

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

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

Instructions for Use

Part I

**Quantitative NAD⁺ and NADH assay kit
for 40 cell 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

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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



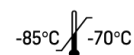
Catalogue number



Batch code



Manufacturer



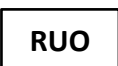
Upper limit of storage temperature



Do not use if package is damaged



Number of reactions



Research-use only



Protect from direct light



Keep dry

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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 setup, 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 its 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 cultured cells, 1.5-2 million cells at minimum are needed for reliable signal in the assays (determined using cultured fibroblasts and HeLa cells as model system). Optimal cell number depends on the cell type, because levels of the metabolites are cell-specific and also affected by culturing conditions. We recommend testing for optimal amount within range from 2 to 10 million cells.

Collection, storage, and analysis methods:

Choose the collection and extraction approach most suitable for your cell culture from the options below. Each option has detailed instructions in this IFU.

1. **Quenching adhesive cell culture on the plate followed by collection of the homogenate for the analysis.** This is recommended approach as it quenches cellular metabolism enabling to assess unaffected steady-state NAD⁺ and NADH levels in cultured cells.
2. **Extraction from frozen cell pellets.** Extraction of NAD⁺ and NADH metabolites can be performed from the frozen cell pellets obtained either by scraping or trypsinization. This approach is less favorable, because NAD⁺ and NADH levels are dynamic and can change in response to collection procedure.

IMPORTANT: Cultured cells should be washed with excess of PBS (PBS should be either RT or of the same temperature as the culture medium) to remove protein in the culture medium before quenching on the plate or collection for pelleting. The washing step is critical for normalization of the results on the sample protein and to avoid interference from the extracellular protein.

Floating or non-adherent cells can be collected directly by centrifugation at ambient temperature followed by a washing step with PBS and centrifugation to remove washing solution. Pellet the cells by centrifugation at 750 x g for 5 minutes and discard the supernatant. Freshly collected pellets should be either extracted immediately or frozen at -70 - -80 C and kept at this temperature before analysis.

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 19.	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 17.	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 **cell type specific**. Levels can be modulated by growth conditions, 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 amount of starting material needs to be increased.

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 <i>in vitro</i> diagnostics (e.g., Sarstedt, ref. 72.690.001). <u>Microtubes NOT</u> 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.
Equipment and Machinery	Aluminium foil	Use foil covers to protect samples, standards, and the plates from light during assay performance as indicated in the instructions.
	Precision scale	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.
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 ₂ HPO ₄ , 38.5 mM KH ₂ PO ₄)
Special	Possibility to work in dim light conditions for the ASSAY part of the measurement. Refer to PRACTICAL CONSIDERATIONS on page 10.

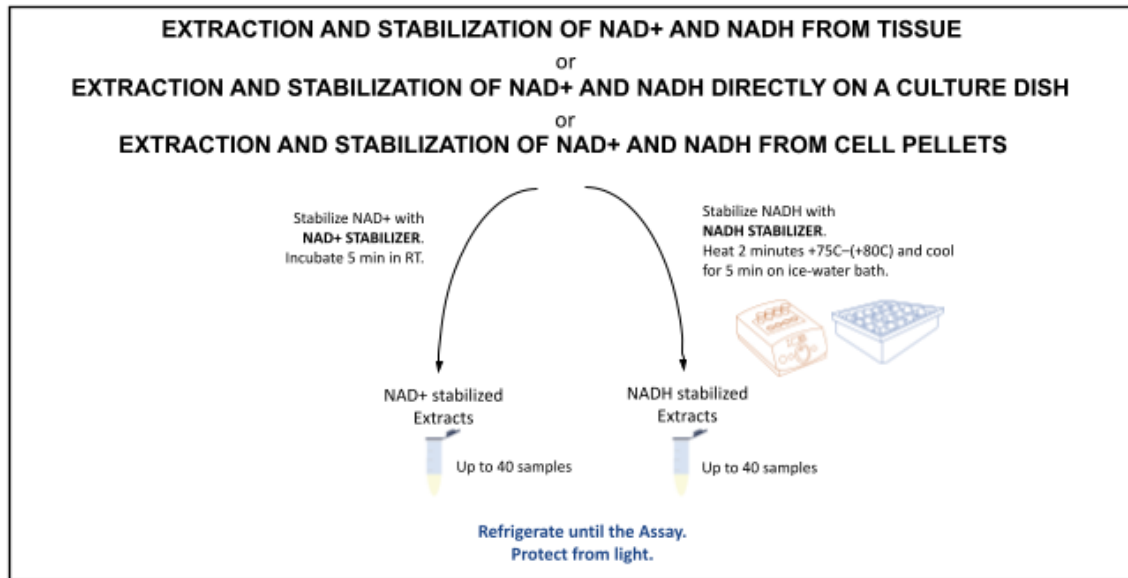
PRACTICAL CONSIDERATIONS

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 <u>sample</u> 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-well 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).

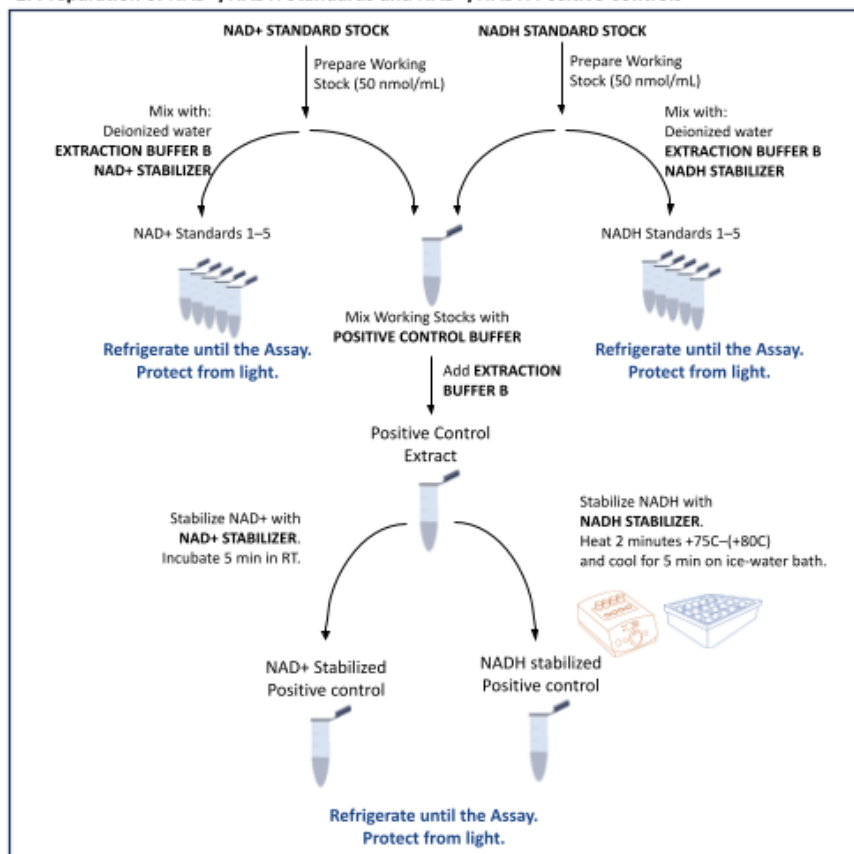
WORKFLOW FOR NAD⁺ AND NADH ASSAYS

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 Extracts 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 CULTURED CELLS ON A CULTURE DISH

This section provides guidance on the simultaneous quenching of cellular metabolism and extraction of NAD+ and NADH from cultured cells directly on the culture dish. 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:

- EXTRACTION BUFFER B must be pre-warmed to 50°-55°C (optimum range) before contacting the cultured cells.
- 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:

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 B	Room temperature
NAD+ STABILIZER	Room temperature
NADH STABILIZER	Room temperature

Guidelines for EXTRACTION BUFFER B volume:

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

Extraction:

IMPORTANT: process one flask, dish or well with the cultured cells at a time.

1. Pre-heat **EXTRACTION BUFFER B** 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. PBS should be either room temperature or of the same temperature as the culture media, to reduce unnecessary stress.
3. Remove PBS and add pre-heated EXTRACTION BUFFER B directly to the cell culture dish. Extraction buffer B is designed to simultaneously quench cell metabolism, break cytoplasmic membrane and denature cellular proteins.
4. Distribute the volume over the cells by tilting the cell culture bottle or dish.

5. Collect the cell ghosts by scraping and carefully transfer obtained homogenate into a clean microtube and place it into the ice-water bath.

↻ Repeat the extraction and collection steps with the next well or dish. The ready homogenates can safely wait in the ice-water bath until all wells or dishes have been extracted. This is the step where you can have a break after preparing of all homogenates.

6. Centrifuge all the homogenates at 20 000 x g for 10 min at 4°C.
7. 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 obtained extract is critical, as this value is required for the final calculation of your results.

8. Obtained extract volume is expected to be lower than the volume of Extraction Buffer B applied to the culture. Minimal volume of the extract needed for analysis of both NAD⁺ and NADH is 250 µl. If extract volume is lower than 250 µl, adjust the volume of the extract to 250 µl with the Extraction Buffer B.
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.

EXTRACTION OF NAD⁺ AND NADH FROM CELL PELLETS

This section provides guidance on the extraction of NAD⁺ and NADH from pelleted cell cultures (fresh or frozen). 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:

- Cell pellets need to be free of culture media proteins, especially if normalization to protein content is desired. Refer to SAMPLE HANDLING AND STORAGE.
- EXTRACTION BUFFER B must be pre-warmed to 50°-55°C before adding to cell pellets. We recommend processing one sample at a time.
- **TIP:** 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 of empty 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
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 B	Room temperature
NAD ⁺ STABILIZER	Room temperature
NADH STABILIZER	Room temperature

Guideline for EXTRACTION BUFFER B volume:

300 µL of EXTRACTION BUFFER B per 1.5-2 million cells (tested on cell pellets of control human fibroblasts and HeLa cells, collected using trypsinization approach). Levels of NAD⁺ and NADH are cell type specific and depend on growth conditions and collection protocol. We suggest testing extraction in 300 µl first (or 400 µl if you have more than 2 million cells per pellet) and then adjust volume of extraction buffer if needed.

Extraction:

1. Pre-heat **EXTRACTION BUFFER B** to 50°-55°C for at least 10 min before starting extraction by placing the bottle with the buffer into a water bath set to 50°-55°C. Keep the bottle at this temperature until you extract all the samples.
2. Pellets can be processed either fresh or frozen. Sample temperature must be equilibrated to the temperature of the ice-water bath before addition of the Extraction Buffer B.
 - a) keep freshly collected cell pellets in an ice-water bath until extraction.

b) place deep-frozen pellets in an ice-water bath for 2 min before extraction to bring the temperature close to 0°C.

NOTE: Extract one sample at a time.

3. Pipette 300 µL (or 400 µL for cell amount higher than 2 million) of warm EXTRACTION BUFFER B to the cell pellet and resuspend cells by a few cycles of up-and-down pipetting to break cell clumps.
4. Incubate the resulting homogenate at 50°-55°C for 2-3 min (use either water bath or dry-bath heat block set at 50°-55°C (this is optimal temperature range)).
5. Place the homogenate into the ice-water bath and keep there for at least 5 min.

↻ Repeat the extraction process for the next cell pellet. 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 extraction of all the 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 and measure their final volumes. Save the pellets for protein content measurement if normalization per protein mass is needed.

TIP: You can measure the 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.

8. Obtained extract volume is expected to be lower than the volume of Extraction Buffer B added to the cell pellet. Minimal volume of the extract needed for analysis of both NAD⁺ and NADH is 250 µL. If extract volume is lower than 250 µL, adjust the volume of the extract to 250 µL with the Extraction Buffer B.
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 67 µL of **NAD⁺ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
12. To the second 100 µL aliquot, add 67 µL of **NADH STABILIZER**. Vortex, and incubate in a dry bath at 75°-80°C for 2 min. Cool down on ice-water bath for 5 min.

13. Treatment with the stabilizers results in x1.67-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 cell 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 50 µl of the original stabilized extract into clean tube, add 50 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 5-10 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	500	400
NAD ⁺ ST2	1	80	20	500	400
NAD ⁺ ST3	2	60	40	500	400
NAD ⁺ ST4	3	40	60	500	400
NAD ⁺ ST5	5	0	100	500	400

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	500	400
NADH ST2	0.5	90	10	500	400
NADH ST3	1.0	80	20	500	400
NADH ST4	1.5	70	30	500	400
NADH ST5	2.0	60	40	500	400

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 2.8 - 3.2 nmol/mL and NADH 0.9-1.1 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 67 µL of **NAD⁺ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
5. To the second 100 µL aliquot, add 67 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 cell type, sample blanks can be prepared from four representative stabilized sample extracts. If several cell types are analyzed, every sample type should have at least one well with a sample blank representing this sample 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 aluminum 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 μ L 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 μ L 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	ST1	ST1	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	ST2	ST2	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	ST3	ST3	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	ST4	ST4	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	ST5	ST5	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	PC	PC	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	BL S1	BL S2	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	BL S3	BL S4	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

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. For cultured cells, the protein content will provide much more accurate normalization than cell count. 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 100 µL of SOLUBILIZATION BUFFER D to each cell protein pellet. Guideline: use 100 µL of SOLUBILIZATION BUFFER D for pellet obtained from 1.5-2 million cells. Adjust accordingly for pellets obtained from higher cell amount.
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-fold 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 total amount of protein in the initial pellet (mg) using the following formula:

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

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 2.8 -3.2 nmol/ml.

NADH:

In the NADH assay, concentration of NADH in the stabilized Positive Control extract is expected to be within 0.9 - 1.1 nmol/ml.

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

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 cell count 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 one mg of protein, use the following formula if original stabilized extract was added to the assay:

$$\text{Conc.} \frac{\text{nmol}}{\text{mg}} \text{protein} = \frac{\text{Conc. in stabilized extract, } \frac{\text{nmol}}{\text{mL}} * 1.67 * V, \text{mL of the extract}}{\text{total protein, mg}}$$

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

$$\text{Conc.} \frac{\text{nmol}}{\text{mg}} \text{protein} = \frac{\text{Conc. in stabilized extract, } \frac{\text{nmol}}{\text{mL}} * 1.67 * 2 * V, \text{mL of the extract}}{\text{total protein, mg}}$$

For normalization per million cells, use the following formula:

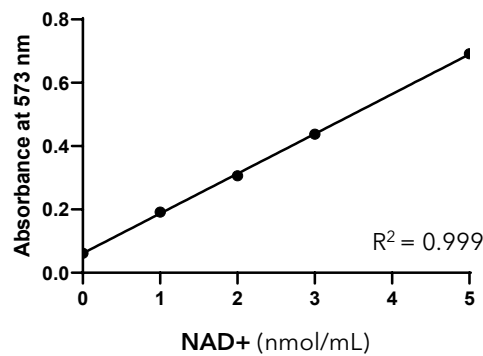
$$\text{Conc.} \frac{\text{nmol}}{\text{million}} \text{cells} = \frac{\text{Conc. in stabilized extract, } \frac{\text{nmol}}{\text{mL}} * 1.67 * V, \text{mL of the extract}}{(\text{cell amount}), \text{million}}$$

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

TYPICAL DATA

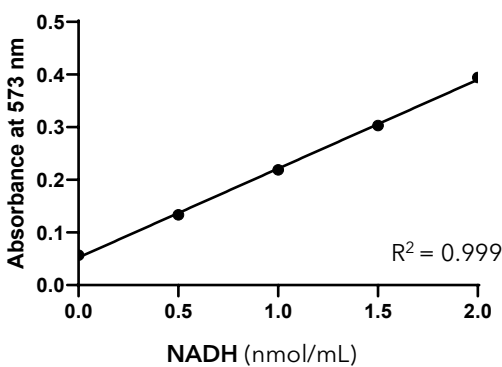
The standard curve and the concentrations in the stabilized sample extracts are provided for demonstration only and should never be used instead of the real-time calibration curve.

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

[illegible]

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H