SIGMA QUALITY CONTROL TEST PROCEDURE

ProductInformation

Enzymatic Assay of PHOSPHODIESTERASE, 3':5'-CYCLIC NUCLEOTIDE ACTIVATOR (Calmodulin)
Sigma Prod. Nos. P-0270, P-1431, P-1915, P-2277, P-3922, P-4046, P-5779, P-6654, and P-9707

PRINCIPLE:

$$3':5'\text{-cAMP} + H_2O \xrightarrow[\text{PDE-3':5'-CNA}]{\text{PDE-3':5'-CN}} \text{AMP}$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{Myokinase}} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{PEP} \xrightarrow{\text{Pyruvate Kinase}} 2\ \text{ATP} + 2\ \text{Pyruvate}$$

$$2\ \text{Pyruvate} + 2\ \beta\text{-NADH} \xrightarrow{\text{Lactic Dehydrogenase}} 2\ \text{Lactate} + 2\ \beta\text{-NAD}$$

Abbreviations used:
3':5'-cAMP = Adenosine 3':5'-Cyclic Monophosphate
PDE-3':5'-CN = Phosphodiesterase, 3':5'-Cyclic Nucleotide
PDE-3':5'-CNA = Phosphodiesterase, 3':5'-Cyclic Nucleotide Activator (Calmodulin)
AMP = Adenosine 5'-Monophosphate
ATP = Adenosine 5'-Triphosphate
ADP = Adenosine 5'-Diphosphate
PEP = Phospho(enol)pyruvate
β-NADH = β-Nicotinamide Adenine Dinucleotide, Reduced Form
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

REAGENTS:

A. 200 mM Tris HCl Buffer, pH 7.5 at 30°C
(Prepare 50 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.5 at 30°C with 1 M HCl.)

B. 1.0 M Potassium Chloride Solution (KCl)
(Prepare 1 ml in deionized water using Potassium Chloride, Sigma Prod. No. P-4504.)

REAGENTS: (continued)

C. 60 mM Magnesium Sulfate Solution ($MgSO_4$)
(Prepare 10 ml in deionized water using Magnesium Sulfate, Heptahydrate, Sigma Prod. No. M-1880.)

D. 0.665 mM Phospho(enol)pyruvate Solution (PEP)
(Prepare by dissolving Phospho(enol)pyruvate, Monopotassium Salt, Sigma Prod. No. P-7127, in 3.7 ml of Reagent A. Then add: 0.6 ml of Reagent B, 1.2 ml of Reagent C, and 4.5 ml of deionized water. **PREPARE FRESH.**)

E. 30 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 10 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394. Adjust to pH 7.5 with solid Sodium Bicarbonate, Sigma Prod. No. S-8875. **PREPARE FRESH.**)

F. 60 mM Adenosine 3':5'-Cyclic Monophosphate Solution (3':5'-cAMP)
(Prepare 1 ml in deionized water using Adenosine 3':5'-Cyclic Monophosphate, Sodium Salt, Sigma Prod. No. A-6885. **PREPARE FRESH**.)

G. PK/LDH Enzymes Solution[1]
(Use PK/LDH Enzymes Solution in 50% Glycerol, Sigma Prod. No. P-0294.)

H. Myokinase Enzyme Solution (MK)
(Immediately before use, prepare a solution containing 200 units/ml of Myokinase, Sigma Prod. No. M-3003, in cold deionized water.)

I. 0.70 mM Calcium Acetate Solution ($Ca(OAc)_2$)
(Prepare 10 ml in deionized water using Calcium Acetate, Sigma Prod. No. C-1000.)

J. β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
(Use β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-101. **PREPARE FRESH.**)

K. Phosphodiesterase, 3':5'-Cyclic Nucleotide-Activator Solution (PDE-3':5'-CNA)
(Immediately before use, prepare a solution containing 100 units/ml of Phosphodiesterase, 3':5'-Cyclic Nucleotide-Activator, Sigma Prod. No. P-0270, in cold deionized water.)

REAGENTS: (continued)

L. 100 mM Tris HCl Buffer, pH 7.5 at 30°C (Enz Dil)
(Prepare 25 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.5 at 30°C.)

M. Phosphodiesterase, 3':5'-Cyclic Nucleotide Enzyme, Solution (PDE-3':5'-CN)
(Immediately before use dissolve Phosphodiesterase 3':5'cyclic Nucleotide, Activator Deficient, Sigma Prod. No. P-0520, 0.5 unit vial in 0.5 ml Reagent L.)

N. Phosphodiesterase 3':5'-Cyclic Nucleotide Enzyme, Working Solution (PDE-3':5'-CN Working Solution)
Assay of Reagent M:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a 1 mg vial of Reagent J.:

Reagent D (PEP)	9.00
Reagent E (ATP)	0.10
Reagent F (3':5'-cAMP)	0.10
Reagent G (PK/LDH)	0.05
Reagent H (MK)	0.05
Reagent I ($Ca(OAc)_2$)	0.15

Mix by inversion.

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

	Activated
Reaction Cocktail	3.00
Reagent K	0.10

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

Reagent M	0.015

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 15 minutes. Obtain the maximum linear rate for the Activated solution.

REAGENTS: (continued)

N. Continued:

Calculation:

I. PDE-3':5'-CN

$$\text{PDE-3':5'-CN Activated units/ml} = \frac{(\Delta A_{340}/\text{min Activated})(3.115)}{2(6.22)(0.015)}$$

PDE-3':5'-CN Activated units/ml equal units/ml Reagent M.

Dilute Reagent M, (with known activated units/ml) to 0.17 units/ml with Reagent L. This resulting 0.17 units/ml solution is now Reagent N, Phosphodiesterase 3':5' Cyclic Nucleotide Enzyme working solution.

O. Phosphodiesterase 3':5'-Cyclic Nucleotide Activator Sample Solution (PDE-3':5'-CNA/Sample) (Immediately before use, prepare a solution containing approximately 10 units/ml of Phosphodiesterase 3':5'-Cyclic Nucleotide Activator in cold deionized water.)

PROCEDURE:

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

	Non-Activated	Activated	Test
Reaction Cocktail	3.00	3.00	3.00
Deionized Water	0.15	0.05	0.05
Reagent K (PDE-3':5'-CNA)	------	0.10	------
Reagent O (PDE-3':5'-CNA/Sample)	------	------	0.10

Mix by inversion and equilibrate to 30°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Each cuvette must contain exactly 0.0085 unit of PDE-3':5'-CN (Reagent M) in a total volume of 3.20 ml. Then add:

	Non-Activated	Activated	Test
Reagent N (PDE-3':5'-CN)	0.05	0.05	0.05

PROCEDURE: (continued)

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 20 minutes. Obtain the maximum linear rate for the Non-Activated, Activated, and Test solutions.

The Test activity should be as close to 1.0 unit of PDE-3':5'-CNA in the cuvette as possible (acceptable range is 0.90-1.10 units). If not in this range, change the sample preparation scheme appropriately and repeat the assay.

CALCULATIONS:

I. PDE-3':5'-CN

$$\text{PDE -3':5'-CN Non-Activated Units/ml} = \frac{(\Delta A_{340nm}/\text{min Non-Activated})(3.2)}{(2)(6.22)(0.05)}$$

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

$$\text{PDE-3':5'-CN Test Units/ml} = \frac{(\Delta A_{340nm}/\text{min Test})(3.2)}{(2)(6.22)(0.05)}$$

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

$$\text{PDE-3':5'-CN Activated Units/ml} = \frac{(\Delta A_{340nm}/\text{min Activated})(3.2)}{(2)(6.22)(0.05)}$$

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

CALCULATIONS: (continued)

II. PDE-3':5'-CNA

$$\text{Ratio} = \frac{\text{(Units/ml PDE-3':5'-CN Test - Units/ml PDE-3':5'-CN Non-Activated)}}{\text{(Units/ml PDE-3':5'-CN Activated - Units/ml PDE-3':5'-CN Non-Activated)}}$$

This ratio should be as close to 0.50 as possible. Repeat the assay until the ratio is in the range of 0.45 - 0.55.

$$\text{Units/ml PDE-3':5'-CNA Sample} = \frac{(\text{Ratio})(2)(3.2)}{3.0} \times \frac{\text{df}}{\text{sample volume (ml)}}$$

or

$$= \frac{(\text{Ratio})(2.1333)(\text{df})}{\text{sample volume (ml)}}$$

df = Dilution factor
2 = Conversion factor since 50% of the maximum activity is obtained as per the Unit Definition
3.2 = Total volume (in milliliters) of assay
3 = Conversion factor to 3 ml assay

UNIT DEFINITION:

One unit will stimulate 0.008 activated units 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^{2+}.

FINAL ASSAY CONCENTRATIONS:

In a 3.20 ml reaction mix (Test), the final concentrations are 66 mM Tris, 54 mM potassium chloride, 6.4 mM magnesium sulfate, 0.6 mM phospho(enol)pyruvate, 0.30 mM adenosine 5'-triphosphate, 0.6 mM adenosine 3':5'-cyclic monophosphate, 0.01 mM calcium acetate, 11 units pyruvate kinase, 16 units L-lactic dehydrogenase, 3 units myokinase, 0.1 mM β-nicotinamide adenine dinucleotide, reduced form, 0.0085 unit phosphodiesterase 3':5'-cyclic nucleotide, and 1.0 unit phosphodiesterase 3':5'-cyclic nucleotide activator.

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REFERENCE:

Chock, S.P. and Huang, C.Y. (1984) *Analytical Biochemistry* **138**, 34-43

NOTES:

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

2. Values greater than 0.17 unit/ml are acceptable. However, the volume of PDE-3':5'-CN added to the cuvette must be adjusted so that each assay contains 0.0085 unit of PDE-3':5'-CN in a final volume of 3.2 ml.

3. This reaction is very sensitive to chelators which sequester Ca^{++} ions at low Ca^{++} concentrations. EDTA or EGTA concentrations of 0.005 mM in the cuvette will almost completely inhibit the reaction. The concentration of EDTA or EGTA should be less then 0.001 mM in the cuvette. Cuvettes should be completely cleaned with 3% (w/v) NaOH before each use, since PDE-3':5'-CNA can bind to glass or quartz cuvettes.

4. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.

5. Pyruvate Kinase Unit Definition: One unit will convert 1.0 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.

6. Myokinase Unit Definition: One unit will convert 2.0 µmoles of ADP to ATP and AMP per minute at pH 7.6 at 37°C.

7. Phosphodiesterase 3':5'-Cyclic Nucleotide Unit Definition: One unit will hydrolyze 1.0 µmole of 3':5'-cyclic-AMP to 5'-AMP per minute at pH 7.5 at 30°C.

8. This assay is based on the cited reference.

7. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.