Version 10d Last updated 26 September 2023

ab176723
NAD/NADH Assay Kit (Fluorometric)

For the rapid, sensitive and accurate measurement of NAD/NADH in cell extracts.

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

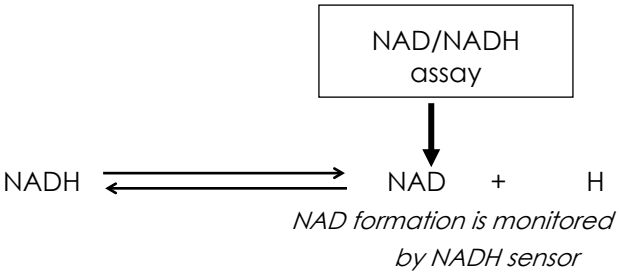
Table of Contents

1. Overview 1
2. Protocol Summary 3
3. Precautions 4
4. Storage and Stability 4
5. Limitations 5
6. Materials Supplied 5
7. Materials Required, Not Supplied 6
8. Technical Hints 7
9. Reagent Preparation 8
10. Standard Preparation 9
11. Sample Preparation 10
12. Assay Procedure 12
13. Protocol for 384-well plate assay 15
14. Calculations 16
15. Typical Data 18
16. Quick Assay Procedure 19
17. Troubleshooting 20
18. Interferences 22
19. FAQs 22
20. Notes 24

1. Overview

NAD/NADH Assay Kit (Fluorometric) (ab176723) provides a convenient method for sensitive detection of NAD, NADH and their ratio. The enzymes in the system specifically recognize NAD/NADH 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.



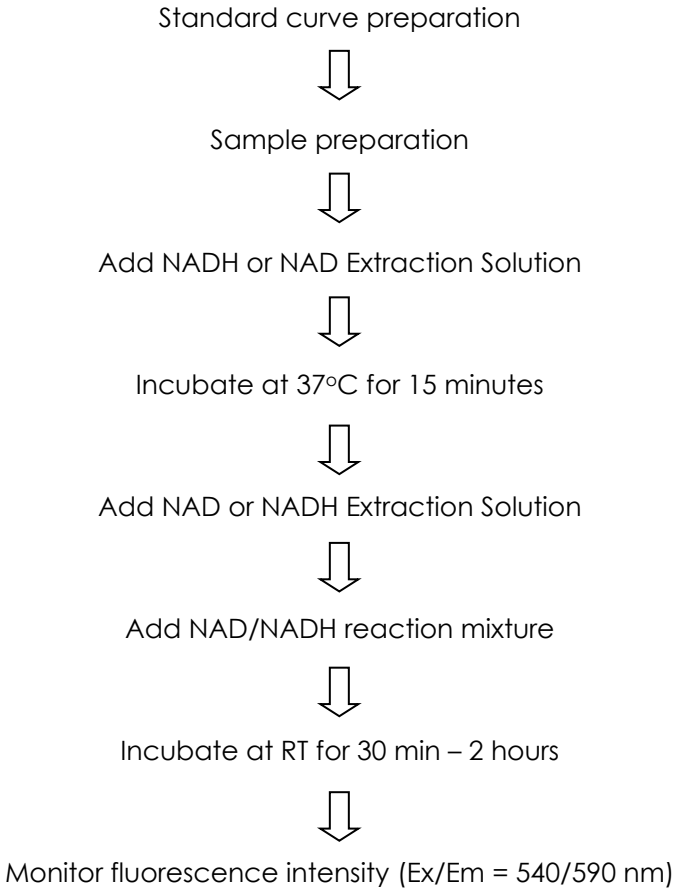
Traditional NAD/NADH assays are done by monitoring the changes in NADH absorption at OD 340 nm. These methods suffer low sensitivity and high interference since the assays are done in the UV range. The low sensitivity of the absorption-based NAD/NADH tests makes the assays difficult to automate for high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

Key features:

- Broad application: can be used for quantifying NAD/NADH in a variety of cell extracts.
- Sensitive: detect as low as 10 picomoles of NAD/NADH 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 (NAD+) are two important cofactors found in cells. NAD forms NAD with the addition of a phosphate group to the 2' position of the adenyl-nucleotide through an ester linkage. NADH is the reduced form of NAD+, while NAD+ is the oxidized form of NADH. NADH plays a key role in the production of energy through redox reactions. NAD+ serves as a cofactor for dehydrogenases, reductases and hydroxylases, making it a major carrier of hydrogen and electrons in major metabolic pathways such as glycolysis, TCA cycle and fatty acid synthesis.

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)
NAD Extraction Solution	1 x 10 mL	-20°C	-20°C
NAD/NADH Control Solution	1 x 10 mL	-20°C	-20°C
NAD/NADH Lysis Buffer	1 x 10 mL	-20°C	-20°C
NAD/NADH Recycling Enzyme Mixture (lyophilized)	2 bottles	-20°C	-20°C
NADH Extraction Solution	1 x 10 mL	-20°C	-20°C
NADH Sensor Buffer	1 x 20 mL	-20°C	-20°C
NADH Standard (142 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/38- 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 NADH Standard (lyophilized, 142 µg):

Reconstitute the NADH Standard in 200 µL of PBS to generate a 1 mM (1 nmol/µL) NADH 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 NAD/NADH Lysis Buffer:

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

9.3 NADH Extraction Solution:

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

9.4 NAD Extraction Solution:

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

9.5 NAD/NADH Control Solution:

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

9.6 NADH Sensor Buffer:

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

9.7 NAD/NADH Recycling Enzyme Mixture:

Add 10 mL of NADH Sensor Buffer (Step 9.6) to the bottle and mix well by inversion. Label this as **NAD/NADH Reaction Mixture**. Aliquot reconstituted mixture so that you have enough to perform the desired number of assays. Store at -20°C.

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 30 µL of 1 mM NADH stock solution (Step 9.1) into 970 µL PBS solution to generate a 30 µM (30 pmol/µL) NADH standard solution.

10.2 Using 30 µM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Sample to dilute	Volume of standard (µL)	PBS (µL)	End conc NADH in well
1	30 µM	400		30 µM
2	Std #1	200	400	10 µM
3	Std #2	200	400	3.3 µM
4	Std #3	200	400	1.1 µM
5	Std #4	200	400	0.37 µM
6	Std #5	200	400	0.12 µM
7	Std #6	200	400	0.04 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 (~1100 *g*) 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 Collect bacterial cells by centrifugating them at 10,000 x *g* for 15 minutes at 4°C.
11.2.2 Resuspend cells in Lysis Buffer to a final concentration of 10^7 – 10^8 cells/mL.
11.2.3 Incubate cells at room temperature for 15 minutes.
11.2.4 Centrifuge lysate at 2,500 rpm (~1100 *g*) for 5 minutes at RT.
11.2.5 Transfer supernatant to a new tube. Discard pellet.
11.2.6 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 (~1100 *g*) 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 (~400 *g*) for 5 minutes at RT.
11.4.3 Resuspend pelleted cells in 100 µL Lysis Buffer.
11.4.4 Incubate cells at 37°C for 15 minutes.
11.4.5 Centrifuge lysate at 1500 rpm (~400 *g*) 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 (~400 *g*) 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 37°C for 15 minutes.
11.5.6 Centrifuge lysate at 1500 rpm (~400 *g*) for 5 minutes and use supernatant for the assay.

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.

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

Note: It is highly recommended to incubate the cells with Lysis Buffer at 37oC and use the supernatant for the experiment.

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 NAD compared to NADH. For mammalian cells, we recommend to use Total (NAD + NADH) minus NAD to calculate the amount of NADH existing in the sample.

12.1 Set up Reaction wells:

- Standard wells = 25 µL standard dilutions.
- Sample wells (NADH) = 25 µL samples.
- Sample wells (NAD) = 25 µL samples.
- Sample wells Total NAD/NADH = 25 µL samples.

See example plate layout for assay in the table below.

BL: Blank control.

NS: NAD/NADH standards.

TS: test sample wells Total NAD/NADH.

TS (NADH): teste sample wells NADH.

TS (NAD): test sample wells NAD.

BL	BL	TS	TS	TS (NADH)	TS (NADH)	TS (NAD)	TS (NAD)
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 NADH extraction:

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

12.2.2 Incubate 37°C for 10 – 15 minutes.

12.2.3 Add 25 µL NAD Extraction Solution to neutralize NADH extracts.

12.3 NAD extraction:

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

12.3.2 Incubate at 37°C for 10 – 15 minutes.

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

12.4 Total NAD + NADH:

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

12.4.2 Incubate at 37°C for 10 – 15 minutes.

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

Note In healthy mammalian cells, there is more NAD compare to NADH, so one can simply use total NAD and NADH minus the NAD to calculate the amount of NADH.

Table 1. Reaction summary layout.

NADH standard	Blank control	Test Sample (total)	Test Sample (NADH)	Test sample (NAD)
Standard dilutions: 25 µL	PBS: 25 µL	Sample: 25 µL	Sample: 25 µL	Sample: 25 µL
NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NADH Extraction solution: 25 µL	NAD Extraction solution: 25 µL
Incubate at 37°C for 10 – 15 minutes				
NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NAD Extraction solution: 25 µL	NADH Extraction solution: 25 µL
Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL

12.5 Run NAD/NADH assay:

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

12.5.2 Incubate reaction at room temperature for 15 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 NADH (> 100 µM final concentration) may cause reduced fluorescence signal due to over oxidation of NADH 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.

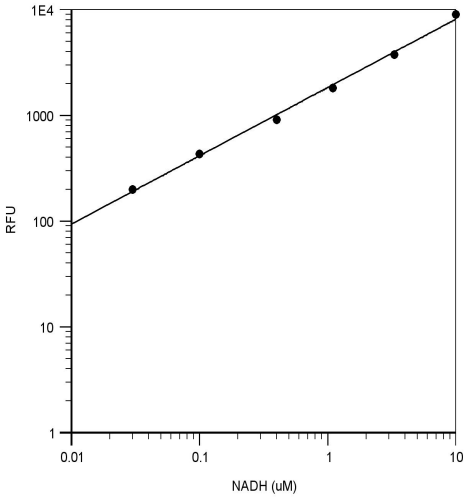


Figure 1. NADH response was measured with Fluorimetric NAD/NADH Assay Kit (Fluorimetric) in a 96-well solid black plate using a Gemini microplate reader. After the addition of the reaction mix the sample was incubated at room temperature for 60 minutes.

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:

NADH standard	Blank control	Test Sample (total)	Test Sample (NADH)	Test sample (NAD)
Standard dilutions: 12.5 µL	PBS: 12.5 µL	Sample: 12.5 µL	Sample: 12.5 µL	Sample: 12.5 µL
NAD/NADH control solution: 12.5 µL	NAD/NADH control solution: 12.5 µL	NAD/NADH control solution: 12.5 µL	NADH Extraction solution: 12.5 µL	NAD Extraction solution: 12.5 µL
Incubate at 37°C for 10 – 15 minutes				
NAD/NADH control solution: 12.5 µL	NAD/NADH control solution: 12.5 µL	NAD/NADH control solution: 12.5 µL	NAD Extraction solution: 12.5 µL	NADH 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 NADH Reaction Mixture (Step 9.7) into each well (final total assay volume = 75 µL).
13.5 Incubate reaction at room temperature for 15 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 NADH.

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 NAD in the test samples is calculated as:

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

Where:

B = NAD amount in the sample well calculated from standard curve (µmol).

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

D = sample dilution factor.

14.6 Concentration of Total NAD/NADH in the test samples is calculated as:

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

Where:

B = Total NAD/NADH amount in the sample well (µmol).

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

D = sample dilution factor.

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

$$NADH = Total\ (NAD/NADH) - NAD$$

15. Typical Data

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

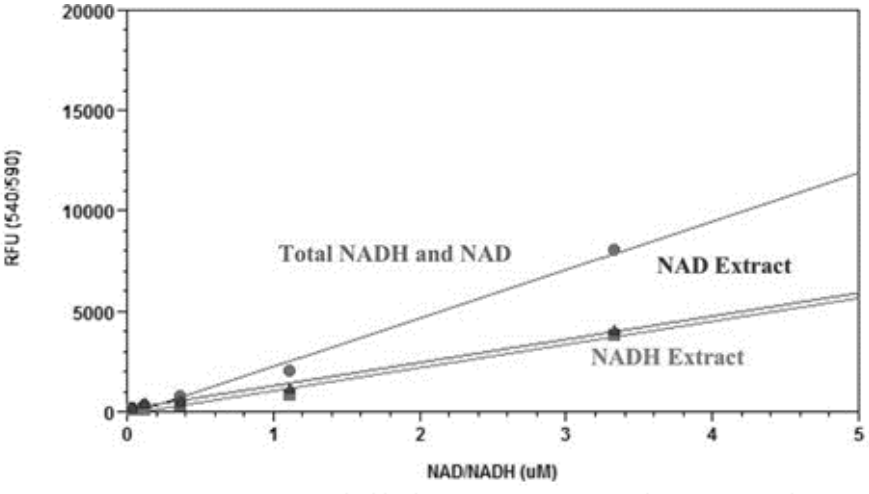


Figure 2. Total NADH and NAD (circles), and their extract dose response (NADH –triangles –; NAD – squares) were measured in a 96-well black plate using a Gemini microplate reader (Molecular Devices). Signal was acquired at Ex/Em = 540/590 nm (cut off at 570 nm) 30 minutes after addition of NAD/NADH reaction mixture.

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 NADH standard and prepare NAD/NADH reaction mixture (aliquot if necessary); get equipment ready.
- Prepare NADH standard dilution: [3 – 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 NAD/NADH samples (25 µL), NADH samples (25 µL) and NAD samples (25 µL).
- Set up reactions in a 96-wp as described in the table below:

NADH standard	Blank control	Test Sample (total)	Test Sample (NADH)	Test sample (NAD)
Standard dilutions: 25 µL	PBS: 25 µL	Sample: 25 µL	Sample: 25 µL	Sample: 25 µL
NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NADH Extraction solution: 25 µL	NAD Extraction solution: 25 µL
Incubate at 37°C for 10 – 15 minutes				
NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NAD/NADH control solution: 25 µL	NAD Extraction solution: 25 µL	NADH Extraction solution: 25 µL
Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL

- Add 75 µL NAD/NADH Reaction mixture to all wells.
- Incubate reaction at RT for 15 – 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 NAD plus NADH does not equal the concentration of total NAD/NADH. What is wrong?

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

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 NAD and NADH in your samples fit within the standard curve readings.

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

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)