Amplite® Fluorimetric NADP/NADPH Ratio Assay Kit *Red Fluorescence*

Catalog number: 15264
Unit size: 250 Tests

Component	Storage	Amount (Cat No. 15264)
Component A: NADP/NADPH Recycling Enzyme Mix	Freeze (< -15 °C), Minimize light exposure	2 bottles (lyophilized powder)
Component B: NADPH Sensor Buffer	Freeze (< -15 °C), Minimize light exposure	1 bottle (20 mL)
Component C: NADPH Standard	Freeze (< -15 °C), Minimize light exposure	1 vial (167 µg)
Component D: NADPH Extraction Solution	Freeze (< -15 °C), Minimize light exposure	1 bottle (10 mL)
Component E: NADP Extraction Solution	Freeze (< -15 °C), Minimize light exposure	1 bottle (10 mL)
Component F: NADP/NADPH Control Solution	Freeze (< -15 °C), Minimize light exposure	1 bottle (10 mL)
Component G: NADP/NADPH Lysis Buffer	Freeze (< -15 °C), Minimize light exposure	1 bottle (10 mL)

OVERVIEW

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. 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. Our 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 NAD/NADH 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 has demonstrated high sensitivity and low interference. This Amplite® Fluorimetric NADP/NADPH Assay Kit can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation with no separation steps required.

AT A GLANCE

Protocol Summary

1. Prepare 25 µL NADPH standards and/or test samples
2. Add 25 µL of NADPH or NADP Extraction Solution
3. Incubate at 37^{o}C for 15 minutes
4. Add 25 µL of NADP or NADPH Extraction Solution
5. Add 75 µL NADP/NADPH working solution
6. Incubate at 37 °C for 15 minutes – 2 hours
7. Monitor fluorescence intensity at Ex/Em = 540/590 nm (Cutoff = 570 nm)

Important

It is highly recommended to incubate the cells with Lysis Buffer (Component G) at 37^{o}C and use the supernatant for the experiment.

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

KEY PARAMETERS

Absorbance microplate reader

Absorbance	576 ± 5 nm
Recommended plate	Clear bottom

Fluorescence microplate reader

Cutoff	570 nm
Emission	590 nm
Excitation	540 nm
Recommended plate	Solid black

CELL PREPARATION

For guidelines on cell sample preparation, please visit https://www.aatbio.com/resources/guides/cell-sample-preparation.html

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles

NADPH standard solution (1 mM)

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

PREPARATION OF STANDARD SOLUTIONS

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/15264

NADPH standard

Add 10 µL of 1 mM NADPH standard solution to 990 µL PBS buffer (pH 7.4) to generate 10 µM (10 pmols/µL) NADPH standard solution (NS7). Take 10 µM NADPH standard solution and perform 1:3 serial dilutions in PBS to get serial dilutions of NADPH standard (NS6 - NS1). Note: Diluted NADPH standard solution is unstable, and should be used within 4 hours.

PREPARATION OF WORKING SOLUTION

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

Note: This NADP/NADPH working solution is enough for 125 assays in 96-well plate.

SAMPLE EXPERIMENTAL PROTOCOL

Table 1. Layout of NADPH standards and test samples in a solid black 96-well microplate. NS= NADPH Standards (NS1 - NS7, 0.01 to 10 µM); BL=Blank Control; TS=Test Samples; TS (NADPH) = Test Samples treated with NADPH Extraction Solution, then neutralized by NADP Extraction Solution; TS (NADP) = Test Samples treated with NADP Extraction Solution, then neutralized by NADPH Extraction Solution.

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

Table 2. Reagent composition for each well. *Note:* 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).

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 37 °C 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

1. Prepare NADPH standards (NS), blank controls (BL), and test samples (TS) according to the layout provided 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. One can simply use total NADP and NADPH minus the NADP to calculate the amount of NADPH.

   Note: It is highly recommended to incubate the cells at 37 °C and use the supernatant for the experiment.

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 37 °C 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 NADP 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 37 °C for 10 to 15 minutes, and then add 25 µL of Control Solution (Component F) as described in Tables 1 & 2.For NADPH Extraction (NADPH): Add 25 µL of NADPH Extraction Solution (Component D) into the wells of NADP/NADPH containing test samples. Incubate at 37 °C 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.
3. Add 75 µL of NADPH working solution into each well of NADPH standard, blank control, and test samples to make the total NADPH assay volume of 150 µL/well.
4. Incubate the reaction at 37 °C for 15 minutes to 2 hours (We tested 60 minutes in the figure shown), protected from light.

   Note: In some cases, incubation time can be increased to more than 2 hours.
5. 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.

EXAMPLE DATA ANALYSIS AND FIGURES

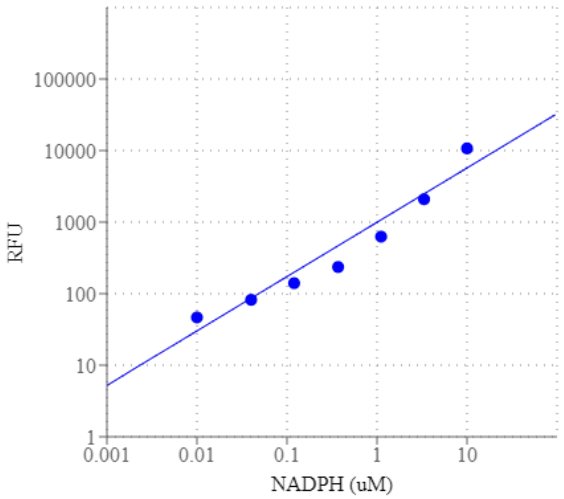

Figure 1. NADPH dose response was measured with Amplite® Fluorimetric NADP/NADPH Ratio Assay Kit in a 96-well solid black plate using a Gemini microplate reader (Molecular Devices).

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