NADMED

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Q-NAD BLOOD
NAD+ and NADH assay kit

Quantitative assay kit for whole blood

Version 5.0

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 Blood NAD+ and NADH assay kit: quantitative assay kit for whole blood
Catalog numbers:
RUO_001
Storage:
-85°– -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

For Research Use Only


Protect from direct light


Keep dry

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INTRODUCTION

NAD metabolites are derivatives of vitamin B3 and exist in cells in four forms – NAD+, NADH, NADP+, and NADPH. NAD metabolites are essential for the functional adaptation of cell metabolism to environmental conditions. Their levels are dynamic and change in response to different endogenous and exogenous stimuli. This kit is designed for separate measurement of systemic levels of NAD+ and NADH in whole blood (human or animal).

RESEARCH BACKGROUND

NAD+ and NADH metabolites play a pivotal role in adjusting human metabolism and energy homeostasis in response to various internal and external stimuli. Research has demonstrated that systemic NAD+ levels diminish in correlation with the onset of diseases, signaling a disruption in the body's energy equilibrium (Covarrubias et al. 2021 doi: 10.1038/s41580-020-00313-x). The extent of this NAD+ reduction is not uniform and varies across different individuals and pathologies. A significant decline in NAD+ impairs the body's ability to sustain essential metabolic functions, a condition that persists even with ongoing therapy. Ongoing research on the contribution of NAD+ and NADH to the mechanisms and progression of different diseases is very active. A list of pathologies with suspected changes in NAD+ and NADH concentrations is constantly expanding with already published evidence for mitochondrial disease, aging, sepsis, viral infections, cardiovascular and kidney disease, diabetes types I and II, neurological disorders, and cancer (e.g., Pirinen et al. 2020 doi: 10.1016/j.cmet.2020.04.008, Verdin 2015 doi: 10.1126/science.aac4854, Fan et al 2020 doi: 10.1111/jdi.13303, Navas & Carnero 2021 doi: 10.1038/s41392-020-00354-w).

PRINCIPLE OF THE ASSAY

The kit measures intracellular NAD+ and NADH content. The principle of the assay is a cyclic enzymatic reaction with a colorimetric end-point detection. First, NAD+ and NADH metabolites are extracted together from a whole blood 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.

SAMPLE HANDLING AND STORAGE

Requirements and Limitations:

- This kit is designed for NAD+ and NADH measurement in whole blood. This assay is NOT suitable for measuring in plasma or serum, cultured cells, or tissues.
- For measuring NAD+ and NADH, 100 µL of whole blood is needed. However, a volume of 150–200 µL is optimal to perform the assay reliably.
- Samples can be analyzed either fresh or frozen.
  a) Fresh blood can be analyzed within 72 hours after collection. Store at 4°– 8°C after withdrawal, before the analysis.
  b) Frozen samples must be continuously maintained frozen before the assay. Subsequent freeze-thawed cycles are not allowed. Storage time of aliquots is one month at -20°C, or approximately one year at -80 – -70°C.
- In clinical trials and longitudinal studies, it is highly important to have consistent sampling and handling of the whole blood. Aim for consistency in analysis type (fresh or frozen) and storage practices before the assay. Refer to Blood collection instructions and Important precautions below.

Blood collection:

Collection: Whole blood samples taken from a vein (using methods like venipuncture) and whole blood samples taken from other parts of the body (using a lancet-type device) are suitable. Detailed instructions on aliquoting and freezing blood samples can be found at https://www.nadmed.com/documents/

Sample volume: The analysis itself requires small volumes of whole blood. Thus, if analyzing frozen samples, we recommend aliquoting a larger volume of blood (e.g., 2–3 mL) into 150–200 µL aliquots before freezing. Collecting the blood directly into a collection tube with anticoagulants is vital to keep the target concentration of anticoagulant in the sample.

Anticoagulants: In general, whole blood samples should be collected into collection tubes with K2 EDTA or Lithium heparin (LH) as anticoagulants and properly mixed by up-and-down rotation. Final concentrations of anticoagulants should be 1.2–2 mg of K2 EDTA per 1 mL of collected blood, or 17–18 IU of LH per 1 mL of collected blood. For venous blood collection, we recommend blood collection vacutainers with a spray coating of K2 EDTA or LH designed to result in anticoagulant concentrations described above (e.g. BD Vacutainer® or Vacuette®).

Important precautions to ensure the integrity and reliability of the results:

Mixing the Sample: When whole blood remains stationary, it separates into different phases. Therefore, it is essential to thoroughly mix a fresh sample during processing.

Practices to avoid: Avoid freezing large (2–3 mL) volumes of blood in the collection tubes. Avoid using skirted double-wall microtubes. These practices can significantly increase the time needed for both freezing and thawing, which is especially critical. Long thawing times can cause a lot of variability in assay results, affecting the accuracy and reliability of the analysis.

Timing of Analysis and Aliquoting: If you cannot analyze the blood sample immediately after collection, make sure to divide (aliquot) the sample within 72 hours. Preferably use aliquots of 150 to 200 µL.

Storing aliquots: Store the aliquots in non-sterile, single-wall transparent polypropylene microtubes. The tubes should have a capacity of 0.5 to 2 mL. After aliquoting, freeze the samples quickly. Use temperatures from -80°C to -20°C for freezing.

REAGENT STORAGE, STABILITY AND PREPARATION

REAGENT	DESCRIPTION (*)	PREPARATION (**)	STORAGE and STABILITY (**, ***)
BUFFER A	28 mL Sufficient for 40 samples	Ready for use. Equilibrate to room temperature.	Stable for two weeks at room temperature.
NAD+ STABILIZING REAGENT	8 mL Sufficient for 40 samples		
NADH STABILIZING REAGENT	8 mL Sufficient for 40 samples		
POSITIVE CONTROL (BUFFER)	200 µL Sufficient for two plates		
DEIONIZED WATER	10 mL Sufficient for two plates		
STOP SOLUTION	3 mL Sufficient for two plates	Ready for use. Equilibrate to room temperature. 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. 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 (1mM) Sufficient for Standards and Positive control	Equilibrate to room temperature. See preparation guide on page 13.	Should be used immediately after thawing. Standards should be protected from light.
NADH STANDARD STOCK	40 µL (1mM) Sufficient for Standards and Positive control		
ENZYME	2x 40 µL One aliquot per 96-well plate	Equilibrate to room temperature. 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

*** 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. Not for use in diagnostic procedures. 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.

BUFFER A 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.

The Q-NADMED 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-NADMED 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/.

MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

CATEGORY	ITEM	SPECIFICATIONS/REQUIREMENTS
Consumables	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 Q-NAD 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.
	Aluminium foil	Use foil to protect samples, standards, and the plates from light during assay as specified in the instructions.
Equipment and Machinery	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).
	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 minutes: a) ensure the tubes fit the block tightly b) increase the target temperature of your device
Special	Possibility to work in dim light conditions for the ASSAY part of the measurement. Refer to PRACTICAL CONSIDERATIONS and WORKFLOW OF Q-NAD BLOOD NAD+ AND NADH ASSAY.	

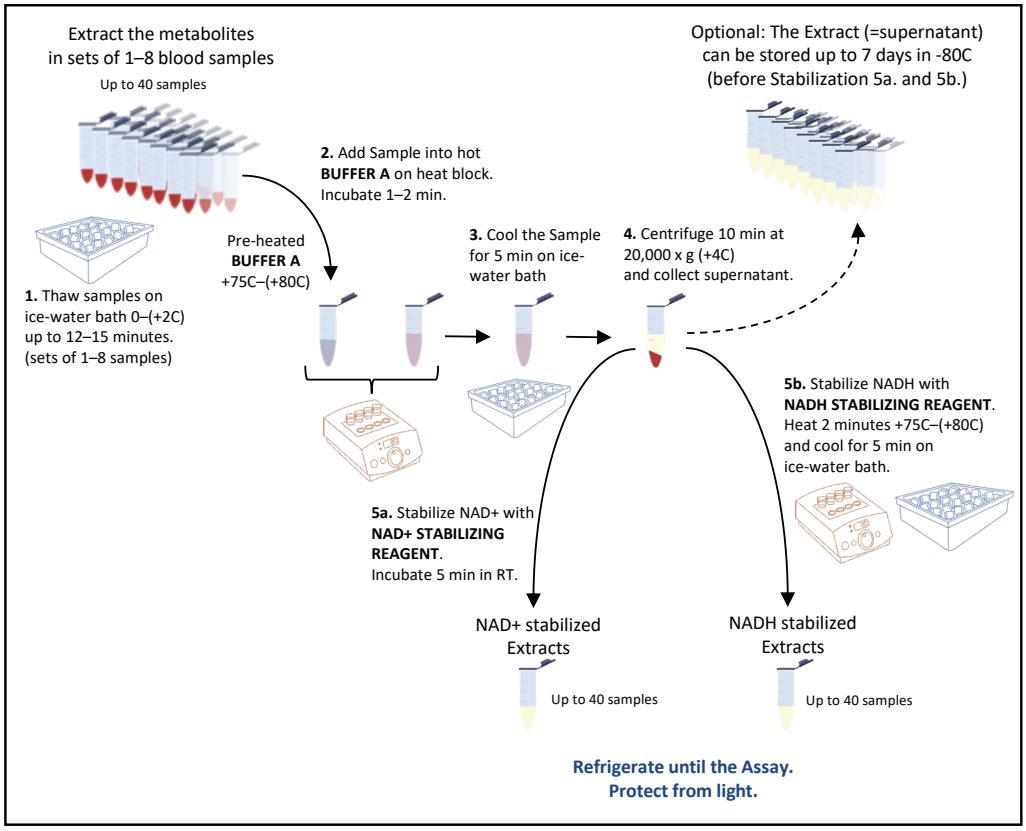
PRACTICAL CONSIDERATIONS

 Please refer to (https://www.nadmed.com/products/nad-nadh-kit-ruo/) for visual instructions.

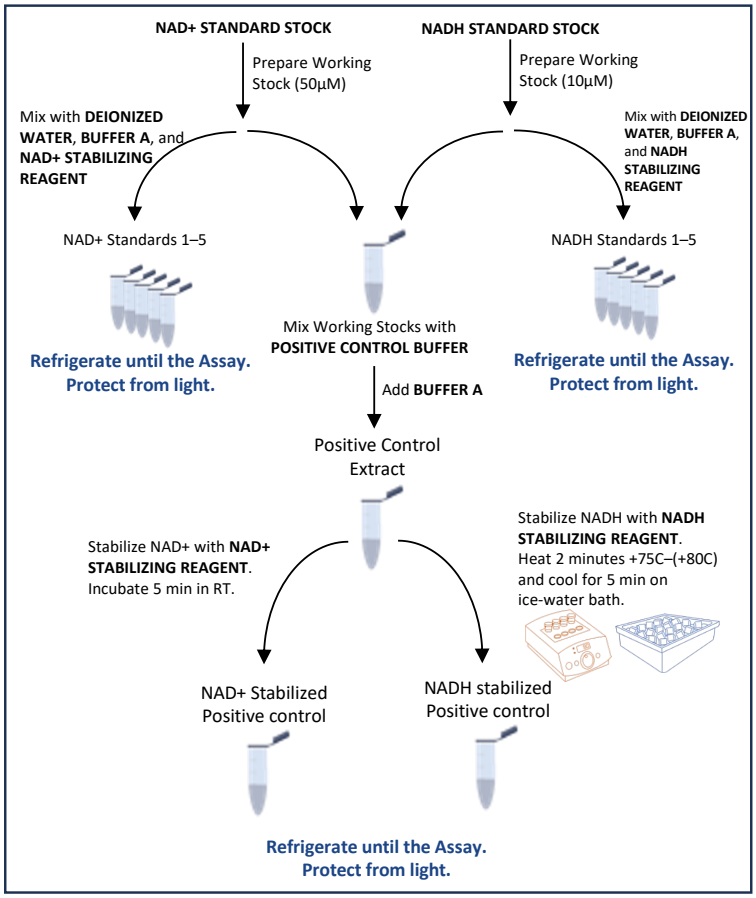
CATEGORY	INSTRUCTIONS
Limitations	Read the SAMPLE HANDLING AND STORAGE carefully. This assay is designed for whole blood and is NOT suitable for measuring NAD+ and NADH in plasma or serum, cultured cells, or tissues. Do not use kit components beyond the expiry date. Do not mix materials from different 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 DEIONIZED WATER, BUFFER A, NAD+ STABILIZING REAGENT, NADH STABILIZING REAGENT, 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.
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 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 the 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 OF Q-NAD BLOOD NAD+ AND NADH ASSAY

1. Extraction of Metabolites and NAD+/NADH stabilization



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



3. NAD+ and NADH Assays

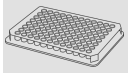
1. Equilibrate reagents, Sample Extracts, Positive Control Extracts and Standards to RT.

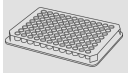
2. Pipette Standards, Sample Exteracts and Positive Control Extracts on the NAD and NADH plate(s) according to plate Layout. **Work on one plate at the time.**

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

3. Prepare the Assay buffer (**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).

EXTRACTION AND STABILIZATION OF NAD+ AND NADH

This section provides guidance on the extraction of NAD+ and NADH from whole blood. Following their 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.

TIP: Please refer to video guidance (https://www.nadmed.com/products/nad-nadh-kit-ruo/)

NOTE: Final dilution of the original whole blood sample will be 10 times. In the case of supplementation with NAD-precursors, the levels of NAD+ may increase in the subject's blood. Thus, the NAD+ stabilized extract should be further diluted 1:2 using DEIONIZED WATER (provided) before the colorimetric assay. In this case, the dilution of the original blood sample will be 20 times for NAD+. The NADH-stabilized extracts do not require dilution.

Materials:

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 steps
BUFFER A	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature
DEIONIZED WATER	Room temperature

Extraction:

1. Pipette 500 µL of **BUFFER A** into 1.5 mL microtubes for all your samples.
2. a) If you work with fresh blood samples, cool them on ice and proceed to extraction with BUFFER A.

   b) If you work with frozen whole blood samples, thaw them in the ice-water bath as follows:

   - Work with sets of 1–8 samples at a time.
   - During the first minutes of thawing, remove any ice formed on the tube walls with tissue paper.
   - Thawing should be completed within 12–15 minutes. Monitor the thawing and facilitate if necessary: hold the sample for 2-3 seconds and place it back in the ice-water bath, repeat every 2 minutes.

3. Pre-heat BUFFER A (in sets of 1–8 samples) in the dry bath heat block set to 80°C. Keep for 5 minutes before the extraction.
4. Mix the thawed whole blood sample with a few up-and-down pipetting cycles, avoid foaming.
5. Without removing the BUFFER A microtube from the heat block, inject the sample as follows:

   - Pipette 100 µL of blood into the BUFFER A without touching the bottom of the tube.
   - Quickly mix with 2–3 intensive up-and-down pipetting cycles and simultaneous rotation of the tip for efficient mixing of the cold sample and hot BUFFER A.

6. Incubate each reaction at 75°–80°C for 1–2 min. Keep the incubation time constant for all your samples.

7. Cool down the extract in the ice-water bath for at least 5 min. Check the sample for successful extraction. After cooling on ice, homogenate should polymerize without any free liquid.
8. Centrifuge the extracts at 20 000 x g at 4°C for 10 min. Transfer the supernatant into a clean microtube and discard the pellet.
9. Protect the sample extracts (supernatants) from light and keep them refrigerated (4°–8°C) until the Stabilization steps.

↻ Repeat the extraction for the next batch(es) of 1–8 samples.

10. Proceed to the Stabilization step without delay.

Optional: The supernatants 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.

Stabilization:

11. Equilibrate the extract to room temperature and prepare two 150 µL aliquots into clean microtubes.
12. To the first 150 µL aliquot, add 100 µL of **NAD+ STABILIZING REAGENT**. Vortex, and incubate at room temperature for 5 min.
13. To the second 150 µL aliquot, add 100 µL of **NADH STABILIZING REAGENT**. Vortex, and incubate for 2 min in a dry-bath at 75°–80°C. Cool down on ice for 5 min.
14. 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. 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 NAD+ working stock to improve accuracy.

Materials:

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.
BUFFER A	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature
DEIONIZED WATER	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 µM 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.

NAD+ STANDARD PREPARATION

STANDARD ID	NAD+ CONCENTRATION (µM)	DEIONIZED WATER (µL)	**50 µM NAD+ working stock (µL)**	BUFFER A (µL)	NAD+ STABILIZING REAGENT (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 **10 µM NADH working stock** by adding 10 µL of 1 mM NADH STANDARD STOCK into 990 µL of DEIONIZED WATER, vortex. Proceed to the preparation of NADH standards according to the table below, pipette the reagents in the indicated order.

NADH STANDARD PREPARATION

STANDARD ID	NADH CONCENTRATION (µM)	DEIONIZED WATER (µL)	**10 µM NADH working stock (µL)**	BUFFER A (µL)	NADH STABILIZING REAGENT (µL)
NADH ST1	0.0	100	**0**	500	400
NADH ST2	0.2	80	**20**	500	400
NADH ST3	0.4	60	**40**	500	400
NADH ST4	0.6	40	**60**	500	400
NADH ST5	1.0	0	**100**	500	400

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 on the day of the assay right after the preparation of the Standards due to the limited stability of 10 µM NADH working stock. The Positive control is mimicking the level of NAD metabolites in a blood sample of a healthy human subject. Positive control undergoes extraction and stabilization like the whole blood samples.

The final dilution of the Positive control will be 10 times. The expected concentration of NAD+ in the Positive control is 25 ± 2 µM, and NADH is 2 ± 0.3 µM after calculation of results.

Materials:

Dry bath heat block set at 75°–80°C	Refer to MATERIALS REQUIRED-Table
Ice-water bath	Refer to MATERIALS REQUIRED-Table
50 µM NAD+ working stock	From Preparation of standards, room temperature
10 µM NADH working stock	From Preparation of standards, room temperature
POSITIVE CONTROL (BUFFER)	Room temperature
BUFFER A	Room temperature
NAD+ STABILIZING REAGENT	Room temperature
NADH STABILIZING REAGENT	Room temperature

Protocol:

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

   45 µL of **POSITIVE CONTROL (BUFFER)**
   75 µL of **50 µM NAD+ working stock**
   30 µL of **10 µM NADH working stock**

2. Pipette 500 µL of **BUFFER A** into a clean microtube.
3. Add 100 µL of Positive control mix into the BUFFER A. Vortex.

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

4. Prepare two 150 µL aliquots of the Positive control extract into clean microtubes.
5. To the first 150 µL aliquot, add 100 µL of **NAD+ STABILIZING REAGENT**. Vortex, and incubate at room temperature for 5 min.
6. To the second 150 µL aliquot, add 100 µL of **NADH STABILIZING REAGENT**. Vortex, and incubate for 2 min in a dry bath at 80°C. Cool down on ice for 5 min.
7. 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. (Positive control does not require a separate blank.) Sample blanks are to be prepared (at minimum) from four representative stabilized sample extracts. If subjects with known NAD supplementation and non-supplemented subjects will be analyzed on the same plate, we recommend two wells of sample blanks per condition.

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
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 whole blood.

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 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
St1	St1	UNK1	UNK1	UNK9	UNK9	UNK17	UNK17	UNK25	UNK25	UNK33	UNK33
St2	St2	UNK2	UNK2	UNK10	UNK10	UNK18	UNK18	UNK26	UNK26	UNK34	UNK34
St3	St3	UNK3	UNK3	UNK11	UNK11	UNK19	UNK19	UNK27	UNK27	UNK35	UNK35
St4	St4	UNK4	UNK4	UNK12	UNK12	UNK20	UNK20	UNK28	UNK28	UNK36	UNK36
St5	St5	UNK5	UNK5	UNK13	UNK13	UNK21	UNK21	UNK29	UNK29	UNK37	UNK37
PosCtr	PosCtr	UNK6	UNK6	UNK14	UNK14	UNK22	UNK22	UNK30	UNK30	UNK38	UNK38
BL UNK1	BL UNK2	UNK7	UNK7	UNK15	UNK15	UNK23	UNK23	UNK31	UNK31	UNK39	UNK39
BL UNK3	BL UNK4	UNK8	UNK8	UNK16	UNK16	UNK24	UNK24	UNK32	UNK32	UNK40	UNK40

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.

TROUBLESHOOTING

If you are facing any issues during the extraction or assay performance, check the NADMED troubleshooting guide here.

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 the 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 23–27 µM (after correction of 10x 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 1.7–2.3 µM (after correction of 10x 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.

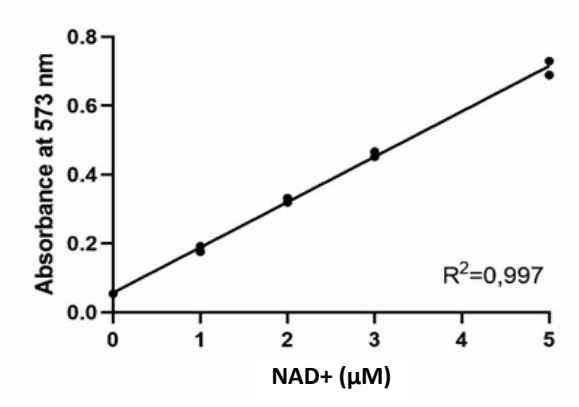
1. Calculate the average of the absorbance readings for each standard (ST1–ST5).
2. Create a standard curve by plotting the mean absorbance for each Standard on the y-axis against the known Standard concentration (in µM) on the x-axis. Calculate a simple linear regression fitting of the standard curve.
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.
7. Calculate the average of duplicates and multiply by 10 to obtain the concentration (µM) of NAD+ and NADH in blood.

NOTE: If the NAD+ stabilized extracts have been additionally diluted due to known supplementation usage, the concentration must be multiplied by the additional dilution factor.

TYPICAL DATA

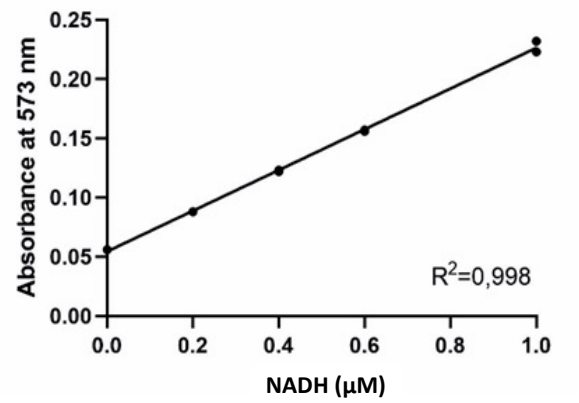
The standard curve and the concentrations in the stabilized sample extracts are provided for demonstration only and should never be used instead of the real-time calibration curve.

A) STANDARD CURVE FOR NAD+



Standard	NAD+ (µM)	Absorbance (573 nm) Assay time: 4 min
ST1	0	0.054 0.054
ST2	1	0.176 0.192
ST3	2	0.319 0.332
ST4	3	0.452 0.466
ST5	5	0.689 0.730

B) STANDARD CURVE FOR NADH



Standard	NADH (µM)	Absorbance (573 nm) Assay time: 6 min
ST1	0	0.056 0.056
ST2	0.2	0.088 0.088
ST3	0.4	0.122 0.123
ST4	0.6	0.157 0.156
ST5	1	0.223 0.232

C) CALCULATION OF RESULTS FOR NAD+

Concentration values in the stabilized sample extracts (UNK) and sample blanks (BL UNK1–4) are determined from the linear fit formula of the NAD+ standard curve.

Unknown	Concentration in stabilized extracts (µM)	Concentration in stabilized extracts corrected by average of sample blanks (BL UNK 1–4, µM)	Final NAD+ concentration in the original sample (µM)*
UNK 1	2.944 3.151	3.008	30.08
UNK 2	2.841 3.129	2.945	29.45
UNK 3	2.686 2.730	2.668	26.68
UNK 4	1.895 1.999	1.907	19.07
UNK 5	2.346 2.420	2.343	23.43
UNK 6	3.432 3.499	3.425	34.25
BL UNK 1	0.040	-	
BL UNK 2	0.048		
BL UNK 3	0.026		
BL UNK 4	0.048		

*Corrected by dilution factor x10

D) CALCULATION OF RESULTS FOR NADH

Concentration values in the stabilized sample extracts (UNK) and sample blanks (BL UNK1–4) are determined from the linear fit formula of the NADH standard curve.

Unknown	Concentration in stabilized extracts (µM)	Concentration in stabilized extracts corrected by average of sample blanks (BL UNK 1–4, µM)	Final NADH concentration in the original sample (µM)*
UNK 1	0.239 0.239	0.086	0.86
UNK 2	0.284 0.290	0.133	1.33
UNK 3	0.228 0.234	0.077	0.77
UNK 4	0.234 0.239	0.083	0.83
UNK 5	0.200 0.195	0.044	0.44
UNK 6	0.228 0.245	0.083	0.83
BL UNK 1	0.156	-	
BL UNK 2	0.161		
BL UNK 3	0.150		
BL UNK 4	0.150		

*Corrected by dilution factor x10

PERFORMANCE AND LIMITATIONS

LIMITS OF DETECTION

The Limit of Blank (LoB) for Q-NAD Blood is presented in the table below (LoB ± standard deviation [SD]).

Limit of Blank (pmol/well)	
NAD+	1.84 ± 0.9
NADH	2.10 ± 0.5

The Limit of Detection (LoD) was calculated from NAD+ and NADH standard curves and is presented in the table below (LoD ± SD).

Limit of Detection (µM in whole blood)	
NAD+	0.33 ± 0.2
NADH	0.19 ± 0.05

The Limit of Quantitation (LoQ) is presented in the table below (LoQ ± SD).

Limit of Quantitation (µM in whole blood)	
NAD+	0.66 ± 0.3
NADH	0.40 ± 0.1

PRECISION AND REPRODUCIBILITY

Intra-assay variation in measurement determined the precision of the assay performance. The table below presents the intra-assay precision (CV=coefficient of variation).

Intra-assay precision (CV (%) ± SD)	
NAD+	1.48 ± 0.8
NADH	3.33 ± 1.5

The table below summarizes the results of the assay reproducibility.

Reproducibility

	NAD+			NADH		
Sample	Ctr1	Ctr2	Ctr3	Ctr1	Ctr2	Ctr3
N of measurements *	9	9	9	9	9	9
Mean (µM)	27.41	29.41	22.00	0.55	0.71	0.64
Standard deviation	0.62	1.31	0.87	0.03	0.05	0.05
CV (%)	2.28	4.45	3.95	5.20	7.06	8.45

(N=number, * 3 aliquots of the same sample were analyzed in triplicates).

ACCURACY

The accuracy of the assay was calculated from samples with known amounts of pure NAD+ and NADH. The table below summarizes the results (assay accuracy +/- SD).

		Accuracy (%)
NAD+	N = 32	97.13 ± 7.6
NADH	N = 25	104.22 ± 16.5

ASSAY CUT-OFF

The low and high cut-off values represent the smallest and highest concentrations observed in 5–7% of Finnish individuals of a given population extract. The table below summarizes the cut-off values.

	Cut-off value	
	Low	High
NAD+ (µM)	20	36
NADH (µM)	0.6	1.8

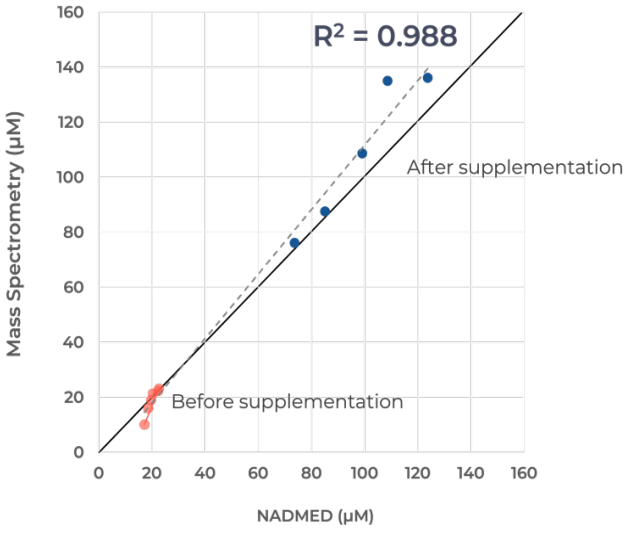
PERFORMANCE CHARACTERISTICS

The interference of other metabolites in the extract was not separately investigated, as their contribution is low and taken into account by performing the blank correction without added enzyme.

Warning: Potassium sorbate, borate, pyridine, and bismuth in a sample can cause enzyme inhibition, thus causing underestimation of the results.

METHOD VALIDATION

To validate the performance of NADMED, we measured NAD+ concentration in a set of control human blood samples that were also analyzed by mass spectrometry. Frozen blood samples of five healthy subjects (before and after 16 weeks of niacin supplementation) were analyzed in parallel by NADMED and mass spectrometry. Results from NADMED were concordant with those obtained by mass spectrometry.



NOTES

PLATE LAYOUT

Use this plate layout to record your samples on the plate.