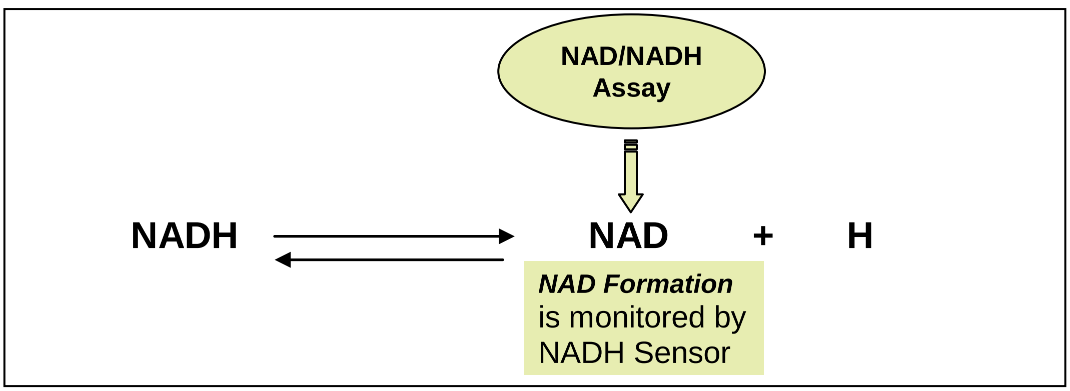
Amplite™ Fluorimetric NAD/NADH Assay Kit
Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 15257 (400 assays)	Keep in freezer Avoid exposure to light	Fluorescence 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. NAD 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 agent. In chloroplasts, NADP is an oxidizing agent 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 run by monitoring the changes in NADH or NADPH absorption at 340 nm. This Amplite™ Fluorimetric NAD/NADH Assay Kit provides a convenient method for sensitive detection of NAD and NADH. The enzymes in the system specifically recognize NAD/NADH in an enzyme cycling reaction that significantly increases detection sensitivity. In addition, this assay has very low background since it is run in the red visible range that considerably reduces the interference resulted from biological samples. The assay has demonstrated high sensitivity and low interference at Ex/Em = 540/590 nm.



The Amplite™ Fluorimetric NAD/NADH Assay Kit provides a sensitive, one-step assay to detect as little as 10 picomoles of NAD(H) in a 100 µL assay volume (100nM; Figure 1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and readily adapted to automation without a separation step. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = 530-570 nm/590-600 nm (maximum Ex/Em = 540/590 nm) or an absorbance microplate reader at ~576 nm.

Kit Key Features

Broad Application: Used for quantifying NAD/NADH in solutions and in cell extracts.
Sensitive: Detect as low as 10 picomoles of NAD/NADH in solution.
Continuous: Easily adapted to automation without a separation step.
Convenient: Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive: No special requirements for waste treatment.

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

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 minutes – 2 hours → Monitor fluorescence intensity at Ex/Em = 540/590 nm

Note: Thaw one of each kit component at room temperature before starting the experiment.

1. Prepare NADH stock solution:

Add 200 µL of of PBS buffer into the vial of NADH standard (Component C) to have 1 mM (1 nmol/µL) NADH stock solution.

Note: The unused NADH stock solution should be divided into single use aliquots and stored at -20 ^{o}C.

2. Prepare NAD/NADH reaction mixture:

Add 10 mL of NAD/NADH sensor buffer (Component B) into the bottle of NAD/NADH Recycling Enzyme Mixture (Component A), and mix well.

Note: The NAD/NADH reaction mixture is enough for two 96-well plates. The unused NAD/NADH reaction mixture should be divided into single use aliquots and stored at -20 ^{o}C.

3. Prepare serial dilutions of NADH standard (0 to 10 µM):

3.1 Add 10 µL of NADH stock solution (from Step 1) into 990 µL PBS buffer (pH 7.4) to generate 10 µM (10 pmol/µL) NADH standard solution.

Note: Diluted NADH standard solution is unstable, and should be used within 4 hours.

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

3.3 Add serial dilutions of NADH standard and NAD/NADH containing test samples into a solid black 96-well microplate as described in Tables 1 and 2.

Note: Prepare cells or tissue samples as desired.

Table 1 Layout of NADH standards and test samples in a solid black 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 fluorescence signal due to the over oxidation of NADH sensor (to a non-fluorescent product).*

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4. Run NAD/NADH assay in supernatants reaction:

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

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

4.3 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540/590 nm.
Note1: The contents of the plate can also be transferred to a white clear bottom plate and read by an absorbance microplate reader at the wavelength of 576 ±5 nm. The absorption detection has lower sensitivity compared to fluorescence reading.
Note2: To detect NADH only, aliquot 200 µL of samples into Eppendorf tubes. Heat samples to 60 °C for 30 minutes in a heating block or a water bath. All NAD will be deactivated while NADH will be still intact under these conditions. Cool samples on ice and quickly spin samples if precipitates occur. Transfer 50 µL of NADH samples into the wells as indicated in Tables 1 and 2.

Data Analysis

The fluorescence 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. A NADH standard curve is shown in Figure 1.
Note: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

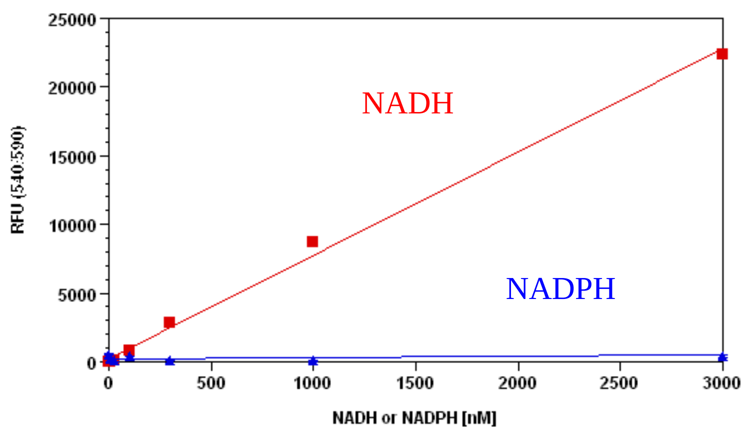

Figure 1. NADH dose response was measured with Amplite™ NAD/NADH Assay Kit in a solid black 96-well plate using a NOVOStar microplate reader (BMG Labtech). As low as 100 nM (10 pmol/well) of NADH can be detected with 1 hour incubation (n=3) while there is no response from NADPH.

References

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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.