NADH oxidase
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Summary

NADH oxidase catalyzes the oxidation of NADH to NAD with water as a co-product. This protocol describes a direct enzyme assay measuring the quantity of NADH consumed. The co-substrate is oxygen, and therefore the assay mixture must contain dissolved oxygen.

Solutions Required

1. 125 mM phosphate buffer pH = 7.0
   0.827 g KH_2PO_4 plus
   2.349 g $K_2HPO_4 \cdot 3H2O$
   in 100 mL DI water

2. 2.9 mM NADH
   must be prepared fresh
   per assay need 0.0002 g / 0.05 mL

3. 5 mM dipotassium EDTA (FW = 404.47)
   0.0041 g/ 2 mL

Preparation of Cell Extract

Follow general protocol **Preparation of Cell Extract**.

1. Centrifuge sufficient cells so that the volume diluted down to 5 mL would give an optical density of 20-30. For example, for a broth of OD=1, use 100 mL. For a broth of OD=10, use 10 mL.
2. After first pelletization of cells, resuspend in 5-15 mL of tricine buffer.
3. After second pelletization of cells, resuspend in 5 mL of tricine buffer, and break with French Press.

Spectrophotometer

Turn on the ultraviolet bulb on the spectrophotometer (Beckman DU50) and wait 30 minutes for warm-up. Select the kinetics-time window on the instrument. Load the method "A:/nadh". This method has a run-time of 60 s, a temperature of 37°C, a wavelength of 340 nm and uses 2 autosamplers.

Procedure

1. For each assay, prepare a cocktail shown in the following table into one UV-translucent cuvettes, and keep on ice. There is no control. "Blank" the spectrophotometer with DI water.

Solution	Volume (µL) added to:
DI H_2O	400
Phosphate	400
EDTA	100
NADH	50

2. Directly from the ice when ready to commence the assay, place the cuvette (containing 950 µL†) into the spectrophotometer holder.
3. Wait 10 minutes to allow the temperature of the solutions in the cuvettes to equilibrate.
4. Aspirate air into the cuvette with a pipettor.
5. Simultaneously add 50 µL† of the cell extract to the cuvettes.
6. To mix solutions, immediately and simultaneously aspirate and dispense the contents of the cuvettes with a pipettor. Mix the solutions in this way ten times. (Count!)
7. Promptly depress "start" on the monitor.
8. Record the rates for the two (control and experimental) cuvettes.

† Dilution of the cell extract may be adjusted so that change in absorbance is between about 0.05 and 0.7 AU in one minute. This dilution should be accomplished externally in a microcentrifuge tube (for example, by adding 50 µL of cell extract to 950 µL DI water to achieve a dilution of 20). The volume of 50 µL should always be used in the enzyme assay mixture.

Calculation of Activity

One unit (U) of NADH oxidase is defined as the amount of enzyme required to oxidase 1.0 µmole of NADH in one minute.

1. $dA/dt\ (min^{-1}) = [Rate]_{experimental} - [Rate]_{control} = dA/dt$

2. $$\text{Activity} = \frac{1000 \times TV \times D \times dA/dt}{\varepsilon \times V \times CF}$$

Activity:	Volumetric Activity (U/L)
TV:	Total volume in cuvette (1000 µL)
D:	Dilution of the cell extract. (For example, if 50 µL of cell extract were add to 950 µL DI water prior to using a volume of cell extract in the assay, then D=20)
V:	Volume of cell extract used (50 µL)
ε:	Molar extinction coefficient for NADH (6.22 L/mmol for a path length of 1.0 cm)
CF:	Concentration Factor of cell extract (For example, if a 100 mL sample is concentrated to a 2 mL volume for the French Press, then CF=50)

3. $$\text{Specific Activity} = \frac{Activity}{Protein\ Concentration} 1$$

Activity:	Volumetric Activity, as calculated in #2 above (U/L)
Protein Concentration:	Protein concentration, as calculated in protocol **Total Protein Concentration** (mg/L)
Specific Activity:	(U/mg protein)