

## **Calcineurin Cellular Activity Assay Kit**

Catalog number: BML-AK816

A complete colorimetric assay for measuring cellular calcineurin (PP2B) phosphatase activity

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# Product Manual

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## TABLE OF CONTENTS

INTRODUCTION .....	2
MATERIALS SUPPLIED .....	3
STORAGE .....	5
ADDITIONAL MATERIALS NEEDED .....	5
EXPERIMENTAL NOTES .....	5
BIOMOL <sup>®</sup> GREEN PHOSPHATASE ASSAY .....	11
DATA ANALYSIS .....	14
LITERATURE CITATIONS OF CALCINEURIN ASSAY KITS ..	16
CONTACT INFORMATION .....	18



Carefully note the handling and storage conditions of each kit component.



Please read entire booklet before proceeding with the assay.



Please contact Enzo Life Sciences Technical Support if necessary.

## INTRODUCTION

Calcineurin (CaN) is the neuronal form of the widely distributed  $\text{Ca}^{2+}$ /calmodulin-dependent Ser/Thr protein phosphatase 2B (**PP2B**). 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**, binding sites for both calmodulin and CaN B-regulatory subunit, and a C-terminal autoinhibitory domain.

The *Calcineurin Cellular Activity Assay Kit* is a complete colorimetric assay kit for measuring cellular calcineurin (**PP2B**) phosphatase activity. It employs a convenient 96-well microtiter-plate format with all reagents necessary for measuring calcineurin (**PP2B**) phosphatase activity in tissue/cellular extracts PLUS, human recombinant calcineurin<sup>5</sup> is included as a positive control! The RII phosphopeptide substrate, supplied with this kit, is the most efficient and outstanding peptide substrate known for calcineurin<sup>1,2</sup>. The detection of free-phosphate released is based on the classic Malachite green assay<sup>3,4</sup> and offers the following advantages: Non-radioactive, convenient 1-step detection, excellent sensitivity.

### References:

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

## MATERIALS SUPPLIED

- 1. Calcineurin Enzyme (Human, Recombinant), 500 U, Component No. BML-SE163-9090**  
50 mM Tris, pH 7.5, 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 5 mM DTT, 0.025% NP-40, 0.5 mM CaCl<sub>2</sub>.  
1 U=1 pmol/min @30°C.  
Lot-specific Activity Concentration listed on vial.  
Store at -80°C; Avoid Freeze/Thaw Cycles.
- 2. Calmodulin (human, recombinant), 100 µL, Component No. BML-SE325-9090**  
25 µM in deionized water (dH<sub>2</sub>O)  
Store at -80°C.
- 3. RII phosphopeptide, (substrate), 1.5 mg, Component No. BML-P160-9090**  
Sequence Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu; MW=2192.0  
Store at -20°C.
- 4. Calcineurin Assay Buffer (2X), 20 mL, Component No. BML-KI128-0020**  
100 mM Tris, pH 7.5, 200 mM NaCl, 12 mM MgCl<sub>2</sub>, 1mM DTT, 0.05% NP-40, 1 mM CaCl<sub>2</sub>  
Store at -80°C (or -20°C).
- 5. EGTA (2X) Buffer, 1 mL, Component No. BML-KI136-0001**  
100 mM Tris, pH 7.5, 20 mM EGTA, 200 mM NaCl, 12 mM MgCl<sub>2</sub>, 1 mM DTT, 0.05% NP-40  
Store at -80°C.
- 6. Lysis Buffer, 40 mL, Component No. BML-KI135-0040**  
50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2% NP-40  
Store at -80°C (or -20°C).

- 7. Protease Inhibitor Cocktail, 2 tablets, Component No. BML-KI103-0001**  
Solid tablet  
Store at +4°C.
- 8. BIOMOL® Green, 20 mL, Component No. BML-AK111-9090**  
Liquid in screw-cap plastic bottle  
Store at +4°C.
- 9. Phosphate Standard, 0.5 mL, Component No. BML-KI132-0500**  
80 µM in dH<sub>2</sub>O  
Store at -80°C (or -20°C).
- 10. Okadaic Acid, 325 µL, Component No. BML-EI181-9090**  
**HARMFUL! AVOID ALL FORMS OF CONTACT.**  
5 µM in 2X Assay Buffer (BML-KI128)  
Store at -80°C.
- 11. Desalting Column and Resin, 1 column and 1 g of resin, Component No. BML-KI100-0001**  
5 mL polypropylene disposable column and P6 DG desalting resin  
Store at ambient temperature. After rehydration, store at 4°C.
- 12. 1/2 Volume Microplates, 2 plates, Component No. 80-2405**  
Clear, 96-well  
Store at ambient temperature.



Storage temp.

## STORAGE

Store all components except the microtiter plate and desalting columns/resin at **-80°C** for the highest stability. All other components may be stored at **-80°C** or the alternate suggested temperature. The calcineurin enzyme component BML-SE163-9090 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 **-80°C**. To minimize the number of freeze/thaw cycles, aliquot the calcineurin into separate tubes and store at **-80°C**.

## ADDITIONAL MATERIALS NEEDED

- Microplate reader capable of measuring  $A_{620}$  to  $\geq 3$ -decimal accuracy.
- Centrifuge capable of 100k x g RCF.
- Swing bucket centrifuge capable of 800 x g RCF.
- Pipetter capable of pipetting 5-100  $\mu\text{L}$  accurately.
- Multi-channel Pipetter capable of pipetting 100  $\mu\text{L}$  (optional).
- Ice bucket to keep reagents cold until use.
- 16 g needle/syringe
- TBS buffer, 100 mL (20 mM Tris, pH 7.2, 150 mM NaCl)
- 15 and 50 mL conical centrifuge tubes
- Biological test material (e.g., tissue, cells)

## EXPERIMENTAL NOTES

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

**PRECAUTIONS:** The BIOMOL<sup>®</sup> 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  $\text{dH}_2\text{O}$  or employ unused plasticware. Do not use phosphate buffered saline (PBS) for any tissue/cell rinses - use TBS (Tris buffered saline)!

### Preparation of tissue/cell extracts

To prepare a tissue/cell extract for calcineurin activity assay:

Calcineurin activity is highly dependent on the experimental conditions and the cell/tissue source. Therefore, the amount of material required for an assay should be determined empirically by the user. Typically, between **0.5 - 5**  $\mu\text{g}$  of total protein or **5,000** and **50,000** cells per assay will provide sufficient signal for detection.

**NOTE ON BIOLOGICAL SAMPLE MATERIAL:** The following procedures have been tested for rat and mouse brain tissue. Other tissue or cell culture samples employed may require adjustment to this protocol for satisfactory results.

1. Add protease inhibitor tablets to lysis buffer (BML-KI135) immediately before use (1 tablet/10 mL buffer). Vortex.
2. Obtain tissue; if fresh, excise quickly.
3. Rinse tissue quickly in ice-cold TBS and shake-off/blot excess wetness.
4. Weigh the tissue in centrifuge tube.
5. Add lysis buffer (BML-KI135) with protease inhibitors to tissue. Use 0.33-0.5 mL per gram of tissue.
6. Loosely break up cells by passing them through a 16 g needle. Avoid air bubbles.
7. **Optional:** Sediment at 100-200k x g in centrifuge at 4°C for 45 min. Save supernatant=HSS (high-speed supernatant). Please note that this step will sediment the nucleus and any associated nuclear calcineurin.
8. Freeze immediately at -70°C.

### Removal of Free Phosphate from Extracts

To desalt tissue samples by gel filtration:

**NOTE:** This procedure is intended to remove excess phosphate and nucleotides (which are slowly hydrolyzed to release free phosphate in the presence of the BIOMOL<sup>®</sup> Green reagent) in the high speed supernatant (HSS) extract.

1. Pour the Desalting Column Resin (BML-KI100) out of the column, into a 50 mL conical tube. Rehydrate the resin by adding 20 mL of phosphate free dH<sub>2</sub>O and vortexing briefly. Allow the resin to set for 4 hours at RT or overnight at 4°C.
2. Decant the dH<sub>2</sub>O carefully, then add fresh dH<sub>2</sub>O at a 1:1 ratio to the rehydrated resin (~10 mL).



3. Add enough of the rehydrated resin to the Chromatography Column (BML-KI100) to obtain a 5 mL settled-bed volume (~5.5 cm bed height – roughly half of the total resin quantity provided). Remove tip from column and allow dH<sub>2</sub>O to drain by gravity.
4. Equilibrate the column by adding 8 mL of lysis buffer (BML-KI135) (without protease inhibitors) and allow the liquid to drain by gravity.
5. Place the column in a 15 mL centrifuge tube. Centrifuge at 800 x g for 3 min at 4°C to displace column buffer. Discard flow-through buffer.
6. Place column in a clean 15 mL centrifuge tube.
7. Add up to 350 µL HSS sample, from above, to column.
8. Centrifuge at 800 x g for 3 min. Save extract flow-through. This is the desalted cell lysate material to be tested for calcineurin activity.
9. Freeze sample immediately at -80°C.

NOTE: The desalting column resin can be reused. Between samples, the column should be rinsed with 4-5 volumes of phosphate free water, and then re-equilibrated with lysis buffer (return to step 4 in the protocol) before using the next sample. At least 4 or 5 samples may be used before efficiency drops.

TIP: The effective removal of phosphate/nucleotides from the extract should be tested qualitatively by adding 100 µL BIOMOL® Green reagent to 1 µL extract, and a separate sample of 1 µL dH<sub>2</sub>O. If no phosphate/nucleotides are present, both samples should remain yellow in color over a time period of 30 minutes at room temperature. The development of a visible green color indicates phosphate contamination, which must be eliminated from the samples before proceeding further!

## CaN Assays:

D.A. Fruman et al. Methods: A Companion to Meth. Enzymol. 1996 9 146

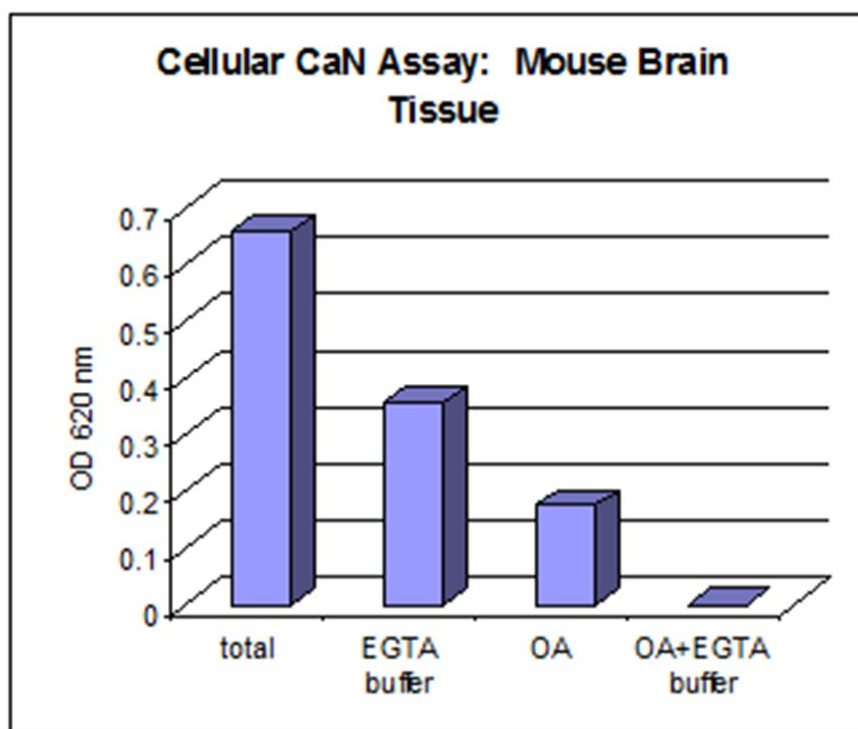
1. Subtract the "Background" phosphate released from each sample except the "Positive control".
2. Plot a graph analogous to Figure 1, below. Use either OD620nm or phosphate released for the Y-axis.
3. Determine the contribution of calcineurin:

$$\text{eq. 1 CaN (PP2B)} = \text{Total} - \text{EGTA buffer}$$

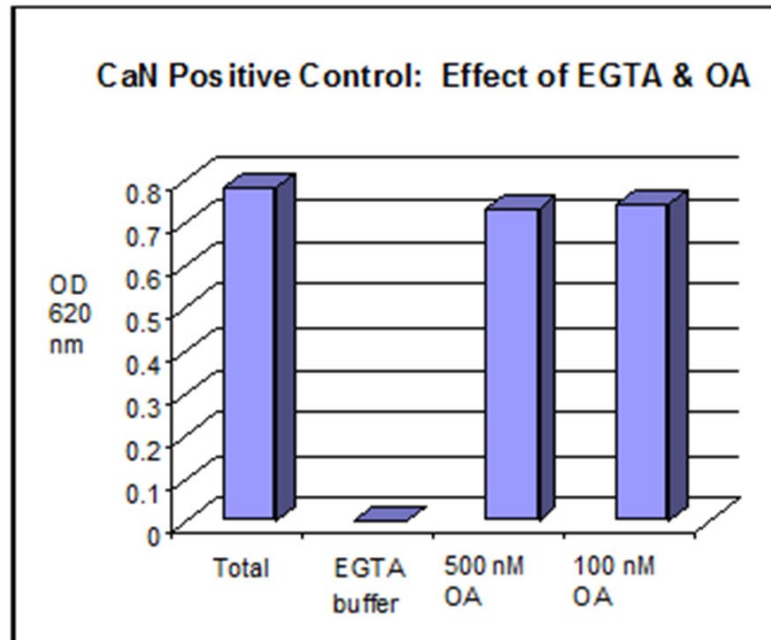
or

$$\text{eq. 2 CaN (PP2B)} = \text{OA} - (\text{OA} + \text{EGTA})$$

Eq. 1 is a conventional method to report CaN activity. However, the user must determine the most appropriate analysis for their specific experimental goal.



**Figure 1: Cellular CaN Assay Using Mouse Brain.** Phosphatase activity from a freshly prepared mouse brain extract. Prior to gel filtration the extract was diluted 1:1 in lysis buffer containing protease inhibitors. After gel filtration and prior to the phosphatase assay, the extract was diluted 1/25 in lysis buffer. Well contents were as in Table 2. The reaction was incubated for 30 minutes at 30°C.



**Figure 2: CaN Positive Control.** Purified calcineurin (BML-SE163-9090) positive control. The CaN was incubated 1 hour at 30°C under various buffer conditions. The results demonstrate that CaN activity is inhibited by the EGTA buffer, but not inhibited by 100 nM or 500 nM concentrations of okadaic acid.

**Table 1: Example of Microtiter plate samples**

Sample <sup>†</sup>	Standard Curve	Extract/CaN Samples
Well Number	1,2	3,4
A	2 nmoL PO <sub>4</sub>	Background
B	1	Total
C	0.5	EGTA buffer
D	0.25	OA (okadaic acid)
E	0.125	OA + EGTA
F	0.063	Positive Control
G	0.031	
H	0	

<sup>†</sup> For highest accuracy, perform all samples in duplicate.

**Table 2: Typical Assay Components**

	H <sub>2</sub> O	2X Assay Buffer with/CaM	2X EGTA Buffer	OA	Substrate (0.75 mM)	Extract/ CaN (dilute to 8 U/μL)
Background	20 μL	25 μL	0	0	0	5 μL <sup>a</sup>
Total	10 μL	25 μL	0	0	10 μL	5 μL <sup>a</sup>
EGTA buffer	10 μL	0	25 μL	0	10 μL	5 μL <sup>a</sup>
OA (okadaic acid)	5 μL	25 μL	0	5 μL	10 μL	5 μL <sup>a</sup>
OA + EGTA	5 μL	0	25 μL	5 μL	10 μL	5 μL <sup>a</sup>
Positive	10 μL	25 μL	0	0	10 μL	5 μL <sup>b</sup>

a) Add cellular extract (HSS)

b) Add calcineurin enzyme (BML-SE163-9090)

## **BIOMOL® GREEN PHOSPHATASE ASSAY**

### **To prepare reagents for the assay:**

1. Thaw all kit components and hold on ice bath, except BIOMOL® Green reagent at room temperature.
2. Dilute calmodulin (BML-SE325-9090) 1/50 in 2X assay buffer (BML-KI128) 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, BML-P160) with dH<sub>2</sub>O to 0.75 mM (1.64 mg/mL): Add 915 µL dH<sub>2</sub>O per 1.5 mg vial (10 µL are needed per assay well).

### **To prepare phosphate standard curve sample wells:**

1. Prepare 1 mL of 1X assay buffer (dilute 500 µL of 2X assay buffer with 500 µL of dH<sub>2</sub>O)
2. 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<sub>4</sub> (see Table 1):
  - a) Add 50 µL of assay buffer (BML-KI128) to wells A1, and A2 (2 nmoL PO<sub>4</sub> standards)
  - b) Add 50 µL of 1X assay buffer (prepared in step 1 above) to wells B1-H1 and wells B2-H2 (remaining standard concentrations).
  - c) Add 50 µL of 80 µM phosphate standard to wells 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 calcineurin activity assay sample wells:**

Assay Preparation (See Tables 1 & 2). Activity Analysis (See Figures 1 & 2).

**Background (no substrate):**

(Control for background phosphate/interfering substances)

1. Add 20  $\mu\text{L}$   $\text{dH}_2\text{O}$  to appropriate wells.
2. Add 25  $\mu\text{L}$  2X assay buffer (BML-KI128) with calmodulin to each well.

**Total phosphatase activity wells:**

(Total phosphatase activity in the extract)

1. Add 10  $\mu\text{L}$   $\text{dH}_2\text{O}$  to each well.
2. Add 25  $\mu\text{L}$  2X assay buffer (BML-KI128) with calmodulin to each well.

**EGTA buffer ( $\text{Ca}^{2+}$ /CaM free):**

(Total activity in the absence of active PP2B (calcineurin))

1. Add 10  $\mu\text{L}$   $\text{dH}_2\text{O}$  to each well.
2. Add 25  $\mu\text{L}$  2X EGTA buffer (BML-KI136) to each well.

**OA (Okadaic acid):**

(Total activity in the absence of active PP1 & PP2A)

1. Add 5  $\mu\text{L}$   $\text{dH}_2\text{O}$  to each well.
2. Add 25  $\mu\text{L}$  2X assay buffer (BML-KI128) with calmodulin to each well.
3. Add 5  $\mu\text{L}$  okadaic acid (BML-EI181-9090; 5 $\mu\text{M}$ )

**OA + EGTA:**

(Total activity in the absence of active PP1, PP2A & PP2B)

1. Add 5  $\mu\text{L}$   $\text{dH}_2\text{O}$  to each well.
2. Add 25  $\mu\text{L}$  2X EGTA buffer (BML-KI136) to each well.
3. Add 5  $\mu\text{L}$  okadaic acid (BML-EI181-9090; 5  $\mu\text{M}$ )

**Positive control (calcineurin enzyme):**

(Purified CaN enzyme positive control)

1. Add 10  $\mu\text{L}$   $\text{dH}_2\text{O}$  to each well.
2. Add 25  $\mu\text{L}$  2X assay buffer (BML-KI128) with calmodulin to each well.

Add phosphopeptide substrate:

3. Add 10  $\mu\text{L}$  phosphopeptide substrate BML-P160 to each well of the calcineurin samples except the "Background" control. DO NOT ADD SUBSTRATE TO THE PHOSPHATE STANDARD CURVE SAMPLES!
4. Equilibrate microtiter plate to reaction temperature (e.g., 30°C) for 10 minutes.

To initiate calcineurin assay:

5. Add 5  $\mu\text{L}$  sample extract or diluted calcineurin (dilute to 8 U/ $\mu\text{L}$  prior to use) to appropriate wells. For sample extract wells, it may be necessary to dilute the HSS tissue extract (e.g., 1/5-1/10 in lysis buffer BML-KI135). For calcineurin "Positive control" add 5  $\mu\text{L}$  BML-SE163-9090 (40 U/well).
6. Incubate plate at reaction temperature for desired duration (e.g., 30 minutes at 30°C).

To terminate reactions:

7. After incubating wells for desired duration, terminate reactions by adding 100  $\mu\text{L}$  BIOMOL<sup>®</sup> Green reagent (BML-AK111-9090) to ALL samples including the phosphate standard curve.
8. Allow color to develop 20-30 minutes, making sure all wells spend approximately the same time with the reagent before reading on microplate reader.
9. Read OD<sub>620nm</sub> on microplate reader.
10. Perform data analysis (see below).

**NOTE:** Retain microplate for future use of unused wells!

## DATA ANALYSIS

### Phosphate (PO<sub>4</sub>) Standard Curve

1. Plot standard curve data as OD<sub>620nm</sub> versus nmol PO<sub>4</sub> (see Figure 3).
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 the experimental data (see below).

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<sup>®</sup> Green reagent, and other experimental factors.

### Conversion of OD<sub>620nm</sub> to Amount Phosphate Released

1. Convert OD<sub>620nm</sub> 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:

$$\text{Std curve slope} = 0.3 \text{ OD}_{620\text{nm}} / \text{nmol phosphate}$$

$$\text{Std curve } Y_{\text{int}} = 0.001 \text{ OD}_{620\text{nm}}$$

$$\text{Sample OD} = 0.4$$

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

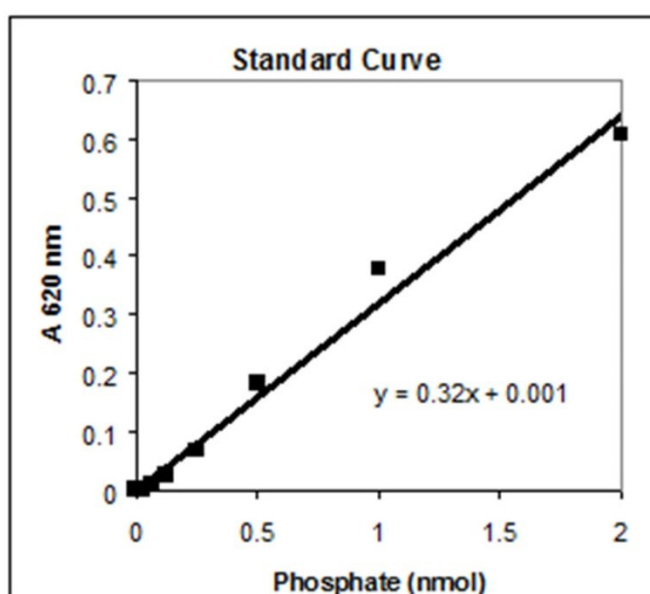


Figure 3: BIOMOL<sup>®</sup> Green Phosphate Standard Curve



## **Data Reduction to Determine Calcineurin Phosphatase**

**PRECAUTION:** The procedures for data analysis that follow are intended only as a guideline. The individual user must determine the suitability of this analysis for their particular experimental protocol. Additional controls and other samples may be appropriate for accurate analysis.

**ANALYSIS DESCRIPTION:** This assay uses the RII phosphopeptide, the best known substrate for CaN (PP2B). Nonetheless, in cellular extracts, the phospho-group is cleaved by other competing phosphatases. Thus, a series of conditions must be employed to discriminate between the contribution of other phosphatases. CaN requires calcium for its activity, thus the "EGTA buffer" sample represents total phosphatase activity less CaN. Okadaic acid (OA) at 100 and 500 nM is known to completely inhibit PP1 and PP2A, while it has no effect on CaN (see Figure 1). Finally, OA + EGTA buffer inhibits PP1, PP2A and PP2B, but not PP2C. Thus, for a given biological system, the analysis of these samples allows the quantification of calcineurin (PP2B) activity in a cellular extract. These methods have been described in the literature. Additional experimental modulation of the cell extracts may be desirable. Inhibitors of calcineurin, calmodulin and calcium ion modulators may be appropriate.

### Suggested reading

#### **Mini-review:**

C.B. Klee et al. J. Biol. Chem. 1998 273 13367

#### **Brain cellular extracts:**

P.M. Stemmer et al. FEBS Lett. 1995 374 237

X. Wang et al. Nature 1996 383 434

#### **Human recombinant CaN:**

A. Mondragon et al. Biochem. 1997 36 4934

## **LITERATURE CITATIONS OF CALCINEURIN ASSAY KITS**

1. B. Mehul et al. J. Biol. Chem. 2000 275 12841
2. T. Taigen et al. Proc. Natl. Acad. Sci. 2000 97 1196
3. G. Mallert et al. Cell 2001 104 675
4. M. Ichida and T. Finkel J. Biol. Chem. 2001 276 3524
5. O. Bueno et al. Proc. Natl. Acad. Sci. 2002 99 4586
6. K.J. Sellar et al. Anal. Biochem. 2006 358 104



# Product Manual

## NOTES

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# Product Manual

## **GLOBAL HEADQUARTERS**

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