Enzymatic Assay of NAD-PYROPHOSPHORYLASE (EC 2.7.7.1)

PRINCIPLE:

ß-NMN + ATP $\xrightarrow{\text{NAD-Pyrophosphorylase}}$ ß-NAD + PP

ß-NAD + Ethanol $\xrightarrow{\text{ADH}}$ ß-NADH + Acetaldehyde

Abbreviations used:
ATP = Adenosine 5'-Triphosphate
ADH = Alcohol Dehydrogenase
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
ß-NMN = ß-Nicotinamide Mononucleotide
PP = Pyrophosphate

CONDITIONS: T = 37°C, pH = 7.4, A_{340nm}, Light path = 1 cm

METHOD: Spectrophotometric Stop Rate Determination

REAGENTS:

A. 250 mM Glycylglycine, Buffer pH 7.4 at 37°C. (Gly-Gly) (Prepare 10 ml in deionized water using Gly-Gly, Free Base, Sigma Prod. No. G-1002. Adjust the pH to 7.4 at 37°C with 5 M NaOH.)

B. 22 mM Adenosine Triphosphate Solution (ATP) (Prepare 1 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394. Neutralize with Sodium Bicarbonate, Sigma Prod. No. S-8875)

C. 60 mM Nicotinamide Mononucleotide Solution (NMN) (Prepare 1 ml in Reagent A using ß-Nicotinamide Mononucleotide, Sigma Prod. No. N-3501.)

D. 150 mM Magnesium Chloride Solution ($MgCl_2$) (Prepare 10 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

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REAGENTS: (continued)

E. 75 mM Pyrophosphate Buffer with 75 mM Semicarbazide HCl, 23 mM Glycine, and 1% (v/v) Ethanol (Nondenatured), pH 8.7 at 25°C
(Prepare 10 ml in deionized water using Pyrophosphate Tetrasodium, Anhydrous, Sigma Prod. No. P-8010, Semicarbazide, Hydrochloride, Sigma Prod. No. S-4125, Glycine, Free Base, Sigma Prod. No. G-7126. Adjust the pH to 8.7 using 1 M HCl solution.)

F. Alcohol Dehydrogenase Enzyme Solution (ADH)
(Immediately before use prepare a solution containing 30 mg/ml of Alcohol Dehydrogenase, Sigma Prod. No. A-3263 in cold deionized water.)

G. NAD-Pyrophosphorylase Enzyme Solution (NAD-PP)
(Immediately before use prepare a solution containing 0.13 - 0.30 unit/ml of NAD-Pyrophosphorylase in cold deionized water.)

PROCEDURE:

Step 1:

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

	Test	Blank
Reagent A (Gly-Gly)	0.20	0.20
Reagent B (ATP)		0.10
0.10		
Reagent C (NMN)	0.20	0.20
Reagent D ($MgCl_2$)	0.10	0.10
Deionized Water	0.30	0.30

Mix by swirling and equilibrate to 37°C. Then Add:

Reagent G (NAD-PP Enzyme solution)	0.10	------
Deionized Water	------	0.10

Immediately mix by swirling and incubate at 37°C for exactly 30 minutes. Stop the reaction by heating both the Test and Blank solutions for 1 minute at 100°C. Remove from the boiling water bath and let cool to room temperature. Filter the supernatant using 0.45 µm syringe

filters.

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PROCEDURE: (continued)

Step 2:

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

	Test	Blank
Reagent E	2.49	2.49
Test Supernatant (Step 1)	0.50	------
Blank Supernatant (Step 1)	------	0.50

Mix by inversion and equilibrate to 25°C. Monitor the A_{340nm} until constant using a suitable thermostatted spectrophotometer and record the initial A_{340nm} for the Test and Blank solutions. Then add:

Reagent F (ADH)	0.01	0.01

Immediately mix by inversion and record the increase in A_{340nm} for approximately 5 - 10 minutes until constant. Obtain the final A_{340nm} for both the Test and Blank solutions.

CALCULATIONS:

$$r\ A_{340nm}\ Test = A_{340nm}\ Test_{\ Final} - A_{340nm}\ Test_{\ Initial}$$

$$r\ A_{340nm}\ Blank = A_{340nm}\ Blank_{\ Final} - A_{340nm}\ Blank_{\ Initial}$$

$$\text{Units/ml enzyme} = \frac{(r_{\ 340nm}\ Test) - (r_{\ 340nm}\ Blank)(3.0)(1)(df)}{(6.22)(30)(0.1)(0.5)}$$

3.0 = Total volume (in milliliters) of reaction mixture in Step 2
1.0 = Total volume (in milliliters) of reaction mixure in Step 1
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADPH at 340 nm
30 = Reaction time (in minutes) of Step 1
0.5 = Volume (in milliliter) of Step 1 Test used in Step 2
0.1 = Volume (in milliliter) of enzyme used in Step 1

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

CALCULATIONS: (continued)

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

UNIT DEFINITION:

One unit will form 1.0 µmole of ß-NAD from nicotinamide mononucleotide and ATP per minute at pH 7.4 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 1.00 ml reaction mix, the final concentrations are 100 mM glycylglycine, 2.2 mM adenosine 5'-triphosphate, 12 mM nicotinamide mononucleotide, 15 mM magnesium chloride, and 0.013 - 0.03 unit of NAD-Pyrophosphorylase.

NOTES:

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