brain cytosolic protein in the amounts shown. **Panel A.** The presence of autophosphorylated CaM KII was detected using Anti-ACTIVE® CaM KII pAb (Cat.# V1111), diluted 1:5,000. **Panel B.** Total CaM KII was detected using a commercially available anti-CaM KII antibody that binds both phosphorylated and nonphosphorylated CaM KII. Detection was performed using a Donkey Anti-Rabbit IgG, AP-conjugated secondary antibody (Cat.# V7971) and Western Blue® Stabilized Substrate (Cat.# S3841). The results demonstrate the specificity of Anti-ACTIVE® CaM KII pAb for phosphorylated CaM KII, as indicated by the high signal-to-noise ratio obtained.

4. Preparation of Brain Tissue Extract for Use with Anti-ACTIVE® CaM KII Antibody

Rat brain tissue provides an excellent source of CaM KII. This protocol describes the procedure for preparation (Section 4.A) and autophosphorylation (Section 4.B; reference 12) of CaM KII in rat brain extract. Table 1 provides component and quantity listings for control and autophosphorylation reactions.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 6.)

- tabletop centrifuge
- centrifuge tubes
- heating block
- vortex
- tissue homogenizer
- spectrophotometer
- 2X control buffer
- 2X phosphorylation buffer
- homogenization buffer
- 10X dye solution
- distilled water

4.A. Protocol for Preparation of Rat Brain Tissue Extract

! Steps 1-3 should be performed on ice.

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, thaw the brain tissue, cut into small pieces (approximately 0.5-1.0cm²/piece) and weigh.
3. Add PMSF to the homogenization buffer immediately prior to use, as described in Section 6. Add homogenization buffer to the brain tissue, 3ml/g tissue. Homogenize on ice.
4. Centrifuge at 350 × g for 5 minutes in a refrigerated centrifuge. Dispense the supernatant (50-100µl/vial) into aliquots and freeze at -80°C, or proceed immediately to Step 5 and Section 4.B.
5. Determine the protein concentration of the supernatant in preparation for the autophosphorylation reaction, described in Section 4.B. The final concentration of rat brain extract must be ≥8mg/ml.

Table 1. Components of the Control and Autophosphorylation Reactions for CaM KII (total reaction volume of 100µl).

| Component | Control Reaction | Autophosphorylation Reaction |
|---------------------------|------------------|------------------------------|
| brain tissue extract | Xµl (400µg) | Xµl (400µg) |
| 2X control buffer | 50µl | — |
| 2X phosphorylation buffer | — | 50µl |
| distilled water | (50-X)µl | (50-X)µl |

4.B. Protocol for Autophosphorylation of CaM KII

1. Mix all reaction components, and incubate for 15 minutes on ice (4°C). Add 100µl of 2X stop buffer to terminate the reactions.
2. Vortex well, then heat the samples at 97°C for 5 minutes. Cool samples on ice for 2-3 minutes.
3. Add 25µl of 10X dye solution to each sample, vortex well and heat at 97°C for 5 minutes. Cool samples on ice for 2-3 minutes.

5. Protocol for Western Blot Analysis using Anti-ACTIVE® CaM KII pAb

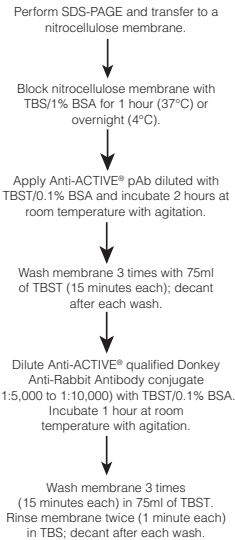
Figure 3 outlines the procedure for the use of Anti-ACTIVE® CaM KII pAb in Western blot analysis, using PVDF or nitrocellulose membranes. In addition, reference 16 contains protocols for use of the Anti-ACTIVE® antibodies in Western blot analysis and compares the results of Western blot analysis using our secondary antibodies to those with other commercially available secondary antibodies. These protocols have resulted in strong signals and very low background levels with a variety of experimental systems; however, some optimization may be required for best results with your particular application.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 6.)

- TMB Reagent for horseradish peroxidase (HRP) colorimetric detection (KPL Cat.# 50-77-00)
- Western Blue® Stabilized Substrate for alkaline phosphatase (AP) colorimetric detection (Promega Cat.# S3841) or Western-Star™ Substrate for chemiluminescent detection (Applera Cat.# WL10RS) or ECL™ Detection Reagent for chemiluminescent detection (GE Healthcare Cat.# RPN2109)
- secondary antibodies (either Donkey Anti-Rabbit IgG, (H + L), HRP, Anti-ACTIVE® Qualified, Cat.# V7951, or Donkey Anti-Rabbit IgG, (H + L), AP, Anti-ACTIVE® Qualified, Cat.# V7971)
- TBST buffer
- TBS buffer
- PVDF buffer

A. Nitrocellulose



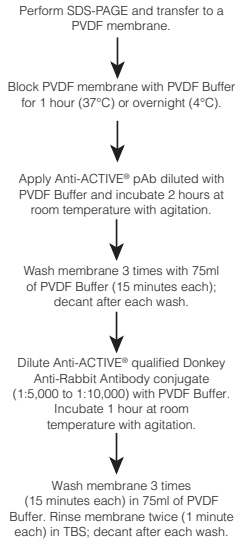
Colorimetric Detection

Incubate with detection reagent until appropriate signal level is obtained.
HRP: KPL TMB Reagent
AP: Promega Western Blue® Substrate.

Chemiluminescent Detection

HRP: Soak blot for 1 minute in ECL™ Detection Reagent. Expose blot to film.
AP: Soak blot for 5 minutes in Applera Western-Star™ Substrate. Remove excess reagent and expose blot to film.

B. PVDF



Colorimetric Detection

Incubate with detection reagent until appropriate signal level is obtained.
HRP: KPL TMB Reagent
AP: Promega Western Blue® Substrate.

Chemiluminescent Detection

HRP: Soak blot for 1 minute in ECL™ Detection Reagent. Expose blot to film.
AP: Soak blot for 5 minutes in Applera Western-Star™ Substrate. Remove excess reagent and expose blot to film.

100000151A

Figure 3. Schematic diagram illustrating the use of nitrocellulose and PVDF membranes in Western blot analysis with Anti-ACTIVE® pAbs. Protocols for use with nitrocellulose (Panel A) and PVDF (Panel B) membranes. Recommended dilution of the Anti-ACTIVE® CaM KII pAb is 1:5,000 and of the Anti-ACTIVE® qualified Donkey Anti-Rabbit IgG (H + L) secondary antibodies (both HRP- and AP-conjugated) is 1:10,000. KPL is an abbreviation for Kirkegaard and Perry Laboratories.

Note: It may be necessary to empirically determine the optimal dilutions of primary and secondary antibodies for your system. Use of secondary antibodies other than those available from Promega may require additional optimization.

5.A. Probing Membranes with Anti-ACTIVE® pAb

1. Perform SDS-PAGE, and transfer the proteins to a nitrocellulose or PVDF membrane (Figure 3).
2. Block the **nitrocellulose** membrane with TBS buffer/1% BSA for 1 hour at 37°C or overnight at 4°C.

Block the **PVDF** membrane with PVDF buffer for 1 hour at 37°C or overnight at 4°C.

3. The recommended dilution is 1:5,000 for Anti-ACTIVE® CaM KII pAb.

Note: It may be necessary to empirically determine the optimal dilution of antibody for your system.

Nitrocellulose membranes: Add the Anti-ACTIVE® pAb, diluted with TBST/0.1% BSA, and incubate for 2 hours at room temperature with agitation.

PVDF membranes: Dilute the Anti-ACTIVE® pAb with PVDF buffer, then incubate as described above.

4. **Nitrocellulose membranes:** Wash three times with 75ml of TBST buffer, 15 minutes per wash. Decant and replace the buffer after each wash.

PVDF membranes: Wash the PVDF membrane three times, as described above, using 75ml of PVDF buffer for each wash. Decant and replace the buffer after each wash.

5. Prepare the secondary antibody for incubation with the membrane. The recommended dilution for the Anti-ACTIVE® Qualified Donkey Anti-Rabbit antibody conjugate is 1:10,000.

Note: It may be necessary to empirically determine the optimal dilution of antibody for your system.

Nitrocellulose membranes: Dilute the Donkey Anti-Rabbit Antibody conjugate (1:10,000) with TBST/0.1% BSA. Incubate for 1 hour at room temperature with agitation.

PVDF membranes: Dilute the Donkey Anti-Rabbit conjugate (1:10,000) with PVDF buffer, and incubate as described above.

6. **Nitrocellulose membranes:** Wash the membrane three times, 15 minutes each wash, in 75ml TBST buffer. Rinse membrane two times, for 1 minute each, in TBS buffer. Decant the solution after each wash and after each rinse.

PVDF membranes: Wash three times as described above, using PVDF buffer. Rinse two times, 1 minute each, in TBS buffer. Decant after each wash and after each rinse.

5.B. Detection Protocols

To analyze results on nitrocellulose or PVDF membranes, select either colorimetric or chemiluminescent detection and follow the appropriate instructions below.

Colorimetric Detection: For both nitrocellulose and PVDF membranes, incubate with the detection reagent until appropriate signal level is obtained.

Chemiluminescent Detection using Horseradish Peroxidase: For both nitrocellulose and PVDF membranes, soak the blot for 1 minute in ECL™ Detection Reagent. Expose the blot to film.

Chemiluminescent Detection using Alkaline Phosphatase: Soak the nitrocellulose or PVDF blot for 5 minutes in Applera Western-Star™ Substrate. Remove the excess reagent and expose the blot to film.

6. Composition of Buffers and Solutions

TBS buffer

20mM Tris-HCl (pH 7.5)
150mM NaCl

TBST buffer

TBS buffer with 0.05% Tween® 20.

homogenization buffer

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

Add the PMSF immediately before use. Store at 4°C.

2X control buffer

100mM HEPES-KOH (pH 7.4)
2mM DTT
20mM MgCl₂
2mM EGTA

Prepare immediately before use. Keep on ice (4°C) during use.

PVDF buffer

TBS buffer with 0.2% I-Block™ and 0.1% Tween® 20.

2X phosphorylation buffer

100mM HEPES-KOH (pH 7.4)
2mM DTT
20mM MgCl₂
2mM CaCl₂
1.2µM calmodulin
100µM ATP

Prepare immediately before use. Keep on ice (4°C) during use.

2X stop buffer

0.125M Tris-HCl (pH 6.8)
3% SDS
5% β-mercaptoethanol

Store at ambient temperature.

10X dye solution

0.1ml 2M Tris (pH 7.5)
0.1g SDS
0.5ml 80% glycerol
10mg bromophenol blue

Make up solution to a final volume of 1ml with sterile distilled water. Store at ambient temperature.

7. References

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8. Related Products

| Product | Size | Cat.# |
|---|--------------|-------|
| SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System | 96 reactions | V8161 |
| Anti-ACTIVE® MAPK pAb, Rabbit, (pTEpY) | 40µl | V8031 |
| Anti-ACTIVE® JNK pAb, Rabbit (pTPpY) | 40µl | V7931 |
| | 120µl | V7932 |
| Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY) | 100µl | V1211 |
| Anti-ACTIVE® MAPK Family Sampler | | V3281 |

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