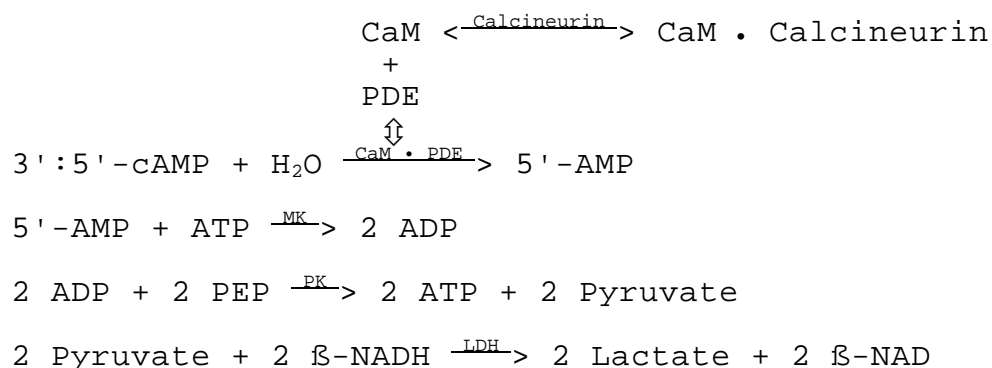


Enzymatic Assay of CALCINEURIN

PRINCIPLE:

The assay for Calcineurin is based on its ability to bind to Calmodulin. Calmodulin which is bound to Calcineurin is no longer available to activate Phosphodiesterase 3':5'-Cyclic Nucleotide. This loss of activation is a measure of the amount of Calcineurin present in the assay.



Abbreviations Used:

CaM = Calmodulin (Phosphodiesterase 3':5'-Cyclic Nucleotide Activator)

CaM • Calcineurin = Calmodulin-Calcineurin Complex

PDE = Phosphodiesterase 3':5'-Cyclic Nucleotide

3':5'-cAMP = Adenosine 3':5'-Cyclic Monophosphate

CaM • PDE = Activated form of Phosphodiesterase 3':5'-Cyclic Nucleotide

5'-AMP = Adenosine 5'-Monophosphate

ATP = Adenosine 5'-Triphosphate

MK = Myokinase

ADP = Adenosine 5'-Diphosphate

PEP = Phospho(enol)pyruvate

PK = Pyruvate Kinase

β -NADH = β -Nicotinamide Adenine Dinucleotide, Reduced Form

LDH = L-Lactic Dehydrogenase

β -NAD = β -Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 30°C, pH = 7.5, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

Enzymatic Assay of CALCINEURIN

DISCUSSION:

General:

Before assaying for Calcineurin, the activity of the PDE and CaM working solutions must be determined. This has to be done every time a new solution is made and at the start of each day. The working range for PDE is 0.012 - 0.016 units per assay and for CaM, 2.0 to 2.2 units per assay.

An activated control, either fully activated PDE (when assaying for CaM) or PDE activated with 2 units of CaM (when assaying for Calcineurin), must be run with each set of 4 Tests. The assay is very sensitive to the amount of PDE added. This is the reason a control is run with each set of Tests. The PDE activity generally changes slowly during the day but doesn't usually cause a problem as long as the controls are run with each set of cuvettes.

Assay for PDE:

The preparation of the Working PDE Solution (Reagent O) is carried out in two steps. The first step is the estimation of the activity of the PDE Stock Solution (Reagent N). Based on this estimation, the second step is the dilution of a portion of the PDE Stock Solution (Reagent N) to 0.24 - 0.32 unit/ml with Reagent J (BSA). The Working PDE Solution (Reagent O) is assayed and adjusted to 0.24 - 0.32 unit/ml, if necessary. Once the proper Working PDE Solution (Reagent O) is prepared, proceed to the assay for CaM (Procedure Part II).

Assay for CaM:

Once Reagent O (Working PDE Solution) is prepared, the final solution required before assaying for Calcineurin is the Reagent Q (Working CaM Solution). The activity of Reagent Q (Working CaM Solution) has to be determined so that the number of CaM units added to the Test during the assay for Calcineurin is known. The activity of CaM present in the Reagent Q (Working CaM Solution) is determined graphically.

Enzymatic Assay of CALCINEURIN

Assay for CaM: (continued)

$$\frac{\text{The ratio of Corrected } \Delta A_{340}/\text{min (CaM unknown)}}{\text{Corrected } \Delta A_{340}/\text{min (fully activated)}}$$

is plotted against volumes used of the CaM unknown. For the results to be valid, three different volumes with ratios between 0.35 - 0.65 are required with at least one ratio above 0.5 and another one below 0.5. With every set of cuvettes, one of the four **MUST** be a fully activated PDE assay (see assay for PDE, Procedure Part I, Section B (Activated Test)).

The other three Tests are different volumes of the same CaM solution, the Reagent Q (CaM Working Solution). The activity of Reagent Q (CaM Working Solution) has to be between 40 - 44 u/ml CaM.

Since the assay requires Ca^{++} a sample containing a high concentration of metal chelator will give false high results.

Assay for Calcineurin

The order of addition of CaM, Calcineurin, and PDE will affect the activity. CaM and Calcineurin are mixed together first and incubated at 30°C for 5 minutes before adding PDE.

The amount of Calcineurin (Reagent R) present in the sample is also determined graphically. The ratio of

$$\frac{\text{Corrected } \Delta A_{340}/\text{min (Calcineurin unknown)}}{\text{Corrected } \Delta A_{340}/\text{min (PDE Activated by 2 units of CaM)}}$$

is plotted against the volumes of the sample used. Three different volumes with ratios between 0.35 - 0.65 are required with at least one of the 3 valid ratios above 0.5 and another one below 0.5. With every set of cuvettes, one of the four must be a PDE Activated by 2 units CaM assay (Test 4 below). The other three Tests are different volumes of the same Calcineurin sample.

REAGENTS:

- A. 200 mM Tris HCl Buffer, pH 7.5 at 30°C
(Prepare 100 ml in deionized water, using Trizma Base,

Sigma Prod. No. T-1503. Adjust to pH 7.5 at 30°C with
1 M HCl.)

Enzymatic Assay of CALCINEURIN

REAGENTS: (continued)

- B. 1 M Potassium Chloride Solution (KCl)
(Prepare 100 ml in deionized water using Potassium Chloride, Sigma Prod. No. P-4504.)
- C. 60.0 mM Magnesium Sulfate Solution (MgSO₄)
(Prepare 100 ml in deionized water using Magnesium Sulfate, Anhydrous, Sigma Prod. No. M-7506.)
- D. 78 mM Tris HCl with 65 mM Potassium Chloride and 7.8 mM Magnesium Sulfate (Assay Buffer)
(Prepare 57.3 ml by combining 22.5 ml of Reagent A, 3.75 ml of Reagent B, 7.5 ml of Reagent C, and 20 ml of deionized water. Equilibrate mixture to 30°C and adjust to pH 7.5 with 2 M HCl. Bring to a final volume of 57.3 ml with deionized water.)
- E. 83.5 mM Phospho(enol)pyruvate Solution (PEP)
(Prepare 1 ml in deionized water using Phospho(enol)pyruvate, Trisodium Salt, Hydrate, Sigma Prod. No. P-7002.)
- F. 42.0 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 5 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-6144. Adjust to pH 7.5 at 30°C with solid Sodium Bicarbonate, Sigma Prod. No. S-8875.)
- G. 76.8 mM Adenosine 3':5'-Cyclic Monophosphate Solution (3':5'-cAMP)
(Prepare 2 ml in deionized water using Adenosine 3':5'-Cyclic Monophosphate, Sodium Salt, Sigma Prod. No. A-6885.)
- H. 14 mM Calcium Chloride Solution (CaCl₂)
(Prepare 300 ml in deionized water using Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881.)
- I. 18.3 mM β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
(Dissolve the contents of one 5 mg vial of β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-105, in the appropriate volume of deionized water.)
- J. 0.3% (w/v) Bovine Serum Albumin Solution (BSA)
(Prepare 10 ml in Reagent D using Albumin, Bovine, Sigma Prod. No. A-7638.)

Enzymatic Assay of CALCINEURIN

REAGENTS: (continued)

- K. PK2/LDH3 Enzyme Suspension¹
(Use PK/LDH Enzymes Suspension, Sigma Stock No. 40-7.)
- L. 3.2 M Ammonium Sulfate Solution (NH₄SO₄)
(Prepare 10 ml in deionized water using Ammonium Sulfate, Sigma Prod. No. A-4915.)
- M. Myokinase Enzyme Solution
(Immediately before use, prepare a solution containing 1667 units/ml in cold Reagent L using Myokinase, Sigma Prod. No. M-3003.)
- N. Phosphodiesterase 3':5'-Cyclic Nucleotide Stock Solution (PDE Stock)
(Immediately before use, prepare 0.6 ml of a solution containing 0.83 unit/ml by dissolving a 0.5 unit vial of Phosphodiesterase 3':5'-Cyclic Nucleotide, Sigma Prod. No. P-9529, in 0.6 ml of Reagent D.)
- O. Phosphodiesterase 3':5'-Cyclic Nucleotide Working Solution (PDE Work)
(Immediately before use prepare a solution containing 0.24-0.32 unit/ml of Phosphodiesterase 3':5'-Cyclic Nucleotide by diluting Reagent N in cold Reagent J. See Procedure, Part I.)
- P. Calmodulin Stock Solution (CaM Stock)
(Prepare a solution containing 1000 units/ml Phosphodiesterase 3':5'-Cyclic Nucleotide Activator, Sigma Prod. No. P-2277, in cold deionized water.)
- Q. Calmodulin Working Solution (CaM Work)
(Prepare a solution of Phosphodiesterase 3':5'-Cyclic Nucleotide Activator by diluting Reagent P such that under the conditions of this assay system (see Assay for Calmodulin), the solution contains 40-44 units/ml. The assay described in "Part II Assay for Calmodulin" usually gives between 50-75% of the units obtained using the Bovine Heart PDE assay. A starting point for dilution is 0.08 ml of Reagent P with 1.0 ml of Reagent J. Follow the assay procedure for Calmodulin and determine the units/ml. Based on this information, adjust the solution to 40 - 44 units/ml and repeat the determination for the Calmodulin activity.)
- R. Calcineurin Solution (Calcin)
(Prepare a solution of Calcineurin as described in Procedure, Part III.)

Enzymatic Assay of CALCINEURIN

PROCEDURE:

Due to the length of the assay (approximately 30 minutes) the reaction cocktail is prepared as needed, 4 cuvette volumes at a time. Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

Reagent D (Assay Buffer)	11.45
Reagent E (PEP)	0.10
Reagent F (ATP)	0.10
Reagent H (CaCl ₂)	0.10
Reagent I (β-NADH)	0.15
Reagent K (PK/LDH)	0.07
Reagent M (Myokinase)	0.02

Part I: Assay for PDE

A. Estimation of Stock PDE Solution (Reagent N) activity.

The Stock PDE solution (Reagent N) is assayed with 25 units of (CaM Stock) Reagent P (these are Label Units).

Pipette (in milliliters) the following reagents into a suitable cuvette:

	<u>Activated</u> <u>Test</u>
Reaction Cocktail	2.80
Deionized Water	0.315
Reagent P (CaM Stock)	0.025
Reagent N (PDE Stock)	0.01

Mix by inversion and equilibrate to 30°C for 5 minutes. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent G (3':5'-cAMP)	0.05
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Immediately mix by inversion and record the decrease in A_{340nm} for approximately 15 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate.

Enzymatic Assay of CALCINEURIN

CALCULATION:

$$\text{Units/ml PDE (Fully activated)} = \frac{(\Delta A_{340}/\text{min Test})(3.2)}{(6.22)(2)(0.01)}$$

- 3.2 = Total volume (in milliliters) of assay
- 6.22 = Millimolar extinction coefficient of β -NADH at 340 nm
- 2 = 2 μ moles of β -NAD produced per mole of 3':5'-cAMP hydrolyzed
- 0.01 = volume (in milliliter) of enzyme used

B. Determination of the Working PDE Solution Activity (Reagent O)

Based on the information obtained above, dilute Reagent N (PDE Stock) with Reagent J (BSA) to 0.24 - 0.32 unit/ml. Assay Reagent O (PDE Work) to verify its concentration by pipetting (in milliliters) the following reagents into suitable cuvettes:

	Non-Activated	Acti vated
	<u>Test</u>	<u>Test</u>
Reaction Cocktail	2.80	2.80
Deionized Water	0.30	0.275
Reagent P (CaM Stock)	-----	0.025
Reagent O (PDE Work)	0.05	0.05

Mix by inversion and equilibrate to 30°C for 5 minutes. Monitor the $A_{340\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent G (3':5'-cAMP)	0.05	0.05
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Immediately mix by inversion and record the decrease in $A_{340\text{nm}}$ for approximately 15 minutes. Obtain the $\Delta A_{340\text{nm}}/\text{minute}$ using the maximum linear rate for both the Non-Activated Test and Activated Test.

CALCULATION:

(For Activated and Non-activated)

$$\text{Units/ml PDE} = \frac{(\Delta A_{340\text{nm}}/\text{min Test})(3.2)}{(6.22)(2)(0.05)}$$

- 3.2 = Total volume (in milliliters) of assay
- 6.22 = Millimolar extinction coefficient of β -NADH at 340nm
- 2 = 2 μ moles of β -NAD produced per mole of 3':5'-cAMP hydrolyzed

0.05 = Volume (in milliliter) of enzyme used

Enzymatic Assay of CALCINEURIN

CALCULATIONS: (Part I, continued)

Determine the number of units/ml for both the Activated and Non-Activated PDE. There should be 0.24 - 0.32 unit/ml of activated PDE, if not, adjust to 0.24 - 0.32 unit/ml and repeat Procedure Part I, Section B.

If the ratio of $\frac{\text{Activated Test}}{\text{Non-Activated Test}}$ is less than 5, a new PDE

working solution (Reagent O) must be prepared.

PROCEDURE: Part II, Assay for Calmodulin (CaM)

Pipette (in milliliters) the following reagents into suitable cuvettes:

	<u>Test 1</u>	<u>Test 2</u>	<u>Test 3</u>	Fully Acti vated <u>Test 4</u>
Reaction Cocktail	2.80	2.80	2.80	2.80
Deionized Water	0.28	0.275	0.27	0.275
Reagent P (CaM Stock)	-----	-----	-----	0.025
Reagent Q (CaM Work)	0.02	0.025	0.03	-----
Reagent O (PDE Work)	0.05	0.05	0.05	0.05

Mix by inversion and equilibrate to 30°C for 5 minutes. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent G (3':5'-cAMP)	0.05	0.05	0.05	0.05
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Immediately mix by inversion and record the decrease in A_{340nm} for approximately 15 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate for each Test.

CALCULATIONS:

- Determine the corrected ΔA₃₄₀/min for each cuvette by

$$[\Delta A_{340}/\text{min}] - [\Delta A_{340}/\text{min}]_{\text{nonactivated PDE}} = \text{Corrected } \Delta A_{340}/\text{min}$$

See assay for PDE, Procedure Part I, Section B (Non-Activated Test) for $[\Delta A_{340}/\text{min}]_{\text{nonactivated PDE}}$

Enzymatic Assay of CALCINEURIN

CALCULATIONS: (continued)

2. Determine the ratio of

$$\frac{\text{Corrected } \Delta A_{340}/\text{min (CaM unknown)}}{\text{Corrected } \Delta A_{340}/\text{min (Fully Activated)}} = \text{Ratio}$$

Tests 1,2, and 3 are the CaM unknowns and Tests 4 is the fully activated. Only ratio values between 0.35 - 0.65 can be used. Repeat the assay with different volumes of Reagent Q (CaM Work) until at least three ratios are found that meet the 0.35 - 0.65 requirements.

3. Plot the ratio values versus the volumes (ml) of Working CaM Solution used. Draw the best straight line through these points.
4. Find the volume which corresponds exactly to the ratio value of 0.50. This volume is $V_{0.5}$.
5. Determine the U/ml of the Working CaM Solution by

$$\frac{1.0 \text{ CaM unit}}{V_{0.5}} = \text{U/ml of Working CaM Solution}$$

$V_{0.5}$ = Value (in milliliter) of CaM giving activation up to 50% of the maximum activity as per the Unit Definition

If the working CaM solution is not between 40 and 44 units/ml, adjust the concentration of Reagent Q (CaM Work) and reassay accordingly.

PROCEDURE: Part III: Assay for Calcineurin

Note: The order of addition, CaM and Calcineurin, then PDE is very important and **MUST NOT** be changed. The amount of Calcineurin activity added should be between 0.8 and 1.2 units per assay.

Pipette (in milliliters) the following reagents into suitable cuvettes:

	<u>Test 1</u>	<u>Test 2</u>	<u>Test 3</u>	Fully Activated <u>Test 4</u>
Reaction Cocktail	2.80	2.80	2.80	2.80
Deionized Water*	-----	-----	-----	0.25
Reagent R (Calcin) up to:	0.25	0.25	0.25	-----
Reagent Q (CaM Work)	0.05	0.05	0.05	0.05

Enzymatic Assay of CALCINEURIN

PROCEDURE: (Part III: Assay for Calcineurin)

Note: *The volume of deionized water and Reagent R added should have a combined volume of 0.25 ml.

Mix by inversion and equilibrate at 30°C for 5 minutes. Then add:

Reagent O (PDE Work)	0.05	0.05	0.05	0.05
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Mix by inversion and equilibrate to 30°C for 5 minutes. Monitor the $A_{340\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent G (3':5'-cAMP)	0.05	0.05	0.05	0.05
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Immediately mix by inversion and record the decrease in $A_{340\text{nm}}$ for approximately 15 minutes. Obtain the $\Delta A_{340\text{nm}}/\text{minute}$ using the maximum linear rate for each Test.

CALCULATIONS:

1. Determine the corrected $\Delta A_{340}/\text{min}$ for each of the four Tests by:

$$\text{Corrected } \Delta A_{340}/\text{min} = [\Delta A_{340\text{nm}}/\text{min}] - [\Delta A_{340\text{nm}}/\text{min}]_{\text{nonactivated PDE}}$$

See assay for PDE, Part I Section B (Non-Activated Test) for

$$[\Delta A_{340}/\text{min}]_{\text{nonactivated PDE}}$$

2. Determine the ratio of

$$\text{Corrected } \Delta A_{340}/\text{min} \text{ (Calcineurin unknown)}$$

$$\text{Corrected } \Delta A_{340}/\text{min} \text{ (Fully Activated by 2 units CaM)}$$

Only ratio values between 0.35 - 0.65 can be used. Repeat the assay with different volumes of the Calcineurin unknown until at least three ratios are found that meet the 0.35 - 0.65 requirement.

3. Plot the ratio values versus the volumes (ml) of Calcineurin unknown used. Draw the best straight line through these points.
4. Find the volume which corresponds exactly to the ratio value of 0.50. The volume is $V_{0.5}$.

Enzymatic Assay of CALCINEURIN

CALCULATIONS: (Part III, continued)

5. Determine the units/ml of Calcineurin by:

$$\text{Units/ml} = \frac{(\text{Working CaM Concentration})(0.05)(df)}{2 (V_{0.5})}$$

Working CaM Concentration = units/ml of Reagent Q (CaM Work)

from Part II of the Procedure

0.05 = Volume (in milliliter) of Reagent Q (CaM Work)

df = Dilution factor

2 = Units of activator (as per the Unit Definition)

$V_{0.5}$ = The volume (in milliliter) of Calcineurin which will cause 50% inhibition (as per the Unit Definition)

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITIONS (Calcineurin):

One unit will cause a 50% inhibition of the activated phosphodiesterase, 3':5'-cyclic nucleotide (P-9529) activity when assayed with two units of activator (P-2777) and

0.1 mM Ca^{++} in an enzyme coupled system at pH 7.5 at 30°C.

FINAL ASSAY CONCENTRATIONS (Calcineurin):

In a 3.20 ml reaction mix the final concentrations are 65 mM Tris, 54 mM potassium chloride, 6.5 mM magnesium sulfate, 0.61 mM phospho(enol)pyruvate, 0.31 mM adenosine 5'-triphosphate, 0.10 mM calcium chloride, 0.13 mM β -nicotinamide adenine dinucleotide, reduced form, 11.4 units pyruvate kinase, 16.3 units L-lactic dehydrogenase, 7.8 units myokinase, 2.0 - 2.2 units calmodulin, 0.012 - 0.016 unit phosphodiesterase 3':5'-cyclic nucleotide, 1.2 mM adenosine 3':5'-cyclic monophosphate, and varying amounts of units of calcineurin.

NOTES:

1. Contains not less than 700 units/ml of Pyruvate Kinase units and 1000 units/ml of L-Lactic Dehydrogenase units/ml.

Enzymatic Assay of CALCINEURIN

NOTES: (continued)

2. Pyruvate Kinase Unit Definition: One unit will convert 1.0 μ mole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.
3. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 μ mole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.
4. Myokinase Unit Definition: One unit will convert 2.0 μ moles of ADP to ATP and AMP per minute at pH 7.6 at 37°C.
5. Phosphodiesterase 3':5'-Cyclic Nucleotide Unit Definition: One unit will hydrolyze 1.0 μ mole of 3':5'-cyclic-AMP to 5'-AMP per minute at pH 7.5 at 30°C.
6. Phosphodiesterase 3':5'-Cyclic Nucleotide Activator (Calmodulin) Unit Definition: One unit will stimulate 0.016 activated unit of phosphodiesterase, 3':5'-cyclic nucleotide, P-0520, in a 3 ml reaction volume at pH 7.5 and 30°C, to 50% of the maximum activity of the enzyme when saturated with activator, in the presence of 0.01 mM Ca⁺⁺.
7. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.