NADMED

EN

Q–NAD Tissue/Cells NAD+ and NADH Assay kit

Q40 NAD+ & NADH Tissue/Cells Kit (RUO)

Instructions for use

Part II

Quantitative NAD+ and NADH assay kit for 40 tissue samples

Version 1.0

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

Proprietary name:

Q-NAD Tissue/Cells NAD+ and NADH Assay kit: quantitative assay kit for tissues and cells

Catalog number:

RUO_003; RUO_003_US

Storage:

-85°C- -70°C upon arrival

IFU issued:
August 2026

Manufacturer:
NADMED Ltd / Oy
www.nadmed.com
info@nadmed.com
Tukholmankatu 8
Biomedicum 2U
00290 Helsinki
FINLAND

SYMBOLS ON THE PACKAGING


Contains flammable liquid and vapor. Refer to PRECAUTIONS AND WARNINGS


Warning/danger. Refer to PRECAUTIONS AND WARNINGS

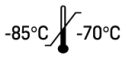

Consult instructions for usage


Use-by date

Catalogue number

Batch code


Manufacturer



Upper limit of storage temperature


Do not use if package is damaged

Number of reactions

Research-use only


Protect from direct light


Keep dry

TABLE OF CONTENTS

INTRODUCTION

NAD+ and its reduced form NADH are bioactive forms 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 kit measures intracellular NAD+ and NADH content. 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. In the analytical process, the sample extract undergoes separate measurements for NAD+ and NADH. In the first segment, the procedure focuses on stabilizing NAD+ while actively eliminating NADH. Conversely, in the second segment, the emphasis shifts to stabilizing NADH, concurrently ensuring the removal of NAD+. The NAD+ and NADH stabilized extracts 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.

SAMPLE CAPACITY AND SETUP

Maximum Capacity: 40 samples per kit. In this set up sample extracts are analyzed in duplicates with 4 joined (shared) blanks.

Flexible Setup: Fewer sample amounts can be analyzed. Depending on sample number each sample extract can be analyzed in duplicate or triplicate with own (dedicated) blank per sample.

SAMPLE HANDLING AND STORAGE

For analysis, fresh and frozen samples can be used.

Limitations:

- This kit is NOT suitable for measuring NAD+ and NADH in blood and in erythrocytes.
- Samples for analysis should be stored at -80 – -70°C. Freeze-thaw cycles are not allowed. Long term storage conditions at temperatures higher than -60°C are not compatible with stability of metabolites in the biological matrix and will result in inaccurate measurement.

Requirements for sample size and handling

Sample size: For tissues, we recommend using 20 mg of tissue per 1 ml of Extraction Buffer for efficient extraction and measurement of both metabolites. Minimal sample amount ensuring reliable extraction is 10 mg (established using mouse tissue samples as model). If possible, weigh the tissue piece at the time of collection and aim for a sample size in the range of 10–25 mg. If frozen pieces of tissue need to be aliquoted and weighted for the assay, the cutting must be done under liquid nitrogen or on dry ice to maintain sample deeply frozen.

Tissue collection and storage conditions: Tissue samples must be snap-frozen in liquid nitrogen immediately after collection. To minimize sample-to-sample variability, harvest tissues consistently from the same anatomical region of the organ and remain uniform processing times across all samples of the same type. If the tissue piece has significant blood contamination, rinse it with cold PBS and gently blot it dry with a paper towel before freezing.

REAGENT STORAGE, STABILITY AND PREPARATION

REAGENT	DESCRIPTION (*)	PREPARATION (**)	STABILITY (**)
EXTRACTION BUFFER B	2x 25 mL Sufficient for 40 samples	Equilibrate to room temperature before use. Ready for use.	Stable for one month at 4°–8°C, protect from light.
NAD+ STABILIZER	8 mL Sufficient for 40 samples		Stable for two weeks at room temperature after thawing.
NADH STABILIZER	8 mL Sufficient for 40 samples		
POSITIVE CONTROL (BUFFER)	200 µL Sufficient for two plates		
SOLUBILIZATION BUFFER D	12 mL Sufficient for protein concentration measurement of 40 samples		
STOP SOLUTION	3 mL Sufficient for two plates	Ready for use. Equilibrate to room temperature before use. (If precipitates have formed, warm in 37°C and cool to room temperature before the assay).	
ASSAY BUFFER C	2x 19 mL One aliquot per 96-well plate	Equilibrate to room temperature. Assay BUFFER C + ASSAY COLOR REAGENT = Assay Master Mix. See preparation guide on page 17.	Stable for 12 hours at room temperature after thawing. Keep in the original amber bottle.
ASSAY COLOR REAGENT	2x 3 mL One aliquot per 96-well plate		Stable for 3 hours at room temperature after thawing. Keep in the original amber bottle.
NAD+ STANDARD STOCK	40 µL (1 mM) Sufficient for Standards and Positive control	See preparation guide on page 15.	**Should be used immediately after thawing.** Should be protected from light.
NADH STANDARD STOCK	40 µL (1 mM) Sufficient for Standards and Positive control		
NAD ENZYME	2x 40 µL One aliquot per one 96-well plate	Equilibrate to room temperature for 5-10 min before use. Add to the Master mix after processing of plate blanks.	**Should be used immediately after thawing.**

* Accepted variation of the filling volume +/-5%.

** Room temperature: 15–25°C

PRECAUTIONS AND WARNINGS

For research use only. For trained personnel use only. Do not smoke, drink, eat, or apply cosmetics in the working area. Wear protective gloves, clothing, and eye protection. Wash hands thoroughly after handling.

EXTRACTION BUFFER B may cause eye irritation. Handle with care; use goggles.

NAD+ STABILIZER may cause skin and eye irritation. Handle with care; use gloves and goggles.

NADH STABILIZER may cause skin, eye, and respiratory irritation. Avoid inhaling fumes.

STOP SOLUTION may cause skin, eye, and respiratory irritation. Avoid inhaling fumes.

ASSAY COLOR REAGENT may cause skin irritation. Handle with care; use gloves.

SOLUBILIZATION BUFFER D may cause skin, eye, and respiratory irritation. Avoid inhaling fumes.

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

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

TROUBLESHOOTING

If you are facing any issues during the assay performance, refer to the NADMED troubleshooting guide at www.nadmed.com/support.

NOTE: Levels of NAD+ and NADH are **tissue type specific.** Levels can be modulated by disease, supplementation with NAD precursors or drugs. We recommend first analyzing samples according to the IFU guidelines. If the signal in the Assay of the stabilized sample extracts is:

a. very low (close to the absorbance of ST1 with 0 nmol/mL of NAD+ or NADH), the concentration of the analyte in the extract needs to be increased by either increasing initial sample amount or decreasing volume of Extraction Buffer B.
NOTE: Make sure that the sample did not undergo a thaw-freeze cycle at any point before extraction. Melting of the sample results in enzymatic degradation of NADs.

b. high (considerably higher than the absorbance of the ST5 in NAD+ or NADH standard curve), the stabilized sample extract should be diluted with **deionized water** before the assay. Take the additional dilution into account in the results calculation.

MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

CATEGORY	ITEM	SPECIFICATIONS/REQUIREMENTS
Consumables	Deionized water	Deionized water from a water purification system (e.g. Millipore, Elga) or commercially available deionized water, e.g., Sigma cat #38796
	Microtubes, 1.5 mL	Use non-sterile microcentrifuge tubes made from transparent/natural color polypropylene (PP) intended for *in vitro diagnostics* (e.g., Sarstedt, ref. 72.690.001). Microtubes NOT compatible with NADMED assay: a) chemically sterilized microtubes b) molecular biology grade sterile microtubes that are free of endotoxin, pyrogen, human DNA, and low retention c) special microtubes, e.g. microtubes intended for protein work marked "LoBind".
	96-well plates (2 pieces)	Use non-sterile, transparent, polystyrene flatbottom plates with medium or low protein binding intended for colorimetric assays (e.g. Revvity, ref. 6055640).
	Liquid reservoirs for multichannel pipetting (2 pieces)	Use non-sterile polystyrene plastic. Use separate reservoirs for Assay Master Mix and STOP SOLUTION.
	Pipette tips	Use non-sterile, bevelled pipette tips with low retention.
	Ice (Ice-water bath)	Fill a container with packed laboratory ice and pour cold tap water to reach a slush-like state. Insert tubes with the samples into the ice-water bath so that part of the microtube with the sample is firmly hold by ice pieces and surrounded by ice-cold water. Avoid tubes with the samples floating in the water.
	Dry ice	When frozen samples are analyzed, they must be kept frozen until extraction.
	Aluminium foil	Use foil covers to protect samples, standards, and the plates from light during assay performance as indicated in the instructions.
Equipment and Machinery		
	Precision scale	For weighing samples before extraction Volume measurement of the obtained sample extract in 1.5 ml microtube can be done by weighing the tubes with the extract following subtraction of average weight for the empty 1.5 mL microtube.
	Calibrated Pipettes	Single channel for volumes of e.g. 5–50 µL, 20–200 µL and 100–1000 µL. Multichannel pipettes for volumes of e.g. 5–50 µL and 30–300 µL.

	Microcentrifuge	Use centrifuge with cooling to +4°C and speed of 20,000 x g
	Spectrophotometric Microplate Reader	a) Capable to measure absorbance at 570–573 nm wavelength b) In the Microplate Readers with an adjustable scanning brightness/intensity: select beam intensity "low", or alternatively, adjust the brightness based on the number of flashes per measurement to 5–10 flashes.
	Warm water bath	Adjustable temperature to 55°C is required.
	Dry bath Heat Block fitting 1.5 mL Microtubes	Adjustable temperature up to 80°C is required. To ensure consistent and reliable results, **test the heat transfer and calibrate the time needed to reach target temperature**: 1. Set your heat block at 80°C and wait until it reaches 75°–80°C. 2. Add 500 µL of water into a microtube and place the tube on your heat block. Make sure the microtube fits tightly to the block. 3. Insert a conventional lab thermometer into the microtube with water. 4. Measure the time needed to reach 75°C. The heat transfer is considered sufficient if the temperature is reached within 5 minutes. If 75°C is NOT reached in 500 µl of water after 5 min incubation of the tube in the heat block set at 80°C : a) ensure the tubes fit the block tightly b) increase the target temperature of your device. Recalibrate.
	Automated tissue homogenization system (e.g. Precellys)	Machine for homogenization tissue pieces in special lysis tubes with beads Lysis tubes with beads suitable for homogenization of dedicated tissue type
Optional (protein concentration measurement)	BCA Protein assay kit or similar Sonicator suitable for use with microtubes with a tip fitting into 1.5 ml microtube 100 mM PB, pH 7.0 (61.5 mM K_2HPO_4, 38.5 mM KH_2PO_4)	
Special	Possibility to work in dim light conditions for the ASSAY part of the measurement. Refer to PRACTICAL CONSIDERATIONS on page 10.	

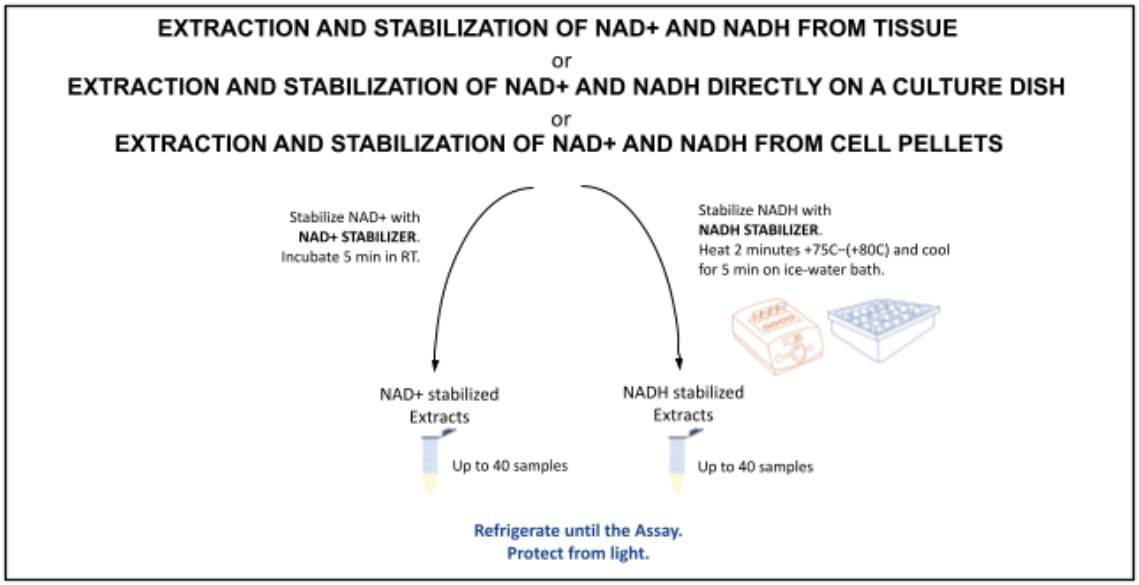
PRACTICAL CONSIDERATIONS

CATEGORY	INSTRUCTIONS
Limitations	Read the SAMPLE HANDLING AND STORAGE carefully. This assay is designed for analysis of NAD+ and NADH in cells and tissue samples. Use IFU for selected sample type. This kit is NOT suitable for measuring NAD+ and NADH in whole blood and in erythrocytes. Do not use kit components beyond the expiry date. Do not mix materials from different kit lots. Subsequent freeze-thaw cycles of reagents are not allowed.
Usability	Thoroughly mix all reagents by gentle swirling. Small microtubes should be quickly centrifuged at low speed before opening. We recommend taking the EXTRACTION BUFFER B (protect from light), NAD+ STABILIZER, NADH STABILIZER, SOLUBILIZATION BUFFER D, and STOP SOLUTION to room temperature one day before the assay. Take ASSAY BUFFER C and ASSAY COLOR REAGENT to room temperature on the day of the assay. These bottles take about 2–3 hours to melt. We recommend extracting one sample at a time. Obtained homogenates can be kept on ice-water bath until all the samples are treated with extraction buffer and then centrifuged all at once to separate extracts.
Accuracy	The analyses of NAD+ and NADH are done on two separate plates. We recommend performing both assays on the same day, one assay at a time. To avoid cross-contamination, change to new pipette tips between the additions of each standard, samples, and reagents. Avoid touching the content of the wells in the 96-well plate with pipette tips when working with multi-channel pipettes. High-precision pipettes and beveled tips with less retention will improve the precision. ASSAY BUFFER C and STOP SOLUTION contain detergents. To avoid bubbles, pipette the Master mix and STOP SOLUTION into the wells on 96-weel plate by pressing the pipette to the first stop position only when ejecting the liquid into the wells. In case of introduced air bubbles, remove them with a small needle before inserting the plate into plate reader.
Protection from light	Protect the stabilized sample extracts, standards, and positive controls from light when they are not being actively processed. However, for convenience, extraction, preparation, and pipetting of them onto the 96-well plates can be performed under normal light conditions. ASSAY COLOR REAGENT is a yellow, light-sensitive compound that turns brown upon enzymatic reaction of the assay. Exposure to excess natural light or direct artificial light causes unspecific color change to green. To minimize the light interference with the assay, the protocol indicates the steps specifically requiring dim conditions. To protect the reactions from both natural and direct artificial light, we recommend the following: Switch off artificial light source directly above your bench. Close blinds or move further away from a window. Use aluminum foil covers for the plate and pipetting reservoirs whenever working with ASSAY COLOR REAGENT and Assay Master Mix. Cover the 96-well plates with aluminum foil covers during Assay incubation steps until the plate is inserted into plate reader. (Do not wrap).

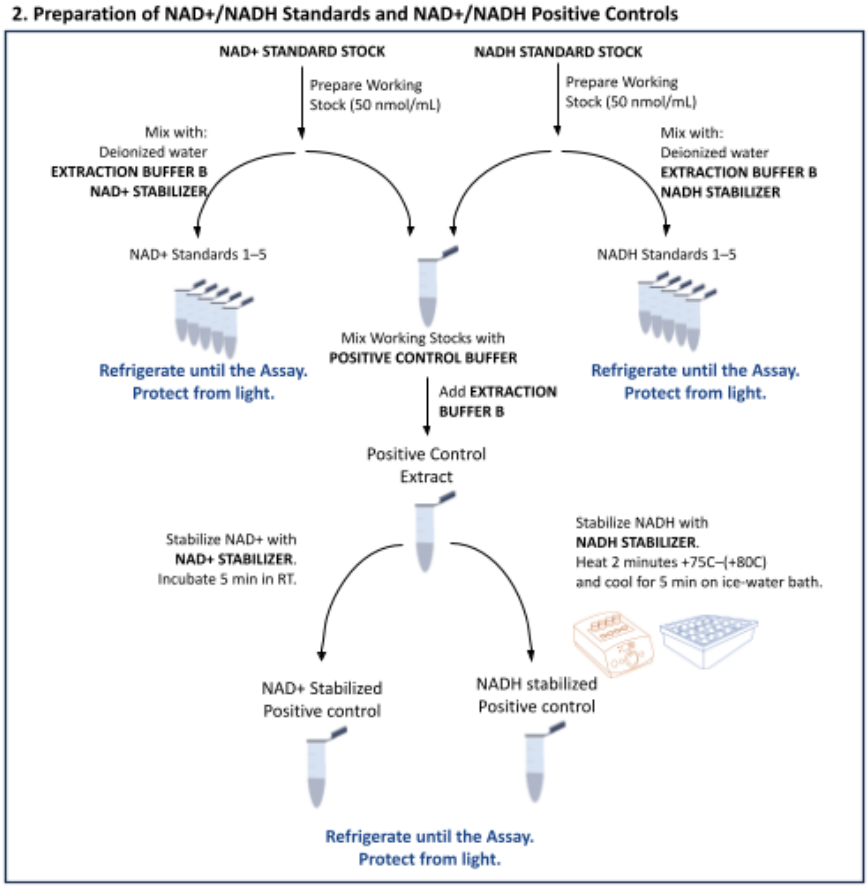
WORKFLOW FOR NAD+ AND NADH ASSAYS

Process overview for NAD+ and NADH analysis from both cells and tissues is shown.

1. Extraction and NAD+/NADH stabilization



2. Preparation of NAD+/NADH Standards and NAD+/NADH Positive Controls



3. NAD+ and NADH Assays

1. Equilibrate reagents, Sample Extracts, Positive Control Extracts and Standards to RT.

2. Pipette Standards, Sample Exteracts and Positive Control Extracts on the NAD and NADH plate(s) according to plate Layout. **Work on one plate at the time.**

Work in dim conditions. Protect the plate and reagent reservoirs from light.

3. Prepare the Assay buffer (**ASSAY BUFFER C** + **ASSAY COLOR REAGENT**). Add Assay buffer to Blank wells.

4. Add **NAD ENZYME** to the Assay buffer (**ASSAY BUFFER C** + **ASSAY COLOR REAGENT**). Add (+Enzyme) Assay buffer to the Standard, Positive Control and Sample wells.


NAD+ 4–6 min


NADH 6–10 min

5. Add **STOP SOLUTION** and measure absorbance at 573 nm.

6. Calculate the results and confirm assay quality (Positive Control readings).

4. Measurement of protein content for normalization

- Solubilization of the cell pellet with **SOLUBILIZATION BUFFER D**
- Sonication
- Standard protein concentration assay

EXTRACTION OF NAD+ AND NADH FROM TISSUE SAMPLES

This section provides guidance on the extraction of NAD+ and NADH from frozen tissue samples. After extraction, NAD+ and NADH are individually stabilized in obtained extract for separate colorimetric assays. If assay cannot be done at the day of the extraction, obtained extracts (after centrifugation) can be stored at -80°C – -70°C for one week before stabilization on the day of assays.

NOTES:

- Tissue samples must be pre-weighted before the extraction to enable normalization of measured NAD+ and NADH concentrations to mg of tissue mass. Refer to SAMPLE HANDLING AND STORAGE for more information.
- EXTRACTION BUFFER B must be equilibrated to 50°–55°C (optimum range) before contacting the samples.
- Keep frozen tissue samples on dry ice (deep frozen) before contact with warm EXTRACTION BUFFER B.
- Extract one sample at a time.
- Depending on the tissue type, e.g. soft or hard, the homogenization time and force can vary considerably. Follow the manufacturers' instructions and guidelines for different tissues when using automated bead-based homogenizing systems or manual homogenizing set-ups.
- The volume of the extract can be measured with a calibrated pipette or by weighing the extract in the 1.5 ml microtube on a precision scale (followed by subtraction of an average weight for the 1.5 mL microtube).

Materials:

Precision scale	Refer to MATERIALS REQUIRED-Table
Tissue homogenizer and lysis tubes	Refer to MATERIALS REQUIRED-Table
Water bath set at 50°–55°C	Refer to MATERIALS REQUIRED-Table
Dry bath heat block set at 75°–80°C	Refer to MATERIALS REQUIRED-Table
Dry ice	Refer to MATERIALS REQUIRED-Table
Ice-water bath	Refer to MATERIALS REQUIRED-Table
Microcentrifuge	Refer to MATERIALS REQUIRED-Table
Microtubes	Marked for all steps
EXTRACTION BUFFER B	Room temperature
NAD+ STABILIZER	Room temperature
NADH STABILIZER	Room temperature

Guideline for EXTRACTION BUFFER B volume: use 1 mL per 20 mg of tissue.

Extraction procedure using automated homogenization system with bead-containing lysis tubes:

1. Calculate volume of the EXTRACTION BUFFER B needed for each sample using the guideline.
2. Pipette calculated volumes of the buffer into homogenization lysis tubes with beads, close them tightly to prevent evaporation.
3. Equilibrate the tubes with the EXTRACTION BUFFER B at 50°–55°C for 10 min. Extract one sample at a time.
4. Add the frozen tissue piece directly into the warm buffer and homogenize immediately. Adjust homogenization intensity and time to disintegrate tissue sample within a minute. Depending on the tissue type, the presence of connective tissue threads is acceptable. It is expected that the temperature of the homogenate will rise during the homogenization.
5. Place tube with obtained tissue homogenate into the ice-water bath for at least 5 min. Upon cooling of the homogenate protein aggregates will be formed.

↻ Repeat the extraction procedure for the next sample. The ready homogenates can safely wait in the ice-water bath until all samples have been extracted. This is the step where you can have a break after preparing of all homogenates.

6. Mix and transfer the homogenate into a clean microtube using a 1 mL pipette. Ensure all protein aggregates and tissue debris are transferred along with the liquid fraction.
7. Centrifuge the homogenates at 20 000 x g at 4°C for 10 min.
8. Transfer the extracts (supernatants) into clean microtubes and measure their final volumes. Save the pellets for protein content measurement if normalization per protein mass is needed.

TIP: You can measure the extract volume using calibrated pipette or by weighing the tubes with supernatants followed by subtraction of the average weight of the empty 1.5 ml microtube. **Critical note:** recording the exact volume of the extract is critical, as this value is required for the final calculation of your results.

9. Proceed to the Stabilization step. If extracts are analyzed on the same day, the stabilization step must happen within an hour after extracts are prepared.

Optional: If samples cannot be assayed on the same day, the obtained extracts can be frozen at -80°C – -70°C and stored at this temperature for up to one week. In this case, thaw the frozen extracts at room temperature for 10 min before proceeding to the stabilization step described below. Protein pellets can be stored at -20°C or -80°C and protein content measurement can be done on a different day.

Stabilization:

10. Equilibrate the extracts at room temperature for 10 min before proceeding to the stabilization step. Prepare two 100 µL aliquots of each extract into clean microtubes.
11. To the first 100 µL aliquot, add 100 µL of **NAD+ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
12. To the second 100 µL aliquot, add 100 µL of **NADH STABILIZER**. Vortex, and incubate for 2 min in a dry bath set at. Cool down on ice for 5 min.
13. Treatment with the stabilizers results in x2-fold dilution of the sample extract. Protect Stabilized sample extracts from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

TIP: When testing a new tissue type with unknown NAD+ and NADH levels, analyse the original stabilized extract alongside its 2-fold dilution. This proactive step ensures that at least one signal falls within the assay's standard curve if the analyte concentration is too high. To prepare the dilution, transfer 100 µl of the original stabilized extract into clean tube, add 100 µl of deionized water, vortex.

PREPARATION OF STANDARDS

Prepare standards on the day of the assay. Prepare one standard set at a time, starting with NAD+. The working standard stocks prepared here are used to prepare the Positive control mix.

NOTE:

- Use the same pipette for deionized water and Standard working stocks to ensure accuracy.

Materials:

Deionized water	Refer to MATERIALS REQUIRED-Table
1 mM NAD+ STANDARD STOCK	Thaw upon usage. Spin down at low speed before opening.
1 mM NADH STANDARD STOCK	Thaw upon usage. Spin down at low speed before opening.
EXTRACTION BUFFER B	Room temperature
NAD+ STABILIZER	Room temperature
NADH STABILIZER	Room temperature

Protocol:

1. Thaw microtubes with 1 mM NAD+ STANDARD and 1 mM NADH STANDARD for 5 min at room temperature. Protect from light with a foil lid during thawing.
2. Prepare **50 nmol/mL NAD+ working stock** by adding 25 µL of 1 mM NAD+ STANDARD STOCK into 475 µL of deionized water, vortex. Proceed to the preparation of NAD+ standards according to the table below, pipette the reagents in the indicated order, ensuring identical chemical conditions for preparation of both samples and the standards.

NAD+ STANDARD PREPARATION					
STANDARD ID	NAD+ CONCENTRATION (nmol/mL)	Deionized water (µL)	**50 nmol/mL NAD+ working stock (µL)**	EXTRACTION BUFFER B (µL)	NAD+ STABILIZER (µL)
NAD+ ST1	0	100	**0**	450	450
NAD+ ST2	1	80	**20**	450	450
NAD+ ST3	2	60	**40**	450	450
NAD+ ST4	3	40	**60**	450	450
NAD+ ST5	5	0	**100**	450	450

3. Prepare **50 nmol/mL NADH working stock** by adding 25 µL of 1 mM NADH STANDARD STOCK into 475 µL of deionized water, vortex. Proceed with the preparation of NADH standards according to the table below, pipette the reagents in the indicated order, ensuring identical chemical conditions for preparation of both samples and the standards.

NADH STANDARD PREPARATION					
STANDARD ID	NADH CONCENTRATION (nmol/mL)	Deionized water (µL)	**50 nmol/mL NADH working stock (µL)**	EXTRACTION BUFFER B (µL)	NADH STABILIZER (µL)
NADH ST1	0.0	100	**0**	450	450
NADH ST2	0.5	90	**10**	450	450
NADH ST3	1.0	80	**20**	450	450
NADH ST4	1.5	70	**30**	450	450
NADH ST5	2.0	60	**40**	450	450

4. Vortex all Standards. Protect the Standards from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

PREPARATION OF POSITIVE CONTROL

The positive control is prepared before the assay right after the preparation of the Standards due to the limited stability of 50 nmol/mL NADH working stock. The purpose of the 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. The expected concentration of NAD+ in the stabilized extract of the Positive control is 2.3 - 2.7 nmol/mL and NADH 0.7 – 1.0 nmol/mL. There is no need to perform any back-calculations for the original Positive Control mix.

Materials:

Dry bath heat block set at 75°–80°C	Refer to MATERIALS REQUIRED-Table
Ice-water bath	Refer to MATERIALS REQUIRED-Table
50 nmol/mL NAD+ working stock	From Preparation of standards, room temperature
50 nmol/mL NADH working stock	From Preparation of standards, room temperature
POSITIVE CONTROL (BUFFER)	Room temperature
EXTRACTION BUFFER B	Room temperature
NAD+ STABILIZER	Room temperature
NADH STABILIZER	Room temperature

Protocol:

1. Prepare the Positive control mix in a microtube. Vortex.

   60 µL of **POSITIVE CONTROL (BUFFER)**
   30 µL of **50 nmol/mL NAD+ working stock**
   10 µL of **50 nmol/mL NADH working stock**

2. Add 200 µL of **EXTRACTION BUFFER B** into the microtube with Positive control mix, vortex.

   NOTE: Positive control is extracted with EXTRACTION BUFFER B at room temperature, no heating is needed.

3. Prepare two 100 µL aliquots of the Positive control extract into clean microtubes.
4. To the first 100 µL aliquot, add 100 µL of **NAD+ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
5. To the second 100 µL aliquot, add 100 µL of **NADH STABILIZER**. Vortex, and incubate for 2 min in the dry bath at 80°C. Cool down on ice-water bath for 5 min.
6. Protect the stabilized NAD+ and NADH Positive control extracts from light and keep them refrigerated (4-8°C) before pipetting on the Assay plates.

ASSAY PROCEDURE

The Assay procedure is the same for both NAD+ and NADH assay plates.

Blanks are used to correct for unspecific background signals from unspecific interactions between the extract components and the ASSAY COLOR REAGENT in the Master mix. Sample blanks are incubated with Master mix without added NAD ENZYME. Positive control does not require a separate blank. If all analyzed samples are of the same tissue type, sample blanks can be prepared from four representative stabilized sample extracts. If several tissue types are analyzed, every sample type should have at least one well with a sample blank representing this tissue type.

NOTE:

Steps 1.–2. are performed under normal light conditions. **Steps from 3. onwards are performed in dim conditions (refer to PRACTICAL CONSIDERATIONS: Protection from light).**

NOTE: Use separate reservoir for Master mix and STOP SOLUTION.

Materials:

Spectrophotometric Reader	Refer to MATERIALS REQUIRED-Table
ASSAY BUFFER C	Room temperature
ASSAY COLOR REAGENT	Room temperature
NAD ENZYME	Thaw upon usage. Spin down at low speed before opening.
STOP SOLUTION	Room temperature

Assay protocol

Perform the NAD+ and NADH assays on separate plates. Work on one Assay at a time.

1. Equilibrate the Standards, Stabilized sample extracts, and Stabilized Positive controls for 10 min at room temperature before pipetting onto the plate.
2. According to the recommended plate layout (see next page), pipette on the 96-well plate:

20 µL Standards (ST 1–5) in duplicates

20 µL of stabilized Positive control and Stabilized sample extracts in duplicates (Samples, S)

20 µL of selected blanks (BL from Samples 1–4) as instructed above.

NOTE: From this step onwards, work in dim conditions.

3. Prepare the Master mix by adding **ASSAY COLOR REAGENT** into **ASSAY BUFFER C**; mix gently by rotation.

NOTE: Protect the Master mix in the reservoir and plate during pipetting with an aluminium foil lid.

4. Add 190 µL of the Master mix WITHOUT NAD ENZYME into each of the four sample blank wells (BL S1–4) using single channel pipette directly from the amber bottle with the Master mix
5. Add 40 µL of NAD **ENZYME** into the bottle with the remaining Master mix. Mix gently by swirling, avoid foaming. Pour the Master mix with the added enzyme into the reservoir.
6. Add 190 µL of the Master mix WITH NAD ENZYME to all remaining wells using a multichannel pipette. Avoid foaming and direct light exposure. Immediately cover the ready plate with the aluminum foil lid.

NAD+ assay: incubate the covered plate for 4–8 min at room temperature.

NADH assay: incubate the covered plate for 10–15 min at room temperature.

Note: Monitor color development closely by opening occasionally the aluminum foil lid. Stop the reaction when a clear color intensity gradient is visible across the standards, and a distinct signal difference appears between the samples and samples blanks. If the sample signal is very weak after the recommended incubation time, the reaction time can be extended up to 15 min for the NAD+ assay, and up to 30 min for the NADH assay. Please note that extended incubation will cause high-concentration NAD+ standards to reach absorbance values close to 2.0 Optical Units; these saturated standards must be excluded from the standard curve calculation.

7. 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. Keep the same pace for pipetting STOP SOLUTION onto the wells as was used for pipetting the Master mix to keep same reaction time across all wells on the plate. Avoid foaming. Gently shake the plate by hand on a table surface and remove any bubbles with a needle.
8. 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 uniformly increase in all the wells. This is expected due to the non-enzymatic background process in the Master mix.

RECOMMENDED PLATE LAYOUT FOR NAD+ OR NADH MEASUREMENTS

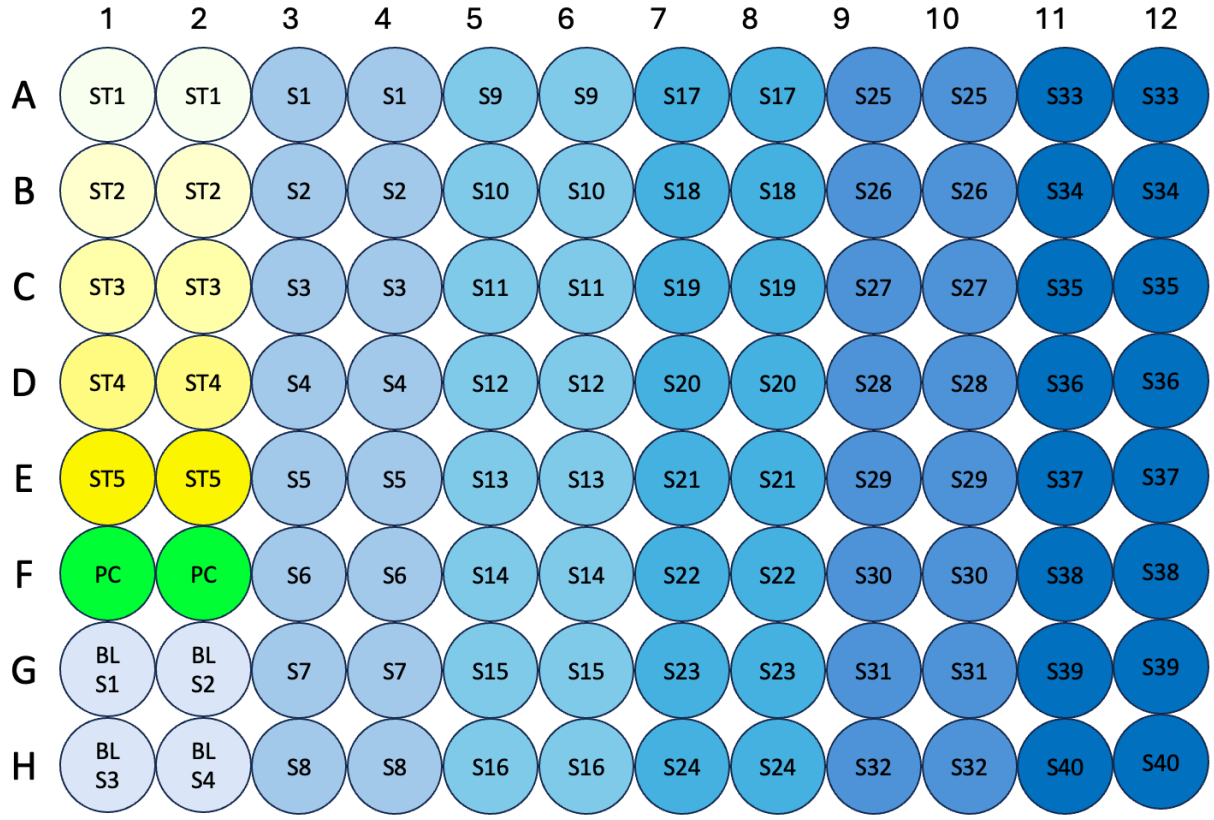


Plate layout for NAD+ or NADH assays: ST = standard, BL S = Sample blank, PC = stabilized Positive control, S = stabilized samples with unknown metabolite concentration. Note that the BL (Sample blanks) of the selected samples are analyzed in the Master mix without added NAD ENZYME.

MEASUREMENT OF PROTEIN CONTENT

Measurement of protein content in the sample is needed for normalization of the NAD+ and NADH assay results on mg of total protein. This kit provides the SOLUBILIZATION BUFFER D for solubilization of the protein pellets obtained after centrifugation of sample homogenates for subsequent measurement using any standard protein assay.

NOTE:

- Before adding the SOLUBILIZATION BUFFER D, remove all the liquid from the microtube with the pellet obtained after the extract separation.
- Solubilization requires short sonication using a microtip. Do not solubilize by pipetting up and down.

Materials:

Sonicator	Refer to MATERIALS REQUIRED-Table
SOLUBILIZATION BUFFER D	Room temperature
100 mM Phosphate Buffer (PB)	Room temperature
Protein assay kit	Refer to MATERIALS REQUIRED-Table

Protocol:

1. Thaw **SOLUBILIZATION BUFFER D** at room temperature.
2. If frozen pellets are analyzed, they must be equilibrated to room temperature for 10 min before adding SOLUBILIZATION BUFFER D.
3. Add 250 µL of SOLUBILIZATION BUFFER D to each tissue protein pellet (if sample mass used for extraction was in the range of 10-25 mg).
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 Phosphate Buffer, pH 7.0 (e.g., mix 10 µL of the sample with 90 µL of PB, 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

POSITIVE CONTROL (ASSAY QUALITY CONTROL)

Positive control aims to monitor the efficiency of the NAD+ and NADH stabilization and the performance of the colorimetric assay. Before calculating your sample results, confirm that your stabilized Positive control extracts perform as expected.

NAD+:

In the NAD+ assay, concentration of NAD+ in the stabilized Positive Control extract is expected to be within 2.3 - 2.7 nmol/ml.

NADH:

In the NADH assay, concentration of NADH in the stabilized Positive Control extract is expected to be within 0.7 - 1.0 nmol/ml.

CALCULATION OF ANALYTE CONCENTRATION IN STABILIZED SAMPLE EXTRACTS AND NORMALIZATION ON PROTEIN OR TISSUE MASS

Calculate results for each plate separately as instructed below. The TYPICAL DATA section below presents examples of standard curves and the calculation formulas.

1. Calculate the average of the absorbance readings for each standard (ST1–ST5).
2. Create a standard curve by plotting the mean absorbance for each Standard on the y-axis against the known Standard concentration (in nmol/mL) on the x-axis. Fit the response in the standards with the linear regression curve.
3. Using the formula of linear regression fit, calculate the analyte concentration in each well on the plate (S and BL S).
4. Calculate the average of duplicates of each stabilized sample extract.
5. Calculate the average of the sample blanks (BL S1–4). The obtained value represents an unspecific signal of the stabilized extract used for sample normalization.
6. Correct for unspecific signals by subtracting the average of blanks from the average of sample concentrations. The obtained concentration represents nmol/ml concentration of analyte in stabilized sample extract. Next, this concentration must be normalized on one mg of total protein or tissue mass considering all dilution steps during preparation of the stabilized extract. Use formulas shown below for normalization process.

NOTE: If the stabilized extracts have been additionally diluted, this dilution has to be taken into account during normalization.

NORMALIZATION

For normalization per mg of tissue, use the following formula if original stabilized extract was added to the assay:

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

For normalization per mg of tissue, use the following formula if x2-fold diluted stabilized extract was added to the assay:

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

For normalization per mg of protein, use the following formula, if original stabilized extract was added to the assay:

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

For normalization per mg of protein, use the following formula, if x2-fold diluted stabilized extract was added to the assay:

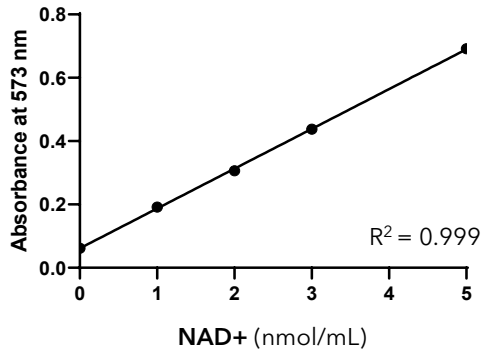
$$Conc.\frac{nmol}{mg} protein = \frac{Conc.\ in\ stabilized\ extract, \frac{nmol}{mL} * 2 * 2 * V, mL\ of\ the\ extract}{total\ protein, mg}$$

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

TYPICAL DATA

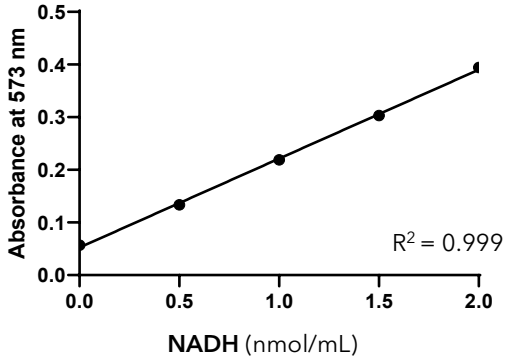
The standard curve and the concentrations in the stabilized sample extracts are provided for demonstration only and should never be used instead of the real-time calibration curve.

STANDARD CURVE FOR NAD+



Standard	NAD+ (nmol/mL)	Absorbance (573 nm) 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 (573 nm) Assay time: 6 min
ST1	0	0.057 0.057
ST2	0.5	0.133 0.134
ST3	1	0.224 0.214
ST4	1.5	0.300 0.306
ST5	2	0.395 0.394

NOTES

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.