NADMED

EN

Q – NAD TISSUES/CELLS

NAD+ and NADH assay kit

Quantitative assay for TISSUES and CELLS

Version 2.0

FOR SINGLE-USE ONLY

These instructions must be read in their entirety before using this product.

RUO

For Research Use Only.
Not for use in diagnostic procedures.

GENERAL INFORMATION

A. Proprietary name: Q-NAD Tissues/Cells NAD+ and NADH assay kit: quantitative assay kit for TISSUES and CELLS
B. Catalog number: RUO_003, 40 samples (96-well format)
C. Storage: -80˚C
D. IFU issued: October 2023

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INTRODUCTION

NAD+ and its reduced form NADH are derivatives of vitamin B3. NAD metabolites are essential for the maintenance of healthy metabolic balance in all living organisms. Their levels are dynamic and change in response to different endogenous and exogenous stimuli. This kit is designed for selective measurement of NAD+ and NADH in tissues and cells (human or animal).

PRINCIPLE OF THE ASSAY

The principle of the assay is a cyclic enzymatic reaction with colorimetric detection. First, NAD+ and NADH metabolites are extracted together from a sample in a single step. Then, the extract is divided into two parts. In the first part, NAD+ is stabilized while NADH is removed; in the second part, NADH is stabilized while NAD+ is removed. Next, NAD+ and NADH metabolites are analyzed on two separate plates by an enzymatic reaction coupled with a color change. The intensity of the color change in the assay is linearly proportional to the concentration of NAD+ or NADH in the reaction mixture. Obtained concentration values are then corrected for dilution factor and normalized on protein amount or tissue mass.

REAGENTS PROVIDED

REAGENTS	DESCRIPTION*	BEFORE THE ASSAY
Extraction BUFFER	2 bottles with 25 mL of extraction buffer	Equilibrate to room temperature (15–25°C).
NAD+ stabilizing reagent	1 bottle with 8 mL of buffered solution for NAD+ measurement	Equilibrate to room temperature (15–25°C).
NADH stabilizing reagent	1 bottle with 8 mL of buffered solution for NADH measurement	Equilibrate to room temperature (15–25°C).
NAD+ standard stock	1 microtube with 40 µL of 1mM NAD+	See preparation guide.
NADH standard stock	1 microtube with 40 µL of 1mM NADH	See preparation guide.
BUFFER C	2 bottles with 19 mL of assay buffer. One per plate.	Equilibrate to room temperature (15–25°C).
Assay color reagent	2 bottles with 3 mL of assay color. One per plate	Equilibrate to room temperature (15–25°C). Should be used within 3 hours after equilibration.
Enzyme	2 microtubes with 40 µL of enzyme. One per plate	Thaw only before adding into master mix.
Stop solution	1 bottle with 3 mL of Stop solution, for both plates	Equilibrate to room temperature (15–25°C).
BUFFER D	12 mL of buffered solution to solubilize protein pellet for protein measurement.	Equilibrate to room temperature (15–25°C) at the day of use.
Positive control (buffer)	1 microtube with 200 µL of buffer to prepare positive control	Equilibrate to room temperature (15–25°C).

*Note: Accepted variation of the filling volume 5%.

PRECAUTIONS AND WARNINGS

Only for use by trained personnel

The Extraction BUFFER, Stop solution, and BUFFER D may cause skin, eye, and respiratory irritation in case of direct contact. Avoid inhaling fumes. Handle with care. Wear protective clothing and use gloves and goggles.

Assay color reagent may cause skin irritation in case of direct contact. Handle with care; use gloves and wear protective clothing.

The Safety Data Sheet Q-NAD Tissues/cells (SDS) presents the identified hazards of the chemicals in this kit and the appropriate warning information associated with those hazards.

The Safety Data Sheet Q-NAD Tissues/Cells (SDS) describes the disposal of used kit components.

STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening, all kit components should be stored at -80°C until use. Avoid temperature fluctuations in the freezer.
- After thawing, NAD+ and NADH stabilizing reagents, BUFFER D, Positive Control (buffer), and Stop solution are stable for two weeks at room temperature.
- After thawing, Extraction BUFFER should be protected from direct light and stored at 4°–8°C for two weeks.
- BUFFER C should be thawed and used on the day of the assay.
- Assay color reagent is stable for up to 3 hours at room temperature after thawing.
- Enzyme should be used directly after thawing.
- Standards and the Positive control should be prepared and used on the day of the assay.
- Standards should be protected from light.

OTHER MATERIALS REQUIRED

The following materials are required but not provided in the kit:

- Deionized water (milli-Q water from a water purification system or commercially available deionized water, e.g., Sigma cat #38796)
- Microtubes, 1.5 mL, for sample preparation, extraction, stabilization, and assay standards preparation. Requirements for the material of the microtubes: use basic non-sterile microcentrifuge tubes made from transparent/natural color polypropylene (PP) intended for in vitro diagnostics (e.g., Sarstedt Ref 72.690.001). Do not use microtubes intended for molecular biology labeled as sterile (chemically sterilized), free of endotoxin, pyrogen, human DNA, and low retention; do not use microtubes intended for work with proteins labeled as LoBind.
- Two plastic multichannel pipette reservoirs (from non-sterile polystyrene): one for pipetting the master mix, the other for pipetting the Stop solution.
- Two 96-well transparent polystyrene microplates with medium protein binding affinity suitable for colorimetric and absorbance assays.
- Dry bath (heat block) with adjustable temperature (up to 80°C).
- Table-top cooling microcentrifuge (max speed 20 000 x g).
- Ice-water bath (packed ice with added tap water to a slush state, which firmly holds inserted microtubes, preventing them from floating).
- Dry ice
- Lysis tubes with beads and Tissue homogenizer (e.g., Precellys) OR Dounce (glass) homogenizer to homogenize tissues.
- Calibrated single-channel pipettes (e.g. 5–50 µL, 20–200 µL, 100–1000 µL) and multichannel pipettes (5–50 µL, 30–300 µL) and beveled tips, low retention
- Spectrophotometric microplate reader capable of measuring absorbance at 570–573 nm.
- Aluminum foil to protect microtubes and plates from light.
- (Optional) Water bath with adjustable temperature to 50 °C
- (Optional) BCA Protein assay kit or similar
- (Optional) Sonicator with a tip fitting into 1.5 ml microtube
- (Optional) 100 mM PBS, pH 7.0 (61.5 mM K_2HPO_4, 38.5 mM KH_2PO_4)

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix the reagents of the kit with those from other sources.
- Variations in sample collection, processing, and storage may cause variations in metabolite values.
- This assay kit is NOT suitable for the measurement of NAD+ and NADH in whole blood. For NAD+ and NADH measurement in blood, please use “Q-NADMED BLOOD NAD+ and NADH assay kit”, RUO_001.

REAGENTS PREPARATION

Thaw all components for NAD+ and/or NADH assay according to the instructions in the table on page 5.

A. **Extraction BUFFER**: Ready to use. Do not shake vigorously.

B. **NAD+ stabilizing reagent**: Ready to use

C. **NADH stabilizing reagent**: Ready to use

D. **Enzyme**: Ready to use

E. **Stop solution**: Ready to use. If precipitate forms in Stop solution upon thawing, it should be re-dissolved by incubation at 37°C for 5 min and then cooled back to ambient temperature before use. Do not shake the Stop solution vigorously.

F. **BUFFER D**: Ready to use. If precipitate forms in BUFFER D upon thawing, it should be re-dissolved by incubation at +37°C for 5 min and then cooled back to 25°C before use. Do not shake vigorously.

G. **Positive control (buffer)**: Ready to use. (For further instructions see preparation of Positive control.)

H. **50 µM NAD+ standard stock**: add 25 µL of 1 mM NAD+ standard stock (provided) into 475 µL of deionized water, vortex. Protect from light. (For further instructions see preparation of standards and preparation of Positive control.)

I. **50 µM NADH standard stock**: add 25 µL of 1 mM NADH standard stock (provided) into 475 µL of deionized water, vortex. Protect from light. (For further instructions see preparation of standards and preparation of Positive control.)

J. **Master mix**: 1 bottle of **Assay color reagent** (3 mL) should be mixed into 1 bottle of **BUFFER C** to create 1 bottle of the master mix required for 1 plate of either NAD+ or NADH assay. Protect from light by keeping it in the original amber bottle. Do not shake vigorously. Discard leftovers. Do not re-freeze.

SAMPLE COLLECTION AND STORAGE

Tissue samples must be snap-frozen in liquid nitrogen as soon as possible after collection. If the tissue has significant blood contamination, rinse it with ice-cold PBS and tap it with a paper towel before freezing. We recommend freezing small pieces of material (10–20 mg per tube). **Important:** collect samples consistently from the same area of an organ to minimize variability. Weigh each piece either before freezing or in a frozen state. Sample aliquots should be stored at -80°C before analysis. **Requirement for sample amount:** 10–20 mg of tissue is sufficient (tested for mouse tissues). For extraction, use 1 mL of Extraction BUFFER per 20 mg of tissue aliquot.

Cultured cells should be washed with excess PBS before collection to remove protein from the culture media (this is especially important if results are normalized per protein). Collect cells by scraping or using enzymes, such as trypsin. Pellet the cells in a tube by centrifugation at 750 x g for 5 min and discard the supernatant. Snap-freeze the pellet in liquid nitrogen and store at -80°C until analysis. **Requirement for sample amount:** 1.5–2 million cells is sufficient (tested for cultured fibroblasts and HeLa cells). For extraction: use 300 µL of Extraction BUFFER per 1.5–2 million cells.

PRACTICAL CONSIDERATIONS

- The content of the Q-NAD tissues/cells assay kit is sufficient for the extraction and analysis of NAD+ and NADH in 40 samples. The number of samples is defined by the provided volume of Extraction BUFFER calculated for tissues (e.g., 1 mL of Extraction BUFFER for 20 mg of tissue). If cells are used, the volume of Extraction BUFFER is provided in excess (i.e., 0.3 mL of Extraction BUFFER is used for 1.5–2 million cells.)
- Both fresh and frozen samples can be used for the assay. Frozen samples should be stored at -80°C all the time before the assay. If melting has occurred during storage/shipping time the sample is not suitable for the assay due to metabolite deterioration. Subsequent freezing will not save the sample for NAD measurement.
- Do not mix materials from different kit lots. Subsequent freeze-thaw cycles are not allowed.
- We recommend homogenizing two samples at a time when using an automated homogenizer OR one when using a glass homogenizer. Obtained homogenates can be kept on ice until all the samples are homogenized and then centrifuged all at once to separate extracts.
- For analysis of both NAD+ and NADH, 10–20 mg of tissue, or 1.5–2 million cells is sufficient (depending on the cell type).
- The analyses of NAD+ and NADH are done on two separate plates. We recommend performing the NAD+ and NADH assays on the day of extraction.
- The kit allows normalization of the results on the tissue mass or cell number, and optionally on the protein content.
- To avoid cross-contamination, change to new pipette tips between the additions of each standard, sample, and reagent. Also, use separate reservoirs for the master mix and Stop solution.
- High-precision pipettes and beveled tips with less retention will improve the precision.
- Assay Color Reagent is a yellow, light-sensitive compound that turns brown upon enzymatic reaction. Exposure to direct sunlight or direct artificial light causes its unspecific color change to green. To minimize the light interference with the assay, we recommend the following:
    - Pipetting of the master mix and then Stop solution into the plate in the conditions of natural indirect light (dim conditions). To achieve these conditions, switch off artificial indoor lights and avoid pipetting close to the window. By natural indirect light (dim conditions), we mean the amount of environmental light one can experience when driving a car under a bridge on a cloudy day.
    - Covering the 96-well plate with an aluminum foil lid during the reaction time after adding the master mix and while transferring to the plate reader after adding Stop solution. Do not wrap the plate with aluminum foil due to the risk of contamination and sample mixing during unwrapping.
    - The protocol indicates the steps requiring the work under dim conditions.
- Positive control is an artificial sample containing known amounts of pure NAD+ and NADH.
- Technical requirements for a dry bath (heat block), especially important for the NADH stabilization step:

- Test the efficiency of heat transfer in the dry bath as follows:

1. Add 200 µL of water into a microtube that fits into the dry bath you are using.
2. Insert a conventional lab thermometer into the microtube with the liquid and place it into the dry bath set to 80°C.
3. Measure the time needed to increase the temperature of the liquid to 70°C.
4. All dry batch incubation times mentioned in this protocol are given for a dry bath equilibrated to 80°C that heats 200 µL of water to 70°C in 2 min. This is enough for the chemical reaction to reach 70°C in the solution upon incubation in the dry bath.

- Technical requirements for the spectrophotometric plate reader:
    1. Measurement of light absorption at 570–573 nm,
    2. Option to adjust the scanning light brightness/intensity to **low**. In some plate readers, the brightness can be adjusted as the number of flashes per measurement. In the latter case, set the number of flashes from 5 to 10.
- Extraction BUFFER, master mix, Stop solution, and BUFFER D contain detergents. Do not shake vigorously. To avoid bubbles, pipette the master mix and Stop solution by pressing the pipette to the first stop position; remove all bubbles in the wells with a small needle. Avoid touching the content of the wells with pipette tips.
- We recommend the following order of steps:
    1. Bring Extraction BUFFER, BUFFER D, NAD+ stabilizing reagent, NADH stabilizing reagent, and Stop solution to room temperature the day before the assay. These solutions are stable at room temperature for two weeks.
    2. Prepare the standards and Positive control on the day of the assay. Keep them refrigerated and light-protected until the assay.
    3. Thaw bottles with BUFFER C and Assay color reagent at room temperature on the day of the assay. These bottles take about 2–3 hours to melt. During this time, extract the samples, and prepare two aliquots of the sample extracts for separate measurements of NAD+ and NADH. Perform NAD+ and NADH assays on separate plates, one at a time.

PREPARATION OF STANDARDS

Note: this section can be done under normal light conditions.

1. Prepare standards on the day of the assay. Thaw microtubes with 1 mM standard stocks for 5 min at room temperature. Protect from light with a foil lid during thawing.
2. Prepare **50 nmol/mL NAD+ stock** by adding 25 µL of 1 mM NAD+ standard stock (provided) into 475 µL of deionized water, vortex. This stock is used to prepare NAD+ standards and Positive control.
3. Prepare **NAD+ standards** according to the scheme below by mixing indicated volumes of reagents in the following order: deionized water, 50 nmol/mL NAD+ stock, Extraction BUFFER, and NAD+ stabilizing reagent. The final volume of each standard is 1 mL. (**Tip**: use the same 20–200 µL pipette for pipetting 50 nmol/mL NAD+ stock and water).

STANDARD NUMBER	CONCENTRATION of NAD+ (nmol/mL)	STANDARD PREPARATION			
		50 nmol/mL NAD+ (µL)	dH2O (µL)	Extraction BUFFER (µL)	NAD+ stabilizing reagent (µL)
ST1	0	0	100	450	450
ST2	1	20	80	450	450
ST3	2	40	60	450	450
ST4	3	60	40	450	450
ST5	5	100	0	450	450

4. Prepare **50 nmol/mL NADH stock** by adding 25 µL of 1 mM NADH standard stock (provided) into 475 µL of deionized water, vortex. This stock is used to prepare NADH standards and Positive control.
5. Prepare **NADH standards** according to the scheme below by mixing indicated volumes of reagents in the following order: deionized water, 50 nmol/mL NADH stock, Extraction BUFFER, and NADH stabilizing reagent. The final volume of each Assay standard is 1 mL. (**Tip**: use the same 20–200 µL pipette for pipetting 50 nmol/mL NADH standard and water).

STANDARD NUMBER	CONCENTRATION of NADH (nmol/mL)	STANDARD PREPARATION			
		50 nmol/mL NADH (µL)	dH2O (µL)	Extraction BUFFER (µL)	NADH stabilizing reagent (µL)
ST1	0	0	100	450	450
ST2	0.5	10	90	450	450
ST3	1	20	80	450	450
ST4	1.5	30	70	450	450
ST5	2	40	60	450	450

6. Cover the stand with ready standards with an aluminum foil lid to protect from light and keep refrigerated before pipetting on the plate.

PREPARATION OF POSITIVE CONTROL

Note: this section is done under normal light conditions

Positive Control is prepared before the assay by mixing known amounts of NAD+ and NADH standards with the Positive control buffer (provided). The purpose of Positive control is to monitor the efficiency of the stabilization step when one of the metabolites is removed from the extract to allow selective measurement of either NAD+ or NADH.

1. Thaw the microtube with Positive control (buffer) for 5 min at room temperature.
2. To prepare Positive control, add 30 µL of Positive control buffer into a microtube.
3. Add 50 µL of **50 nmol/mL NAD+ stock** (for preparation, see pg. 10) and 20 µL of **50 nmol/mL NADH stock** (for preparation, see pg. 10) into the Positive control buffer and vortex (total volume: 100 µL).
4. Positive control does not contain proteins; therefore, it can be extracted with Extraction BUFFER at room temperature. Add 200 µL of Extraction BUFFER at room temperature into the prepared Positive control to create an "extract" of Positive control, vortex, and proceed to step 5.
5. Prepare two separate aliquots of the Positive Control "extract" (from step 4) into clean microtubes: 100 µL/tube.
6. To the first 100 µL aliquot, add 100 µL of **NAD+ stabilizing reagent**, vortex, and incubate at room temperature for 5 min. Then keep refrigerated and covered with foil until the assay. This stabilized Positive control aliquot will be used in **NAD+ assay**.
7. To the second 100 µL aliquot, add 100 µL **NADH stabilizing reagent**, vortex. Heat this solution at 80°C for 2 min; cool on ice. Then keep refrigerated and covered with foil until the assay. This stabilized Positive control aliquot will be used in **NADH assay**.

NOTE: Final dilution of the Positive control will be 10 times.

EXTRACTION OF NAD+ AND NADH FROM TISSUES

IMPORTANT:

- Tissue samples must be pre-weighted before the homogenization to allow normalization of obtained NAD+ and NADH values on tissue mass. The weight can be obtained either from fresh samples before freezing or from frozen samples while cooled with liquid nitrogen.

- Extraction BUFFER should be warmed to 50°–55°C (the optimal temperature range) before the extraction process. In this protocol, frozen samples are added into a warmed Extraction BUFFER followed by mechanical homogenization.

1. Keep tissue samples frozen on dry ice before the extraction. We recommend homogenizing one sample at a time or a maximum of two if tubes with beads and an automated Homogenizer are used.
2. Calculate the volume of Extraction BUFFER needed for homogenization of each sample based on the ratio: 1 mL of Extraction BUFFER per 20 mg of tissue.
3. Equilibrate Extraction BUFFER to 50°–55°C before extraction. To do so, there are two options: A) Pipette the calculated volume of Extraction BUFFER needed for homogenization into a homogenization tube, close the tube, and place it for 5 min into a dry bath equilibrated to 50°–55°C.

   B) Place the whole bottle with Extraction BUFFER into a water bath equilibrated to 50°–55°C for 10 min before extraction and pipette the calculated volumes of warmed buffer into homogenization tubes or directly into a glass homogenizer.
4. Add the frozen tissue piece with the known weight directly into the warm buffer. Homogenize the tissue sample in an automated homogenizer OR Dounce homogenizer until the solution is homogenous without visible tissue fragments. The presence of white threads of connective tissue is acceptable.
5. Cool the homogenate in an ice-water bath for at least 5 min. NAD+ and NADH metabolites are stable in obtained homogenate when kept on ice. Repeat steps 4 and 5 with the next sample and continue until all samples are homogenized. Proceed to step 6 when all homogenates are ready on ice.
6. From this step, process all samples at the same time. Mix each homogenate with a 1 mL pipette and transfer it into a clean microtube trying to collect all protein precipitate formed during the homogenization/extraction process.
7. Centrifuge at 20 000 x g for 10 min at 4°C. Transfer the extracts into clean microtubes and save the pellets for protein measurement, if normalization per protein content is needed. *Optional:* freeze the pellets at -80°C to measure the protein content on a different day.
8. Prepare two separate aliquots from the obtained extracts into clean microtubes: 100 µL/tube.
9. To the first 100 µL aliquot, add 100 µL of **NAD+ stabilizing reagent** to get a stabilized extract with NAD+ (keeps NAD+ and removes NADH). Vortex and incubate at room temperature for 5 min. If the assay is not performed immediately, refrigerate the stabilized extracts until the assay. **Protect from light by covering with an aluminum foil lid**.
10. To the second 100 µL aliquot, add 100 µL of **NADH stabilizing reagent** to get a stabilized extract with NADH (keeps NADH and removes NAD+). Vortex and incubate in a dry bath equilibrated at 80°C for 2 min. Cool on ice for 5 min. If the assay is not performed immediately, refrigerate the stabilized extracts until the assay. **Protect from light by covering with an aluminum foil lid**.

EXTRACTION OF NAD+ AND NADH FROM CULTURED CELLS

Fresh and frozen cell pellets can be used for analysis. When fresh cell pellets are to be analyzed, keep them on ice before homogenization. When frozen cells are used, keep them on dry ice before the procedure. We recommend processing a maximum of two samples at a time.

1. Equilibrate Extraction BUFFER to 50°–55°C before extraction. To do so, there are two options:

   A) make small aliquots, e.g., 1.2 mL, of Extraction BUFFER in microtubes and place them for 5–10 min in a dry bath equilibrated to 50°–55°C.

   B) place the bottle with Extraction BUFFER into a water bath equilibrated to 50°–55°C for 10 min before extraction.

2. Keep deeply frozen cell pellets for 2 min on ice before the extraction. This is needed to reduce the temperature drop of the Extraction BUFFER upon addition to the sample.
3. Add 300 µL of warm Extraction BUFFER to every 1.5–2 million cells and resuspend by a few cycles of up-and-down pipetting until there are no cell clumps.
4. Incubate the homogenate in a dry bath equilibrated to 50°–55°C for 2-3 min. Cool the homogenate in the ice-water bath for at least 5 min. NAD+ and NADH metabolites are stable in the obtained homogenate when kept on ice. Repeat steps 3 and 4 with the next two samples and continue until all samples are homogenized. Proceed to step 5 when all homogenates are ready on ice.
5. Centrifuge at 20 000 x g for 10 min at 4°C. Transfer the extracts into clean microtubes and save the pellets for protein measurement, if normalization per protein content is needed. *Optional*: freeze the pellets at -80°C to measure the protein content on a different day.
6. Prepare two separate aliquots from the obtained extracts into clean microtubes: 100 µL/tube.
7. To the first 100 µL aliquot, add 100 µL of **NAD+ stabilizing reagent** to get a stabilized extract with NAD+ (keeps NAD+ and removes NADH). Vortex and incubate at room temperature for 5 min. If the assay is not performed immediately, refrigerate the stabilized extracts until the assay. **Protect from light by covering with an aluminum foil lid.**
8. To the second 100 µL aliquot, add 100 µL of **NADH stabilizing reagent** to get a stabilized extract with NADH (keeps NADH and removes NAD+). Vortex and incubate in a dry bath equilibrated at 80°C for 2 min. Cool on ice for 5 min. If the assay is not performed immediately, refrigerate the stabilized extracts until the assay. **Protect from light by covering with an aluminum foil lid.**

NOTE: Final dilution of the initial extract is 2 times.

NOTE: Levels of NAD+ and NADH are tissue and cell-type specific. Levels also can be modulated by supplementation with NAD precursors or drugs. We recommend first extracting samples according to the provided guidelines (sample amount per volume of Extraction BUFFER). If the light absorbance in reactions with added stabilized extract is very low (close to 0 nmol/mL standard) or significantly higher than the absorbance of the highest standard, then the amount of starting material needs adjustment. In case of a high concentration of metabolite in the stabilized extract, the stabilized extract should be diluted with deionized water before the assay. Take the additional dilution into account during the result calculation.

ASSAY PROCEDURE

Notes:

- Steps 1–3 are performed under normal light conditions.
- **Steps 4–9 are performed in dim conditions (when direct artificial light is turned off).**
- Every assay contains sample blanks to correct for all unspecific background signals. Each sample type must have its own sample blank. If all analyzed samples are of the same type (e.g., 40 muscle samples or fibroblast cultures), sample blanks are prepared from four stabilized sample extracts (e.g., BL UNK1–4 of samples 1–4) to correct for unspecific interaction between the extract components and Assay color reagent in the master mix. If several tissue types are analyzed, every sample type should have at least one well with a sample blank representing this sample type. Positive control does not require a separate blank.
- **All Sample Blanks are incubated with the master mix WITHOUT added enzyme.**
- The enzyme should be thawed just before being added to the master mix. Briefly centrifuge the microtube with the enzyme at a low speed before opening.

Steps:

1. Incubate ready standards, stabilized sample extracts, and stabilized Positive control for 5 min at room temperature before pipetting onto the plate.
2. Pipette 20 µL of each NAD+ or NADH standard in duplicates starting from ST1 (0 nmol/mL) according to the plate scheme on pg. 19.
3. Pipette 20 µL of stabilized Positive control and stabilized sample extracts in duplicates (see the scheme on pg. 19). For the first four stabilized sample extracts, pipette one extra replicate to the indicated well (BL UNK1–4) according to the scheme on pg. 19. These four wells are sample blanks needed for the analysis without enzyme to correct for the unspecific interaction between the extract and Assay color reagent within the master mix.
4. From this step onwards, work in dim conditions (avoiding direct artificial light). Prepare the master mix by adding Assay color reagent into BUFFER C; mix gently by rotation.
5. Add 190 µL of the master mix without enzyme into each of the four sample blank wells (BL UNK1–4).
6. Add 40 µL of enzyme into the bottle with the remaining master mix. Mix gently; avoid foaming. Pour the master mix with added enzyme into the reservoir for the multichannel pipette; protect the master mix in the reservoir during pipetting with an aluminum foil lid. (Please see further instructions in our instructional video at www.nadmed.com.)
7. Add 190 µL of the master mix with the added enzyme to all remaining wells, including stabilized Positive control, using a multichannel pipette; avoid foaming and direct light. Immediately cover the ready plate with the aluminum foil lid.
8. **For NAD+ assay:** incubate the covered plate for 4 - 6 min at room temperature.

   For NADH assay: incubate the covered plate for 6 - 8 min at room temperature.

NOTE: The provided times for the assays are the guidelines. The reaction can be stopped when there is a distinct color gradient in the standards and differences in color intensity between samples with added enzyme and sample blanks. The longer the reaction time, the more intense signal will be observed. The color intensity in the NADH assay is lower than in the NAD+ because the physiological concentration of NADH is lower than NAD+ in tissues and cells. Therefore, the NADH standards range from 0 to 2 nmol/mL, while the NAD+ standards range from 0 to 5 nmol/mL.

9. Stop the reactions by adding 10 µL of Stop solution to each well in the same order as the master mix using a multichannel pipette. Avoid foaming by gently shaking the plate by hand on a table surface removing bubbles.
10. Measure light absorbance at 573 nm immediately after adding Stop solution. If possible, shake the plate inside the microplate reader for 5 sec before the measurement. Note: after adding Stop solution, the color intensity can slowly increase uniformly in all the wells. This is expected due to the non-enzymatic background process in the master mix after the addition of Stop solution.

MEASUREMENT OF PROTEIN CONTENT

Important! Remove all liquid from the microtube with the pellet obtained after the metabolite extraction before adding the solubilization BUFFER D. Do not solubilize by pipetting up and down. Solubilization requires short sonication using a microtip.

1. Equilibrate BUFFER D to room temperature.
2. If frozen pellets are analyzed, they must be equilibrated to room temperature for 10 min before adding BUFFER D.
3. **Tissues:** add 250 µL of BUFFER D to each tissue protein pellet.

   Cells: add 100 µL of BUFFER D to each cell protein pellet.
4. Sonicate the samples using a microtip for 2–5 sec. Place the tip of the sonicator so that it touches the protein pellet.
5. Dilute samples 10 times with 100 mM PBS, pH 7.0 (e.g., mix 10 µL of the sample with 90 µL of PBS, vortex).
6. Measure the protein concentration in the diluted solution using any available protein assay kits (e.g., BCA Protein assay kit).
7. Using the obtained result, calculate the absolute amount of protein in the initial pellet (mg) using the following formula:

$$total\ protein, mg = obtained\ concentration\ \frac{mg}{mL} * 10 * V(mL)\ used\ for\ pellet\ solubilization$$

CALCULATION OF RESULTS

Perform calculations for each plate separately using the following algorithm:

1. Calculate the average of the absorbance readings for each standard (ST1-ST5). Create a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration (in nmol/mL) on the x-axis and perform a simple linear regression fitting of the standard curve. Note: concentration units for NAD+ and NADH are nmol/mL, representing the concentration in the solution added into the well.
2. Find the concentration (in nmol/mL) of metabolite in stabilized sample extract added to each well using the formula of linear regression of the standard curve.
3. Calculate the average of four sample blanks (BL UNK1-4). The obtained value represents an unspecific signal of the stabilized extracts.
4. Calculate the average concentration value for duplicates of each stabilized sample extract (UNK).
5. Calculate the average of duplicates of the stabilized Positive control and compare obtained values with the expected concentrations described below (section Positive control on pg. 17).
6. Correct the obtained concentrations in stabilized sample extracts for the mean of sample blanks (calculated in step 3). Obtained concentration values are: nmol/mL of the target metabolite in the stabilized sample extract added into the assay. Normalize obtained concentration values to tissue mass, total protein, and cell number as shown below.

For normalization per mg of tissue use the following formula:

$$Conc.\frac{nmol}{mg}\ tissue = \frac{Concentration\ in\ stabilized\ extract, \frac{nmol}{mL} * 2}{20mg/mL}$$

For normalization per mg of protein use the following formula:

$$Conc.\frac{nmol}{mg} protein = \frac{Conc.\ in\ stabilized\ extract, \frac{nmol}{mL} * 2 * V, mL\ of\ added\ Extraction\ BUFFER}{total\ protein, mg}$$

For normalization per million cells use the following formula:

$$Conc.\frac{nmol}{million} cells = \frac{Conc.\ in\ stabilized\ extract, \frac{nmol}{mL} * 2 * 0{,}3\ mL\ of\ Extraction\ BUFFER}{(cell\ amount)}$$

7. For convenience, the obtained concentration in nmol/mg can be converted to pmol/mg by multiplying by 1000.

POSITIVE CONTROL

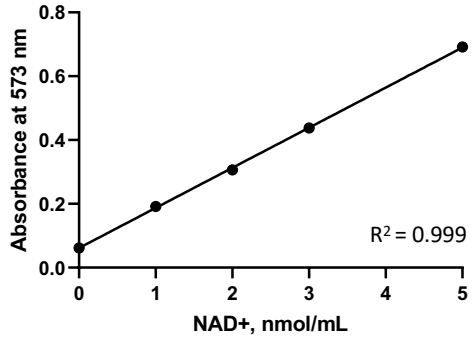
Positive Control aims to monitor the efficiency of the stabilization step when one of the metabolites is removed from the extract. The concentration of measured NAD+ in Positive control stabilized “extract” is expected to be within 4.0–4.5 nmol/mL; the concentration of NADH is expected to be within 1.5–1.9 nmol/mL.

1. Find the concentration of NAD+ in stabilized Positive control “extract” on NAD+ plate as nmol/mL using the formula of linear regression of the NAD+ standard curve.
2. Find the concentration of NADH in stabilized positive control “extract” on NADH plate as nmol/mL using the formula of linear regression of the NADH standard curve.

TYPICAL DATA

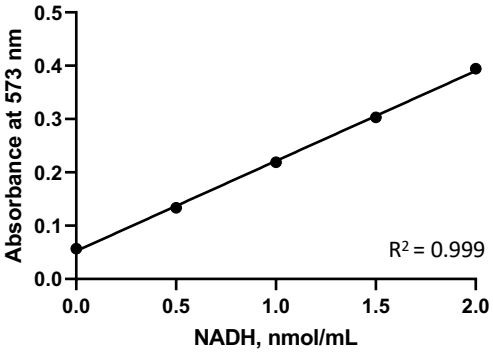
The standard curve values are provided for demonstration only. A standard curve for each NAD+ and NADH plate is generated based on the obtained absorbances on a target plate. Do not use the standard curve from one plate to calculate concentrations in samples measured on a different plate.

STANDARD CURVE FOR NAD+



Standard	NAD+ (nmol/mL)	Absorbance, 573nm Assay time – 4 min
ST1	0	0.064 0.006
ST2	1	0.192 0.192
ST3	2	0.309 0.304
ST4	3	0.432 0.444
ST5	5	0.703 0.681

STANDARD CURVE FOR NADH



Standard	NADH (nmol/mL)	Absorbance, 573nm Assay time – 6 min
ST1	0	0.057 0.057
ST2	1	0.133 0.134
ST3	2	0.224 0.214
ST4	3	0.300 0.306
ST5	5	0.395 0.394

RECOMMENDED PLATE LAYOUT FOR NAD+ OR NADH MEASUREMENT

An example plate for an experiment with 40 samples of the same type. If the experiment includes different sample types on one plate, each sample type should have at least one sample blank (BL UNK) well per sample type.

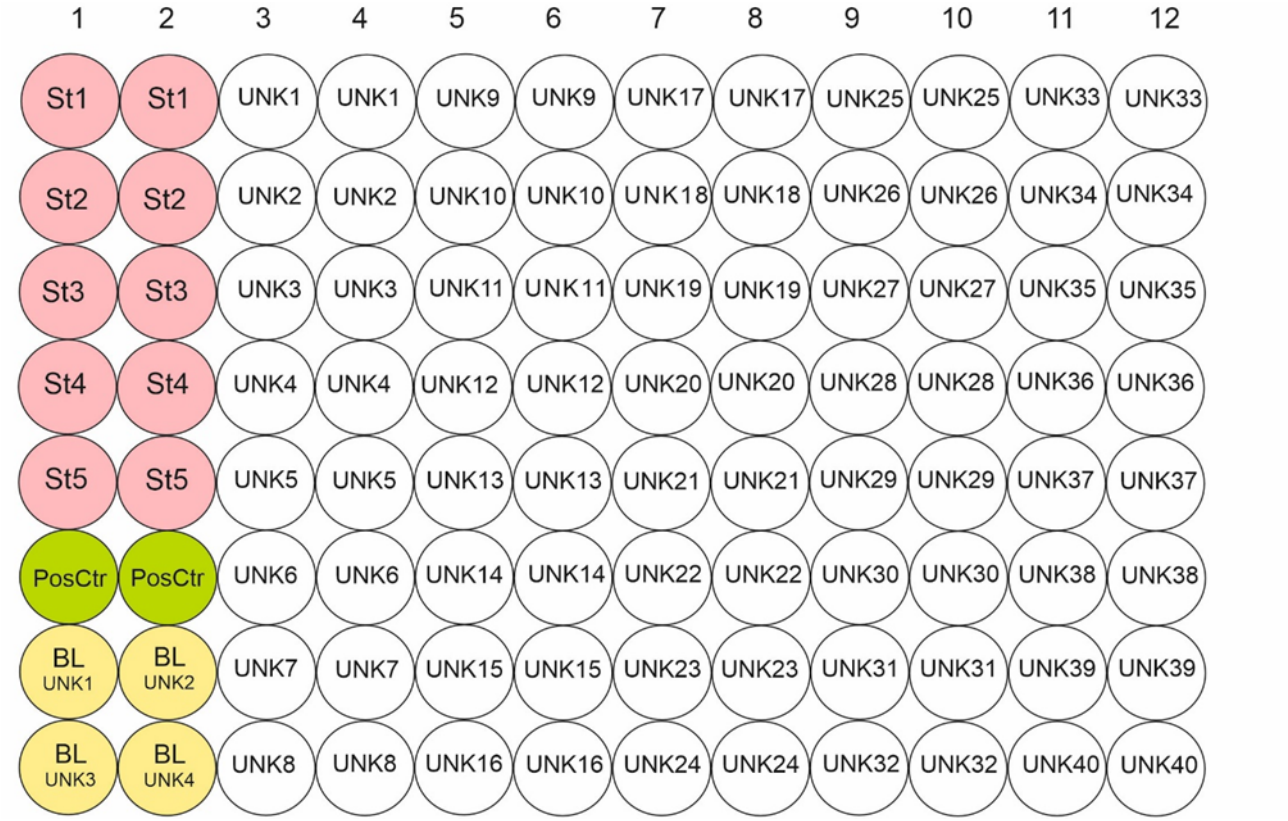


Plate layout for NAD+ or NADH assay: St – standard, BL - blanks with indicated samples used for the blank reading, UNK – unknown samples, stabilized extracts from the samples with unknown metabolite concentration, PosCtr – stabilized Positive control. Use 20 µL of standards, stabilized Positive control, and stabilized sample extracts per well. Sample blanks of the first four samples are analyzed in the master mix without added enzyme.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.