NADMED

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Q-NAD BLOOD
NAD+ and NADH assay kit

Quantitative assay kit for whole blood

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

A. Proprietary name: Q-NAD Blood NAD+ and NADH assay kit: quantitative assay kit for whole blood
    a. Catalog number: RUO_001, 40 samples (96-well format)
B. Storage: -80˚C
C. IFU issued: May 2023

Manufacturer:

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

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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 functional adaptation of cell metabolism to environmental conditions. Their levels are dynamic and change in response to different endogenous and exogeneous 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 adapt human body metabolism and energy homeostasis to changing endogenous and exogenous conditions. Accumulated research data show that systemic levels of NAD+ decrease in response to manifested disease creating a signal of an imbalance in body energy homeostasis. The degree of NAD+ decrease varies in different patients and pathologies. The progressive decline of NAD+ levels makes it impossible for the body to maintain its basic metabolic functions to survive, even in conditions of ongoing therapy. Q-NAD allows screening patients for NAD+ and NADH deficiency to correct it and increase the efficiency of treatment. 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.

PRINCIPLES OF THE ASSAY

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 blood sample in a single step. Then, the extract is divided into two parts. In the first part, NAD+ is stabilized while NADH is removed; in the second part, NADH is stabilized while NAD+ is removed. Next, NAD+ and NADH metabolites are analyzed on two separate plates by an enzymatic reaction coupled with a color change. The intensity of the color change in the assay is linearly proportional to the concentration of NAD+ or NADH in the reaction mixture.

REAGENTS PROVIDED

REAGENTS	DESCRIPTION*	BEFORE THE ASSAY
BUFFER A	28 mL of extraction buffer	Equilibrate to room temperature (15–25˚C).
NAD+ stabilizing reagent	8 mL of buffered solution for NAD+ measurement	Equilibrate to room temperature (15–25˚C).
NADH stabilizing reagent	8 mL of buffered solution for NADH measurement	Equilibrate to room temperature (15–25˚C).
NAD+ standard stock	1 microtube with 40 µL of 1 mM NAD+	See preparation guide.
NADH standard stock	1 microtube with 40 µL of 1 mM NADH	See preparation guide.
BUFFER C	2 bottles with 19 mL of assay buffer	Equilibrate to room temperature (15–25˚C).
Assay color reagent	2 bottles with 3 mL of reagent with the assay color	Equilibrate to room temperature (15–25˚C). Should be used within 2 h after equilibration.
Enzyme	2 microtubes with 40 µL of enzyme. One per plate.	Thaw only before adding into master mix.
Stop solution	3 mL of solution to stop the assay reaction	Equilibrate to room temperature (15–25˚C).
Positive control (buffer)	1 microtube with 200 µL of buffer for preparation of Positive control	Equilibrate to room temperature (15–25˚C). See preparation guide.

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

PRECAUTIONS AND WARNINGS

Safety

For research use only. Not for use in diagnostic procedures.

The 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 A can cause eye irritation. Handle with care; use goggles.

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

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.

STORAGE AND EXPIRATION DATING OF REAGENTS

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

OTHER MATERIAL REQUIRED

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

- Deionized water (milli-Q water from a water purification system or commercially available deionized water, e.g., Sigma cat #38796)
- Microtubes, 1.5 mL, for sample preparation, extraction, stabilization, and assay standards preparation. Requirements for the material of the microtubes: use basic non-sterile microcentrifuge tubes made from transparent/natural color polypropylene (PP) intended for *in vitro diagnostics* (e.g., Sarstedt Ref 72.690.001). Do not use microtubes intended for molecular biology labeled as sterile (chemically sterilized), free of endotoxin, pyrogen, human DNA, and low retention; do not use microtubes intended for work with proteins labeled as LoBind.
- Two plastic multichannel pipette reservoirs (from non-sterile polystyrene): one for pipetting the master mix, the other for pipetting the Stop solution.
- Two 96-well transparent polystyrene microplates with medium protein binding affinity intended for colorimetric and absorbance assays.
- Dry bath (heat block) with adjustable temperature (up to 80˚C).
- Table-top cooling microcentrifuge (max speed 20 000 x g).
- Ice-water bath (packed ice with added tap water to a slush state, which firmly holds inserted microtubes, preventing them from floating).
- Calibrated single channel pipettes (0.5–10 µL, 5–50 µL, 20–200 µL, 100–1000 µL) and multichannel pipettes (5–50 µL, 30–300 µL) and beveled pipette tips, low retention.
- Spectrophotometric microplate reader capable of measuring absorbance at 570–573 nm.
- Aluminum foil to protect microtubes and plates from light.

REAGENT PREPARATION

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

A. **BUFFER A** - Ready to use

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

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

D. **Enzyme** - Ready to use

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

F. **Positive control (buffer)** - Ready to use. (For further instructions, see preparation of Positive control.)

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

H. **10 µM NADH standard working stock** - add 10 µL of 1 mM NADH standard stock (provided) into 990 µL of deionized water, vortex. Protect from light. (For further instructions, see preparation of standards and preparation of Positive control.)

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

SAMPLE COLLECTION AND STORAGE

The kit is for analyzing whole blood. Venous blood, collected by vein puncture, and peripheral blood, collected by lancet-type blood collection devices, are suitable sample types. Whole blood samples should be collected into collection tubes spray-coated with lithium heparin or K2 EDTA as anticoagulants and **properly mixed** by up-and-down rotation.

Requirements for the venous blood collection tubes with anticoagulants (BD Vacutainer or Vacuette): use K2 EDTA blood tubes containing spray-coated K2 EDTA resulting in a concentration of 1.2–2 mg of K2 EDTA per 1 mL of collected blood or Lithium Heparin (LH) blood tubes containing spray-coated LH resulting in a concentration of 17–18 IU of LH per 1 mL of collected blood. The analysis requires small volumes of whole blood; thus, we recommend using small blood collection tubes, e.g., 2–3 mL. Collecting the intended volume of blood into the collection tube is vital to keep the target concentration of anticoagulant in the sample.

Samples can be analyzed fresh or frozen. Fresh blood can be analyzed within 72 hours if continuously maintained at 4°–8°C after being drawn. As blood undergoes phase separation in the collection tube, carefully mixing the sample before taking an aliquot for extraction is critical. If blood cannot be analyzed fresh, we strongly recommend aliquoting the sample within 72 hours after the draw into 150–200 µL aliquots using non-sterile, single-wall transparent polypropylene 0.5–2 mL microtubes and freezing them at -20°C or -80°C. Freezing collection tubes with a larger sample volume (e.g., > 2mL) or in skirted double-

wall microtubes will increase the freezing time and, especially, the thawing time before extraction, which can cause high variability in results. Storage time for small aliquots is one month at -20°C and approximately a year at -80°C. The kit measures intracellular NAD content. Thus, adequately mixing each fresh sample before and during aliquoting is crucial to ensure every aliquot contains approximately the same number of cells. Frozen samples must be continuously maintained frozen before the assay. For clinical trials, we strongly recommend keeping the same time interval between sample draw and aliquoting/freezing for all the samples in the study. For measuring NAD+ and NADH, 100 µL of whole blood is needed. Subsequently, freeze-thawed blood samples cannot be used for the analysis.

PRACTICAL CONSIDERATIONS

- Do not use kit components beyond the expiry date.
- Do not mix materials from different kit lots. Subsequent freeze-thaw cycles are not allowed.
- This assay is NOT suitable for measuring NAD+ and NADH in plasma or serum, cultured cells, or tissues (human or animal).
- Thoroughly mix all reagents by gentle swirling. Small microtubes should be quickly centrifuged at low speed before opening.
- Fresh and frozen blood samples can be used for the assay. A homogenous (properly mixed) sample is essential for analysis.
- We recommend extracting a maximum of eight samples at a time to minimize handling time.
- The analyses of NAD+ and NADH are done on two separate plates. We recommend performing the NAD+ and NADH assays on the day of extraction.
- To avoid cross-contamination, change to new pipette tips between the additions of each standard, sample, and reagent. Also, use separate reservoirs for the master mix and Stop solution.
- High-precision pipettes and beveled tips with less retention will improve the precision.
- Assay color reagent is a yellow, light-sensitive compound that turns brown upon enzymatic reaction. Exposure to direct sunlight or direct artificial light causes its unspecific color change to green. To minimize the light interference with the assay, we recommend the following:
    - Pipetting of the master mix and then Stop solution into the plate under conditions of natural indirect light (dim conditions). To achieve these conditions, switch off artificial indoor lights and avoid pipetting close to the window. By natural indirect light (dim conditions), we mean the amount of environmental light one can experience when driving a car under a bridge on a cloudy day.
    - Covering the 96-well plate with an aluminum foil lid during the reaction time after adding the master mix and while transferring to the plate reader after adding Stop solution. Do not wrap the plate with aluminum foil due to the risk of contamination and sample mixing during unwrapping.
    - The protocol indicates the steps requiring the work under dim conditions.
- Positive control is an artificial sample containing known amounts of pure NAD+ and NADH.

- Technical requirements for a dry bath (heat block):
    - Test the efficiency of heat transfer in the dry bath as follows:
    1. Add 250 µL of water into a microtube that fits into the dry bath you are using.
    2. Insert a conventional lab thermometer into the microtube with the liquid and place it into the dry bath set to 80°C.
    3. Measure the time needed to increase the temperature of the liquid to 70°–75°C.
    4. All dry bath incubation times mentioned in this protocol are given for a dry bath equilibrated to 80°C that heats 250 µL of water to 75°C in 2 minutes. This is enough for the chemical reaction to reach 70°–75°C in the solution upon incubation in the dry bath.
- Technical requirements for the spectrophotometric plate reader:
    1. Measurement of light absorption at 570–573 nm.
    2. Option to adjust the scanning light brightness/intensity to **low**. In some plate readers, the brightness can be adjusted as the number of flashes per measurement. In the latter case, set the number of flashes from 5 to 10.
- Master mix and Stop solution contain detergents. To avoid bubbles, pipette the master mix and Stop solution by pressing the pipette to the first stop position; remove any bubbles in the wells with a small needle. Avoid touching the content of the wells with pipette tips.
- We recommend the following order of steps:
    1. Bring BUFFER A, NAD+ stabilizing reagent, NADH stabilizing reagent, and Stop solution to room temperature the day before the assay. These solutions are stable at room temperature for two weeks.
    2. Prepare the standards and Positive control on the day of the assay.
    3. Thaw bottles with BUFFER C and Assay color reagent and bring them to room temperature on the day of the assay. These bottles take about 2–3 hours to melt. During this time, extract the samples and prepare two aliquots of the sample extracts for separate measurements of NAD+ and NADH. Perform NAD+ and NADH assays on separate plates, one at a time.

EXTRACTION OF NAD+ AND NADH FROM BLOOD

Note: this section is done under normal light conditions.

1. Bring BUFFER A, NAD+ stabilizing reagent, and NADH stabilizing reagent to room temperature before extraction.
2. Use fresh blood cooled on ice or thaw frozen blood samples in the ice-water bath for 12–15 min before the extraction. (**Tip**: use occasional quick cycles of warm hands while thawing). We recommend extracting a maximum of 8 samples at a time.
3. Pipette 500 µL of BUFFER A into 1.5 mL microtubes.
4. Heat the aliquots of BUFFER A in a dry bath set at 75°–80°C for 2–3 min (up to 5 min). Keep in the dry bath until step 6.
5. Quickly mix blood samples by a couple of up-and-down pipetting cycles with simultaneous rotation of the tip before extraction; avoid foaming.
6. Add 100 µL of blood in one quick, firm move directly into the microtube with hot BUFFER A in the dry bath. Immediately mix by a few intensive up-and-down pipetting cycles with simultaneous rotation of the tip to efficiently mix the cold sample with the hot BUFFER A.
7. Incubate homogenate at 75°–80°C for 1 min.
8. Cool the mixture in an ice-water bath for at least 5 min. After cooling on ice, homogenate should polymerize without any free liquid.
9. Centrifuge at 20 000 x g for 10 min at 4°C. Transfer the supernatant into a clean microtube and discard the pellet. Keep obtained total extracts (supernatants) containing NAD+ and NADH at 4°C, covered with a foil lid until the next step. Continue from step 2 to step 9 with the next batch of 8 samples as long as you have samples to extract. Once all samples are extracted, continue to step 10. *Optional:* The supernatants can be stored at -80°C for one week. In this case, thaw at room temperature for 10 min before preparing aliquots in step 10.
10. Prepare two separate aliquots from the obtained extracts into clean microtubes: 150 µL / tube.
11. To the first 150 µL aliquot, add 100 µL of **NAD+** stabilizing reagent to get stabilized extract with NAD+ (keeps NAD+ and removes NADH). Vortex and incubate at room temperature for 5 min. If the assay is not performed immediately, refrigerate the stabilized extracts until the assay. **Protect from light by covering with an aluminum foil lid.**
12. To the second 150 µL aliquot, add 100 µL of **NADH** stabilizing reagent to get stabilized extract with NADH (keeps NADH and removes NAD+). Vortex and incubate for 2 min in a dry bath set at 80°C. Cool on ice for 5 min. If the assay is not performed immediately, refrigerate the stabilized extracts until the assay. **Protect from light by covering with an aluminum foil lid.**

NOTE: Final dilution of the original whole blood sample will be 10 times.

NOTE: In case of individual supplementation with NAD precursors, the levels of NAD+ may increase in the blood; thus, the NAD+ stabilized extract should be further diluted (twice) using deionized water before the assay. In this case, the dilution of the original blood sample will be 20 times for NAD+. The NADH-stabilized extract does not require dilution.

PREPARATION OF STANDARDS

Note: this section is done under normal light conditions.

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

STANDARD NUMBER	CONCENTRATION of NAD+ (µM)	STANDARD PREPARATION			
		50 µM NAD+ stock (µL)	dH_2O (µL)	BUFFER A (µL)	NAD+ stabilizing reagent (µL)
ST1	0	0	100	500	400
ST2	1	20	80	500	400
ST3	2	40	60	500	400
ST4	3	60	40	500	400
ST5	5	100	0	500	400

4. Prepare **10 µM stock of NADH** by adding 10 µL of 1 mM NADH standard stock (provided) into 990 µL of deionized water, vortex. This stock is used to prepare NADH standards and Positive control.
5. Prepare **NADH standards** according to the scheme below by mixing indicated volumes of reagents in the following order: deionized water, 10 µM NADH stock, BUFFER A, and NADH stabilizing reagent. The final volume of each standard is 1 mL. (**Tip**: use the same single 20–200 µL pipette for pipetting 10 µM NADH stock and water.)

STANDARD NUMBER	CONCENTRATION of NADH (µM)	STANDARD PREPARATION			
		10 µM NADH stock (µL)	dH_2O (µL)	BUFFER A (µL)	NADH stabilizing reagent (µL)
ST1	0	0	100	500	400
ST2	0.2	20	80	500	400
ST3	0.4	40	60	500	400
ST4	0.6	60	40	500	400
ST5	1	100	0	500	400

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

PREPARATION OF POSITIVE CONTROL

Note: this section is done under normal light conditions

Positive control is prepared before the assay by mixing known amounts of NAD+ and NADH standards with the Positive control buffer (provided). The volume of the Positive control and concentration of NAD metabolites mimic a blood sample of a healthy human subject. Prepared Positive control undergoes the same extraction and stabilization steps as whole blood samples.

1. Thaw the microtube with Positive control (buffer) for 5 min at room temperature.
2. To prepare Positive control, add 45 µL of Positive control buffer into a microtube.
3. Add 75 µL of **50 µM NAD+ stock** (for preparation, see pg. 11) and 30 µL of **10 µM NADH stock** (for preparation, see pg. 11) into Positive control buffer and vortex. The expected concentration of NAD+ in Positive control is 25 ± 3 µM, and NADH is 2 ± 0.3 µM.
4. Positive control does not contain proteins; therefore, it can be extracted with BUFFER A at room temperature. Add 100 µL of the prepared Positive control into 500 µL of BUFFER A at room temperature to create an "extract" of Positive control, vortex, and proceed to step 5.
5. Prepare two separate aliquots of the Positive control "extract" (from step 4) into clean microtubes: 150 µL/tube.
6. To the first 150 µL aliquot, add 100 µL of **NAD+ stabilizing reagent**, vortex, and incubate at room temperature for 5 min. Then keep refrigerated and covered with foil until the assay. This stabilized Positive control aliquot will be used in **NAD+** assay.
7. To the second 150 µL aliquot, add 100 µL of **NADH stabilizing reagent**, vortex. Heat this solution at 75°–80°C for 2 min; cool on ice. Then keep refrigerated and covered with foil until the assay. This stabilized Positive control aliquot will be used in **NADH** assay.

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

ASSAY PROCEDURE

Notes:

- Steps 1–3 are performed under normal light conditions.
- **Steps 4–9 are performed in dim conditions (when direct artificial light is turned off).**
- Every assay contains sample blanks to correct for all unspecific background signals. Sample blanks are prepared from four stabilized sample extracts (e.g., BL UNK1–4 of samples 1–4) to correct for unspecific interaction between the extract components and Assay color reagent in the master mix. Positive control does not require a separate blank.
- If the blood of subjects undergoing supplementation with NAD+ precursors is analyzed, these samples require at least two wells with sample blanks for this sample type. If samples of supplemented and non-supplemented subjects will be analyzed on the same plate, we recommend preparing two wells of sample blanks per condition. For this, randomly select two stabilized extracts from supplemented individuals and two from non-supplemented. Use these extracts to prepare sample blanks: two wells per condition.
- **All sample blanks are incubated with master mix WITHOUT added enzyme**.
- The enzyme should be thawed just before being added to the master mix. Briefly centrifuge the microtube with the enzyme at low speed before opening.

Steps:

1. Incubate ready standards, stabilized sample extracts, and stabilized Positive control for 5 min at room temperature before pipetting onto the plate.
2. Pipette 20 µL of each NAD+ or NADH standard in duplicates starting from ST1 (0 µM), according to the plate scheme on pg. 23.
3. Pipette 20 µL of stabilized Positive control and stabilized sample extracts in duplicates (see the scheme on pg. 23). For the first four stabilized sample extracts, pipette one extra replicate to the indicated well (BL UNK1–4) according to the scheme on pg. 23. These four wells are sample blanks needed for the analysis without enzyme to correct for the unspecific interaction between the extract and Assay color reagent within the master mix.
4. From this step onwards, work in dim conditions. Prepare the master mix by adding Assay color reagent into BUFFER C; mix gently by rotation.
5. Add 190 µL of the master mix without enzyme into each of the four sample blank wells (BL UNK1–4).
6. Add 40 µL of enzyme into the bottle with the remaining master mix. Mix gently; avoid foaming. Pour the master mix with added enzyme into the reservoir; protect the master mix in the reservoir during pipetting with an aluminum foil lid. (Please see further instructions in our instructional video at www.nadmed.com.)
7. Add 190 µL of the master mix with added enzyme to all remaining wells, including stabilized Positive control, using a multichannel pipette; avoid foaming and direct light. Immediately cover the ready plate with the aluminum foil lid.
8. **For NAD+ assay**: incubate the covered plate for 4–6 min at room temperature.

   For NADH assay: incubate the covered plate for 6–10 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 intense signal will be observed. The color intensity in NADH assay is lower than in NAD+ because the concentration of NADH is much lower than NAD+ in the blood. Therefore, the NADH standards range is from 0 to 1 µM, while the NAD+ standards range from 0 to 5 µM.

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

CALCULATION OF RESULTS

1. Calculate the average of the absorbance readings for each standard (ST1–ST5). Create a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration (in µM) on the x-axis and perform a simple linear regression fitting of the standard curve.
2. Find the concentration of each metabolite in each well using the formula of linear regression of the standard curve.
3. Calculate the average of four sample blanks (BL UNK1-4). The obtained value represents an unspecific signal of the stabilized extracts.
4. Calculate the average of duplicates of each stabilized sample extract (UNK).
5. Calculate the average of duplicates of the stabilized Positive control and multiply by 10 to obtain the concentration (µM) of NAD+ and NADH metabolite in the original 150 µL of Positive control.
6. Correct the obtained concentrations in stabilized sample extracts for the sample blank value (calculated in step 3) and multiply by 10 to obtain the concentration (µM) of NAD+ or NADH metabolite in the original blood sample (see details on page 16). If NAD+ stabilized extracts have been additionally diluted, the concentration must be multiplied by the additional dilution factor.

POSITIVE CONTROL

Positive control aims to monitor the efficiency of the stabilization step when one of the metabolites is removed from the mixture. The concentration of measured NAD+ in Positive control is expected to be within 22–28 µM; the concentration of NADH is expected to be within 1.7–2.3 µM.

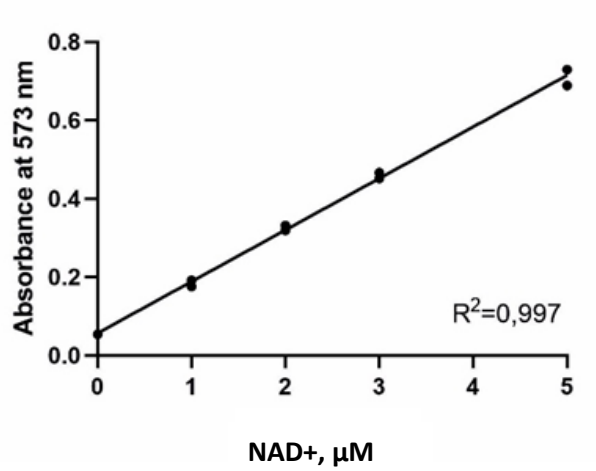
- Absorbance values of stabilized Positive control on the NAD+ plate is expected to be between the absorbance of ST3 and ST4.
- Absorbance values of stabilized Positive control on the NADH plate is expected to be equal to ST2 with a variation of 0.05 optical units.

PERFORMANCE AND LIMITATIONS

A. TYPICAL DATA

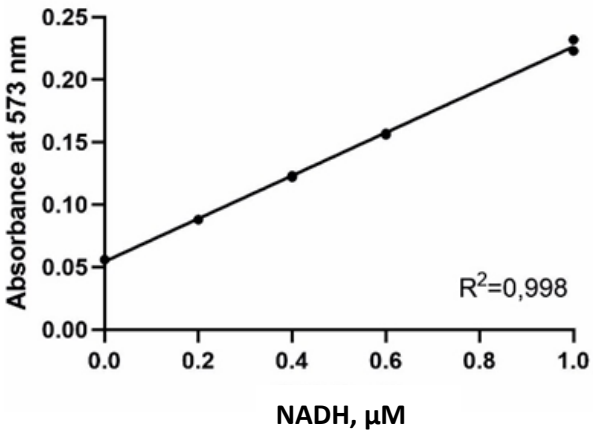
The standard curve and the concentrations in the stabilized sample extracts are provided for demonstration only and should never be used instead of the real-time calibration curve.

STANDARD CURVE FOR NAD+



Standard	NAD+ (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

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

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	3.008	30.08
	3.151		
UNK 2	2.841	2.945	29.45
	3.129		
UNK 3	2.686	2.668	26.68
	2.730		
UNK 4	1.895	1.907	19.07
	1.999		
UNK 5	2.346	2.343	23.43
	2.420		
UNK 6	3.432	3.425	34.25
	3.499		
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

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	0.086	0.86
	0.239		
UNK 2	0.284	0.133	1.33
	0.290		
UNK 3	0.228	0.077	0.77
	0.234		
UNK 4	0.234	0.083	0.83
	0.239		
UNK 5	0.200	0.044	0.44
	0.195		
UNK 6	0.228	0.083	0.83
	0.245		
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

B. LIMITS OF DETECTION

The Limit of Blank (LoB) for Q-NADMED 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

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

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

D. 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

E. ASSAY CUT-OFF

The low and high cut-off values represent the smallest and highest concentrations observed in 5–7% of 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

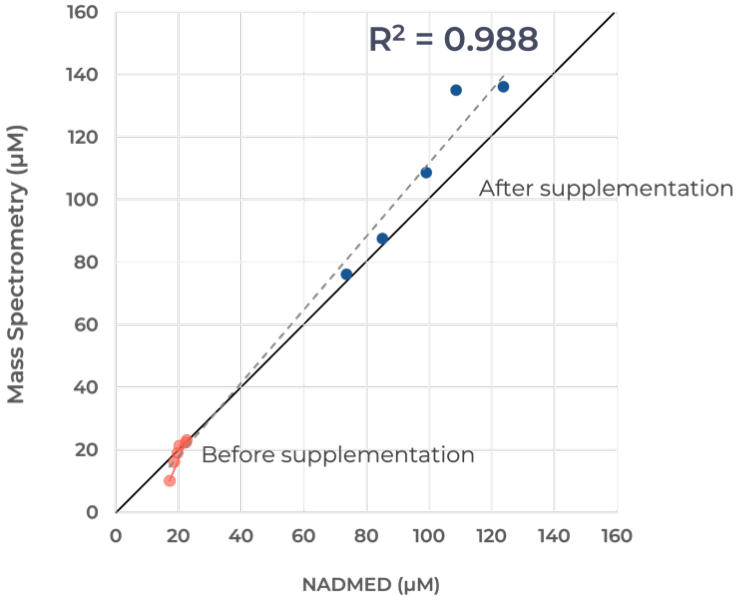
F. PERFORMANCE CHARACTERISTICS

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

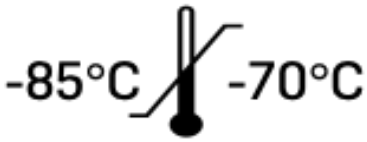
Warning: Potassium sorbate, borate, pyridine, and bismuth in a sample can cause enzyme inhibition, thus causing underestimation of the results.

G. METHOD COMPARISON

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.



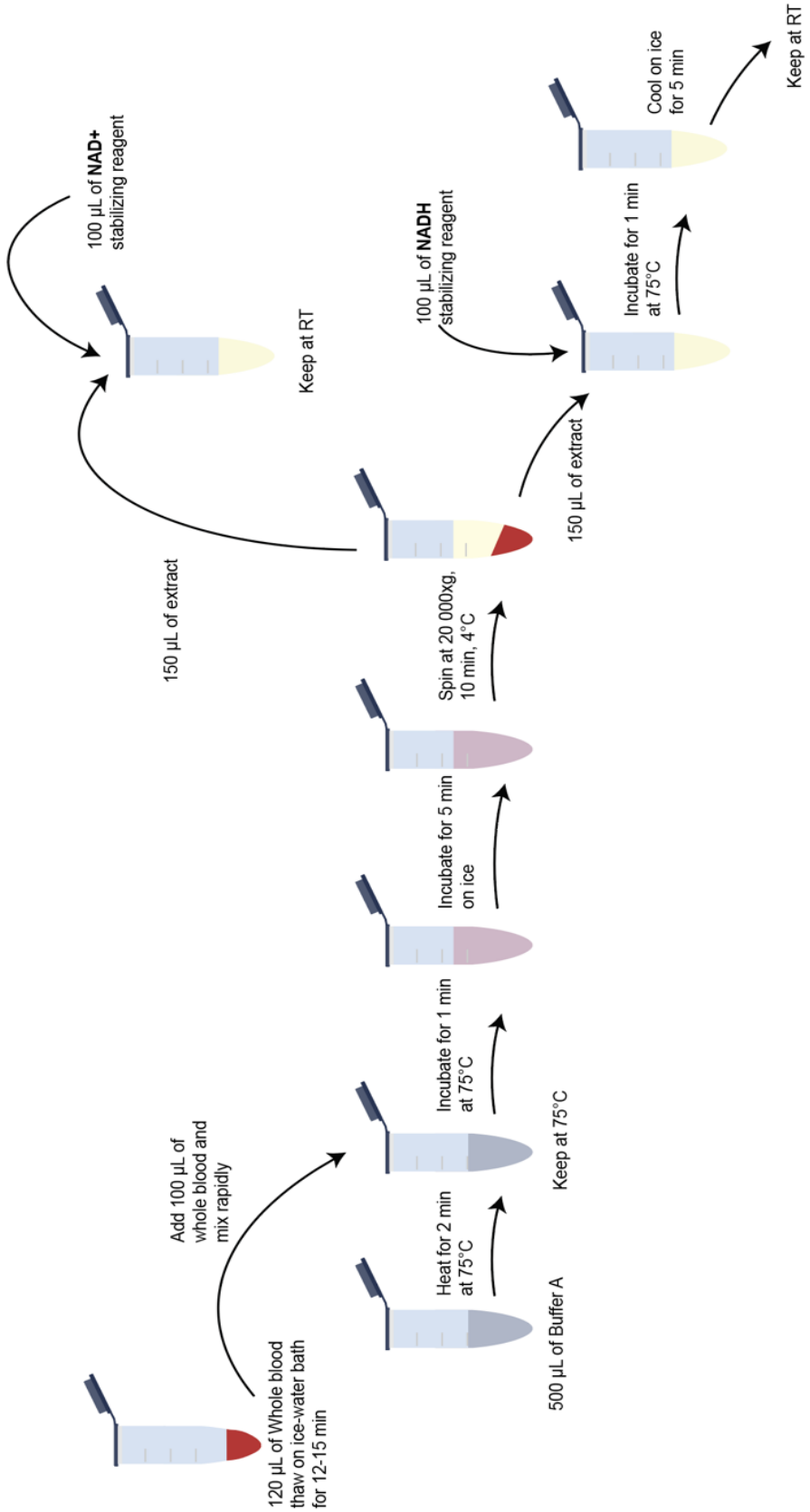
SYMBOLS

Symbol	
	Flammable liquid and vapor
	Warning/danger
	Consult instructions for use
	Use-by date
	Catalogue number
	Batch code
	Manufacturer
	Upper limit of temperature

	Do not use if package is damaged
	Keep dry
	Number or reactions
	For Research Use Only
	Protect from direct light

SCHEMATIC PICTURES

A. EXTRACTION OF NAD+ AND NADH FROM BLOOD



B. RECOMMENDED PLATE LAYOUT FOR NAD+ OR NADH MEASUREMENT

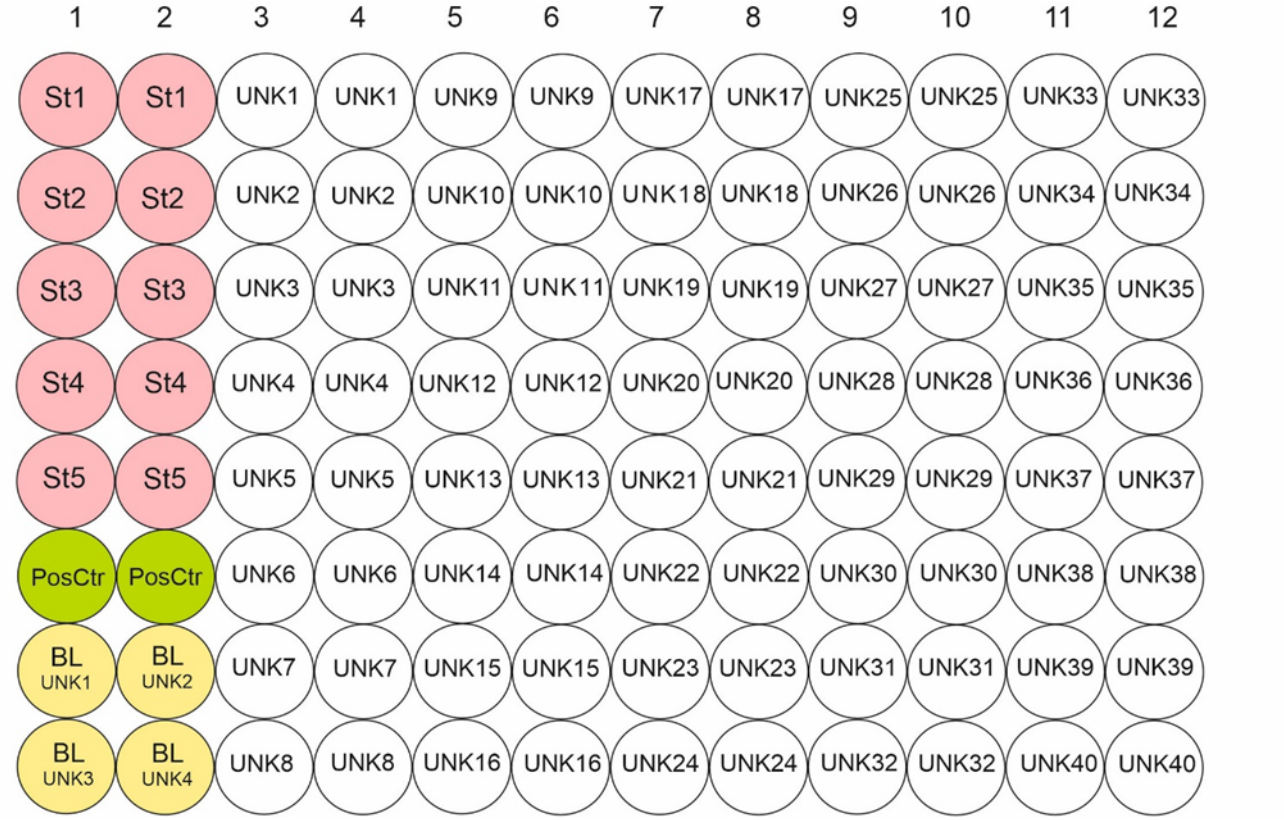


Plate layout for NAD+ or NADH assay: St – standard, BL – blanks with indicated samples used for the blank reading, UNK – unknown samples, stabilized extracts from the samples with unknown metabolite concentration, PosCtr – stabilized Positive control. Use 20 µL of standards, Positive control, and stabilized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

NOTES

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.