SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of PHOSPHODIESTERASE, 3':5'-CYCLIC NUCLEOTIDE
Crude Complex
Sigma Prod. No. P-0134

PRINCIPLE:

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

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

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

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

Abbreviations used:
3':5'-cAMP = Adenosine 3':5'-Cyclic Monophosphate
PDE-3':5'-CN = Phosphodiesterase, 3':5'-Cyclic Nucleotide
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 Hydrochloride, Sigma Prod. No. T-3253. Adjust to pH 7.5 at 30°C with 2 M NaOH.)

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

C. 60 mM Magnesium Sulfate Solution ($MgSO_4$)
(Prepare 10 ml in deionized water using Magnesium Sulfate, Anhydrous, Sigma Prod. No. M-7506.)

REAGENTS: (continued)

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 at 30°C 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. 0.70 mM Calcium Acetate Solution ($Ca(OAc)_2$)
(Prepare 10 ml in deionized water using Calcium Acetate, Sigma Prod. No. C-1000.)

H. β-Nicotinamide Adenine Dinucleotide, Reduced Form, Preweighed vial 1 mg (β-NADH)
(Use β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-101. **PREPARE FRESH**.)

I. PK/LDH Enzymes Suspension[1]
(Use PK/LDH Enzymes Suspension, Sigma Stock No. 40-7.)

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

K. Phosphodiesterase, 3':5'-Cyclic Nucleotide Crude Complex Enzyme Solution (PDE-3':5'-CN)
(Immediately before use, prepare a solution containing 0.05 - 0.1 unit/ml of Phosphodiesterase 3':5'-Cyclic Nucleotide, Crude Complex, in cold deionized water.)

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PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into Reagent H (β-NADH):

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

Mix by inversion.

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

	Test	Blank
Reaction Cocktail	3.00	3.00

Equilibrate to 30°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent K (PDE-3':5'-CN)	0.10	------
Deionized Water	------	0.10

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 both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(3.1)(df)}{(2)(6.22)(0.1)}$$

3.1 = Total volume (in milliliters) of assay
df = Dilution factor
2 = 2 μmoles of β-NAD produced per μmole of 3':5'-cAMP hydrolyzed
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm
0.1 = Volume (in milliliter) of enzyme used in the assay

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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.

FINAL ASSAY CONCENTRATIONS:

In a 3.10 ml reaction mix, the final concentrations are 68 mM Tris, 55 mM potassium chloride, 7 mM magnesium sulfate, 0.61 mM phospho(enol)pyruvate, 0.31 mM adenosine 5'-triphosphate, 0.61 mM adenosine 3':5'-cyclic monophosphate, 11 units pyruvate kinase, 16 units lactic dehydrogenase, 3.1 units myokinase, 0.01 mM calcium acetate, 0.1 mM β-nicotinamide adenine dinucleotide, reduced form, and 0.005 - 0.01 unit phosphodiesterase, 3':5'-cyclic nucleotide, crude complex.

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 per ml.

2. 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.

3. 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.

4. 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.

5. This reaction is very sensitive to chelators, which sequester the calcium ions at low Ca^{2+} 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 than 0.001 mM in the cuvette. Cuvettes should also be completely cleaned with 3% (w/v) NaOH solution before each use, as phosphodiesterase 3':5' cyclic activator can bind to quartz or glass cuvettes.

NOTES: (continued)

6. This assay is based on the cited reference.

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