

## EnzyFluo™ NAD<sup>+</sup>/NADH Assay Kit (EFND-100)

### Quantitative Fluorimetric Determination of NAD<sup>+</sup>/NADH

#### DESCRIPTION

Pyridine nucleotides play an important role in metabolism and, thus, there is continual interest in monitoring their concentration levels. Quantitative determination of NAD<sup>+</sup>/NADH has applications in research pertaining to energy transformation and redox state of cells or tissue. BioAssay Systems' EnzyFluo™ NAD<sup>+</sup>/NADH assay kit is based on a lactate dehydrogenase cycling reaction, in which the formed NADH reduces a probe into a highly fluorescent product. The fluorescence intensity of this product, measured at  $\lambda_{\text{ex/em}} = 530/585$  nm, is proportional to the NAD<sup>+</sup>/NADH concentration in the sample. This assay is highly specific for NAD<sup>+</sup>/NADH with minimal interference (<1%) by NADP<sup>+</sup>/NADPH and is a convenient method to measure NAD, NADH and their ratio.

#### APPLICATIONS

**Direct Assays:** NAD<sup>+</sup>/NADH concentrations and ratios in cell or tissue extracts.

#### KEY FEATURES

**Sensitive and accurate.** Detection limit of 0.02  $\mu\text{M}$  and linearity up to 1  $\mu\text{M}$  NAD<sup>+</sup>/NADH in 96-well plate assay.

**Convenient.** The procedure involves adding a single working reagent, and reading the fluorescence at time zero and 10 min.

**High-throughput.** Can be readily automated as a high-throughput 96-well plate assay for thousands of samples per day.

#### KIT CONTENTS

<b>Assay Buffer:</b>	10 mL	<b>Enzyme A:</b>	120 $\mu\text{L}$
<b>Lactate:</b>	1.5 mL	<b>Enzyme B:</b>	120 $\mu\text{L}$
<b>Probe:</b>	750 $\mu\text{L}$	<b>NAD Standard:</b>	0.5 mL

**NAD/NADH Extraction Buffers:** each 12 mL

**Storage conditions.** The kit is shipped on ice. Store all reagents at -20°C. Shelf life: 6 months after receipt.

**Precautions:** reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

#### GENERAL CONSIDERATIONS

- At these concentrations, the standard curves for NAD and NADH are identical. Since NADH in solution is unstable, we provide only NAD as the standard.
- This assay is based on an enzyme-catalyzed kinetic reaction. Addition of Working Reagent should be quick and mixing should be brief but thorough. Use of multi-channel pipettor is recommended.
- The following substances interfere and should be avoided in sample preparation. EDTA (>0.5 mM), ascorbic acid, SDS (>0.2%), sodium azide, NP-40 (>1%) and Tween-20 (>1%).
- For samples containing higher than 100  $\mu\text{M}$  pyruvate, we recommend using an internal standard.

#### PROCEDURES

- Sample Preparation.** For tissues weigh ~20 mg tissue for each sample, wash with cold PBS. For cell samples, wash cells with cold PBS and pellet  $\sim 10^5$  cells for each sample. Homogenize samples (either tissue or cells) in a 1.5 mL Eppendorf tube with either 100  $\mu\text{L}$  NAD extraction buffer for NAD determination or 100  $\mu\text{L}$  NADH extraction buffer for NADH determination. Heat extracts at 60°C for 5 min and then add 20  $\mu\text{L}$  Assay Buffer and 100  $\mu\text{L}$  of the opposite extraction buffer to neutralize the extracts. Briefly vortex and spin the samples down at 14,000 rpm for 5 min. Use supernatant for NAD/NADH assays. Determination of both NAD and NADH concentrations requires extractions from two separate samples.
- Calibration Curve.** Prepare 500  $\mu\text{L}$  1  $\mu\text{M}$  NAD Premix by mixing 5  $\mu\text{L}$  1 mM Standard and 4995  $\mu\text{L}$  distilled water. Dilute standard as follows.

No	Premix + H <sub>2</sub> O	NAD ( $\mu\text{M}$ )
1	100 $\mu\text{L}$ + 0 $\mu\text{L}$	1.0
2	60 $\mu\text{L}$ + 40 $\mu\text{L}$	0.6
3	30 $\mu\text{L}$ + 70 $\mu\text{L}$	0.3
4	0 $\mu\text{L}$ + 100 $\mu\text{L}$	0

Transfer 50  $\mu\text{L}$  standards into wells of a clear flat-bottom 96-well plate.

- Samples.** Add 50  $\mu\text{L}$  of each sample in separate wells.
- Reagent Preparation.** For each reaction well, prepare Working Reagent by mixing 40  $\mu\text{L}$  Assay Buffer, 1  $\mu\text{L}$  Enzyme A, 1  $\mu\text{L}$  Enzyme B, 10  $\mu\text{L}$  Lactate and 5  $\mu\text{L}$  Probe. Fresh reconstitution is recommended.
- Reaction.** Add 50  $\mu\text{L}$  Working Reagent per well quickly. Tap plate to mix.
- Read fluorescence at  $\lambda_{\text{ex/em}} = 530/585$  nm for time "zero" ( $F_0$ ) and  $F_{10}$  after a 10-min incubation at room temperature. Protect plate from light during this incubation.

#### CALCULATION

First compute the  $\Delta F$  for each standard and sample by subtracting  $F_0$  from  $F_{10}$ . Plot the standard  $\Delta F$ 's and determine the slope. The NAD(H) concentration of the sample is computed as follows:

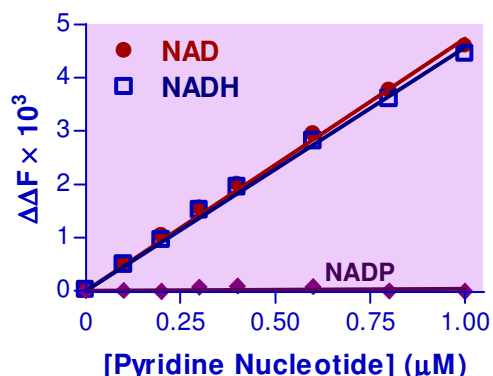
$$[\text{NAD(H)}] = \frac{\Delta F_{\text{SAMPLE}} - \Delta F_{\text{BLANK}}}{\text{Slope } (\mu\text{M}^{-1})} \times n \quad (\mu\text{M})$$

where  $\Delta F_{\text{SAMPLE}}$  and  $\Delta F_{\text{BLANK}}$  are the change in fluorescence intensity values of the Sample and Blank (STD 4) respectively. Slope is the slope of the standard curve and  $n$  is the dilution factor (if necessary).

Note: If the sample  $\Delta F$  values are higher than the  $\Delta F$  value for the 1  $\mu\text{M}$  standard, dilute sample in distilled water and repeat this assay. Multiply the results by the dilution factor.

#### MATERIALS REQUIRED, BUT NOT PROVIDED

Pipetting (multi-channel) devices. Black, flat bottom 96-well plates and fluorescent plate reader capable of reading at  $\lambda_{\text{ex/em}} = 530/585$  nm.



#### LITERATURE

- Zhao, Z, Hu, X and Ross, CW (1987). Comparison of Tissue Preparation Methods for Assay of Nicotinamide Coenzymes. *Plant Physiol.* 84: 987-988.
- Matsumura, H. and Miyachi, S (1980). Cycling assay for nicotinamide adenine dinucleotides. *Methods Enzymol.* 69: 465-470.
- Vilcheze, C et al. (2005). Altered NADH/NAD<sup>+</sup> Ratio Mediates Coresistance to Isoniazid and Ethionamide in Mycobacteria. *Antimicrobial Agents and Chemotherapy.* 49(2): 708-720.