

ab65349– NADP/NADPH Assay kit (Colorimetric)

For rapid, sensitive and accurate measurement of NADP/NADPH levels in various samples.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: <http://www.abcam.com/ab65349> (use <http://www.abcam.cn/ab65349> for China, or <http://www.abcam.co.jp/ab65349> for Japan)

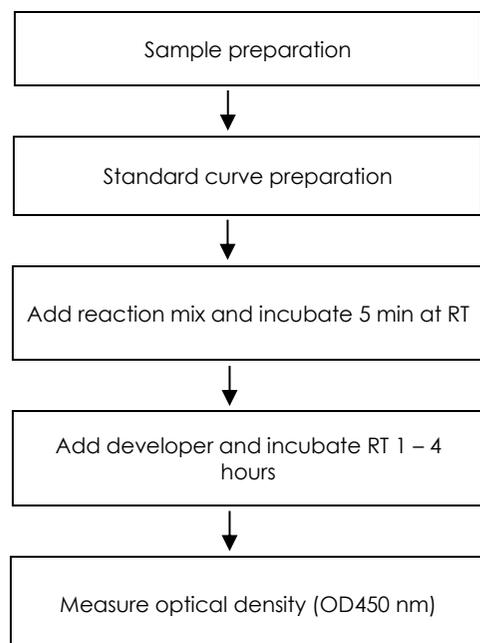
Background:

NADP/NADPH Assay Kit (Colorimetric) ab65349 provides a convenient tool for sensitive detection of the intracellular nucleotides NADP⁺, NADPH and their ratio, in samples such as tissue extracts and cell lysates.

The NADP cycling enzyme mix in the kit specifically acts on NADPH/NADP⁺, but not NAD nor NADH, in a cycling reaction which significantly increases sensitivity and specificity. There is no requirement to purify NADP⁺/NADPH from samples. The levels of both NADP^t (total NADP⁺ and NADPH) and NADPH can be easily measured; the level of NADP⁺ can be calculated by subtracting NADPH from NADP^t. The assay is read by absorbance at 450 nm.

Assay Summary:

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.



QUICK ASSAY PROCEDURE

- Solubilize NADPH Standard, NADP Cycling Enzyme Mix and Developer Solution II. Thaw Stop Solution II, Extraction Buffer II and NADP Cycling Buffer I (aliquot if necessary); get equipment ready.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Prepare standard curve.
- Set up plate for standard (50 µL) and samples (50 µL).
- Prepare Reaction Mix and add 100 µL to each well.
- Incubate plate at RT for 5 mins.
- Add 10 µL of Developer Solution II each well.
- Measure plate at OD 450 nm in kinetics mode during 1 – 4 hours.
- OPTIONAL: Stop the reaction by adding 10 µL of Stop Solution II into each well.

Precautions & Limitations:

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit.

- Modifications to the kit components or procedures may result in loss of performance.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

Storage and Stability:

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Reconstituted components are stable for 2 months. Do not use kit or components if they have exceeded the expiry date.

Materials Supplied:

Item	Quantity	Storage Temperature (on receipt)	Storage temperature (reconstituted)
Extraction Buffer II	50 mL	-20°C	-20°C
NADP Cycling Buffer	15 mL	-20°C	-20°C
NADP Cycling Enzyme Mix	200 µL	-20°C	-20°C
Developer Solution II	1 vial	-20°C	-20°C
Stop Solution II	1.2 mL	-20°C	-20°C
NADPH Standard	1 vial	-20°C	-20°C

Materials Required, Not Supplied:

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance (OD) at 450 nm (colorimetric)
- 96 well clear plate with clear flat bottom (colorimetric assay)
- Orbital shaker
- DMSO
- 10 kDa Spin Column (ab93349): for deproteinization step
- Microcentrifuge
- Dounce homogenizer (if using cells or tissue)
- 1 x PBS pH 7.4
- MilliQ water or other type of double distilled/deionized water (ddH₂O)
- (Optional) 0.5 M Tris HCl, pH 8.0 to neutralize acidic samples

Reagent Preparation:

- Briefly centrifuge small vials at low speed prior to opening.
- Equilibrate reagents to room temperature before use.
- Aliquot reagents so that you have enough volume to perform the desired number of assays.

NADPH Standard: Reconstitute NADPH Standard with 200 μL of pure DMSO to generate a 1 nmol/ μL (1 mM) NADPH Standard solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store aliquots at -20°C . Use within two months.

Developer Solution II: Reconstitute Developer Solution II in 1.2ml of ddH₂O. Pipette up and down several times to completely dissolve the pellet in solution. Aliquot so that you have enough volume to perform the desired number of experiments. Store at -20°C . Use within two months.

NADP Cycling Enzyme Mix: Ready to use as supplied. Keep on ice protected from light during the assay. Aliquot so that you have enough volume to perform the desired number of assays. Store aliquots at -20°C .

NADPH Cycling Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C .

Extraction Buffer II: Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C .

Stop Solution II: Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C .

Sample Preparation:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples at -20°C . Alternatively, snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C . When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Interferences:

- $> 5\%$ non-ionic detergents (Triton-X 100, NP-40, etc)
- Ascorbic acid
- NADPH quantification can be compromised after exposure to very acidic pH and therefore, we do not recommend TCA or PCA precipitation for this assay

Cells (adherent or suspension) samples:

1. Harvest the number of cells necessary for each assay (initial recommendation = 2×10^5 cells/well). Any remaining trypsin can inhibit the assay.
2. Wash cells in cold PBS.
3. Pellet cells in a tube by spinning at 2,000 rpm) for 5 minutes, and discard supernatant.
4. Lyse cells with 800 μL of Extraction Buffer II by performing two freeze/thaw cycle (20 min on dry ice followed by 10 min at RT) or a homogenization with a Dounce homogenizer sitting on ice.
5. Vortex the extraction for 10 seconds.

6. Centrifuge for 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
7. Collect supernatant (containing extracted NADP/NADPH) and transfer into a new tube. Keep on ice.
8. It is recommended that the DNA is thoroughly sheared before carrying out the assay procedure using an appropriate method i.e. syringing.
9. Cells may contain enzymes that consume NADH rapidly. Remove enzymes by filtering the samples through a 10 kDa Spin Column (ab93349) before performing the assay.
 - Add sample to the spin column, centrifuge at $10,000 \times g$ for 10 minutes at 4°C .
 - Collect the filtrate. We recommend testing the cell samples neat or at 1/5 dilution.

Tissue Samples:

1. Harvest the amount of tissue necessary for each assay (initial recommendation = 50 mg).
2. Wash tissue in cold PBS.
3. Homogenize in 500 μL Extraction Buffer II using a Dounce homogenizer (30 – 50 passages).
4. Centrifuge 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.
5. Collect supernatant (containing extracted NADP/NADPH) into a new tub and keep on ice.
6. It is recommended that the DNA is thoroughly sheared before carrying out the assay procedure using an appropriate method i.e. syringing.
7. Tissues may contain enzymes that consume NADH rapidly. Remove enzymes by filtering the samples through a 10 kDa Spin Column (ab93349) before performing the assay.
 - Add sample to the spin column, centrifuge at $10,000 \times g$ for 10 minutes at 4°C .
 - Collect the filtrate. We recommend testing the tissue samples neat or at 1/5 dilution.

Standard Preparation:

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.
- Each dilution has enough standard to set up duplicate readings (2 x 50 μ L).

Prepare 10 μ M dilution (10 pmoles/ μ L) NADPH Standard by adding 5 μ L of NADPH Standard to 495 μ L Extraction Buffer II. Gently pipette up and down a few times to ensure all standard is removed from tip. Mix well by inversion. Using 10 pmoles/ μ L NADH standard, add 0, 2, 4, 6, 8, 10 μ L NADPH Standard into a series of wells, generating 0, 20, 40, 60, 80, 100 pmol/well of NADPH Standard. Adjust the volume to 50 μ L/well with Extraction Buffer II. Or prepare triplicate standard curve dilutions as described below.

Using 10 μ M (10 pmoles/ μ L) NADPH standard, prepare standard curve dilution as described in the table below in a microplate or microcentrifuge tubes:

Standard #	Volume of 10 μ M Standard (μ L)	Extraction Buffer II (μ L)	Final volume standard in well (μ L)	End NADPH Amount (pmole/well)
1	0	125	50	0
2	5	120	50	20
3	10	115	50	40
4	15	110	50	60
5	20	105	50	80
6	25	100	50	100

Assay Procedure:

- Keep enzymes and heat labile components and samples on ice during the assay.
- Equilibrate all other materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls, and samples in duplicate.
- To only measure Total NADP^t (total NADP & NADPH): leave your sample as it is.
- To measure NADPH only, NADP⁺ needs to be decomposed before the reaction.

Decomposition Step for NADPH detection in samples:

1. Aliquot 200 μ L of extracted samples into microcentrifuge tubes.
2. Heat samples to 60°C for 30 minutes in a water bath or heating block. Under these conditions, all NADP⁺ will be decomposed while the NADPH will still be intact.
3. Cool samples on ice.
4. If precipitation occurs, centrifuge for 5 minutes at 4°C at top speed in a microcentrifuge to remove any insoluble material.
5. Label samples as NADP⁺ decomposed (NADPH Only) samples.

Set up Reaction wells:

- Standard wells = 50 μ L standard dilutions.
- Sample Background wells = 1 – 50 μ L samples (adjust volume to 50 μ L/well with Extraction Buffer II).
- NADP^t Sample wells = 1 – 50 μ L samples (adjust volume to 50 μ L/well with Extraction Buffer II).

- NADPH Sample wells = 1 – 50 μ L decomposed samples (adjust volume to 50 μ L/well with Extraction Buffer II)
6. Each well (standards, samples, and controls) requires 100 μ L of NADP Reaction Mix To ensure consistency, use the table below to prepare a Master Mix of the appropriate Reaction Mix for your assay using the following calculation:

$$X \mu\text{L component} \times (\text{Number reactions} + 1)$$

Component	Colorimetric Assay Reaction Mix (μ L)	Background Reaction Mix (μ L)
NADP Cycling Buffer	98	100
NADP Cycling Enzyme Mix	2	0

7. Mix Master Reaction Mix by inversion. Add 100 μ L of the Master Reaction Mix to each well. Use a clean tip for each well.
 8. Add 100 μ L of Background Reaction Mix to sample background control sample wells.
 9. Incubate plate at room temperature for 5 minutes to convert NADP to NADPH.
 10. Add 10 μ L of Developer Solution II into each well and mix. Let the reaction cycle at room temperature for 1 – 4 hours or longer depending on the reading.
 11. Measure plate at OD 450 nm multiple times or in kinetic mode during the 1 – 4 hours. The plate can be read multiple times while the color is still developing. Stop Solution eloping. Longer incubation times maybe needed depending on the OD reading.
- OPTIONAL: The reaction can be stopped by adding 10 μ L of Stop Solution II into each well and mixing thoroughly. The color should be stable for 48 hours in a sealed plate after addition of the Stop Solution II.

Calculations:

- For samples producing signals greater than that of the highest standard: dilute further in appropriate buffer and reanalyze. Multiply the concentration found by the appropriate dilution factor.
- 1. Average the duplicate reading for each standard, sample and sample background if required.
- 2. If the absorbance of the sample background control is significant, then subtract the sample background control from the sample reading.
- 3. Subtract the mean absorbance value of the blank (Standard #1) from all standard readings. This is the corrected absorbance.
- 4. Plot the corrected absorbance values for each standard as a function of the final amount of NADPH, and determine the slope of the standard curve using a linear regression based on the corrected standard curve data points.
- 5. Interpolate the amount of NADPt or NADPH in the sample wells based upon the linear standard curve equation, using the sample corrected readings for NADPt and NADPH sample wells.
- 6. Concentration of NADPt or NADPH in the test samples is calculated as:

$$NADPt\ concentration = \frac{NADPt}{V} \times D = pmol/\mu L = \mu M$$

$$NADPH\ concentration = \frac{NADPH}{V} \times D = pmol/\mu L = \mu M$$

Where:

NADPt = amount of NADPt in the sample well, calculated from standard curve (in pmoles).

NADPH = amount of NADPH in the sample well, calculated from standard curve (in pmoles).

V = sample volume added to the reaction well (in μL).

D = sample dilution factor (before addition to the well).

Alternatively, NADPt or NADPH values can be expressed in ng/mg protein, if a protein quantification assay has been previously performed (NADPH MW = 745.4 g/mol).

- 7. NADP/NADPH Ratio is calculated as:

$$NADP/NADPH\ ratio = \frac{NADPt - NADPH}{NADPH}$$

FAQ:

Q: I want to deproteinize my cellular and tissue samples. Can I use PCA precipitation?

A: NADPH quantification could be compromised after exposure to very acidic pH from the perchloric acid. Therefore, we strongly recommend using the 10 kD Spin columns (ab93349) for sample deproteinization.

Q: My neat samples are showing the lowest concentration of NADP but the concentration increases with each dilution. The 1:100 dilution shows a higher reading than the sample diluted 1:10 in reaction buffer. Why is that happening?

A: In this case it is possible that the sample has an inhibitory substance. Therefore, increasing the sample concentration is correspondingly resulting in lower readings.

Q: I have decomposed NADP by heating up sample at 60°C for 30 min as described in the protocol, but the results show that the "NADPH only value" is higher than "total NADP (NADP+ NADPH)". Total NADPH should be higher than NADPH only, so what is wrong with my result?

A: It is possible that in the heating process to decompose NADP there must have been some evaporation that made the resulting solution more concentrated, which might lead to this problem. Please do the heating step in a sealed container.

Q: How can I normalize the number of cells? Should I first estimate the protein concentration and then do the assay or first do assay then do normalization?

A: For the NAD/NADH (ab65348) and the NADP/NADPH quantification kit (ab65349), you need to take a specific number of cells for the assay and do the protein estimation and the assay in parallel or independently of each other. Use the same number of cells as used for the assay in order to do the protein analysis.

Q: I am planning on grounding my sample at -80°C to powder. Do I still need to homogenize the sample after adding Extraction Buffer II?

A: In such case, resuspend the powder in the assay buffer, extract out the NADH/NADPH and quickly deproteinate the samples. Then continue with the recommended protocol.

Q: Is BCA suitable for protein quantification for this assay?

A: Yes, BCA estimation can be used.

Technical Hints

For additional helpful hints and tips on using our assay kits please visit: <https://www.abcam.com/en-us/support/product-support>

Technical Support

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