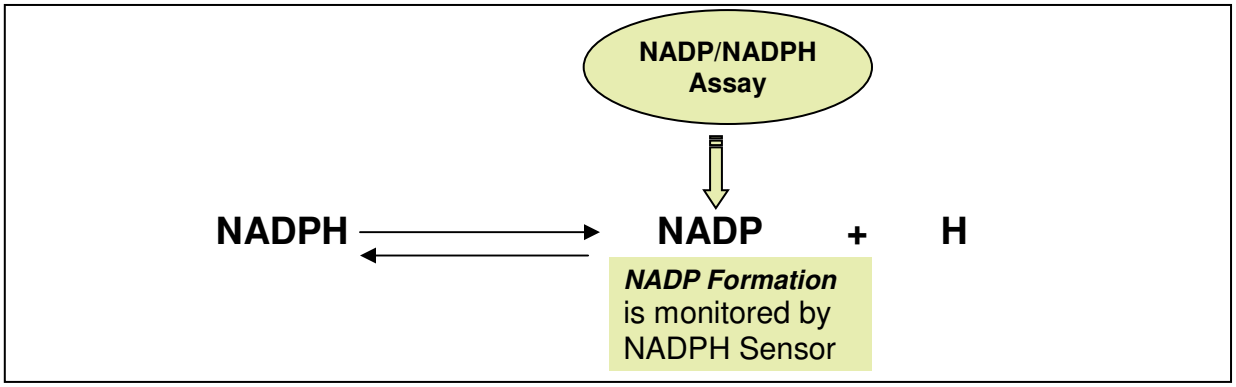
Amplite™ Fluorimetric NADP/NADPH Ratio Assay Kit

Red Fluorescence

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: 15264 (250 assays)	Keep in freezer and avoid 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. 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 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 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™ NADP/NADPH Ratio Assay Kit provides a convenient method for sensitive detection of NADP, NADPH and their ratio. The enzymes in the system specifically recognize NADP/NADPH in an enzyme cycling reaction. There is no need to purify NADP/NADPH from sample mix. The enzyme cycling reaction significantly increases detection sensitivity. In addition, this assay has very low background since it is run in the red visible range that significantly reduces the interference from biological samples. 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 either fluorescence microplate reader with Ex/Em = 530 to 570/590 to 600 nm (maximum Ex/Em = 540/590 nm) or absorbance microplate reader at 576±5 nm. This kit also provides cell lysis buffer for convenience.

Kit Key Features

Broad Application:	Can be used for quantifying NADP/NADPH in solutions, and in cell extracts.
Sensitive:	The kit detects as low as 10 picomoles of NADP/NADPH in solution.
Continuous:	Easily be adapted to automation with no separation required.
Convenient:	Formulated to have minimal hands-on time.
Non-Radioactive:	No special requirements for waste treatment.

Kit Components

Components	Amount
Component A: NADP/NADPH Recycling Enzyme Mixture	2 bottles (lyophilized powder)
Component B: NADPH Sensor Buffer	1 bottle (20 mL)
Component C: NADPH Standard	1 vial (167 µg)
Component D: NADPH Extraction Solution	1 bottle (10 mL)
Component E: NADP Extraction Solution	1 bottle (10 mL)
Component F: NADP/NADPH Control Solution	1 bottle (10 mL)
Component G: NADP/NADPH Lysis Buffer	1 bottle (10 mL)

Assay Protocol (for One 96-Well Plate):

Brief Summary

Prepare NADPH standards or test samples (25 µL) → Add 25 µL of NADPH or NADP Extraction Solution → Incubate at room temperature for 15 min → Add 25 µL of NADP or NADPH Extraction Solution (25 µL) → Add NADP/NADPH reaction mixture (75 µL) → Incubate at RT for 15 min – 2 hr→ Read fluorescence at Ex/Em = 540/590 nm

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

1. Prepare NADPH stock solution:

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

2. Prepare NADP/NADPH reaction mixture:

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

3. Prepare serial NADPH (0 to 10 µM) solutions

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

3.2 Take 200 µL of 10 µM NADPH standard solution (from step 3.1) to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 standard NADPH solutions.

3.3 Add NADPH standards and NADP/NADPH containing test samples into a 96-well solid black microplate as described in Tables 1 and 2.
Note: Prepare your cell or tissue samples as desired. Component G (NADP/NADPH Lysis Buffer) can be used for lysing the cells for convenience.

Table 1. Layout of NADPH standards and test samples in a solid black 96-well microplate:

BL	BL	TS	TS	TS (NADPH)	TS (NADHP)	TS (NADP)	TS (NADP)				
NS1	NS1	….	….	….	….	….	….				
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

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

Table 2. Reagent composition for each well:

NADPH Standard	Blank Control	Test Sample (NADP/NADPH)	Test Sample (NADPH Extract)	Test Sample (NADP 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 min				
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 NADPH standards from 0.01 µM to3 µM into wells from NS1 to NS7 in duplicate. High concentration of NADPH (e.g., >100 µM, final concentration) may cause reduced fluorescence signal due to the over oxidation of NADPH sensor (to a non-fluorescent product).*

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

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

For Total NAPD and NADPH, add 25 µL NADP/NADPH Control Solution (Component F) into the wells of NADPH standards and NADP/NADPH 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.

Note: Prepare your cell or tissue samples as desired. Component G (NADP/NADPH Lysis Buffer) can be used for lysing the cells.

4. Run NADP/NADPH assay in supernatants reaction

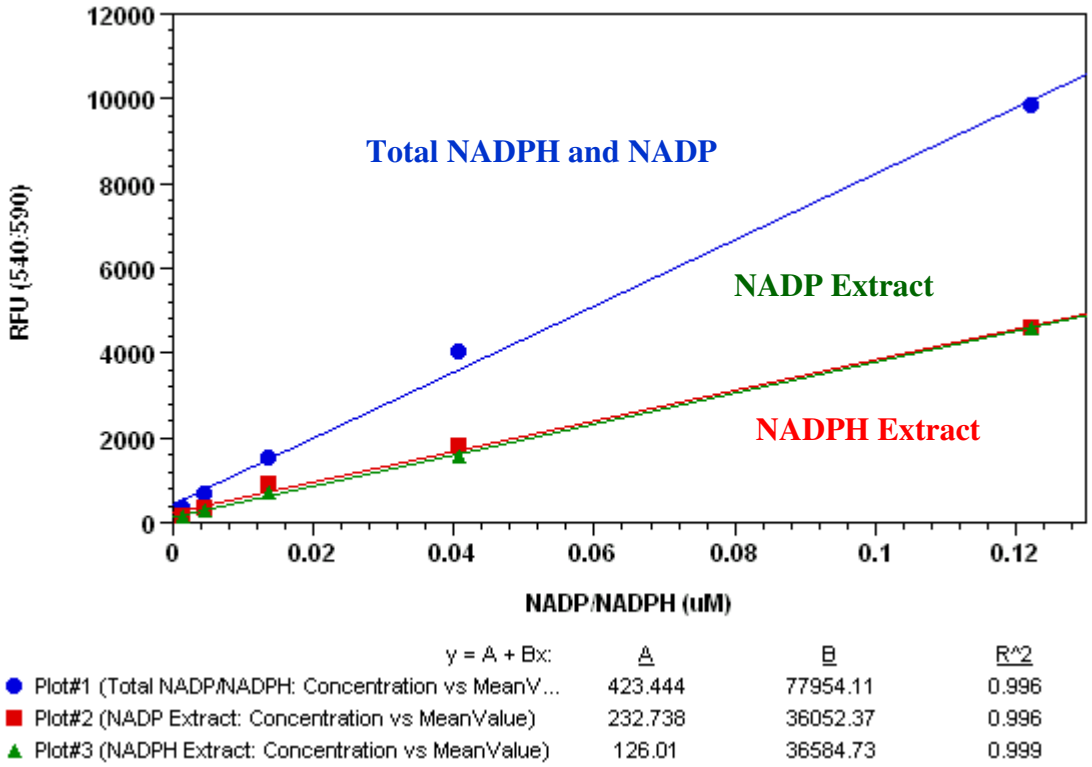
4.1 Add 75 µL of NADPH reaction mixture (from step 2) to each well of the NADPH standard, blank control, and test samples (from step 3.4) so that the total NADPH assay volume is 150 µL/well.

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

4.3 Monitor the fluorescence increase with Ex/Em = 540/590 nm using a fluorescence plate reader.
Note1: The contents of the plate can also be transferred to a white clear bottom plate and read by 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 NADPH reactions. The typical data are shown in Figure 1 (Total NADP and NADPH vs. NADP or NADPH Extract).



	y = A + Bx:	A	B	R^2
●	Plot#1 (Total NADP/NADPH: Concentration vs MeanV...	423.444	77954.11	0.996
■	Plot#2 (NADP Extract: Concentration vs MeanValue)	232.738	36052.37	0.996
▲	Plot#3 (NADPH Extract: Concentration vs MeanValue)	126.01	36584.73	0.999

Figure 1. Total NADPH and NADP, and their extract dose response on 96-well black plate were measured with Amplite™ NADP/NADPH Ratio Assay Kit using a Gemini microplate reader (Molecular Devices). 25 µL of equal amount of NADP and NADPH was treated with or without NADPH or NADP 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 570 nm) after 30 minutes of the addition of 75 µL of NADPH reaction mixture. The blank signal was subtracted from the values for those wells with the NADPH 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).*

References:

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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.

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