NADMED

EN

Q – NAD TISSUES/CELLS

NAD+ and NADH assay kit

Quantitative assay for TISSUES and CELLS

Version 3.0

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 Tissues/Cells NAD+ and NADH assay kit: quantitative assay kit for tissues and cells

Catalog number:

RUO_003

Storage:

-85°C– -70°C upon arrival

IFU issued:

April 2024

Manufacturer:
NADMED Ltd / Oy
www.nadmed.com
info@nadmed.com
Haartmaninkatu 4, Building 14
00290 Helsinki
FINLAND

SYMBOLS ON THE PACKAGING


Contains flammable liquid and vapor. Refer to PRECAUTIONS AND WARNINGS


Warning/danger. Refer to PRECAUTIONS AND WARNINGS

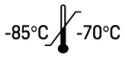

Consult instructions for usage


Use-by date

Catalogue number

Batch code


Manufacturer



Upper limit of storage temperature


Do not use if package is damaged

Number or reactions

Research-use only


Protect from direct light


Keep dry

TABLE OF CONTENTS

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 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 HANDLING AND STORAGE

Limitations:

- This kit is designed for NAD+ and NADH measurement in cultured cells and tissue samples. This assay is NOT suitable for measuring blood.
- Any storage of samples should be done at -80 – -70˚C. Frozen samples must be continuously maintained frozen before the assay. Subsequent freeze-thawed cycles are not allowed.

Tissue requirements and handling:

Sample size: For tissues, we recommend using 20 mg for reliable measurement, with 10 mg as the lower limit (mouse tissues as reference). If possible, weigh the tissue piece at the time of collection and aim for a sample size of approximately 10–25 mg. If frozen pieces of tissue need to be processed for the assay, the tissue must remain frozen during the cutting and weighing.

Collection and storage: Tissue samples must be snap-frozen in liquid nitrogen as soon as possible after collection. Preferably, collect samples consistently from the same area of an organ to minimize variability. If the tissue has significant blood contamination, rinse it with ice-cold PBS and tap it with a paper towel before freezing.

Follow EXTRACTION NAD+ AND NADH FROM TISSUE for processing of the tissue pieces.

Cell requirements and handling:

Sample size: For cultured cells, 1.5-2 million cells at minimum are needed for reliable testing (cultured fibroblasts and HeLa cells as reference).

Collection, storage, and analysis methods:

Choose the collection and extraction method most suitable for your cell type and experiment. Aim for preserving the integrity of the culture and maximal collection efficiency.

1. This IFU contains detailed instructions for NAD+ and NADH collection and simultaneous extraction directly on the culture dish using the EXTRACTION BUFFER provided in the kit. Refer to EXTRACTION OF NAD+ AND NADH DIRECTLY ON A CULTURE DISH.
2. Alternatively, cells can be collected from various types of culture for the analysis of NAD+ and NADH assay following the EXTRACTION OF NAD+ AND NADH FROM CELL PELLETS.

   NOTE: Cultured cells should be washed with excess PBS before NAD+ and NADH assay to remove proteins of the culture medium (this is especially important if results are normalized per protein).

   Floating or non-adherent cells can be collected directly by centrifugation followed by a washing step with PBS. Adherent cells can be collected using enzymatic methods, such as trypsinization. Wash the cells with PBS on the dish before detaching them. Pellet the cells in a microtube by centrifugation at 750 x g for 5 minutes and discard the supernatant.

   Freshly collected pellets should be kept on ice and directed to the EXTRACTION OF NAD+ AND NADH FROM CELL PELLETS without delay, or the collected pellets should be frozen immediately and stored at -80˚– -70˚C until analysis.

REAGENT STORAGE, STABILITY AND PREPARATION

REAGENT	DESCRIPTION (*)	PREPARATION (**)	STORAGE and STABILITY (**, ***)
EXTRACTION BUFFER	2x 25 mL Sufficient for 40 samples	Ready for use. Equilibrate to room temperature before use.	Stable for one month at 4°–8°C, protect from light.
NAD+ STABILIZING REAGENT	8 mL Sufficient for 40 samples	Ready for use. Equilibrate to room temperature before use.	Stable for two weeks at room temperature.
NADH STABILIZING REAGENT	8 mL Sufficient for 40 samples		
POSITIVE CONTROL (BUFFER)	200 µL		
BUFFER D	12 mL Sufficient for 40 protein measurement		
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.	
BUFFER C	2x 19 mL One aliquot per 96-well plate	Equilibrate to room temperature before use. Mix one bottle of ASSAY COLOR REAGENT with one bottle of BUFFER C (= Master mix sufficient for one 96-well plate). Use Master mix immediately. Protect from light. Do not shake vigorously. Discard leftovers.	Stable for 12 hours at room temperature after thawing.
ASSAY COLOR REAGENT	2x 3 mL One aliquot per 96-well plate		Stable for 3 hours at room temperature after thawing.
NAD+ STANDARD STOCK	40 µL (1 mM) Sufficient for Standards and Positive control	Equilibrate to room temperature before use. See preparation guide on page 14.	Should be used immediately after thawing. Standards should be protected from light.
NADH STANDARD STOCK	40 µL (1 mM) Sufficient for Standards and Positive control		
ENZYME	2x 40 µL One aliquot per 96-well plate	Equilibrate to room temperature before use. Add to the Master mix after processing of plate blanks.	Should be used immediately after thawing.

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

** Room temperature: 18–25°C

*** Before opening, all kit components should be stored at -85°C – -70°C. Avoid temperature fluctuations in the freezer.

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 may cause eye irritation. Handle with care; use goggles.

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

NADH STABILIZING REAGENT 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.

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 extraction or assay performance, check the NADMED troubleshooting guide at https://www.nadmed.com/documents/.

NOTE: Levels of NAD+ and NADH are **tissue- and cell type-specific.** Levels can be modulated by supplementation with NAD precursors or drugs. We recommend first extracting samples according to the IFU guidelines. If the absorbance values of stabilized sample extracts in the Assay are:

a. very low (close to the absorbance of ST1 with 0 nmol/mL of NAD or NADH), the amount of starting material needs adjustment.
   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. NADs must be extracted from frozen samples.

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

MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

CATEGORY	ITEM	SPECIFICATIONS/REQUIREMENTS
Consumables	Deionized water	Milli-Q water from a water purification system 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). NOT compatible with NADMED assay: a) molecular biology grade sterile microtubes that are free of endotoxin, pyrogen, human DNA, and low retention (chemically sterilized) b) microtubes intended for protein work marked "LoBind".
	96-well plates (2 pieces)	Use non-sterile, transparent, polystyrene flatbottom plates with medium protein binding intended for colorimetric assays (e.g. Revvity, formerly PerkinElmer, 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 ice and pour cold tap water on top. Liquid part of the sample is immersed, but the tube is stays supported by the ice.
	Dry ice	To keep the tissue samples frozen until homogenization
	Aluminium foil	Use foil to protect samples, standards, and the plates from light during assay as specified in the instructions.
	Homogenization tubes with beads	Mixed (mid + large) or large beads depending on the tissue type (e.g. Precellys lysis kit, ref. P000918-LYSK0-A)
Equipment and Machinery	Tissue homogenizer	To homogenize tissues, e.g. Precellys OR Dounce (glass) homogenizer
	Precision scale	Scale to weigh tissue pieces ranging from 10–25 mg.
	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) Measuring absorbance at 570–573 nm wavelength b) Adjustable scanning light brightness/intensity to "low". Alternatively, possibility to adjust the brightness based on the number of flashes per measurement (set to 5–10 flashes).
	Warm water bath	Adjustable temperature to 55°C is required.

	Dry bath Heat Block fitted for 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 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 the correct temperature is NOT reached with 80°C setting in 5 min: a) ensure the tubes fit the block tightly b) increase the target temperature of your device
Optional (protein concentration measurement)	BCA Protein assay kit or similar Sonicator with a tip fitting into 1.5 ml microtube 100 mM PBS, 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.	

PRACTICAL CONSIDERATIONS

CATEGORY	INSTRUCTIONS
Limitations	Read the SAMPLE HANDLING AND STORAGE carefully. This assay is designed for tissues and cells and is NOT suitable for measuring NAD+ and NADH in blood. 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 (protect from light), NAD+ STABILIZING REAGENT, NADH STABILIZING REAGENT, BUFFER D, and STOP SOLUTION to room temperature one day before the assay. Take 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 homogenizing maximum two samples at a time when using an automated homogenizer OR one when using glass homogenizer. Obtained homogenates can be kept on ice until all the samples are homogenized 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. 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 with pipette tips when working with multi-channel pipettes. High-precision pipettes and beveled tips with less retention will improve the precision. BUFFER C and STOP SOLUTION contain detergents. To avoid bubbles, pipette the Master mix and STOP SOLUTION into the wells by pressing the pipette to the first stop position only. Remove any bubbles in the wells 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, tasks such as 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).

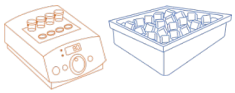
WORKFLOW FOR NAD+ AND NADH ASSAYS

1. Extraction and NAD+/NADH stabilization

EXTRACTION OF NAD+ AND NADH FROM TISSUE
or
EXTRACTION OF NAD+ AND NADH DIRECTLY ON A CULTURE DISH
or
EXTRACTION OF NAD+ AND NADH FROM CELL PELLETS

Stabilize NAD+ with **NAD+ STABILIZING REAGENT**. Incubate 5 min in RT.

Stabilize NADH with **NADH STABILIZING REAGENT**. Heat 2 minutes +75C–(+80C) and cool for 5 min on ice-water bath.



NAD+ stabilized Extracts

Up to 40 samples

NADH stabilized Extracts

Up to 40 samples

Refrigerate until the Assay. Protect from light.

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

NAD+ STANDARD STOCK

Prepare Working Stock (50 nmol/mL)

Mix with: Deionized water **EXTRACTION BUFFER** **NAD+ STABILIZING REAGENT**

NAD+ Standards 1–5

Refrigerate until the Assay. Protect from light.

NADH STANDARD STOCK

Prepare Working Stock (50 nmol/mL)

Mix with: Deionized water **EXTRACTION BUFFER** **NADH STABILIZING REAGENT**

NADH Standards 1–5

Refrigerate until the Assay. Protect from light.

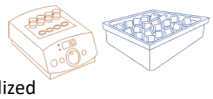
Mix Working Stocks with **POSITIVE CONTROL BUFFER**

Add **EXTRACTION BUFFER**

Positive Control Extract

Stabilize NAD+ with **NAD+ STABILIZING REAGENT**. Incubate 5 min in RT.

Stabilize NADH with **NADH STABILIZING REAGENT**. Heat 2 minutes +75C–(+80C) and cool for 5 min on ice-water bath.



NAD+ Stabilized Positive control

NADH stabilized Positive control

Refrigerate until the Assay. Protect from light.

3. NAD+ and NADH Assays

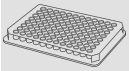
1. Equilibrate reagents, Sample Extracts, Positive Control Extracts and Standards to RT.

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.**

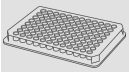
Work in dim conditions. Protect the plate and reagent reservoirs from light.

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

4. Add **ENZYME** to the Assay buffer (**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

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

EXTRACTION OF NAD+ AND NADH FROM TISSUE

This section provides guidance on the extraction of NAD+ and NADH from tissues. After extraction, NAD+ and NADH are individually stabilized in preparation for separate colorimetric assays. Extracts (after centrifugation) can be stored at -80°C – -70°C for one week before stabilization on the day of assays. The final dilution of the initial extract after stabilization is 2x.

NOTE:

- Tissue samples must be pre-weighted before the homogenization to allow normalization of NAD+ and NADH on tissue mass. Refer to SAMPLE HANDLING AND STORAGE for more information.
- EXTRACTION BUFFER needs to stay warm (50°–55°C) when in contact with the sample. Process simultaneously only 1-2 samples.
- Depending on the tissue, the homogenization time and force can vary considerably. Follow the manufacturers' instructions and guidelines for different tissues when using automated bead beating systems or manual homogenizing set-ups.

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	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature

Calculation of EXTRACTION BUFFER volume:

The tissue weight can be obtained either from fresh samples upon collection or from frozen samples. When processing frozen tissues, maintain cooling with liquid nitrogen or dry ice.

Keep tissue samples deep-frozen before contact with warm EXTRACTION BUFFER. The volume of EXTRACTION BUFFER needed for homogenization of each sample based on the ratio:

50 µL of EXTRACTION BUFFER per 1 mg of tissue (= 1 mL per desired 20 mg of tissue).

Extraction:

1. Pre-heat EXTRACTION BUFFER to 50°–55°C:
    a. Pipette the calculated volume of EXTRACTION BUFFER into the homogenization tube, close it, and place it into a dry bath pre-heated to 50°–55°C for 5 minutes.

       OR

b. Pre-heat the EXTRACTION BUFFER bottle for 10 minutes in a warm water bath set at 50°–55°C. Pipette the calculated volumes of warm buffer into the homogenization tube just before adding the sample.

2. Add the frozen tissue piece directly into the warm buffer and homogenize immediately. Process only 1–2 samples simultaneously. Continue the homogenization of the tissue sample until the solution is free of visible tissue fragments. Depending on the tissue type, the presence of connective tissue threads is acceptable.
3. Cool down the homogenate in the ice-water bath for at least 5 min. NAD+ and NADH metabolites are stable in obtained homogenate when kept on ice.

↻ Repeat the homogenization steps with the next batch(es) of a maximum of two samples.

4. Mix and transfer the homogenate into a clean microtube using a 1 mL pipette.
5. Centrifuge the homogenates at 20 000 x g at 4°C for 10 min.
6. Transfer the extracts (supernatants) into clean microtubes. Save the pellets for protein measurement (if normalization per protein content is desired).
7. Proceed to the Stabilization step without delay.

Optional: The extracts can be stored at -80°C – -70°C for 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 to measure the protein content on a different day.

Stabilization:

8. Equilibrate the extracts to room temperature and prepare two 100 µL aliquots into clean microtubes.
9. To the first 100 µL aliquot, add 100 µL of **NAD+ STABILIZING REAGENT**. Vortex, and incubate at room temperature for 5 min.
10. To the second 100 µL aliquot, add 100 µL of **NADH STABILIZING REAGENT**. Vortex, and incubate in a dry bath at 75°–80°C for 2 min. Cool down on ice for 5 min.
11. Protect Stabilized sample extracts from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

EXTRACTION OF NAD+ AND NADH DIRECTLY ON A CULTURE DISH

This section provides guidance on the simultaneous collection and extraction of NAD+ and NADH from cultured cells directly on the culture dish. After extraction, NAD+ and NADH are individually stabilized in preparation for separate colorimetric assays. Extracts (after centrifugation) can be stored at -80°C – -70°C for one week before stabilization on the day of assays. The final dilution of the initial extract after stabilization is 2x.

NOTE: We recommend processing one plate, well, or a bottle at a time to avoid differences in the handling times. EXTRACTION BUFFER needs to stay warm (50°–55°C) when in contact with the sample.

TIP: The volume of the extract can be measured with a calibrated pipette or by weighing the extract on a precision scale (assuming an average weight for the 1.5 mL microtube).

Materials:

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
Ice-water bath	Refer to MATERIALS REQUIRED-Table
Microcentrifuge	Refer to MATERIALS REQUIRED-Table
Microtubes	Marked for all the steps
EXTRACTION BUFFER	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature

Guidelines for EXTRACTION BUFFER volume:

Culture dish	Volume of EXTRACTION BUFFER
T75 flask	2 ml
10 cm culture dish	1 ml
6-well plate	400 µL (per well)

Extraction:

1. Pre-heat **EXTRACTION BUFFER** to 50°–55°C before extraction by placing the bottle into a water bath set to 50°–55°C (at least for 10 min before extraction).
2. Wash the cells with PBS.
3. Remove PBS and add warm EXTRACTION BUFFER directly to the cell culture dish.
4. Distribute the volume over the cells by tilting the cell culture bottle or dish.
5. Collect the cell material by scraping and carefully transfer all material into a clean microtube. Place the homogenate in a microtube in an ice-water bath for the time needed to collect cells from the rest of the dishes.

↻ Repeat the extraction and collection steps with the next well or dish.

6. Centrifuge all the homogenates at 20 000 x g for 10 min at 4°C.

7. Transfer the extracts (supernatants) into clean microtubes. Save the pellets for protein measurement (if normalization per protein content is desired).
8. Measure the volume of the extract. The volume will be needed for the calculation and normalization of the results.
9. Proceed to the Stabilization step without delay.

- Optional: The extracts can be stored at -80°C – -70°C for one week. In this case, thaw the frozen extracts at room temperature for 10 min before proceeding to the stabilization steps described below. Protein pellets can be stored at -20°C or -80°C to measure the protein content on a different day.

Stabilization:

10. Equilibrate the extracts to room temperature and prepare two 100 µL aliquots into clean microtubes.
11. To the first 100 µL aliquot, add 100 µL of **NAD+ STABILIZING REAGENT**. Vortex, and incubate at room temperature for 5 min.
12. To the second 100 µL aliquot, add 100 µL of **NADH STABILIZING REAGENT**. Vortex, and incubate in a dry bath at 75°–80°C for 2 min. Cool down on ice for 5 min.
13. Protect Stabilized sample extracts from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

EXTRACTION OF NAD+ AND NADH FROM CELL PELLETS

This section provides guidance on the extraction of NAD+ and NADH from pre-pelleted cell cultures (fresh and frozen). After extraction, NAD+ and NADH are individually stabilized in preparation for separate colorimetric assays. Extracts (after centrifugation) can be stored at -80°C – -70°C for one week before stabilization on the day of assays. The final dilution of the initial extract after stabilization is 2x.

NOTE:

- Cells need to be free of culture media proteins, especially if normalization to protein amount is desired. Refer to SAMPLE HANDLING AND STORAGE.
- EXTRACTION BUFFER needs to stay warm (50°–55°C) when in contact with the sample. We recommend processing a maximum of two samples at a time.

Materials:

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 the steps
EXTRACTION BUFFER	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature

Guideline for EXTRACTION BUFFER volume:

300 µL of EXTRACTION BUFFER per 1.5–2 million cells (fibroblasts and HeLa cells are reference). If you have collected considerably more, consider adjusting the volume.

Extraction:

1. Pre-heat **EXTRACTION BUFFER** to 50°–55°C before extraction by placing the bottle into a water bath set to 50°–55°C (at least for 10 min before extraction).
2. Pellets can be processed either fresh or frozen.

    a) keep freshly collected cells cooled in an ice-water bath until extraction.

    b) thaw deep-frozen pellets in an ice-water bath for 2 min before extraction.

    NOTE: Homogenize a maximum of two samples at a time.

3. Pipette 300 µL (or adjusted volume based on cell amount) of warm EXTRACTION BUFFER to the cell pellet and resuspend by a few cycles of up-and-down pipetting until there are no cell clumps.
4. Incubate the homogenate at 50°–55°C for 2 min (in the water bath or dry-bath heat block).
5. Cool down 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 the homogenization steps with the next batch(es) of two samples.

6. Centrifuge all the homogenates at 20 000 x g for 10 min at 4˚C.
7. Transfer the extracts (supernatants) into clean microtubes. Save the pellets for protein measurement.
8. Proceed to the Stabilization step without delay.

Optional: The extracts can be stored at -80˚C – -70˚C for one week. In this case, thaw the frozen extracts at room temperature for 10 min before proceeding to the stabilization steps described below. Protein pellets can be stored at -20˚C or -80˚C to measure the protein content on a different day.

Stabilization:

9. Equilibrate the extracts to room temperature and prepare two 100 µL aliquots into clean microtubes.
10. To the first 100 µL aliquot, add 100 µL of **NAD+ STABILIZING REAGENT**. Vortex, and incubate at room temperature for 5 min.
11. To the second 100 µL aliquot, add 100 µL of **NADH STABILIZING REAGENT**. Vortex, and incubate in a dry bath at 75°–80˚C for 2 min. Cool down on ice for 5 min.
12. Protect Stabilized sample extracts from light and keep them refrigerated (4°–8˚C) before pipetting on the Assay plates.

PREPARATION OF STANDARDS

Prepare standards on the day of the assay. 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 improve accuracy.
- The working stocks of NAD+ and NADH should be directed to PREPARATION OF POSITIVE CONTROL right after finalizing standard preparation.

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	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	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 with the preparation of NAD+ standards according to the table below, pipette the reagents in the indicated order.

NAD+ STANDARD PREPARATION					
STANDARD ID	NAD+ CONCENTRATION (nmol/mL)	Deionized water (µL)	**50 nmol/mL NAD+ working stock (µL)**	EXTRACTION BUFFER (µL)	NAD+ STABILIZING REAGENT (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 NAD+ standards according to the table below, pipette the reagents in the indicated order.

NADH STANDARD PREPARATION					
STANDARD ID	NADH CONCENTRATION (nmol/mL)	Deionized water (µL)	**50 nmol/mL NADH working stock (µL)**	EXTRACTION BUFFER (µL)	NADH STABILIZING REAGENT (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 the Standards. Protect Standards from light and keep 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 Positive control is 4.0–4.5 nmol/mL and NADH 1.5–1.9 nmol/mL.

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	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature

Protocol:

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

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

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

   NOTE: Positive control is extracted with EXTRACTION BUFFER 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+ STABILIZING REAGENT**. Vortex, and incubate at room temperature for 5 min.
5. To the second 100 µL aliquot, add 100 µL of **NADH STABILIZING REAGENT**. Vortex, and incubate for 2 min in the dry bath at 80°C. Cool down on ice 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 measurements. Perform the NAD+ and NADH assays on separate plates. Work on one Assay at a time.

Blanks are used to correct for unspecific background signal from unspecific interaction between the extract components and ASSAY COLOR REAGENT in the Master mix. Sample blanks are incubated with Master mix without added ENZYME. If all analyzed samples are of the same type (tissue or cell type), sample blanks are to be prepared at minimum 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 sample type. Positive control does not require a separate blank.

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).**
- Use separate reservoir for Master Mix and STOP SOLUTION.

Materials:

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

Protocol:

1. Equilibrate the Standards, Stabilized sample extracts, and Stabilized Positive controls for 5 min at room temperature before pipetting onto the plate.
2. According to the recommended plate layout below, pipette on the 96-well plate:

   20 µL Standards (ST1–5) in duplicates
   20 µL of stabilized Positive control and Stabilized sample extracts in duplicates (Unknowns, UNK)
   20 µL of selected blanks (BL UNK1–4) as instructed above.

From this step onwards, work in dim conditions.

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

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

4. Add 190 µL of the Master mix WITHOUT ENZYME into each of the four sample blank wells (BL UNK1–4).
5. Add 40 µL of **ENZYME** into the bottle with the remaining Master mix. Mix gently, avoid foaming. Pour the Master mix with the added enzyme into the reservoir.
6. Add 190 µL of the Master mix WITH ENZYME to all remaining wells using a multichannel pipette. Avoid foaming and light. Immediately cover the ready plate with the aluminum foil lid.
7. **NAD+ assay**: incubate the covered plate for 4–6 min at room temperature.

   NADH assay: incubate the covered plate for 6–10 min at room temperature.

NOTE: 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 intensive signal is observed. The color intensity in NADH assay is generally lower than in NAD+ due to lower concentration of NADH in the Standards and the tissues and/or cells.

8. 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. Gently shake the plate by hand on a table surface and remove any bubbles with a needle.
9. 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 and 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

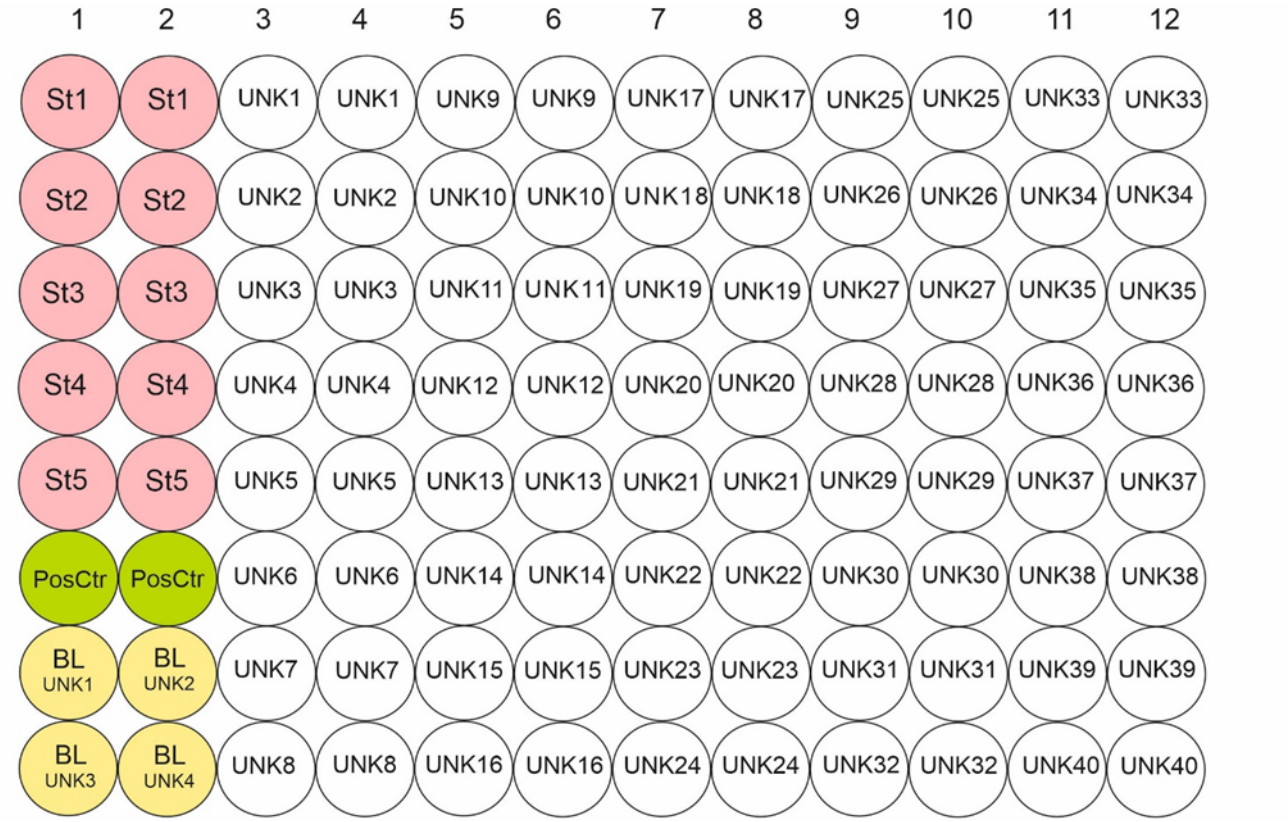


Plate layout for NAD+ or NADH assays: St = standard, BL = blank, PosCtr – stabilized Positive control, UNK = stabilized samples with unknown metabolite concentration. Sample blanks of the selected samples are analyzed in the Master mix without added ENZYME.

MEASUREMENT OF PROTEIN CONTENT

Measurement of protein concentration is needed for normalization of the NAD+ and NADH concentrations to the total protein abundance in the sample. Especially for the cultured cells, the protein content will provide much more accurate normalization than cell count. This IFU provides the BUFFER D for solubilization of the protein pellets for measurement with any standard protein concentration assays.

NOTE:

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

Materials:

Sonicator	Refer to MATERIALS REQUIRED-Table
BUFFER D	Room temperature
100 mM PBS	Room temperature
Protein assay kit	Refer to MATERIALS REQUIRED-Table

Protocol:

1. Thaw **BUFFER D** at 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

POSITIVE CONTROL (ASSAY QUALITY CONTROL)

Positive control is not a reference, but it aims to monitor the efficiency of the NAD+ and NADH stabilization and colorimetric assay. Before calculating your sample results, confirm that your Positive controls perform as expected.

NAD+:
In an NAD+ assay, the amount of light absorbed by the stabilized NAD+ Positive control should be within the range observed for standards ST3 and ST4. This absorbance range corresponds to an NAD+ concentration of between 4.0–4.5 nmol/mL (after correction of 2x dilution).

NADH:
In an NADH assay, the amount of light absorbed by the stabilized NADH Positive control should equal ST2 (+/-0.05 optical units). This absorbance corresponds to an NADH concentration of 1.5–1.9 nmol/mL (after correction of 2x dilution).

SAMPLE RESULTS

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

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. Calculate a simple linear regression fitting of the standard curve.
3. Using the formula of linear regression for the standard curve, calculate the concentration in each of the Sample and Blank wells (UNK and BL UNK).
4. Calculate the average of duplicates of each stabilized sample extract.
5. Calculate the average of the sample blanks (BL UNK1–4). 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 values are nmol/mL in 20 µL of stabilized sample extract added into the assay. Normalize obtained concentration values to tissue mass, total protein, and cell number as shown below.

NOTE: If the stabilized extracts have been additionally diluted, the concentration must be multiplied by the additional dilution factor.

NORMALIZATION

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:

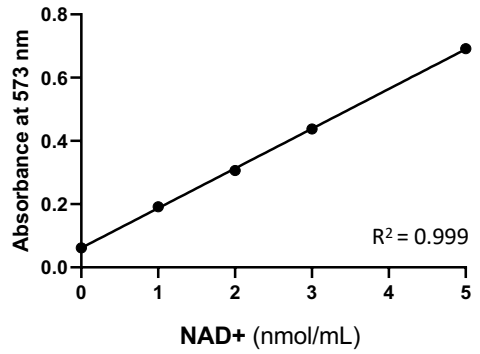
$$Conc.\frac{nmol}{million}cells = \frac{Conc.in\ stabilized\ extract, \frac{nmol}{mL} * 2 * 0{,}3\ mL\ of\ Extraction\ BUFFER}{(cell\ amount)}$$

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

TYPICAL DATA

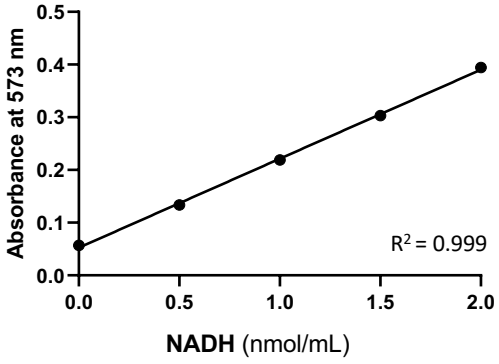
The standard curve and the concentrations in the stabilized sample extracts are provided for demonstration only and should never be used instead of the real-time calibration curve.

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.