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ab186031

NADH and NADPH Assay Kit (Colorimetric)

Instructions for Use

An optimized assay for monitoring NADH and NADPH.

This product is for research use only and is not intended for diagnostic use.

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1. 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+. NAD forms NADP with the addition of a phosphate group to the 2' position of the adenyl nucleotide through an ester linkage. The traditional NAD/NADH and NADP/NADPH assays are based on monitoring the changes in NADH or NADPH absorption at 340 nm. The short UV wavelength of NAD/NADH and NADP/NADPH assays makes the traditional methods suffer low sensitivity and high interference.

Abcam's Colorimetric NADH and NADPH Assay Kit (ab186031) provides a convenient method for detecting NADH and NADPH. The NADPH probe is a chromogenic sensor that has its maximum absorbance at 460 nm upon NADPH reduction. The absorption of the NADPH probe is directly proportional to the concentration of NADPH and NADH in the solution. The Colorimetric NADPH Assay Kit provides a sensitive assay to detect as little as 3 μM NADPH and NADH in a 100 μL assay volume. Note that the kit will measure both NADPH and NADH together, and cannot differentiate them.

2. Protocol Summary

Prepare NADPH Reaction Mixture

Add NADPH standards or test samples

Incubate at room temperature for 15-120 minutes

Monitor Absorbance at 460 nm

3. Kit Components

Item	Quantity
Component A: NADH/NADPH Probe	4 mL
Component B: Assay Buffer	16 mL
Component C: NADPH Standard (Lyophilized)	167 µg
Component D: Lysis Buffer	10 mL

4. Storage and Stability

Upon arrival, store the kit at -20°C and protect from light. Avoid repeated freeze/thaw cycles.

Warm all buffers to room temperature before use. Briefly centrifuge all small vials prior to opening.

5. Materials Required, Not Supplied

- 96 or 384-well plate with clear flat bottoms
- Microplate reader.
- PBS
- Centrifuge

6. Sample Preparation

Cell samples:

1. Remove medium from plate wells and add about 100 μL lysis buffer per 1-5×10^6 cells (or 50-100 μL/well in a 96-well cell culture plate).
2. Incubate the treated solution at room temperature for 15 minutes.
3. Use the cell lysate directly or centrifuge at 1500 rpm for 5 minutes and then use the supernatant for tests.

Tissue samples:

1. Weigh ~20 mg tissue and wash with cold PBS.
2. Homogenize with 400 μL of lysis buffer in a micro-centrifuge tube.
3. Centrifuge at 2500 rpm for 5-10 minutes.
4. Use the supernatant for the assay.

Cell culture supernatant samples:

- Use directly for the assay.

7. Assay Protocol

Please read the entire protocol before performing the assay.

1. **Prepare NADPH stock solution:**
   - Add 200 µL of PBS buffer into the vial of NADPH standard to make a 1 mM (1nmol/ µL) NADPH stock solution.
     Note: The unused NADPH stock solution should be divided into single use aliquots and stored at -20°C, and is stable for 1-2 months.
2. **Prepare NADPH reaction mixture:**
   - Add 1 ml of NADH/NADPH Probe into 4 mL NADPH Assay Buffer and mix well. Only make enough reaction mix to use in the assay, as it is not stable and cannot be stored.

   Note: 5 mL NADPH reaction mixture is for one 96-well. The working solution is not stable, use it promptly and avoid direct exposure to light.

3. **Prepare serial dilutions of NADPH standard (0-100 µM)**

   3.1 Add 100 µL of NADPH stock solution (from Step 1) into 400 µL PBS buffer (pH 7.4) to generate 200 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 200 µM NADPH standard solution to perform 1:2 serial dilutions to get 100, 50, 25, 12.5, 6.25, 3.13 and 0 µM serial dilutions of NADPH standard.

3.3 Add serial dilutions of NADPH standard and NADPH containing test samples into a white/clear bottom 96-well microplate as described in Tables 1 and 2.

Note: Prepare cells or add supernatant as desired.

BL	BL	TS	TS	….	….						
NS1	NS1	….	….	….	….						
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

Table 1. Layout of NADPH standards and test samples in a white/clear bottom 96-well microplate.

Note: NS= NADPH *Standards; BL=Blank Control; TS=Test Samples*

NADPH Standards	**Blank Control**	**Test Sample**
Serial Dilutions*: 50 µl	PBS: 50 µl	50 µl

Table 2. Reagent composition for each well.

**Note: Add the serial dilutions of NADPH standard from 3.13-200* µM *into wells from NS1 to NS7 in duplicate.*

4. Run NADPH assay in supernatants reaction:

4.1 Add 50 µL of NADH/NADPH reaction mixture (from Step 2) into 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

Note1: For a 384-well plate, add 25 µL of sample and 25 µL of NADH/NADPH reaction mixture into each well.

Note2: Prepare cells or tissue samples as desired. Lysis Buffer (Component D) can be used for lysing the cells for convenience.

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

4.3 Monitor the absorbance increase with an absorbance plate reader at 460 nm.

8. Data Analysis

The absorbance 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

Figure 1: NADPH dose response was measured with the Colorimetric NADPH Assay Kit in a 96-well white/clear bottom plate using a microplate reader. As low as 3 μM of NADPH can be detected with 30 minutes of incubation with absorbance measurement at 460nm.

Figure 2: NADH dose response curve.

9. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Problem	Reason	Solution
Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)***
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Refer to datasheet for recommended incubation time and/ or temperature

	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)
Problem	**Reason**	**Solution**
Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

Technical Support

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