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ab176724 NADP/NADPH Assay Kit (Fluorometric)

For the rapid, sensitive and accurate measurement of NADP/NADPH in cell extracts.

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

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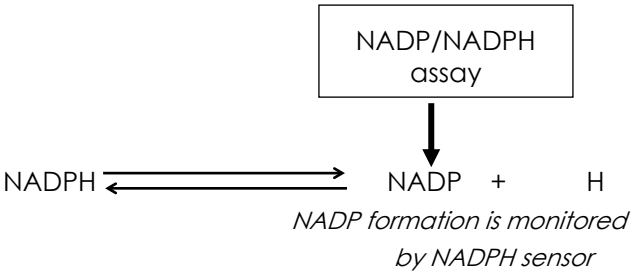
1. Overview

NADP/NADPH Assay Kit (Fluorometric) (ab176724) provides a convenient method for sensitive detection of NADP, NADPH and their ratio. The enzymes in the system specifically recognize NADP/NADPH in an enzyme recycling reaction that significantly increases detection sensitivity. In addition, this assay has very low background since it is run in the red visible range that considerably reduces the sample interference. Its signal can be easily read in a fluorescence microplate reader at Ex/Em = 530 – 570/590 – 600 nm (maximum EX/Em = 540/590 nm). Alternatively, the signal can also be read on a colorimetric plate reader at OD 576 nm.

The assay can be performed in a convenient 96-well or 384-well microtiter-plate format.



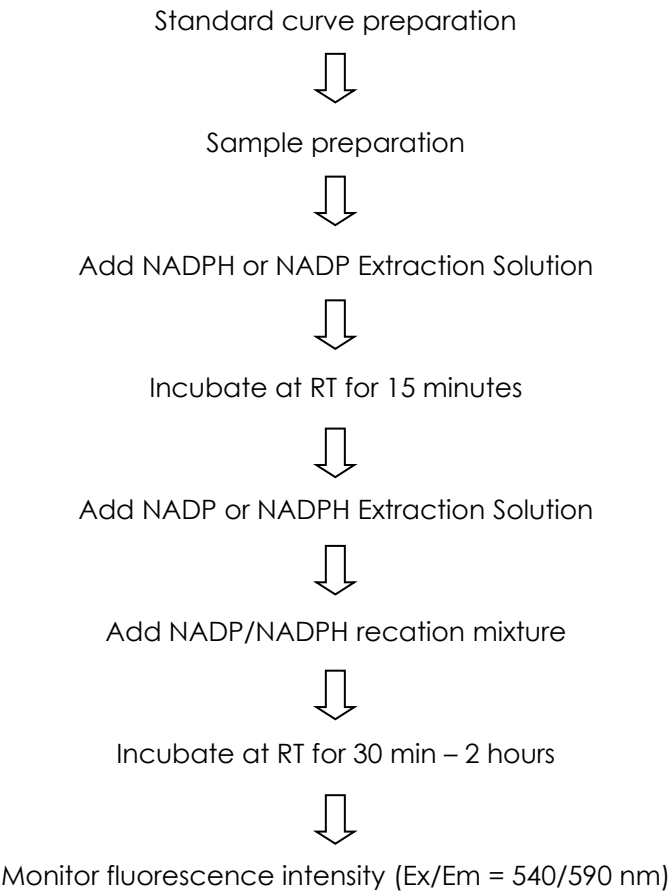
Traditional NADP/NADPH assays are done by monitoring the changes in NADPH absorption at OD 340 nm. These methods suffer low sensitivity and high interference since the assays are done in the UV range. The low sensitivity of the absorption-based NADP/NADPH tests makes the assays difficult to automate for high throughput screening.

Key features:

- Broad application: can be used for quantifying NADP/NADPH in a variety of cell extracts.
- Sensitive: detect as low as 10 picomoles of NADP/NADPH in solution.
- Continuous: suitable for both manual and automated operations without mixing or separation step.
- Convenient: formulated to have minimal hands-on-time.
- Non-radioactive: no special requirements for waste treatment.

Nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) are two important cofactors found in cells. NAD forms NADP with the addition of a phosphate group to the 2' position of the adenyl-nucleotide through an ester linkage. NADPH is the reduced form of NADP+, while NADP+ is the oxidized form of NADPH. NADP is used in anabolic biological reactions, such as fatty acid synthesis, which require NADPH as 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.

2. Protocol Summary



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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

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 the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- 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.

6. Materials Supplied

Item	Quantity	Storage Condition (Before prep)	Storage Condition (After prep)
NADP Extraction Solution	1 x 10 mL	-20°C	-20°C
NADP/NADPH Control Solution	1 x 10 mL	-20°C	-20°C
NADP/NADPH Lysis Buffer	1 x 10 mL	-20°C	-20°C
NADP/NADPH Recycling Enzyme Mixture (lyophilized)	2 bottles	-20°C	-20°C
NADPH Extraction Solution	1 x 10 mL	-20°C	-20°C
NADPH Sensor Buffer	1 x 20 mL	-20°C	-20°C
NADPH Standard (167 µg)	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring fluorescence at Ex/Em = 540/590 nm
- MilliQ water or other type of double distilled water (ddH_2O)
- PBS solution (pH7.4)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Cell scraper (for adherent cells)
- 96 well/384 well plate with clear flat bottom, preferably black. Alternatively, a 96 well/384 well white plate with clear flat bottom can be used if reading assay on a colorimetric plate reader.

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.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 NADPH Standard (lyophilized, 167 µg):

Reconstitute the NADPH Standard in 200 µL of PBS to generate a 1 mM (1 nmol/µL) NADPH stock solution. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C.

9.2 NADP/NADPH Lysis Buffer:

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

9.3 NADPH Extraction Solution:

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

9.4 NADP Extraction Solution:

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

9.5 NADP/NADPH Control Solution:

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

9.6 NADPH Sensor Buffer:

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

9.7 NADP/NADPH Recycling Enzyme Mixture:

Add 10 mL of NADPH Sensor Buffer (Step 9.6) to the bottle and mix well by inversion. Label this as **NADP/NADPH Reaction Mixture**.

One reconstituted bottle is enough to assay 2 x 96 well plates.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Dilute 10 µL of 1 mM NADPH stock solution (Step 9.1) into 990 µL PBS solution to generate a 10 µM (10 pmol/µL) NADPH standard solution.

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

Standard #	Sample to dilute	Volume standard in well (µL)	PBS (µL)	End conc NADPH in well
1	10 µM	300	0	10 µM
2	Std #1	200	400	3.3 µM
3	Std #2	200	400	1.1 µM
4	Std #3	200	400	0.37 µM
5	Std #4	200	400	0.12 µM
6	Std #5	200	400	0.04 µM
7	Std #6	200	400	0.014 µM
8 (Blank)	0	0	200	0 µM

Each dilution has enough amount of standard to set up duplicate readings (2 x 25 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 before storing the samples. Alternatively, if that is not possible, we suggest that you 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.
- Samples prepared by other protocols can be used as well for this assay. Do not use RIPA buffer as it will interfere with the assay. If you have your samples ready, please skip this section and proceed to Assay Procedure section.

11.1 Plant cell samples:

11.1.1 Homogenize leaves with Lysis Buffer at a final concentration of 200 mg/mL.
11.1.2 Centrifuge lysate at 2,500 rpm for 5 – 10 minutes at RT.
11.1.3 Transfer supernatant to a new tube. Discard pellet.
11.1.4 Keep sample on ice.

11.2 Bacterial cell samples:

11.2.1 Ensure cells at in exponential growing phase.
11.2.2 Collect bacterial cells by centrifugating them at 10,000 x *g* for 15 minutes at 4°C.
11.2.3 Resuspend cells in Lysis Buffer to a final concentration of 10^7 – 10^8 cells/mL.
11.2.4 Incubate cells at room temperature for 15 minutes.
11.2.5 Keep on ice.
11.2.6 Centrifuge lysate at 2,500 rpm for 5 minutes at RT.
11.2.7 Transfer supernatant to a new tube. Discard pellet.
11.2.8 Keep sample on ice.

11.3 Tissue samples:

11.3.1 Wash 20 mg tissue with cold PBS.
11.3.2 Homogenize tissue in 400 µL Lysis Buffer.
11.3.3 Centrifuge homogenate at 2,500 rpm for 5 – 10 minutes at RT.
11.3.4 Transfer supernatant to a new tube. Discard pellet.
11.3.5 Keep sample on ice.

11.4 Mammalian cell samples (suspension cells):

11.4.1 Grow suspension cells in appropriate tissue culture vessel.
11.4.2 Collect 0.5 – 1 x 10^7 cells by centrifugation at 1,500 rpm for 5 minutes at RT.
11.4.3 Resuspend pelleted cells in 100 µL Lysis Buffer.
11.4.4 Incubate cells at room temperature for 15 minutes.
11.4.5 You can use the cell lysate directly. Alternatively, centrifuge lysate at 1500 rpm for 5 minutes and use supernatant for the assay.

11.5 Mammalian cell samples (adhesion cells):

11.5.1 Grow adhesion cells in appropriate tissue culture vessel (for example, 10 cm plate dish).
11.5.2 Scrape cells in cold PBS and centrifuge at 1,500 rpm for 5 minutes at RT.
11.5.3 Resuspend cells in 1 mL cold PBS. Count cells and centrifuge again.
11.5.4 Resuspend 5 x 10^6 cells in 100 µL Lysis Buffer.
11.5.5 Incubate cells at room temperature for 15 minutes.
11.5.6 You can use the cell lysate directly. Alternatively, centrifuge lysate at 1500 rpm for 5 minutes and use supernatant for the assay.

Δ Note: This kit has been tested successfully in the following cell lines: HeLa (adherent cells), RAW 264.7 and U937 cells (suspension cells). Jurkat cells did not give any signal.

Δ Note: We suggest using different sample dilutions to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- In healthy mammalian cells there is more NADPH compared to NADP. For mammalian cells, we recommend to use Total (NADP + NADPH) minus NADPH to calculate the amount of NADP existing in the sample.

12.1 Set up Reaction wells:

- Standard wells = 25 µL standard dilutions.
- Sample wells (NADPH) = 25 µL samples.
- Sample wells (NADP) = 25 µL samples.
- Sample wells Total NADP/NADPH = 25 µL samples.

See example plate layout for assay in the table below.

BL: Blank control.

NS: NADP/NADPH standards.

TS: test sample wells Total NADP/NADPH.

TS (NADPH): teste sample wells NADPH.

TS (NADP): test sample wells NADP.

BL	BL	TS	TS	TS (NADPH)	TS (NADPH)	TS (NADP)	TS (NADP)
NS 1	NS 1	….	….	….	….	….	….
NS 2	NS 2						
NS 3	NS 3						
NS 4	NS 4						
NS 5	NS 5						
NS 6	NS 6						
NS 7	NS 7						

12.2 NADPH extraction:

12.2.1 Add 25 µL of NADPH Extraction Solution (Step 9.3) into the NADPH sample wells (labeled "TS (NADPH)").

12.2.2 Incubate at room temperature for 10 – 15 minutes.

12.2.3 Add 25 µL NADP Extraction Solution to neutralize NADPH extracts.

12.3 NADP extraction:

12.3.1 Add 25 µL of NADP Extraction Solution (Step 9.4) into the NADP sample wells (labeled "TS (NADP)").

12.3.2 Incubate at room temperature for 10 – 15 minutes.

12.3.3 Add 25 µL NADPH Extraction Solution (Step 9.3) to neutralize NADP extracts. See table 1 for reaction summary.

12.4 Total NADP + NADPH:

12.4.1 Add 25 µL of NADP/NADPH Control Solution (Step 9.5) into the blank control (labeled "BL"), standard wells (labeled "NS") and into the Total NADP/NDPH sample wells (labeled "TS").

12.4.2 Incubate at room temperature for 10 – 15 minutes.

12.4.3 Add 25 µL NADP/NADPH Control Solution (Step 9.5). See table 1 for reaction summary.

Table 1. Reaction summary layout.

NADPH standard	Blank control	Test Sample (total)	Test Sample (NADPH)	Test sample (NADP)
Standard dilutions: 25 µL	PBS: 25 µL	Sample: 25 µL	Sample: 25 µL	Sample: 25 µL
NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADPH Extraction solution: 25 µL	NADP Extraction solution: 25 µL
Incubate at room temperature for 10 – 15 minutes				
NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADP Extraction solution: 25 µL	NADPH Extraction solution: 25 µL
Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL

12.5 Run NAD/NAPDH assay:

12.5.1 Add 75 µL of NADPH Reaction Mixture (Step 9.7) into each well (final total assay volume = 150 µL).

12.5.2 Incubate reaction at room temperature for 30 minutes – 2 hours, protected from light.

12.5.3 Measure fluorescence increase on a microplate reader at Ex/Em = 540/590 nm.
High concentration of NADPH (> 100 µM final concentration) may cause reduced fluorescence signal due to over oxidation of NADPH sensor to a non-fluorescent product).

Δ Note: This assay can also be done on a white clear bottom plate and read by a colorimetric microplate reader at wavelength λ = 576 ± 5 nm. The absorption detection has lower sensitivity compared to fluorescence detection.

13. Protocol for 384-well plate assay

13.1 Prepare standard as described in Section 10.
13.2 Prepare samples as described in Section 11.
13.3 Set up assay reaction as described in the table below:

NADPH standard	Blank control	Test Sample (total)	Test Sample (NADPH)	Test sample (NADP)
Standard dilutions: 12.5 µL	PBS: 12.5 µL	Sample: 2.5 µL	Sample: 12.5 µL	Sample: 12.5 µL
NADP/NADPH control solution: 12.5 µL	NADP/NADPH control solution: 12.5 µL	NADP/NADPH control solution: 12.5 µL	NADPH Extraction solution: 12.5 µL	NADP Extraction solution: 12.5 µL
Incubate at room temperature for 10 – 15 minutes				
NADP/NADPH control solution: 12.5 µL	NADP/NADPH control solution: 12.5 µL	NADP/NADPH control solution: 12.5 µL	NADP extraction solution: 12.5 µL	NADPH extraction solution: 12.5 µL
Total: 37.5 µL	Total: 37.5 µL	Total: 37.5 µL	Total: 37.5 µL	Total: 37.5 µL

13.4 Add 37.5 µL of NADPH Reaction Mixture (Step 9.7) into each well (final total assay volume = 75 µL).
13.5 Incubate reaction at room temperature for 30 minutes – 2 hours, protected from light.
13.6 Measure fluorescence increase on a microplate reader at Ex/Em = 540/590 nm.

14. Calculations

– Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

14.1 Subtract the mean fluorescence value of the blank (BL) from all standard and sample readings for each data point. This is the corrected fluorescence.

14.2 Average the duplicate reading for each standard and each of the test samples.

14.3 Plot the corrected fluorescence values for each standard as a function of the final concentration of NADPH.

14.4 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).

14.5 Concentration of NADPH in the test samples is calculated as:

$$\text{NADPH concentration} = \left(\frac{B}{V}\right) * D$$

Where:

B = NADPH amount in the sample well calculated from standard curve (µM).

V = sample volume added in the sample wells (µL).

D = sample dilution factor.

14.6 Concentration of Total NADP/NADPH in the test samples is calculated as:

$$\text{Total (NADP/NADPH) concentration} = \left(\frac{B}{V}\right) * D$$

Where:

B = Total NADP/NAPDH amount in the sample well (µM).

V = sample volume added in the sample wells (µL).

D = sample dilution factor.

14.7 Concentration of NADP in the test samples is calculated as:

$$NADP = Total\ (NADP/NADPH) - NADPH$$

Δ Note: When performing the calculations for Total NADPH/NADP, the same NADPH standard curve is used to solve for "B" in the equation. There are not separate standard curves for Total NADPH/NADP or NADP alone.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

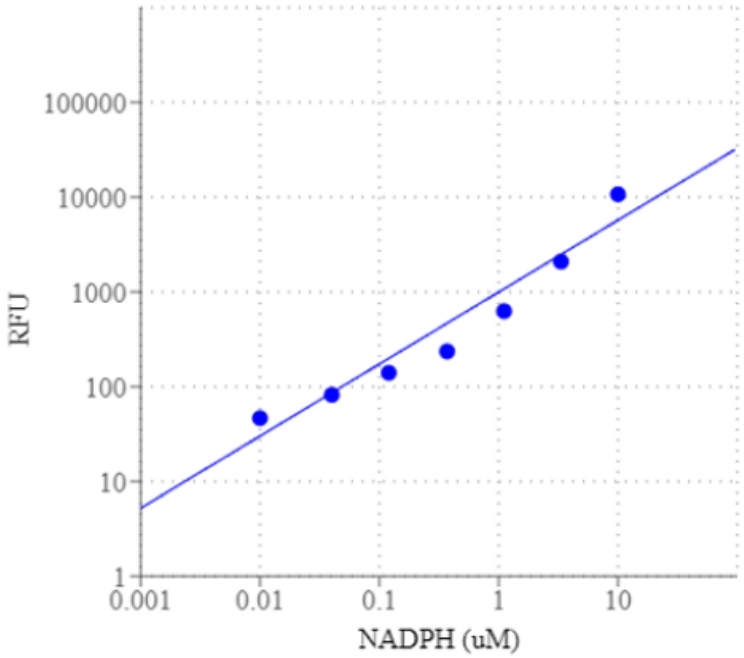


Figure 1. NADPH dose response was measured with ab176724 in a 96-well solid black plate using a Gemini microplate reader.

16. Quick Assay Procedure

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

- Solubilize NADPH standard and prepare NADP/NADPH reaction mixture (aliquot if necessary); get equipment ready.
- Prepare NADPH standard dilution: [10 – 0.01 µM].
- Prepare samples (dilute if necessary to fit standard curve readings).
- Set up plate in duplicate for standard (25 µL), blank (25 µL), Total NADP/NADPH samples (25 µL), NADPH samples (25 µL) and NADP samples (25 µL).
- Set up reactions in a 96-wp as described in the table below:

NADPH standard	Blank control	Test Sample (total)	Test Sample (NADPH)	Test sample (NADP)
Standard dilutions: 25 µL	PBS: 25 µL	Sample: 25 µL	Sample: 25 µL	Sample: 25 µL
NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADPH Extraction solution: 25 µL	NADP Extraction solution: 25 µL
Incubate at room temperature for 10 – 15 minutes				
NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADP/NADPH control solution: 25 µL	NADP Extraction solution: 25 µL	NADPH Extraction solution: 25 µL
Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL

- Add 75 µL NADP/NADPH Reaction mixture to all wells.
- Incubate reaction at RT for 30 – 2 hours protected from light.
- Monitor fluorescence increase in a fluorescence microplate reader at Ex/Em= 540/590 nm.

17. Troubleshooting

Problem	Reason	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 microplate	Colorimetric: clear plates. Fluorometric: black wells/clear bottom plates.
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use provided 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

Problem	Reason	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 described in the 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

18. Interferences

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

- RIPA buffer: we recommend using the lysis buffer provided in the kit. Alternatively, you can use PBS or Mammalian Cell Lysis Buffer 5X (ab179835).

19. FAQs

Q. The concentration of NADP plus NADPH does not equal the concentration of total NADP/NADPH. What is wrong?

A. In healthy mammalian cells, there is generally more NADPH compared to NADP. Therefore, we recommend measuring total NADP/NADPH minus NADPH to calculate the amount of NADP.

Q. I have some extracts I have prepared previously for another assay. Can use them with this kit?

A. Yes, as long as they haven't been prepared in RIPA buffer (see Interferences section). We recommend performing several dilutions of your sample in PBS or Lysis Buffer to ensure the concentration of NADP and NADPH in your samples fit within the standard curve readings.

Q. How can I make sure the cells are completely lysed before measuring NADP/NADPH?

A. You can look at the cells in the tissue culture plate during lysis to ensure they are lysing. If that is the case, scrape them carefully with a pipet tip.

Q. I would like to grow and lyse with cells directly on the assay plate. Is that possible?

A. Yes, you can grow 5 – 8 x 10^4 cells/well in a 96 well plate and lyse them with 100 µL/well. Because the lysis reaction will take in a small volume, we recommend that you check for complete lysis before proceeding with the assay (see above)

This procedure is not recommended if you want to run your samples in a 384 well plate.

Q. The reaction is changing color. Is that normal?

A. Yes. The reagents look dark blue at the beginning of the assay, and turns into pink as the reaction takes place.

20. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)