ab139461

Calcineurin Phosphatase Activity Assay Kit (Colorimetric)

Instructions for Use

For measuring Calcineurin phosphatase activity.

This product is for research use only and is not intended for diagnostic use.
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1. 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 αδ isoform), binding sites for both Calmodulin (residues 391-414) and CaN B-regulatory subunit, and a C-terminal (residues 457-482) autoinhibitory domain.

2. Principle of the Assay

Abcam Calcineurin Phosphatase Activity Assay Kit (Colorimetric) (ab139461) 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 Calcineurin 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 assay offers the following advantages:

1) Non-radioactive
2) Convenient one step detection
3) Microplate format

The 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.

3. Protocol Summary

**Prepare standard curve**

Dilute standards

Prepare standard wells

Add Phosphate Standard to each well and mix thoroughly

Incubate at appropriate temperature and time

Terminate reaction by adding Green Assay Reagent
Prepare time course/linearity assay

Add Calcineurin Assay Buffer with Calmodulin to appropriate wells

\[ \downarrow \]

Dilute Calcineurin enzyme

\[ \downarrow \]

Add Calcineurin enzyme to each well

\[ \downarrow \]

Add H\textsubscript{2}O to each well

\[ \downarrow \]

Equilibrate microtiter plate to reaction temperature

\[ \downarrow \]

Add Calcineurin Substrate to each well at 5 minute intervals

\[ \downarrow \]

Terminate reaction by adding Green Assay Reagent

Prepare test sample/inhibitor assay

Prepare samples containing Calcineurin enzyme, Calcineurin Assay Buffer with Calmodulin, substrate and test compound

\[ \downarrow \]

Incubate samples at appropriate temperature and time

\[ \downarrow \]

Terminate reaction by adding Green Assay Reagent
### 4. Materials Supplied

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active Calcineurin Enzyme (100 U/µl)</td>
<td>1 x 50 µL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Calmodulin (25 µM)</td>
<td>1 x 100 µL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Calcineurin Substrate</td>
<td>1 x 1.5 mg</td>
<td>-80°C</td>
</tr>
<tr>
<td>Calcineurin Assay Buffer</td>
<td>1 x 20 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Green Assay Reagent</td>
<td>1 x 20 mL</td>
<td>4°C</td>
</tr>
<tr>
<td>Phosphate Standard (80 µM)</td>
<td>1 x 0.5 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>96-well Clear Microplate (1/2 Volume)</td>
<td>1</td>
<td>4°C</td>
</tr>
</tbody>
</table>
5. Storage and Stability

- Store all components except the microtiter plate at -80°C for the highest stability.

- Green Assay Reagent should be stored at 4°C short term or at -80°C for long term storage. For long term storage, aliquot to prevent the bottle bursting.

- 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 -80°C. To minimize the number of freeze/thaw cycles, aliquot the Calcineurin into separate tubes and store at -80°C.

- One U of Calcineurin Enzyme (Human, Recombinant) = pmol/min @ 30°C.

- Calcineurin 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)
The Green Assay 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$_2$O or employ unused plasticware.

6. Materials Required, Not Supplied

- Microplate reader capable of measuring $A_{620}$ to $\geq$3-decimal accuracy.
- Pipettes capable of pipetting 5-100 µL accurately.
- Multi-channel pipette capable of pipetting 100 µl (optional).
- Ice bucket to keep reagents cold until use.
7. Assay Protocol

A. Reagent Preparation

1. Thaw all kit components and hold Calcineurin, Calmodulin and Calcineurin Assay Buffer on an ice bath; Store Green Assay Reagent at room temperature (RT).

2. Add Calmodulin to the Calcineurin Assay Buffer: Dilute Calmodulin 1/50 in Calcineurin Assay Buffer to required quantity (25 µl are required per assay well). For example, add 20 µl to 980 µl Calcineurin Assay Buffer.

3. Reconstitute Calcineurin Substrate (RII phosphopeptide) with dH$_2$O to 0.75 mM (1.64 mg/ml): Add 915 µl dH$_2$O per 1.5 mg vial (10 µl are needed per assay well).

B. Preparing a Standard Curve

1. Prepare 1 ml of 1X Assay Buffer (dilute 500 µl of Calcineurin Assay Buffer with 500 µl dH$_2$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$_4$ (see Table 1):
   a) Add 50 µl of Calcineurin Assay Buffer to each wells A1, and A2 (2 nmol PO$_4$ standards).
   b) Add 50 µl 1X Assay Buffer (prepared in step B1 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)
Table 1. example of standard curve and time course/linearity microplate samples.

<table>
<thead>
<tr>
<th>Sample Well</th>
<th>PO\textsubscript{4} Standard Curve nmol (Columns 1,2)</th>
<th>Time course Min. (Columns 3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td>D</td>
<td>0.25</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td>0.125</td>
<td>10</td>
</tr>
<tr>
<td>F</td>
<td>0.063</td>
<td>5</td>
</tr>
<tr>
<td>G</td>
<td>0.31</td>
<td>2</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

For highest accuracy, perform all samples in duplicate. See Figures 1 and 2 for example results.
C. Preparing a Time Course/Linearity Assay

1. Add 25 µl Calcineurin Assay Buffer (step A2) to microtiter plate wells designated for linearity assay (see Table 1).

2. Dilute the Calcineurin to 8 U/µl, in 1X Assay Buffer, and add 5 µl diluted Calcineurin to wells. Final amount of Calcineurin= 40 U per well.

3. Add 10 H₂O µL to each well.

4. 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).

5. Equilibrate microplate to reaction temperature (e.g.: 30°C).

6. Start reaction by addition of 10 µl Calcineurin Substrate (0.75 mM from step A3) 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..
D. Preparing a Test Sample/Inhibitor Assay

1. Add 25 µl Calcineurin Assay Buffer (step A2) to wells in microtiter plate. See Table 1.

2. Add 5 µl diluted Calcineurin to wells (step C2). Final amount of Calcineurin= 40 U per well.

3. Add 10 µl dH_{2}O to control wells.

4. Add 10 µl of test sample/inhibitor (dissolved in dH_{2}O) to test sample wells.

5. Allow test sample/inhibitor to equilibrate to reaction temperature (e.g.: 30°C) for 10 minutes.

6. Start reaction by addition of 10 µl Calcineurin Substrate (0.75 mM from step A3). Final concentration= 0.15 mM. Allow reaction to proceed for a time period in which the reaction is linear (~10 min, see below).

Table 2: Example of Test Samples/Inhibitor Assay Microplate Samples

<table>
<thead>
<tr>
<th></th>
<th>Calcineurin Assay Buffer</th>
<th>Calcineurin (40U)</th>
<th>H_{2}O</th>
<th>Test compound</th>
<th>Substrate (0.75 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>25 µL</td>
<td>5 µL</td>
<td>10 µL</td>
<td>0 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td><strong>Test</strong></td>
<td>25 µL</td>
<td>5 µL</td>
<td>0 µL</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>
E. Terminating Reactions

1. After incubating wells for desired duration, terminate reactions by addition of 100 µL Green Assay Reagent.

2. Allow color to develop for 20-30 minutes. Be careful to assure samples spend approximately the same time with the reagent before reading on the microplate reader.

3. Read OD$_{620nm}$ on microtiter-plate reader.

   Note: Retain microtiter plate for future use of unused wells.

8. Data Analysis

A. PO$_4$ Standard Curve

1. Plot standard curve data as OD$_{620nm}$ versus nmol PO4 (Note that a background OD$_{620}$ value for 0 nmol PO4 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$_{620}$ values before using the standard curve to convert them to nmol of 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 other experimental data (e.g., time course and experimental data).
B. Conversion of OD$_{620\text{nm}}$ to Amount Phosphate Released

Convert OD$_{620\text{nm}}$ data into the amount of phosphate released using the standard curve line-fit data from above.

\[
\text{Phosphate released} = \frac{(\text{OD}_{620\text{nm}} - \text{y-intercept})}{\text{slope}}
\]

**SAMPLE CALCULATION:**

Std curve slope=0.3 OD$_{620\text{nm}}$/nmol phosphate
Std curve Yint=0.001 OD$_{620\text{nm}}$
Sample OD=0.4

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

**Figure 1. Standard Curve.**
C. 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$_{620}$nm versus reaction time. See Figure 2. Alternatively, the OD$_{620}$nm 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](image-url)
D. Calmodulin Activation of Calcineurin Activity

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. In the presence (+) of Calmodulin, Calcineurin’s activity is high. In the absence (-) of Calmodulin, Calcineurin activity of relatively low.
### 9. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>High background (High signal with no added calcineurin)</td>
<td>Interfering substance present.</td>
<td>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.</td>
</tr>
<tr>
<td>Phosphatase contamination of substrate stock solution.</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>Problem</td>
<td>Reason</td>
<td>Solution</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Expected calcineurin activity</td>
<td>Calcineurin has been inactivated.</td>
<td>Use positive control phosphatase or standard curve to check assay performance</td>
</tr>
<tr>
<td>is not detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activators are not present.</td>
<td></td>
<td>Be sure necessary co-factors, such as calcium and calmodulin, are in reaction mix.</td>
</tr>
<tr>
<td>Cloudy precipitate</td>
<td>Interfering substances present.</td>
<td>Identify and remove incompatible metals, phosphate or detergents. Certain divalent cations (Magnesium, Copper, Zinc) and detergents (SDS and deoxycholate) should be avoided.</td>
</tr>
<tr>
<td>Problem</td>
<td>Reason</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Weak signal</td>
<td>Calcineurin is too dilute.</td>
<td>Increase amount of calcineurin used or increase the assay time.</td>
</tr>
<tr>
<td>Tested calcineurin inhibitor fails to</td>
<td>Inhibitor concentration</td>
<td>Perform assay using a broader range of inhibitor concentrations. Verify</td>
</tr>
<tr>
<td>demonstrate expected activity</td>
<td>employed in assay was too</td>
<td>inhibition with other well-characterized inhibitors (e.g. Cyclosporin A,</td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>FK506, Cypermethrin, Deltamethrin, or Fenvalerate)</td>
</tr>
</tbody>
</table>
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