Amplite™ Fluorimetric NADPH Assay Kit

Red Fluorescence

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: #15262 (400 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+, 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 requires 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™ NADPH Assay Kit provides a convenient method for detection of NADPH. The enzymes in the system specifically recognize NADPH in an enzyme recycling reaction. 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 with Ex/Em = 540/590 nm.

NADPH + Amplite™ NADPH Sensor (Non-fluorescent) ⟶ **NADP + Red Fluorescent Product**

The Amplite™ Fluorimetric NADPH Assay Kit provides a sensitive, one-step fluorimetric assay to detect as little as 0.3 nanomoles of NADPH in a 100 µL assay volume (0.03 µM; Figure 1). 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.

Kit Key Features

Broad Application:	Can be used for quantifying NADPH in solutions, and in cell extracts.
Sensitive:	The kit detect as low as 1 nanomoles of NADPH in solution.
Continuous:	Easily adapted to automation with no separation required.
Convenient:	Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive:	No special requirements for waste treatment.

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Kit Components

Components	Amount
Component A: NADPH Recycling Enzyme Mixture	2 bottles (lyophilized powder)
Component B: NADPH Assay Buffer	1 bottle (20 mL)
Component C: NADPH Standard (FW: 833.36)	1 vial (167 µg)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare NADPH reaction mixture (50 µL) → Add NADPH standards or test samples (50 µL) → Incubate at room temperature for 15 min-2hr → 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 Add 200 µL of PBS buffer into the NADPH standard vial (Component C) to make 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 NADPH reaction mixture:

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

3. Prepare serial NADPH (0 to 100 µM) solutions:

3.1 Add 50 µL of NADPH standard stock solution (from step 1) to 450 µL PBS (pH 7.4) buffer to generate 100 µM (100 pmol/µL) standard.
Note: Diluted NADPH standard solution is unstable, should be used within 4 hours
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3.2 Take 200 µL of 100 µM solution to perform 1:3 serial dilutions to get 30, 10, 3, 1, 0.3, 0.1 and 0 standard NADPH solutions.

3.3 Add NADPH standards and 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.

Table 1. Layout of NADPH 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= NADPH Standards, BL=Blank Control, TS=Test Samples.

Table 2. Reagent composition for each well:

NADPH Standard	**Blank Control**	**Test Sample**
Serial dilutions* (50 µL)	PBS: 50 µL	50 µL

**Note: Add the serially diluted NADPH standards from 0.1 µM to 100 µM into wells from NS1 to NS7 in duplicate.*

4. Run NADPH assay in supernatants reaction:

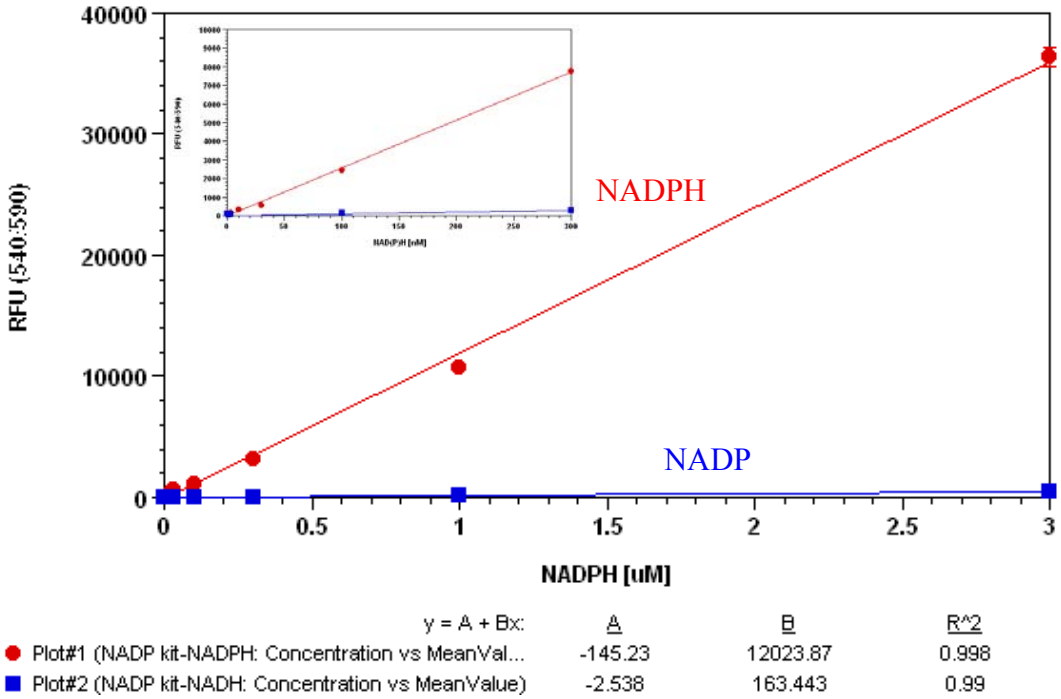
4.1 Add 50 µL of NADPH reaction mixture (from step 2) to each well of the NADPH standard, blank control, and test samples (see step 3.3) so that the total NADPH assay volume is 100 µL/well.
Note: For a 384-well plate, add 25 µL sample, 25 µL of NADPH reaction mixture per well.

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 = 530 to 570/590 to 600 nm ((optimal 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 (NADPH standard curve).
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.



	y = A + Bx:	A	B	R^2
Plot#1 (NADP kit-NADPH: Concentration vs MeanVal...		-145.23	12023.87	0.998
Plot#2 (NADP kit-NADH: Concentration vs MeanValue)		-2.538	163.443	0.99

Figure 1. NADPH dose response on 96-well black plate was measured with Amplite™ NADPH Assay Kit using a NOVOStar microplate reader (BMG Labtech). As low as 0.03μM (0.3 nmol/well) of NADPH can be detected with 1 hour incubation time (n=3) while there is no response from NADP (the insert shows the lower detection limit).

References:

1. Hedeskov CJ, Capito K, Thams P. (1987) Cytosolic ratios of free [NADPH]/[NADP+] and [NADH]/[NAD+] in mouse pancreatic islets, and nutrient-induced insulin secretion. Biochem J, 241, 161.
2. Gaetani GF, Ferraris AM, Sanna P, Kirkman HN. (2005) A novel NADPH:(bound) NADP+ reductase and NADH:(bound) NADP+ transhydrogenase function in bovine liver catalase. Biochem J, 385, 763.
3. Kobayashi K, Miura S, Miki M, Ichikawa Y, Tagawa S. (1995) Interaction of NADPH-adrenodoxin reductase with NADP+ as studied by pulse radiolysis. Biochemistry, 34, 12932.
4. Marino D, Gonzalez EM, Frendo P, Puppo A, Arrese-Igor C. (2006) NADPH recycling systems in oxidative stressed pea nodules: a key role for the NADP(+)-dependent isocitrate dehydrogenase. Planta.