Amplite™ Fluorimetric NADP/NADPH Ratio Assay Kit

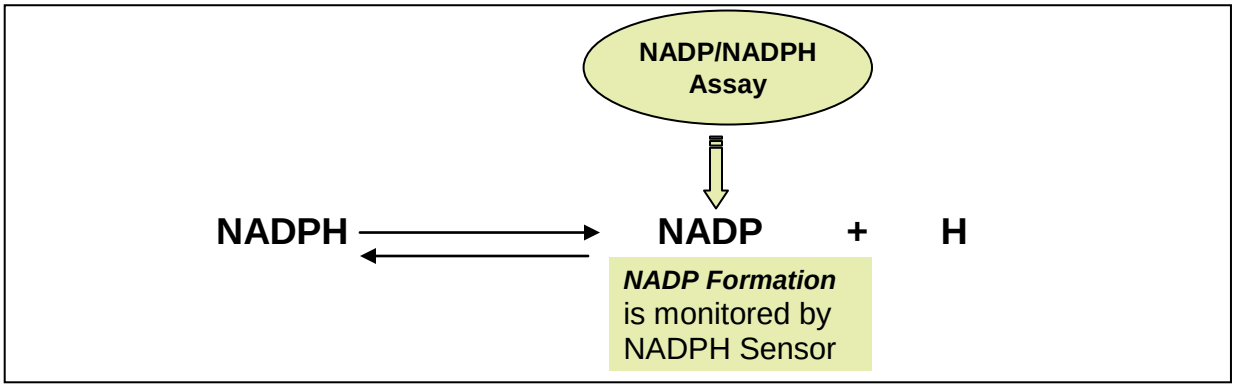
Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 15264 (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. These methods suffer low sensitivity and high interference since the assays are done in the UV range that requires expensive quartz microplates. The low sensitivity of the absorption-based NADP/NADPH assays makes the assays difficult to be automated for high throughput screening that often uses small sample size.



This Amplite™ Fluorimetric 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 recycling 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 sample interference. 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 also provides NADP, NADPH extraction buffer, and cell lysis buffer.

Kit Key Features

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

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

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 RT for 15 minutes → Add 25 µL of NADP or NADPH Extraction Solution (25 µL) → Add NADP/NADPH reaction mixture (75 µL) → Incubate at RT 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 NADPH stock solution:

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

Note: The unused NADPH stock solution should be divided into single use aliquots and stored at -20 °C.

2. Prepare NADP/NADPH reaction mixture:

Add 10 mL of NADPH Sensor Buffer (Component B) into the bottle of NADP/NADPH Recycling Enzyme Mixture (Component A), and mix well.

Note: This NADP/NADPH reaction mixture is enough for two 96-well plates. The unused NADP/NADPH reaction mixture should be divided into single use aliquots and stored at -20 °C.

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

3.1 Add 10 µL of 1 mM NADPH stock solution (from Step 1) to 990 µL PBS buffer (pH 7.4) to generate 10 µM (10 pmols/µL) NADPH standard solution.

Note: Diluted NADPH standard solution is unstable, and 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 µM serial dilutions of NADPH standard.

3.3 Add serial dilutions of NADPH standard and NADP/NADPH 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. NADP/NADPH Lysis Buffer (Component G) 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										

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*Note: NS= NADP/NADPH Standards; BL=Blank Control; TS=Test Samples; TS (NADPH) = Test Samples treated with **NADPH Extraction Solution** for 10 to 15 minutes, then neutralized by **NADP Extraction Solution**; TS (NADP) = Test Samples treated with NADP Extraction Solution for 10 to 15 minutes, 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 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 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 of 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 of 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 of 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 & 2.
Note: Prepare cells or tissue samples as desired. NADP/NADPH Lysis Buffer (Component G) can be used for lysing the cells.

4. Run NADP/NADPH assay in supernatants reaction:

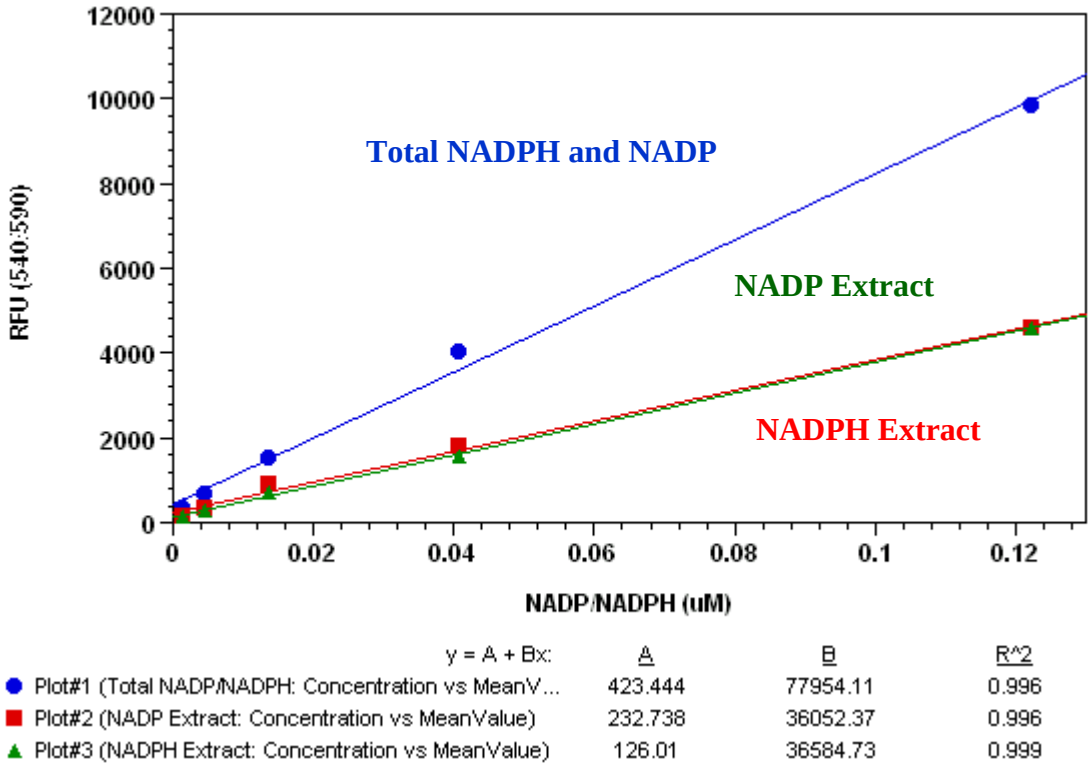
4.1 Add 75 µL of NADPH reaction mixture (from Step 2) into each well of NADPH standard, blank control, and test samples (from Step 3.4) to make the total NADPH assay volume of 150 µL/well.

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.
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 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 were measured with Amplite™ Fluorimetric NADP/NADPH Ratio Assay Kit in a 96-well black plate 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 at 570 nm) 30 minutes after adding 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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