Product Manual

Calcineurin Phosphatase Assay Kit

Catalog #: BML-AK804

A complete colorimetric assay kit for measuring calcineurin phosphatase activity

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Product Manual

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

BACKGROUND

Calcineurin (CaN) is the neuronal form of the widely distributed Ca^{2+}/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 $\alpha\delta$ isoform), binding sites for both calmodulin (residues 391-414) and CaN B-regulatory subunit, and a C-terminal (residues 457-482) autoinhibitory domain.

The *Calcineurin Phosphatase Assay Kit* 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 BML-AK804 kit incorporates human calcineurin Aα (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 protein[5].

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

BML-SE163-5000 CALCINEURIN ENZYME (human, recombinant)
FORM: 100 U/µl in 1X assay buffer (1:1 dilution of BML-KI128, below).
1 U=pmol/min @ 30°C.
STORAGE: -80°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 5000 U

BML-SE325-9090 CALMODULIN (human, recombinant)
FORM: 25 µM in dH2O
STORAGE: -80°C
QUANTITY: 100µl

BML-P160-9090 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: 1.5 mg net peptide/vial
STORAGE: -20°C
QUANTITY: 1 x 1.5 mg

BML-KI128-0020 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: -80°C
QUANTITY: 20 ml

BML-AK111-9090 BIOMOL® Green REAGENT
FORM: Liquid in screw-cap plastic bottle.
STORAGE: +4°C
QUANTITY: 20 ml

BML-KI132-0500 PHOSPHATE STANDARD
FORM: 80 µM in dH2O
STORAGE: -80°C
QUANTITY: 0.5 ml

80-2404 1/2 VOLUME MICROPLATE
1 clear, 96-well
STORAGE: Ambient

Storage temp

STORAGE

Please note that all components, with the exception of Calcineurin (BML-SE163-5000) and Calmodulin (BML-SE325-9090) can be stored at the temperatures listed or at -80°C. The enzymes listed above must be stored at -80°C. *The calcineurin enzyme component BML-SE163 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 A620 to ≥3-decimal accuracy.
- Pipette capable of pipetting 5-100µl accurately
- Multi-channel pipette capable of pipetting 100µl (optional).
- Ice bucket to keep reagents cold until use.

SAFETY WARNINGS & PRECAUTIONS


Important/ Warning

1. Wear appropriate personnel protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
2. Use a safety pipetting device for all pipetting. Never pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.

PROCEDURE

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.*

PREPARING REAGENTS FOR ASSAY

1. Thaw all kit components and hold calcineurin, calmodulin and 2x assay buffer on an ice bath; equilibrate BIOMOL® Green reagent to room temperature.
2. Add calmodulin to the 2x assay buffer: Dilute calmodulin (BML-SE101-9090) 1/50 in 2X assay buffer (BML-KI128) to required quantity (25µl are required per assay well). For example, add 20µl to 980µl 2X assay buffer.
3. Reconstitute substrate (RII phosphopeptide, BML-P160) with dH_2O to 0.75 mM (1.64 mg/ml): Add 915µl dH_2O per 1.5 mg vial (10µl are needed per assay well).

PERFORMING THE ASSAY

To prepare a standard curve:

4. Prepare 1 ml of 1X assay buffer (dilute 500µl of 2X assay buffer with 500µl 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 0.625µM correspond to 2, 1, 0.5, 0.25, 0.125, 0.063 and 0.031 nmol PO_4 (see Table 1):

a. Add 50µl of 2X assay buffer (BML-KI128) to each wells A1, and A2 (2 nmol PO_4 standards).
b. Add 50µl 1X assay buffer (prepared in step 4 above) to wells B1-H1 and wells B2-H2 (remaining standard concentrations)
c. Add 50µl of 80µM phosphate standard to well A1 and A2 of assay plate. Mix thoroughly by pipetting up and down several times.
d. Remove 50µl from well A1 and add it to well B1. Mix thoroughly by pipetting up and down several times.
e. Remove 50µl from well B1 and add it to well C1.
f. Mix thoroughly and repeat for wells D1-G1. At well G1, remove 50µl and discard. DO NOT PROCEED TO WELL H1 (assay buffer blank). Final volume=50µl.
g. Repeat serial dilution for the wells in column 2 (standard curve duplicates)

To prepare a time course/linearity assay:

6. Add 25µl 2X assay buffer (BML-KI128) w/ calmodulin (step 2) to microtiter plate wells designated for linearity assay (see Table 1).
7. Dilute the calcineurin (BML-SE163) to 8 U/µl, in 1X assay buffer, and add 5µl diluted calcineurin to wells. Final amount of calcineurin= 40 U per well.
8. Add 10µl 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°C).
11. Start reaction by addition of 10µl phosphopeptide substrate (BML-P160; 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 25µl assay buffer (BML-KI128) w/ calmodulin (step 2) to wells in microtiter plate. See Table 1.
13. Add 5µl diluted calcineurin (BML-SE163) to wells (step 7). Final amount of calcineurin= 40 U per well.
14. Add 10µl dH_2O to control wells.
15. Add 10µl 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°C) for 10 minutes.
17. Start reaction by addition of 10µl phosphopeptide substrate (BML-P160; 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 100µl BIOMOL® green reagent (BML-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 OD_{620nm} 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_2O	Test compound	Substrate (0.75 mM)
CONTROL	25µl	5µl	10µl	0	10µl
TEST SAMPLE	25µl	5µl	0	10µl	10µl

DATA ANALYSIS

Phosphate (PO_4) Standard Curve

1. Plot standard curve data as OD_{620nm} versus nmol PO_4 (Note that a background OD_{620nm} value for 0 nmol PO_4 has been subtracted from all data. See Figure 1. Data may also be plotted without subtracting the background. In that case, however, one should also not subtract background from experimental OD_{620nm} values before using the standard curve to convert them to nmol of PO_4).
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.*

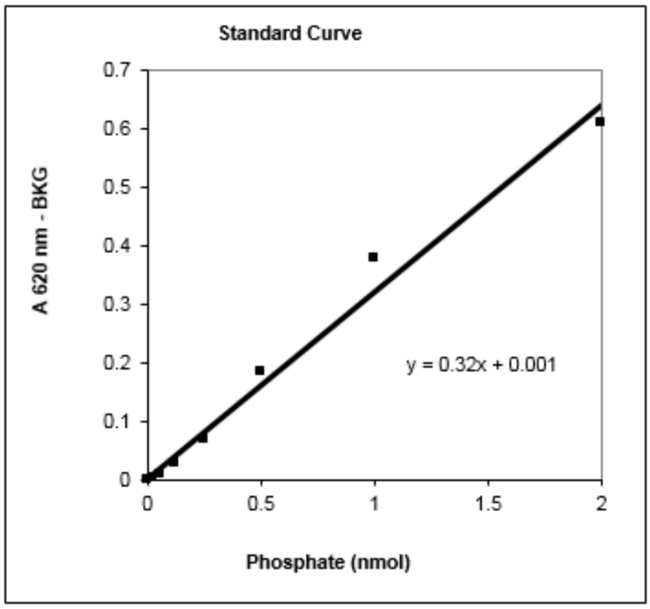

Figure 1. BIOMOL® Green Phosphate Standard Curve

Conversion of OD620nm to Amount Phosphate Released

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

Phosphate released = $(OD_{620nm} – Y_{int})$/slope

EXAMPLE:

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

Std curve Yint = 0.001 OD_{620nm}

Sample OD_{620nm} = 0.4

Phosphate released = (0.4-0.001)/0.3 = 1.33 nmol

Time Course/Linearity Curve

1. If the 0 time (Table 1, wells 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.

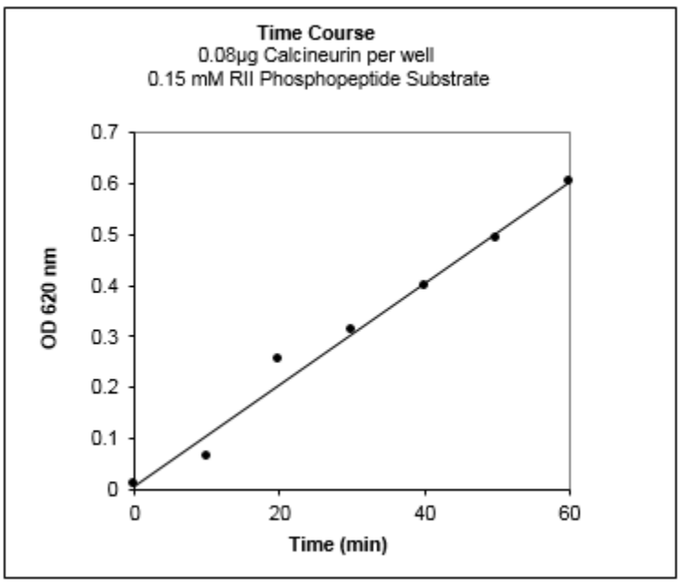

Figure 2. Time Course of Phosphate Released by Calcineurin

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 of relatively low.

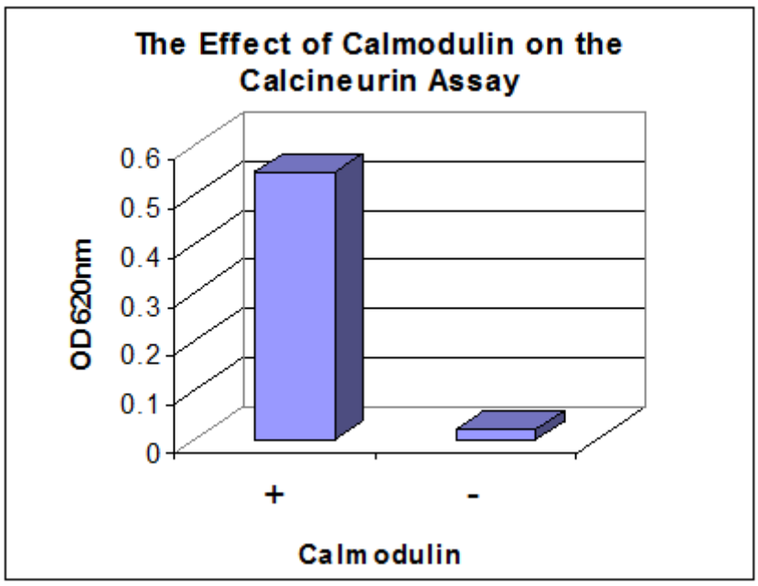

Figure 3. Calmodulin Activation of Calcineurin Phosphatase Activity

TROUBLESHOOTING

Problem	Potential Cause	Solution
High background (High signal with no added calcineurin)	Interfering substance present. Phosphatase contamination of substrate stock solution.	Remove trace amounts of phosphate from assay buffers/reagents. Only use 18 MΩ deionized water, such as Milli-Q™ water, in preparation of all buffers. Soaps and detergents may cause high background. Any container coming into contact with any solutions used in the assay should be triple washed with deionized water prior to use. *Milli-Q is a trademark of Millipore Corporation.*
Expected calcineurin activity is not detected	Calcineurin has been inactivated. Activators are not present.	Use positive control phosphatase or standard curve to check assay performance. Be sure necessary co-factors, such as calcium and calmodulin, are in reaction mix.
Cloudy precipitate	Interfering substances present.	Identify and remove incompatible metals, phosphate or detergents. Certain divalent cations (Magnesium, Copper, Zinc) and detergents (SDS and deoxycholate) should be avoided.
Weak signal	Calcineurin is too dilute.	Increase amount of calcineurin used or increase the assay time.
Tested calcineurin inhibitor fails to demonstrate expected activity	Inhibitor concentration employed in assay was too low.	Perform assay using a broader range of inhibitor concentrations. Verify inhibition with other well-characterized inhibitors (e.g. Cyclosporin A , FK506, Cypermethrin, Deltamethrin, or Fenvalerate)

SELECTED REFERENCES FOR INHIBITORS SCREENED WITH ENZO'S CALCINEURIN KIT

	Inhibitor	Reference
1	FK506 Inhibition of CN activity	Toxicological Sciences (2007) 100: 474–485
2	Targeted inhibition of calcineurin with cain or an adenovirus expressing calcineurin inhibitory domain	PNAS (2000) 97: 1196–1201
3	RCAN1 inhibition of calcineurin	Arch. Biochem and Biophys (2007) 467: 185–192
4	cyclosporin A, FK506	Pharmacology, Biochemistry and Behavior (2008) 90: 763–768
5	Minimal RCAN derived sequence (part of the RCAN CIC motif) that inhibits Cn-NFAT signaling in vivo.	J. Biol Chem (2009) 284: 9394–9401
6	Inhibition of calcineurin by caffeoyl phenylethanoid glycosides	J. of Ethnopharmacology (2011) 137: 1306– 1310
7	Jasminum humile leaf and root ethanolic extracts inhibit CN-dependent gene expression in a yeast model system	J. of Ethnopharmacology (2012) 140: 293– 297
8	Effects of CsA and FK506 on aquaporin-2	Pflugers Arch - Eur J Physiol (2011) 462: 611–622
9	Multiple Domains of MCIP1 Contribute to Inhibition of Calcineurin Activity	J Biol Chem. (2002) 277: 30401-7.
10	Tacrolimus (TRL) calcium-dependent protein phosphatase calcineurin CN	Analytical Biochemistry (2006) 358: 104–110

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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

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