

BIOMOL QuantiZyme™ Assay System
✦ BIOMOL GREEN™ CALCINEURIN ASSAY KIT ✦
AK-804

BACKGROUND

Calcineurin (CaN) is the neuronal form of the widely distributed Ca²⁺/calmodulin-dependent Ser/Thr protein phosphatase 2B (PP-2B). CaN is a heterodimer consisting of a catalytic A subunit (57-61 kDa) and a regulatory B subunit (19 kDa). The catalytic A subunit is composed of four functional domains: the catalytic core with sequence homology to PP-1 and PP-2A (located between residues 71-235 in the rat brain αδ isoform), binding sites for both calmodulin (residues 391-414) and CaN B-regulatory subunit, and a C-terminal (residues 457-482) autoinhibitory domain.

The *BIOMOL GREEN™ Calcineurin Assay Kit* is a product in the *BIOMOL QuantiZyme™ Assay System* series. It is a complete colorimetric assay kit for measuring calcineurin phosphatase activity. It employs a convenient 96-well microtiter-plate format with all reagents necessary for measuring calcineurin (PP2B) phosphatase activity of purified enzyme. The RII phosphopeptide substrate, supplied with this kit, is the most efficient and outstanding peptide substrate known for calcineurin^{1,2}. The detection of free-phosphate released is based on the classic Malachite green assay^{3,4} and offers the following advantages:

- NON-RADIOACTIVE!
- CONVENIENT 1-STEP DETECTION -no mixing!
- MICROTITER-PLATE FORMAT -for high-throughput!

This new, improved version of the AK-804 kit incorporates human calcineurin A α (MW=60 kDa) + calcineurin B (MW=15 kDa) heterodimer expressed in an *E. coli* expression system. Both subunits were coexpressed in a construct with yeast myristoyl-CoA:protein N-myristoyltransferase. The resulting highly active calcineurin (protein phosphatase 2B) is N-myristoylated on the CaNB subunit, similar to the native protein⁵.

Refs: 1. A. Enz *et al. Anal. Biochem.* 1994 **216** 147
 2. A. Donella-Deana *et al. Eur. J. Biochem.* 1994 **219** 109
 3. B. Martin *et al. J. Biol. Chem.* 1985 **260** 14932
 4. K.W. Harder *et al. Biochem. J.* 1994 **298** 395
 5. A. Mondragon *et al. Biochemistry* 1997 **36** 4934

ALSO AVAILABLE SEPARATELY...

PRODUCT	CATALOG #
<i>BIOMOL GREEN™</i> Reagent 250 ml bottle	AK-111
Calcineurin (human, recombinant)	SE-163
RII phosphopeptide substrate	P-160
Cyclophilin A (human, recombinant)	SE-105
Cyclosporin A	A-195
CaN Autoinhibitory Peptide CN412	PR-104

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT BIOMOL® TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

COMPONENTS

NOTE ON STORAGE: Store all components except the microtiter plate at -70°C for the highest stability. The calcineurin enzyme component SE-163 must be handled particularly carefully in order to retain maximal enzymatic activity. Thaw it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused enzyme should be quickly refrozen by placing at -70°C. To minimize the number of freeze/thaw cycles, aliquot the calcineurin into separate tubes and store at -70°C.

SE-163 CALCINEURIN ENZYME (human, recombinant)
 FORM: 100 U/μl in 1X assay buffer (1:1 dilution of KI-128, below). 1 U=pmol/min @ 30°C.
 STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
 QUANTITY: 5000 U

SE101-9090 CALMODULIN (from bovine brain)
 FORM: 25 μM in dH₂O
 STORAGE: -70°C
 QUANTITY: 100 μl

P-160 SUBSTRATE (RII phosphopeptide, sequence Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu; MW=2192.0)
 FORM: 0.5 mg net peptide/vial
 STORAGE: -20 or -70°C
 QUANTITY: 3 x 0.5 mg

KI-128 2X ASSAY BUFFER
 (100 mM Tris, pH 7.5, 200 mM NaCl, 12 mM MgCl₂, 1 mM DTT, 0.05% NP-40, 1 mM CaCl₂)
 FORM: Liquid in screw-cap plastic bottle.
 STORAGE: -70°C
 QUANTITY: 20 ml

AK111-9090 BIOMOL GREEN™ REAGENT
 FORM: Liquid in screw-cap plastic bottle.
 STORAGE: 4°C. Long-term at -70°C.
 QUANTITY: 20 ml

KI-132 PHOSPHATE STANDARD
 FORM: 80 μM in dH₂O
 STORAGE: -20 or -70°C
 QUANTITY: 0.5 ml

KI-101 1/2 VOLUME MICROTITER-PLATE

STORAGE: Room temperature.

INSTRUCTION BOOKLET

OTHER MATERIALS REQUIRED

Microplate reader capable of measuring A_{620} to ≥ 3 -decimal accuracy.

Pipetman capable of pipetting 5-100 μl accurately

Multi-channel pipetman capable of pipetting 100 μl (optional).

Ice bucket to keep reagents cold until use.

EXPERIMENTAL METHODS

NOTE ON HANDLING: Hold all samples on ice bath until use, unless otherwise noted.

PRECAUTIONS: The *BIOMOL GREEN™* reagent is a highly sensitive phosphate detection solution. Free phosphate present on labware and in reagent solutions will greatly increase the background absorbance of the assay. This is detected visually as a change in color from yellow to green. Detergents used to clean labware may contain high levels of phosphate. Use caution by either rinsing labware with dH_2O or employ unused plasticware.

To prepare reagents for the assay:

1. Thaw all kit components and hold calcineurin, calmodulin and 2x assay buffer on an ice bath; Store *BIOMOL GREEN™* reagent at room temperature (RT).
2. Add calmodulin to the 2x assay buffer: Dilute calmodulin (SE101-9090) 1/50 in 2X assay buffer (KI-128) to required quantity (25 μl are required per assay well). For example, add 20 μl to 980 μl 2X assay buffer.
3. Reconstitute substrate (R11 phosphopeptide, P-160) with dH_2O to 0.75 mM (1.64 mg/ml): Add 305 μl dH_2O per 0.5 mg vial (10 μl are needed per assay well).

To prepare a standard curve:

4. Prepare 1 ml of 1X assay buffer (dilute 500 μl of 2X assay buffer with 500 μl dH_2O)
5. Perform 1:1 serial dilutions of phosphate standard and an assay buffer blank. Concentrations of 40, 20, 10, 5, 2.5, 1.25 and 0.625 μM correspond to 2, 1, 0.5, 0.25, 0.125, 0.063 and 0.031 nmol PO_4 (see Table 1):
 - a) Add 50 μl of 2X assay buffer (KI-128) to each wells A1, and A2 (2 nmol PO_4 standards).
 - b) Add 50 μl 1X assay buffer (prepared in step 4 above) to wells B1-H1 and wells B2-H2 (remaining standard concentrations)
 - c) Add 50 μl of 80 μM phosphate standard to well A1 and A2 of assay plate. Mix thoroughly by pipetting up and down several times.
 - d) Remove 50 μl from well A1 and add it to well B1. Mix thoroughly by pipetting up and down several times.
 - e) Remove 50 μl from well B1 and add it to well C1.
 - f) Mix thoroughly and repeat for wells D1-G1. At well G1, remove 50 μl and discard. DO NOT PROCEED TO WELL H1 (assay buffer blank). Final volume=50 μl .
 - g) Repeat serial dilution for the wells in column 2 (standard curve duplicates)

To prepare a time course/linearity assay:

6. Add 25 μl 2X assay buffer (KI-128) w/calmodulin (step 2) to microtiter plate wells designated for linearity assay (see Table 1).
7. Dilute the calcineurin (SE-163) to 8 U/ μl , in 1X assay buffer, and add 5 μl diluted calcineurin to wells. Final amount of calcineurin= 40 U per well.
8. Add 10 μl dH_2O to each well.
9. Designate a reaction time to each well (e.g.: 60 min, 40 min, 30 min, 20 min, 10 min, 5 min, 2 min, 0 min).
10. Equilibrate microtiter plate to reaction temperature (e.g.: 30 $^\circ\text{C}$).
11. Start reaction by addition of 10 μl phosphopeptide substrate (P-160; 0.75 mM from step 3) at appropriate time point. Make the addition in the reverse time order such that all incubations end at the same time (e.g.: Add 60 min time pt. at t=0; add 5 min at t=55 min, etc.). Final substrate concentration= 0.15 mM.

To prepare a test sample/inhibition assay:

12. Add 25 μl assay buffer (KI-128) w/calmodulin (step 2) to wells in microtiter plate. See Table 1.
13. Add 5 μl diluted calcineurin (SE-163) to wells (step 7). Final amount of calcineurin= 40 U per well.
14. Add 10 μl dH_2O to control wells.
15. Add 10 μl of test sample/inhibitor (dissolved in dH_2O) to test sample wells.
16. Allow test sample/inhibitor to equilibrate to reaction temperature (e.g.: 30 $^\circ\text{C}$) for 10 minutes.
17. Start reaction by addition of 10 μl phosphopeptide substrate (P-160; 0.75 mM from step 3). Final concentration= 0.15 mM. Allow reaction to proceed for a time period in which the reaction is linear (~10 min, see below).

To terminate reactions:

18. After incubating wells for desired duration, terminate reactions with 100 μl *BIOMOL GREEN™* reagent (AK111-9090).
19. Allow color to develop 20-30 minutes, making sure all wells spend approximately the same time with the reagent before reading on microplate reader.
20. Read $\text{OD}_{620\text{nm}}$ on microplate reader.
21. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells!

TABLE 1. EXAMPLE OF MICROTITER PLATE SAMPLES.

Sample [†]	Std Curve	Time course	Test Samples
Well #	1,2	3,4	5,6
A	2 nmol PO_4	60 min	Control
B	1	40	Inhibitor/test sample
C	0.5	30	
D	0.25	20	
E	0.125	10	
F	0.063	5	
G	0.031	2	
H	0	0	

[†] For highest accuracy, perform all samples in duplicate.

TABLE 2. TYPICAL ASSAY COMPONENTS

	2X Assay Buffer w/CaM	Calcineurin (40U)	H ₂ O	Test compound	Substrate (0.75 mM)
CONTROL	25 µl	5 µl	10 µl	0	10 µl
TEST SAMPLE	25 µl	5 µl	0	10 µl	10 µl

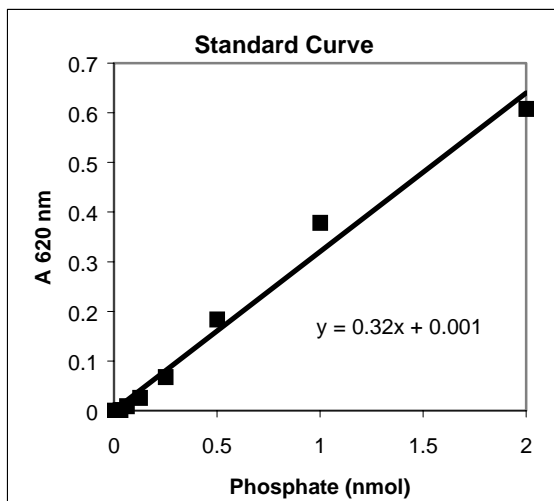
DATA ANALYSIS

Phosphate (PO₄) Standard Curve

1. Plot standard curve data as OD_{620nm} versus nmol PO₄ (see Figure 1).
2. Obtain a line-fit to the data using an appropriate routine.
3. Use the slope and Y-intercept to calculate amount of phosphate released for other experimental data (e.g.: time course and experimental data).

NOTE: For highest accuracy, a standard curve must be performed for each new set of assay data. This will normalize for variations in free phosphate in samples, time of incubation with the BIOMOL GREEN™ reagent, and other experimental factors.

FIGURE 1. BIOMOL GREEN™ PHOSPHATE STANDARD CURVE



Conversion of OD_{620nm} to Amount Phosphate Released

1. Convert OD_{620nm} data into the amount of phosphate released using the standard curve line-fit data, from above:

$$\text{Phosphate released} = (\text{OD}_{620\text{nm}} - Y_{\text{int}}) / \text{slope}$$

EXAMPLE:

Std curve slope=0.3 OD_{620nm}/nmol phosphate

Std curve Yint=0.001 OD_{620nm}

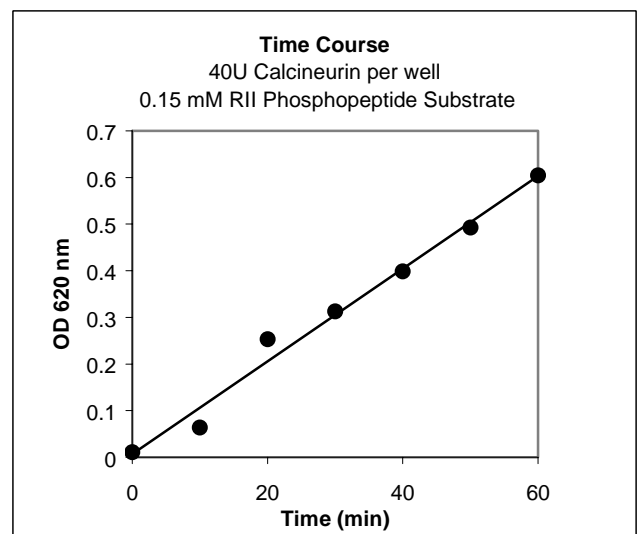
Sample OD=0.4

$$\text{Phosphate released} = (0.4 - 0.001) / 0.3 = 1.33 \text{ nmol}$$

Time Course/Linearity Curve

1. If the 0 time (Table 1, well# H3,4) has a significant value, subtract this number from all samples. This is background phosphate in the samples.
2. Plot OD_{620nm} versus reaction time. See Figure 2. Alternatively, the OD_{620nm} can be converted to phosphate released, as above.
3. Determine the reaction time range in which the amount of phosphate released is linear. In Figure 2, this range is from 0-60 min. This value is variable depending on reaction conditions and storage/handling of the calcineurin. The time range can be lengthened by decreasing the amount of calcineurin in the assay and lowering the assay temperature. For accurate results, it is important to perform inhibitor/agonist assays under linear assay conditions.

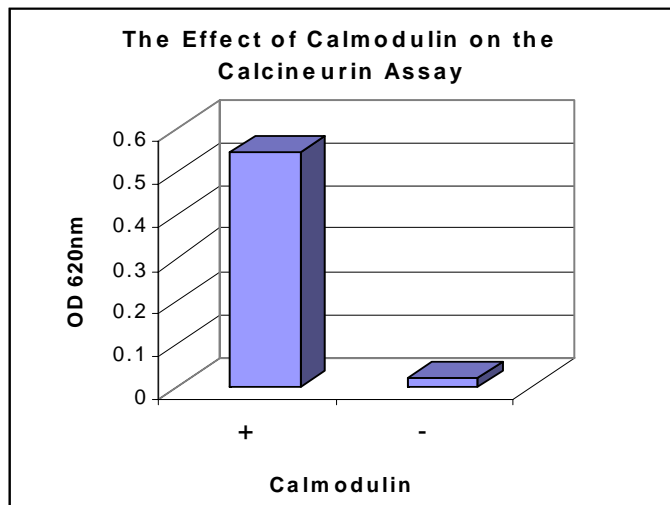
FIGURE 2. TIME COURSE OF PHOSPHATE RELEASED BY CALCINEURIN



Calmodulin Activation of Calcineurin Activity

1. Figure 3 illustrates the activation of calcineurin's phosphatase activity by calmodulin. In the presence (+) of calmodulin, calcineurin's activity is high. In the absence (-) of calmodulin, calcineurin activity is relatively low.

FIGURE 3. CALMODULIN ACTIVATION OF CALCINEURIN PHOSPHATASE ACTIVITY



Literature Citations of BIOMOL Calcineurin Assay Kits

B. Mehl et al. *J. Biol. Chem.* 2000 **275** 12841
T. Taigen et al. *Proc. Natl. Acad. Sci.* 2000 **97** 1196
G. Mallert et al. *Cell* 2001 **104** 675
M. Ichida and T. Finkel *J. Biol. Chem.* 2001 **276** 3524
O. Bueno et al. *Proc. Natl. Acad. Sci.* 2002 **99** 4586

USE OF PRODUCT

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

WARRANTY

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