Amplite™ Colorimetric NAD/NADH Assay Kit

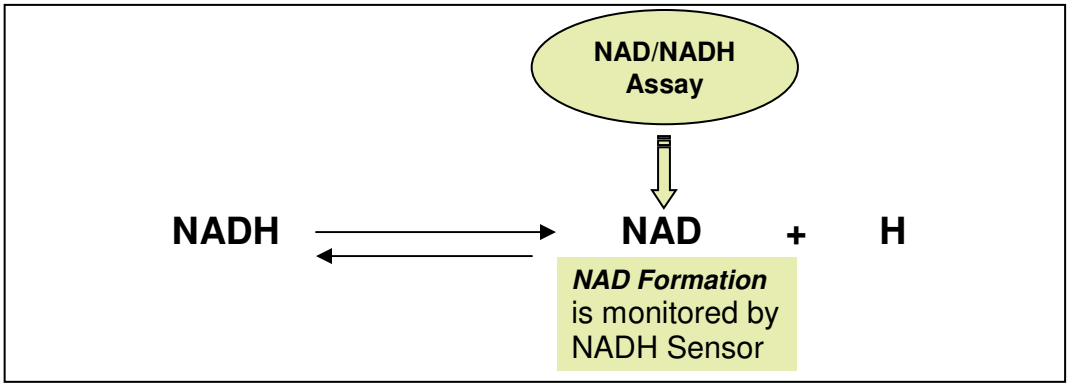
Blue Color

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: #15258 (400 assays)	Keep in freezer and avoid light.	Absorbance microplate readers

Introduction

Nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) are two important cofactors found in cells. NADH is the reduced form of NAD+, and NAD+ is the oxidized form of NADH. It forms NADP with the addition of a phosphate group to the 2' position of the adenyl nucleotide through an ester linkage. NADP is used in anabolic biological reactions, such as fatty acid and nucleic acid synthesis, which require NADPH as a reducing reagent. In chloroplasts, NADP is an oxidizing reagent important in the preliminary reactions of photosynthesis. The NADPH produced by photosynthesis is used as reducing power for the biosynthetic reactions in the Calvin cycle of photosynthesis. The traditional NAD/NADH and NADP/NADPH assays are done by monitoring of NADH or NADPH absorption at 340 nm. This method suffers low sensitivity and high interference since the assay is done in the UV range that requires expensive quartz microplate.

This Amplite™ NAD/NADH Assay Kit provides a convenient method for sensitive detection of NAD, NADH and their ratio. The enzymes in the system specifically recognize NAD/NADH in an enzyme cycling reaction. There is no need to purify NAD/NADH from sample mix. The enzyme cycling reaction significantly increases detection sensitivity. In addition, this assay has very low background since it is done in the red visible range that significantly reduces the interference from biological samples. The assay has demonstrated high sensitivity and low interference with absorbance at 576±5 nm.



The Amplite™ Fluorimetric NAD/NADH Assay Kit provides a sensitive, one-step colorimetric assay to detect as little as 30 picomoles of NAD(H) in a 100 µL assay volume (300 nM; Figure 1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation with no separation steps required. Its signal can be easily read by absorbance microplate reader at 575±5 nm or with absorbance ratio at 570±10 nm to 605±10 nm to increase assay sensitivity.

Kit Key Features

Broad Application:	Can be used for quantifying NAD/NADH in solutions, in cell extracts.
Sensitive:	The kit detect as low as 30 picomoles of NAD/NADH in solution.
Continuous:	Easily adapted to automation with no separation required.
Convenient:	Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive:	No special requirements for waste treatment.

Kit Components

Components	Amount
Component A: NAD/NADH Recycling Enzyme Mixture	2 bottles (lyophilized powder)
Component B: NADH Sensor Buffer	1 bottle (20 mL)
Component C: NADH Standard (FW: 709)	1 vial (142 µg)

Assay Protocol for one 96-well plate

Brief Summary

Prepare NAD/NADH reaction mixture (50 µL) → Add NADH standards or test samples (50 µL) → Incubate at room temperature for 15 min-2 hr → Read absorbance at 575±5 nm

Note: Thaw1 vial (or bottle) each of all the kit components to room temperature before starting the experiment.

1. Prepare NADH stock solution:

1.1 Prepare NADH standard stock solution: Add 200 µL of PBS buffer into the NADH standard vial (Component C) to have 1 mM (1 nmol/µL) stock solution.
Note: The unused NADH solution should be divided as single use aliquots and stored at -20°C.

2. Prepare NAD/NADH reaction mixture:

2.1 Prepare the NAD/NADH reaction mixture: Add 10 mL of NAD/NADH Sensor Buffer (Component B) to the bottle of NAD/NADH Recycling Enzyme Mixture (Component A), mixed well.
Note: This solution is enough for two 96-well plates. The unused NAD/NADH mixture should be divided as single use aliquots and stored at -20°C.

3. Prepare serial NADH (0 to 10 µM) solutions:

3.1 Add 10 µL of NADH standard stock solution (from step 1) to 990 µL PBS (pH 7.4) buffer to generate 10 µM (10 pmol/µL) standard.
Note: Diluted NADH standard solution is unstable, should be used within 4 hours.

3.2 Take 200 µL of 10 µM solution to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 standard NADH solutions.

3.3 Add NADH standards and NAD/NADH containing test samples into a 96-well white/clear bottom microplate as described in Tables 1 and 2
Note: Prepare your cell or tissue samples as desired.

Table 1. Layout of NADH standards and test samples in a white/clear bottom 96-well microplate:

BL	BL	TS	TS	….	….						
NS1	NS1	….	….	….	….						
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

Note: NS= NADH Standards, BL=Blank Control, TS=Test Samples.

Table 2. Reagent composition for each well:

NADH Standard	**Blank Control**	**Test Sample**
Serial dilutions*: 50 µL	PBS: 50 µL	50 µL

**Note: Add the serially diluted NADH standards from 0.01 µM to10 µM into wells from NS1 to NS7 in duplicate.*

High concentration of NADH (e.g., >100 µM, final concentration) may cause reduced signal due to the over oxidation of NADH sensor.

4. Run NAD/NADH assay in supernatants reaction:

4.1 Add 50 µL of NADH reaction mixture (from step 2) to each well of the NADH standard, blank control, and test samples (from step 3.3) so that the total NADH assay volume is 100 µL/well.
Note: For a 384-well plate, add 25 µL sample, 25 µL of NADH reaction mixture per well.

4.2 Incubate the reaction for 15 minutes to 2 hours at room temperature, protected from light.

4.3 Monitor the absorbance increase with 575 $\pm$ 5 nm by using an absorbance plate reader.
Note1: To detect NADH only, aliquot 200 µL samples into Eppendorf tubes. Heat samples to 60^{o}C for 30 min in a heating block or a water bath. All NAD will be decomposed while NADH will be still intact under the conditions. Cool samples on ice, and quick spin samples if precipitates occur. Transfer 50 µL of NADH samples into the wells as indicated in Table 1 and 2.

Data Analysis

The absorbance in blank wells (with the PBS buffer only) is used as a control, and is subtracted from the values for those wells with the NADH reactions. The typical data are shown in Figure1 (NADH standard curve).
Note: The absorbance background increases with time, thus it is important to subtract the absorbance of the blank wells for each data point.

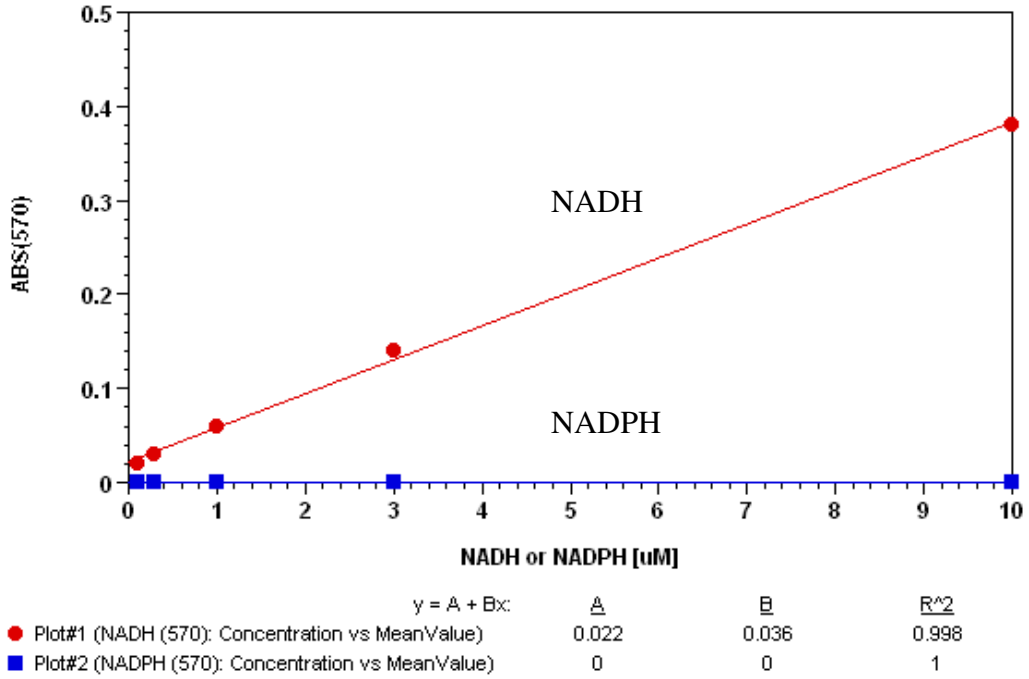


Figure 1. NADH dose response on 96-well white/clear bottom plate was measured with Amplite™ NAD/NADH Assay Kit using a NOVOStar (BMG Labtech) microplate reader. As low as 300 nM (30 pmol/well) of NADH can be detected with 1 hour incubation time (n=3) while there is no response from NADPH.

References:

1. Ziegenhorn J, Senn M, Bucher T. (1976) Molar absorptivities of beta-NADH and beta-NADPH. Clin Chem, 22, 151.
2. Ikegami T, Kameyama E, Yamamoto SY, Minami Y, Yubisui T. (2007) Structure and Properties of the Recombinant NADH-Cytochrome b(5) Reductase of Physarum polycephalum. Biosci Biotechnol Biochem.
3. Kimura N, Fukuwatari T, Sasaki R, Shibata K. (2006) Comparison of metabolic fates of nicotinamide, NAD+ and NADH administered orally and intraperitoneally; characterization of oral NADH. J Nutr Sci Vitaminol (Tokyo), 52, 142.
4. O'Donnell JM, Kudej RK, LaNoue KF, Vatner SF, Lewandowski ED. (2004) Limited transfer of cytosolic NADH into mitochondria at high cardiac workload. Am J Physiol Heart Circ Physiol, 286, H2237.