CREATIVE BIOMART®
Assay Kit

Cellular calcineurin activity assay Kit

Product Information

Cat

Kit-0134

Cat.No.

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Description

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 calcineurin5 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. The detection of free-phosphate released is based on the classic Malachite green assay and offers the following advantages: Nonradioactive; convenient 1-step detection; excellent sensitivity.

Stability

Store all components except the microtiter plate at -70°C for the highest stability. The calcineurin enzyme component 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.

Storage

at -80°C

Synonyms

Cellular calcineurin activity assay Kit; Cellular calcineurin assay Kit; calcineurin activity assay Kit; calcineurin; PP2B

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Size

96 wells

Kit Components

1. Calcineurin enzyme (human, recombinant)
FORM: 50 mM Tris, pH 7.5, 100 mM NaCl, 6 mM MgCl2, 5 mM DTT, 0.025% NP-40, 0.5 mM CaCl2.
1 U=1 pmol/min at 30°C.
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 500 U
2. Calmodulin (human, recombinant)
FORM: 25 µM in dH2O
STORAGE: -70°C
QUANTITY: 100 µL
3. Substrate (RII phosphopeptidee, sequence Asp-LeuAsp-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
4. 2x assay buffer
FORM: 100 mM Tris, pH 7.5, 200 mM NaCl, 12 mM MgCl2, 1mM DTT, 0.05% NP-40, 1 mM CaCl2
STORAGE: -70°C
QUANTITY: 20 mL
5. 2x EGTA Buffer
FORM: 100 mM Tris, pH 7.5, 20 mM EGTA, 200 mM NaCl, 12 mM MgCl2, 1 mM DTT, 0.05% NP-40
STORAGE: -70°C
QUANTITY: 1 mL
6. Lysis Buffer
FORM: 50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2% NP-40
STORAGE: -70°C
QUANTITY: 1 X 40 mL

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7. Protease inhibitor cocktail
FORM: Solid tablet
STORAGE: 4°C
QUANTITY: 2 tablets
8. Biomol Green Reagent
FORM: Liquid in screw-cap plastic bottle.
STORAGE: 4°C
QUANTITY: 20 mL
9. Phosphate standard
FORM: 80 µM in dH2O
STORAGE: -70°C
QUANTITY: 0.5 mL
10. Okadaic acid
HARMFUL! AVOID ALL FORMS OF CONTACT.
FORM: 5 µM in 2X Assay Buffer
STORAGE: -70°C.
QUANTITY: 325 µL
11. Desalting column and resin
FORM: 5 mL polypropylene disposable column and P6 DG desalting resin
STORAGE: RT. After rehydration store at 4°C.
QUANTITY: 1 column and 1 g of resin
12. ½-Volume microplate
2 clear, 96-well
STORAGE: Room temperature

Materials Required but Not Supplied

Microplate reader capable of measuring A620 to ≥3-decimal accuracy.
Centrifuge capable of 100k x g RCF.
Swing bucket centrifuge capable of 800 x g RCF.
Pipetman capable of pipetting 5-100 µL accurately.
Multi-channel Pipetman capable of pipetting 100 µL (optional).

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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)

Technical Notes

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 dH2O or employ unused plasticware. Do not use phosphate buffered saline (PBS) for any tissue/cell rinses - use TBS (Tris buffered saline)!

Detection method Colorimetric

Preparation

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 µg 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 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.

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5. Add lysis buffer 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 GREEN reagent) in the high speed supernatant (HSS) extract.
1. Rehydrate Desalting Column Resin in a 50 mL conical tube by adding 20 mL of phosphate free dH2O and vortexing briefly. Allow to set for 4 hours at RT or overnight at 4°C.
2. Decant the dH2O carefully, then add fresh dH2O at a 1:1 ratio to the rehydrated resin (~10 mL).
3. Add rehydrated resin to the Chromatography Column to obtain a 5 mL settled-bed volume (~5.5 cm bed height). Remove tip from column and allow dH2O to drain by gravity.
4. Equilibrate column by adding 8 mL of lysis buffer (without protease inhibitors) and allow to drain by gravity.
5. Place 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, below.
9. Freeze sample immediately at -70°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.

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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 dH2O. If no phosphate/nucleotides are present, both samples should remain yellow in color over a time period of 30 min at RT. The development of a visible green color indicates phosphate contamination, which must be eliminated from the samples before proceeding further!

Assay Protocol

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 use either OD620nm or phosphate released for the Y-axis.
3. Determine the contribution of calcineurin:
eq. 1 CaN (PP2B) = Total - EGTA buffer or
eq. 2 CaN (PP2B) = OA - (OA + 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.

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 RT.
2. Dilute calmodulin 1/50 in 2X assay buffer to required quantity (25 µL are required per assay well). For example, add 20 µL to 980 µL 2X assay buffer.
3. Reconstitute substrate (RII phosphopeptide) with dH20 to 0.75 mM (1.64 mg/mL): Add 915 µL dH20 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 dH2O)
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 PO4:
a) Add 50 µL of assay buffer to wells A1, and A2 (2 nmol PO4 standards)
b) Add 50 µL of 1X assay buffer (prepared in step 1 above) to wells B1-H1 and wells B2-H2.

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(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:
Background (no substrate): {Control for background phosphate/interfering substances}
1. Add 20 µL dH2O to appropriate wells.
2. Add 25 µL 2X assay buffer with calmodulin to each well.

Total phosphatase activity wells: {Total phosphatase activity in the extract }
1. Add 10 µL dH2O to each well.
2. Add 25 µL 2X assay buffer with calmodulin to each well.

EGTA buffer (Ca2+/CaM free): {Total activity in the absence of active PP2B (calcineurin)}
1. Add 10 µL dH2O to each well.
2. Add 25 µL 2X EGTA buffer to each well.

OA (okadaic acid): {Total activity in the absence of active PP1 & PP2A}
1. Add 5 µL dH2O to each well.
2. Add 25 µL 2X assay buffer with calmodulin to each well.
2+. Add 5 µL okadaic acid (5µM)

OA + EGTA: {Total activity in the absence of active PP1, PP2A & PP2B}
1. Add 5 µL dH2O to each well.

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2. Add 25 µL 2X EGTA buffer to each well.
2+. Add 5 µL okadaic acid (5 µM)

Positive control (calcineurin enzyme): {Purified CaN enzyme positive control}
1. Add 10 µL dH2O to each well.
2. Add 25 µL 2X assay buffer with calmodulin to each well.
Add phosphopeptide substrate:
3. Add 10 µL phosphopeptide substrate 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 min.

To initiate calcineurin assay:
5. Add 5 µL sample extract or diluted calcineurin (dilute to 8 U/µL 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). For calcineurin "Positive control" add 5 µL (40 U/well).
6. Incubate plate at reaction temperature for desired duration (e.g.: 30 min@30°C).

To terminate reactions:
7. After incubating wells for desired duration, terminate reactions by adding 100 µL BIOMOL GREEN reagent 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 OD620nm on microplate reader.
10. Perform data analysis (see below).
NOTE: Retain microplate for future use of unused wells!

Analysis

Phosphate (PO4) Standard Curve
1. Plot standard curve data as OD620nm versus nmol PO4.
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

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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 GREEN reagent, and other experimental factors.

Conversion of OD620nm to Amount Phosphate Released
1. Convert OD620nm data into the amount of phosphate released using the standard curve line-fit data, from above:
Phosphate released = (OD620nm – Yint)/slope
EXAMPLE:
Std curve slope=0.3 OD620nm/nmol phosphate
Std curve Yint=0.001 OD620nm
Sample OD=0.4
Phosphate released=(0.4-0.001)/0.3 = 1.33 nmol