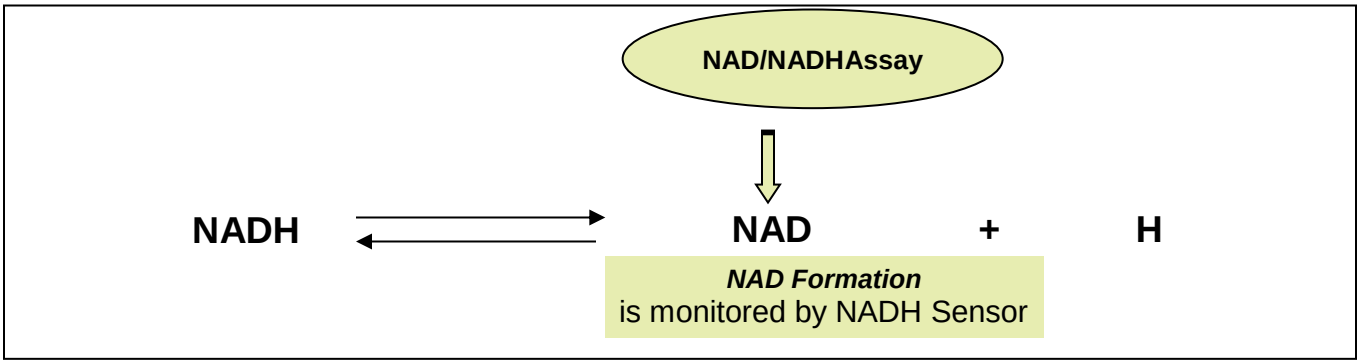
Amplite™ Fluorimetric NAD/NADH Ratio Assay Kit **Red Fluorescence**

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 15263 (250 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 done by monitoring the changes in 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 microplates.



This Amplite™ Fluorimetric NAD/NADH Ratio 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 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 from biological samples. There is no need to purify NAD/NADH from sample mix. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = 530 - 570/590 - 600 nm (maximum Ex/Em = 540/590 nm) or an absorbance microplate reader at ~576 nm. This kit provides NAD and NADH extraction buffer, and cell lysis buffer for your convenience. It has been frequently used for determining NAD/NADH from cell lysates.

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	1 vial (142 µg)
Component D: NADH Extraction Solution	1 bottle (10 mL)
Component E: NAD Extraction Solution	1 bottle (10 mL)
Component F: NAD/NADH Control Solution	1 bottle (10 mL)
Component G: NAD/NADH Lysis Buffer	1 bottle (10 mL)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare 25 µL of NADH standards and/or test samples → Add 25 µL of NADH or NAD Extraction Solution → Incubate at room temperature for 15 minutes → Add 25 µL of NAD or NADH Extraction Solution → Add 75 µL of NAD/NADH reaction mixture → Incubate at RT for 15 minutes to 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.

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Technical Support: support@aatbio.com; Tel: 408-733-1055

1. Prepare NADH stock solution:

Add 200 µL 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°C.

2. Prepare NAD/NADH reaction mixture:

Add 10 mL of NADH Sensor Buffer (Component B) to the bottle of NAD/NADH Recycling Enzyme Mixture (Component A), and mix well.

Note: This 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°C.

3. Prepare serially diluted NADH standards (0 to 10 µM):

3.1 Add 30 µL of 1 mM NADH stock solution (from Step 1) into 970 µL PBS buffer (pH 7.4) to generate 30 µM (30 pmols/µL) NADH standard solution.

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

3.2 Take 200 µL of 30 µM NADH standard solution (from Step 3.1) to perform 1:3 serial dilutions to get 10, 3, 1, 0.3, 0.1, 0.03 and 0 µM serially diltued NADH standards.

3.3 Add serially diluted NADH standards and/or 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	TS (NADH)	TS (NADH)	TS (NAD)	TS (NAD)				
NS1	NS1	….	….	….	….	….	….				
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

*Note: NS= NAD/NADH Standards; BL=Blank Control; TS=Test Samples; TS (NADH) = Test Samples treated with **NADH Extraction Solution** for 10 to 15 minutes, then neutralized by **NAD Extraction Solution**; TS (NAD) = Test Samples treated with NAD Extraction Solution for 10 to 15 minutes, then neutralized by **NADH Extraction Solution.***

Table 2 Reagent composition for each well

NADH Standard	**Blank Control**	**Test Sample (NAD/NADH)**	**Test Sample (NADH Extract)**	**Test Sample (NAD Extract)**
Serial Dilutions*: 25 µL	PBS: 25 µL	Test Sample: 25 µL	Test Sample: 25 µL	Test Sample: 25 µL
Component F: 25 µL	Component F: 25 µL	Component F: 25 µL	Component D: 25 µL	Component E: 25 µL
Incubate at room temperature for 10 to 15 minutes				
Component F: 25 µL	Component F: 25 µL	Component F: 25 µL	Component E: 25 µL	Component D: 25 µL
Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL

**Note: Add the serially diluted NADH standards from 0.03 µM to30 µM into wells from NS1 to NS7 in duplicate. High concentration of NADH (e.g., > 300 µM, final concentration) may cause reduced fluorescence signal due to the over oxidation of NADH sensor (to a non-fluorescent product).*

3.4 **For NADH Extraction (NADH):** Add 25 µL of NADH Extraction Solution (Component D) into the wells of NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, then add 25 µL of NAD Extraction Solution (Component E) to neutralize the NADH extracts as described in Tables 1 & 2.

For NAD Extraction (NAD): Add 25 µL of NAD Extraction Solution (Component E) into the wells of NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, then add 25 µL of NADH Extraction Solution (Component D) to neutralize the NAD extracts as described in Tables 1 & 2.

For Total NAD and NADH: Add 25 µL of NAD/NADH Control Solution (Component F) into the wells of NADH standards and NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, and then add 25 µL of Control Solution (Component F) as described in Tables 1 and 2.
Note1: Prepare cells or tissue samples as desired. NAD/NADH Lysis Buffer (Component G) can be used for lysing the cells (See appendix for details).

Note2: In healthy mammalian cells, there is more NAD compare to NADH, so one can simply use total NAD and NADH minus the NAD to calculate the amount of NADH.

4. Run NAD/NADH assay in supernatants reaction:

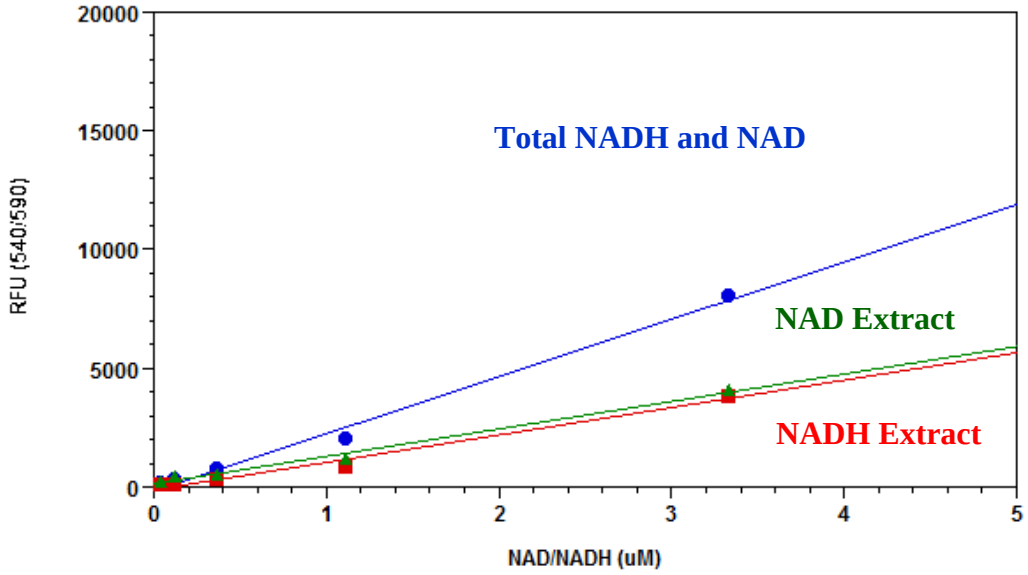
4.1 Add 75 µL of NADH reaction mixture (from Step 2) into each well of NADH standard, blank control, and test samples (from Step 3.4) to make the total NADH assay volume of 150 µL/well.

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 (cutoff 570 nm).
Note: 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.

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. The typical data are shown in Figure 1 (Total NAD and NADH vs. NAD or NADH Extract).



	y = A + Bx:	A	B	R^2
●	Plot#1 (Total NAD/NADH: Concentration vs Values)	-172.086	2411.885	0.993
■	Plot#2 (NADH Extract: Concentration vs Values)	-123.699	1154.729	0.987
▲	Plot#3 (NAD Extract: Concentration vs Values)	130.009	1155.929	0.992

Figure 1. Total NADH and NAD, and their extract dose response were measured with Amplite™ NAD/NADH Ratio Assay Kit in a 96-well black plate using a Gemini microplate reader (Molecular Devices). 25 µL of equal amount of NAD and NADH was treated with or without NADH or NAD extraction solution for 15 minutes, and then neutralized with extraction solutions at room temperature. The signal was acquired at Ex/Em = 540/590 nm (cut off at 570 nm) 30 minutes after adding 75 µL of NADH reaction mixture. The blank signal was subtracted from the values for those wells with the NADH reactions. *(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).*

Appendix: Test Sample Preparations Using Component G (NAD/NADH Lysis Buffer)

1. Plant Cell Samples:

   Homogenize leaves with the lysis buffer at 200 mg/mL, and centrifuge at 2500 rpm for 5-10 minutes, use the supernatant for tests.

2. Bacterial Cell Samples:

   Collect bacterial cells by centrifugation ((10,000 g, 0°C, 15 min). Use about 100 to 10 million cells/mL lysis buffer, keep the treated solution at room temperature for 15 minutes. Centrifuge at 2500 rpm for 5 minutes, and use the supernatant for tests.

3. Mammalian Cell Samples:

   Remove medium from plate wells, use about 100 µL lysis buffer per 1-5 million cells (or 50-100 µL/well in a 96-well cell culture plate), and keep the treated solution at room temperature for 15 minutes. Use the cell lysate directly or centrifuge it at 1500 rpm for 5 minutes, use the supernatant for tests.

4. Tissue Samples:

   Weigh ~20 mg tissue, wash with cold PBS. Homogenize with 400 µl of lysis buffer in a micro-centrifuge tube. Centrifuge at 2500 rpm for 5-10 minutes, use the supernatant for the assay.

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