

ab65349

NADP/NADPH Assay kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of NADP/NADPH in various samples.

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

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

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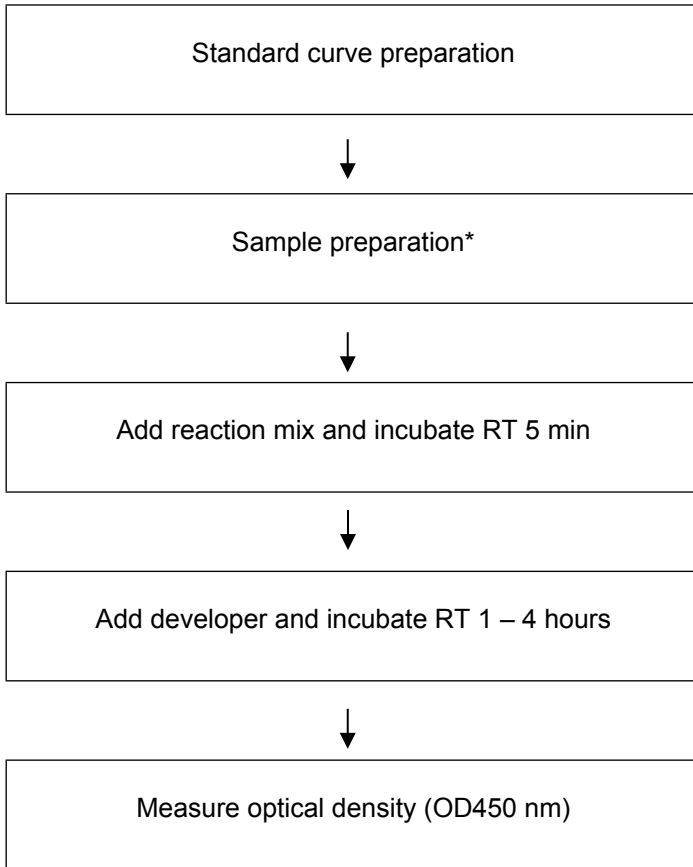
1. BACKGROUND

NADP/NADPH Assay Kit (Colorimetric) (ab65349) provides a convenient tool for sensitive detection of the intracellular nucleotides: NADP, NADPH and their ratio. The enzymes in the system specifically recognize NADP/NADPH in an enzyme cycling reaction. There is no need to purify NADP/NADPH from samples.

The reaction specifically detects NADP and NADPH but it does not recognize NAD⁺/NADH. The enzyme cycling reaction significantly increases detection sensitivity. Results can be quantified using plate reader at OD450 nm.

Nicotinamide Assays of nicotinamide nucleotides are of continual interest in the studies of energy transforming and redox state of cells or tissue.

2. ASSAY SUMMARY



* NADPH detection only: samples need to be decomposed

3. PRECAUTIONS

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.

4. 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.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition
Extraction Buffer II/NADP/NADPH Extraction Buffer	50 mL	-20°C
NADP Cycling Buffer	15 mL	-20°C
NADP Cycling Enzyme Mix	0.2 mL	-20°C
Developer Solution II/NADPH Developer	1 vial	-20°C
Stop Solution II/Stop Solution	1.2 mL	-20°C
NADPH Standard/NADPH Standard (MW:833.36)	166.7 µg	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH₂O)
- DMSO
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD450 nm.
- 96 well plate: clear plates for colorimetric assay
- Orbital shaker
- Heat block or water bath
- Vortex
- Dry ice
- Dounce homogenizer or pestle (if using tissue)

For deproteinization step, additional reagents are required:

- 10kD Spin Columns (ab93349): NADPH quantitation could be compromised after exposure to very acidic pH; therefore, we do not recommend PCA precipitation for this assay.

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- 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.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes and heat labile components and samples on ice during the assay.
- Make sure all buffers and developing solutions are at room temperature before starting the experiment.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.
- Make sure you have the appropriate type of plate for the detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **NADPH Standard:**

Reconstitute NADPH Standard with 200 μ L of pure DMSO to generate a 1 nmol/ μ L (1mM) 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.

9.2 **Developer Solution II/NADPH Developer:**

Reconstitute Developer Solution II/NADPH Developer 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.

9.3 **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.

9.4 **NADPH Cycling Buffer:**

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

9.5 **Extraction Buffer II/NADP/NADPH Extraction Buffer:**

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

9.6 **Stop Solution II/Stop Solution:**

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

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 Prepare a 10 μM NADPH standard by diluting 5 μL NADPH Standard in 495 μL of Extraction Buffer.

10.2 Using 10 μM NADPH standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of NADPH 10 μM Standard (μL)	Extraction Buffer (μL)	Final volume standard in well (μL)	End [NADPH] in well
1	0	150	50	0 pmol/well
2	6	144	50	20 pmol/well
3	12	138	50	40 pmol/well
4	18	132	50	60 pmol/well
5	24	126	50	80 pmol/well
6	30	120	50	100 pmol/well

Each dilution has enough amount of standard to set up duplicate reading (2 x 50 μL)

11. SAMPLE PREPARATION

General Sample information:

- 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 as well as the Deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples 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.

11.1 **Cell (adherent or suspension) Samples:**

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 4×10^6 cells/assay).

11.1.2 Wash cells with cold PBS. Pellet cells in a tube by spinning at low speed for 5 minutes, and discard supernatant.

11.1.3 Extract cells with 800 μL of Extraction Buffer II/NADP/NADPH Extraction Buffer by performing two freeze/thaw cycle (20 min on dry ice followed by 10 min at RT) or a homogenization with a Douncer.

11.1.4 Vortex the extraction for 10 sec.

11.1.5 Centrifuge the sample at 14000 rpm for 5 min.

11.1.6 Transfer the extracted NADP/NADPH supernatant into a labeled tube and keep on ice.

11.1.7 It is recommended that the DNA is thoroughly sheared before carrying out the assay procedure using an appropriate method i.e. syringing.

Cells may contain enzymes that consume NADPH rapidly. The enzymes can be removed by filtering the samples

through a 10 kD Spin Column (ab93349) before performing the assay.

11.2 Tissue Samples:

- 11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 50 mg tissue).
- 11.2.2 Wash with cold PBS. Homogenize the sample using a Dounce homogenizer (30 – 50 passages) with 500 μ L of Extraction Buffer II/NADP/NADPH Extraction Buffer.
- 11.2.3 Transfer samples to a tube and spin the sample at the highest speed for 5 minutes. Transfer the extracted NADP/NADPH supernatant into a new tube and keep on ice.
- 11.2.4 It is recommended that the DNA is thoroughly sheared before carrying out the assay procedure using an appropriate method i.e. syringing.

Tissues may contain enzymes that consume NADPH rapidly. The enzymes can be removed by filtering the samples through a 10 kD Spin Column (ab93349) before performing the assay.

NOTE: We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 Decomposition Step for NADPH detection in samples

Total NADPt (total NADP & NADPH): leave sample as it is.

NADPH: NADP needs to be decomposed before the reaction.

- Aliquot 200 μL of extracted samples into microcentrifuge tubes.
- Heat samples to 60°C for 30 min in a water bath or heating block. Under these conditions, all NADP will be decomposed while the NADPH will still be intact.
- Cool samples on ice. Quickly spin the samples to remove precipitate if precipitation occurs.
- Label samples as **NADP decomposed** samples.

12.2 Set up Reaction wells:

- Standard wells = 50 μL standard dilutions.
- NADPt Sample wells = 1 – 50 μL samples (adjust volume to 50 μL /well with Extraction Buffer).
- NADPH Sample wells = 1 – 50 μL decomposed samples (adjust volume to 50 μL /well with Extraction Buffer)

12.3 NADP Cycling Mix:

Prepare 100 μL Reaction Mix for each reaction:

Component	Reaction Mix (μL)	Background Reaction Mix (μL)
NADP Cycling Buffer	98	100
NADP Cycling Enzyme Mix	2	0

Mix enough reagents for the number of assays (samples, standards) to be performed. Prepare a Master Mix of the

Reaction Mix to ensure consistency. We recommend the following calculation:

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

- 12.4 Add 100 μL of Reaction Mix to each standard and sample well.
- 12.5 Incubate plate at room temperature for 5 minutes to convert NADP to NADPH.
- 12.6 Add 10 μL of Developer Solution II/NADPH Developer into each well and mix. Let the reaction cycle at room temperature for 1 – 4 hours or longer depending on the reading.
- 12.7 Take multiple readings during the 1 – 4 hours at OD450 nm. The plate can be read multiple times while the color is still developing. Longer incubation times maybe needed depending on the OD reading.
- 12.8 **OPTIONAL:** The reaction can be stopped by adding 10 μL of Stop Solution II/Stop Solution 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/Stop Solution.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of NADPt or NADPH (pmol/well).

13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5 Extrapolate sample readings from the standard curve plotted using the following equation:

$$[NADPt] = \left(\frac{\text{Corrected absorbance} - (y \text{ intercept})}{\text{Slope}} \right)$$

$$[NADPH] = \left(\frac{\text{Corrected absorbance} - (y \text{ intercept})}{\text{Slope}} \right)$$

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).

13.6 NADP/NADPH Ratio is calculated as:

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

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

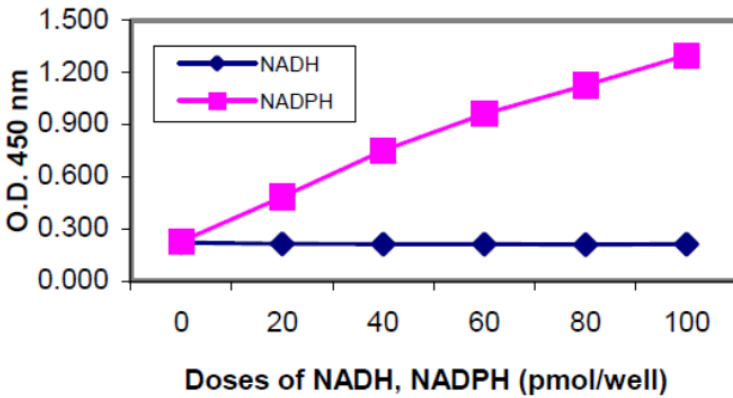


Figure 1. NADPH Standard Curve.

15. QUICK ASSAY PROCEDURE

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

- Prepare NADPH standard, Developer Solution II/Developer and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- To detect NADPH: decompose samples by heating 200 μ L extracted samples at 60°C for 30 min and quickly cooling samples on ice.
- Set up plate for standard (50 μ L), total NADPt samples (50 μ L), and NADPH samples (if detecting - 50 μ L).
- Prepare Reaction Mix and Background Reaction Mix (Number samples + Standards + 1)

Component	Reaction Mix (μ L)	Background Reaction Mix (μ L)
NADPH Cycling Buffer	98	100
NADPH Cycling Enz	2	0

- Add 100 μ L Reaction / Background Reaction Mix to relevant wells.
- Incubate plate at RT 5 min.
- Add 10 μ L Developer Solution II/NADPH Developer into each well.
- Incubate plate at RT 1 – 4 hours. Measure plate at OD450 nm.
- (Optional) Add 10 μ L Stop Solution II/Stop Solution into each well.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

RESOURCES

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQ

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

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.

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?

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

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?

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.

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?

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.

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/NADP/NADPH Extraction Buffer?

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.

Is BCA suitable for protein quantification for this assay?

Yes, BCA estimation can be used.

Protocol step 12.6 (Incubate the plate at room temperature for 5 min to convert NADP to NADPH) – In this step, is all the NADP converted to NADPH?

Yes, if there is any NADP in the samples, it will get converted to NADPH in the step 12.6.

Protocol step 12.7 (Add 10 ml Developer Solution II/NADPH developer into each well and mix. Let the reaction develop for 1 – 4 hours.) – I have measured the sample absorbance after 24 hour, and it is higher than 24 hours before. Is this normal? In this step, what kind of reaction has occurred?

In step 12.7, the NADPH reacts with the probe in the developer to produce a color. Did you perform the incubation for 24 hours before you added the stop solution II/stop solution? If you have incubated your samples for 24 hours, then there will be color development as the NADPH will still react with the probe. However, it is very unusual to observe color development if the stop solution II/stop solution has been added after 1 – 4 hours. The stop solution II/stop solution should be added once the absorbance falls within the linear range of the standard curve. Readings can be taken within 48 hours of adding the stop solution II/stop solution, since the color is stable for that long.

If we choose to lyse cells by homogenizing instead of freeze-thaw, what kind of device or method is the most suitable?

Please use a Dounce homogenizer. About 30-50 passages should be good for the homogenization. You can perform a microscopic examination to ascertain the homogenization. If required, please do 10-20 more passages.

18. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- > 5 % detergents (Triton-X 100, NP-40, etc)
- Ascorbic acid

19. NOTES

Technical Support

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