Amplite™ Fluorimetric NADPH Assay Kit

Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 15262 (400 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+, 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 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 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 microplate.

This Amplite™ Fluorimetric NADPH Assay Kit provides a convenient method for the 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.

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

The Amplite™ Fluorimetric NADPH Assay Kit provides a sensitive, one-step assay to detect as little as 100 picomoles of NADPH in a 100 µL assay volume (1 µM; Figure 1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and readily adapted to automation. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = 540/590 nm or an absorbance microplate reader at ~576 nm. This assay kit has been used for screening enzyme activities that use NADP/NADPH as a cofactor. It has also been used for the sensitive detection of NADPH in cell-based assays. Compared to the other commercial kits, this assay has higher signal/background ratio.

Kit Key Features

Broad Application:	Can be used for quantifying NADPH in solutions and in cell extracts.
Sensitive:	Detect as low as 1 µM of NADPH in solution.
Continuous:	Easily adapted to automation without a separation step.
Convenient:	Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive:	No special requirements for waste treatment.

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)

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

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 minutes – 2 hours → Monitor fluorescence increase 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 make 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 NADPH reaction mixture:

Add 10 mL of Amplite™ NADPH Assay Buffer (Component B) into the bottle of NADPH Recycling Enzyme Mixture (Component A), and mix well.

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

3. Prepare serial dilutions of NADPH standard (0 to 100 µM):

3.1 Add 50 µL of NADPH stock solution (from Step 1) into 450 µL PBS buffer (pH 7.4) to generate 100 µM (100 pmol/µL) NADPH standard solution.

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

3.2 Take 200 µL of 100 µM NADPH standard solution to perform 1:3 serial dilutions to get 30, 10, 3, 1, 0.3, 0.1 and 0 µM serial dilutions of NADPH standard.

3.3 Add serial dilutions of NADPH standard and 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.

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 NADPH standard, blank control, and test samples (see Step 3.3) to make the total NADPH assay volume of 100 µL/well.

Note: For a 384-well plate, add 25 µL of sample and 25 µL of NADPH reaction mixture into each well.

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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 = 530 - 570/590 - 600 nm ((optimal Ex/Em = 540/590 nm).
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.
*Note2: For cell based NADPH measurements, ReadiUse™ mammalian cell lysis buffer *5X* (cat#20012) is recommended to use for lysing the cells.*

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. A NADPH standard curve is shown in Figure 1.
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.

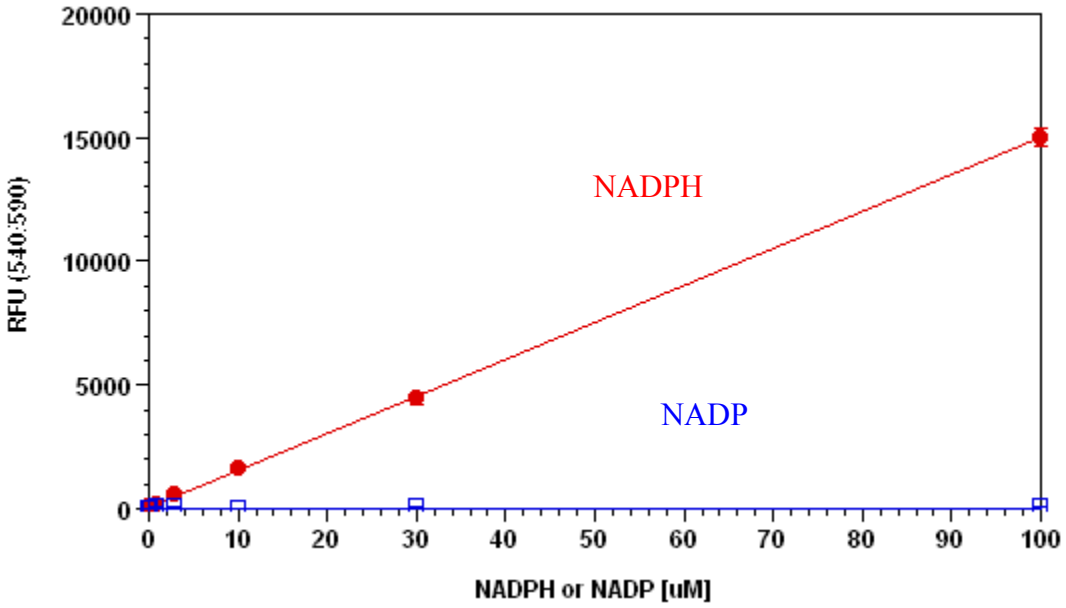

Figure 1. NADPH dose response was measured with Amplite™ Fluorimetric NADPH Assay Kit in a 96-well black plate using a NOVOStar microplate reader (BMG Labtech). As low as 1 μM (100 pmols/well) NADPH can be detected with 1 hour incubation (n=3) while there is no response from NADP.

References

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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.