Enzymatic Assay of NAD^+ KINASE
(EC 2.7.1.23)

PRINCIPLE:

$$\text{ß-NAD} + \text{ATP} \xrightarrow[Mg\ 2^+]{NAD^+\ Kinase} \text{ß-NADP} + \text{ADP}$$

$$\text{ß-NADP} + \text{G-6-P} \xrightarrow{\text{G-6-PDH}} \text{ß-NADPH} + \text{6-Phosphogluconate}$$

Abbreviations used:
ADP = Adenosine 5'-Diphosphate
ATP = Adenosine 5'-Triphosphate
G-6-PDH = Glucose 6-Phosphate Dehydrogenase
ß-NAD = ß-Nicotinamide Adenine Dinucleotide
ß-NADP = ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form
ß-NADPH = ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form

CONDITIONS: T = 37°C, pH = 7.5, A_{340nm}, Light path = 1 cm

METHOD: Spectrophotometric Stop Rate Determination

REAGENTS:

A. 1 M Tris Solution
(Prepare 50 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503.)

B. 28 mM ß-Nicotinamide Adenine Dinucleotide and 33 mM ATP with 20 mM Magnesium Chloride, pH 7.5 at 37°C (Reaction Cocktail)
(Prepare 25 ml in deionized water using ß-Nicotinamide Adenine Dinucleotide, Sigma Prod. No. N-7004, Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394, and Magnesium Chloride Hexahydrate, Sigma Prod.
No. M-0250. Adjust the pH to 7.5 at 37°C using Reagent A.)

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REAGENTS: (continued)

C. 250 mM Glycylglycine Buffer, pH 7.5 at 25°C (Buffer)
(Prepare 50 ml in deionized water using Glycylglycine, Sigma Prod. No. G-1002. Adjust the pH to 7.5 at 25°C with 1 M NaOH.)

D. 60 mM Glucose-6-Phosphate Solution (G-6-P)
(Prepare 5.0 ml in deionized water using Glucose-6-Phosphate, Disodium Salt, Hydrate, Sigma Prod. No. G-7250.)

E. 300 mM Magnesium Chloride ($MgCl_2$)
(Prepare 10 ml in deionized water using Magnesium Chloride Hexahydrate, Sigma Prod. No. M-0250.)

F. 10 mM Tris HCl Buffer, pH 7.5 at 37°C (Enzyme Diluent)
(Prepare 50 ml in deionized water using Reagent A. Adjust to pH 7.5 at 37°C with 1 M HCl.)

G. Glucose-6-Phosphate Dehydrogenase Solution (G-6-PDH)
(Immediately before use prepare a solution containing 100 units per ml of Glucose-6-Phosphate Dehydrogenase, Sigma Prod. No. G-4134, in cold Reagent C.)

H. NAD^+ Kinase Solution (NAD Kinase)
(Immediately before use prepare a solution containing 10 - 30 units per ml in cold Reagent F.)

PROCEDURE:

Step 1:

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

	Test	Blank
Reagent B (Reaction Cocktail)	1.00	1.00

Equilibrate to 37°C. Then add:

Reagent H (NAD^+ Kinase)	1.00	------
Reagent F (Enzyme Diluent)	------	1.00

Immediately mix by swirling and incubate at 37°C for exactly 30 minutes. Immediately transfer to a boiling water bath for 2 minutes. Remove from the boiling water bath and let cool to room temperature. Centrifuge to clarify.

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PROCEDURE: (continued)

Step 2:

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

	Test	Blank
Reagent C (Buffer)	2.50	2.50
Reagent D (G-6-P)	0.10	0.10
Reagent E ($MgCl_2$)	0.10	0.10
Test Supernatant (from Step 1)	0.20	------
Blank Supernatant (from Step 1)	------	0.20

Mix by inversion and equilibrate to 37°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Record the initial A_{340nm} for both the Test and Blank. Then add:

	Test	Blank
Reagent G (G-6-PDH Solution)	0.10	0.10

Immediately mix by inversion and record the increase in A_{340nm} until complete (for approximately 5 minutes). Obtain the final A_{340nm} for both the Test and Blank.

CALCULATIONS:

$r\ A_{340nm}$ Test = (Final A_{340nm} of Test) - (Initial A_{340nm} of Test)

$r\ A_{340nm}$ Blank = (Final A_{340nm} of Blank) - (Initial A_{340nm} of Blank)

$$\text{Units/ml enzyme} = \frac{(r\ A_{340nm}\ \text{Test}) - (r\ A_{340nm}\ \text{Blank})(1000\ \text{nmoles}/\mu\text{mole})(3)(2)}{(6.22)(30)(1)(0.2)}$$

3 = Volume (in milliliters) of reaction in Step 2
2 = Volume (in milliliters) of reaction in Step 1
6.22 = Millimolar extinction coefficient of ß-NADPH at 340 nm
30 = Time (in minutes) of reaction for Step 1
1 = Volume (in milliliter) of enzyme used for Step 1
0.2 = Volume (in milliliter) of Test Supernatant (from Step 1) used in Step 2

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(EC 2.7.1.23)

CALCULATIONS: (continued)

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

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

UNIT DEFINITION:

One unit will phosphorylate 1.0 nanomole of ß-NAD to ß-NADP per minute at pH 7.5 at 37°C in the presence of ATP.

FINAL ASSAY CONCENTRATION:

In a 2.00 ml reaction mix, the final concentrations are 14 mM ß-nicotinamide adenine dinucleotide, 17 mM adenosine 5'-triphosphate, 10 mM magnesium chloride, 5 mM Tris, and 10 - 30 units NAD^+ Kinase.

NOTES:

1. Unit definition for Glucose-6-Phosphate Dehydrogenase: One unit will oxidize 1.0 µmole of glucose-6-phosphate to 6-phosphogluconate per minute in the presence of ß-NADP at pH 7.4 at 25°C.

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