NADMED

EN

Q-NAD BLOOD
NAD+ and NADH assay kit

Quantitative assay kit for whole blood

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
B. Catalog number: RUO_001, 40 samples (96-well format)
C. Storage: -80˚C
D. IFU issued: March 2023

Manufacturer:

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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 are involved in adaptation of 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, signaling of an imbalance of body energy homeostasis. Degree of NAD+ decrease varies in different patients and in different pathologies. 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 with the aim to correct it and increase efficiency of treatment. Ongoing research on the contribution of NAD+ and NADH to mechanisms and progression of different diseases is very active. List of pathologies with suspected changes of NAD+ and NADH concentrations is constantly expanding with already published evidence for mitochondrial disease, aging, sepsis, viral infections, cardiovascular and kidney disease, Diabetes type 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, whereas 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 to 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 in vitro diagnostic use only by trained personnel.

The Stop Solution may cause skin, eye and respiratory irritation. Avoid breathing in 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.

Identified hazards of the chemicals presented in this kit and the appropriate warning information associated with those hazards are presented in NADMED Safety Data Sheet (SDS).

Disposal of used kit components is described in in NADMED Safety Data Sheet (SDS).

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.
- The Enzyme should be used directly after thawing.
- Standards and the 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
- Microtubes (1.5 mL, e.g., microcentrifuge tubes) for dilution of standards and sample preparation
- Two plastic Multichannel Pipette reservoirs, one for pipetting Master Mix, the other for pipetting Stop Solution
- Two 96-well transparent polystyrene microplates with medium protein binding affinity designed 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
- 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 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 a precipitate forms in Stop Solution upon thawing, it should be re-dissolved by 5 min incubation at +37°C, and then cooled back down 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 Master Mix required for 1 plate of either 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 only suitable for whole blood samples. Whole blood samples should be collected into either heparin or EDTA collection tubes (e.g. BD K2E Vacutainer or TASSO+ device©) and **properly mixed**. Samples can be analyzed fresh or frozen. Fresh blood can be analyzed within 72 hours if maintained at 4 – 8°C. Fresh samples can be aliquoted (e.g. 120 - 200 µL) within 72 hours and then stored frozen at -20°C for one month or at -80°C for approximately a year. The kit is measuring intracellular NAD content. Thus, it is very important to adequately mix each fresh sample during the aliquoting to ensure that every aliquot contains approximately the same number of cells. Frozen samples must be maintained frozen before the assay. Time intervals during sample handling should be consistent for all samples. For measurement of 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 measurement of 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 prior to opening.
- Both fresh and frozen blood samples can be used for the assay. It is very important to have homogenous (properly mixed) sample 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 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 which turns brown upon enzymatic reaction. The exposure to direct sunlight or artificial light causes its unspecific color change to green. To minimize the light interference with the assay, we recommend:
  - pipetting of Master Mix and then Stop Solution into the plate under conditions of natural indirect light. To achieve these conditions, switch off artificial indoor lights and avoid pipetting close to the window.
  - covering the 96-well plate with a lid from an aluminum foil during the reaction time after addition of Master Mix, as well as during the transfer to the plate reader after addition of Stop Solution. Do not wrap the plate with the aluminum foil due to risk of contamination and sample mixing during unwrapping.
- Positive Control is an artificial sample containing known amounts of pure NAD+ and NADH.
- Technical requirements for 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 is fitting 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 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 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 one measurement. In the latter case, set the number of flashes in the range from 5 to 10.
- Master Mix and Stop Solution contain detergents. To avoid bubbles, pipette 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. On the day of the assay, first prepare the standards and Positive Control.
    3. On the day of the assay, thaw bottles with BUFFER C and Assay Color reagent and bring them to room temperature. It takes about 2-3 hours to melt. During this time perform extraction of the samples and prepare two aliquots of the sample extracts for separate measurement of NAD+ and NADH. Perform NAD+ and NADH assays on separate plates, one at the time.

EXTRACTION OF NAD+ AND NADH FROM BLOOD

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 prior to the extraction. (**Tip**: use occasional quick cycles of warm hands during thawing time). We recommend extracting a maximum of eight 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 (max 5 min). Keep in the dry bath until step 6.
5. Quickly mix blood samples by couple of up-and-down pipetting cycles with simultaneous rotation of the tip before extraction, avoid foaming.
6. Add 100 µL of blood by one quick firm move directly into the microtube with hot BUFFER A kept 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 both NAD+ and NADH at 4°C, covered with a foil lid until the next step. Optionally, the supernatants can be stored at -80°C for one week. In this case, thaw at room temperature for 10 min before preparation of 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, keep the stabilized extracts refrigerated 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 75°- 80°C. Cool on ice for 5 min. If the assay is not performed immediately, keep the stabilized extracts refrigerated 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 and thus, the NAD+ stabilized extract should be further diluted (two times) 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

Handling notes: Protect the standards from light with aluminum foil.

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 for preparation of NAD+ standards and Positive Control.
3. Prepare **NAD+ standards** according to the scheme below by mixing indicated volumes of 50 µM NAD+ stock, deionized water, BUFFER A and NAD+ stabilizing reagent. 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 for preparation of NADH standards and Positive Control.
5. Prepare **NADH standards** according to the scheme below by mixing indicated volumes of 10 µM NADH stock, deionized water, BUFFER A and NADH stabilizing reagent. 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 prior to pipetting on the plate.

PREPARATION OF POSITIVE CONTROL

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 page 11) and 30 µL of **10 µM NADH stock** (for preparation, see page 11) into Positive Control buffer and vortex. Expected concentration of NAD+ in Positive Control is 25 ± 3 µM, and of NADH is 2 ± 0.3 µM.
4. Positive Control does not contain proteins and 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

Handling notes: Important! Pipette Master Mix and Stop Solution into plates under conditions of natural indirect light (see practical considerations).

- Every assay contains Sample Blanks to correct for all unspecific background signals. Sample Blanks are prepared from the first four stabilized sample extracts (BL UNK1-4 of samples 1-4) to correct for unspecific interaction between the extract components and Assay Color reagent in Master Mix. Positive Control does not require a separate Blank.
- **All Sample Blanks are incubated with Master Mix WITHOUT added enzyme**.

Enzyme should be thawed just before addition into Master Mix. Briefly centrifuge the microtube with enzyme at low speed prior to opening.

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 page 22.
3. Pipette 20 µL of stabilized Positive Control and stabilized sample extracts in duplicates (see the scheme on p.22). For the first four stabilized sample extracts, pipette one extra replicate to the indicated well (BL UNK1-4) according to the scheme on p.22. These four wells are Sample Blanks needed for the analysis without enzyme, to correct for unspecific interaction between the extract and Assay Color reagent within the Master Mix.
4. Prepare Master Mix by adding Assay Color reagent into BUFFER C, mix gently by rotation.
5. From this step onwards, turn off the direct indoor lights. Protect Master Mix in the reservoir during pipetting by using an aluminum foil lid (please see further instructions in our instructional video at www.nadmed.com).
6. Add 190 µL of Master Mix into each of the four Sample Blank wells (BL UNK1-4).
7. Add 40 µL of Enzyme into the remaining Master Mix. Mix gently, avoid foaming.
8. Add 190 µL of 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.
9. **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 difference in color intensity between samples with added enzyme and Sample Blanks. The longer the reaction time the more intense signal will be observed. Color intensity in NADH assay is lower than in NAD+, because concentration of NADH is much lower than NAD+ in the blood. Therefore, NADH standards range is from 0 to 1 µM, while NAD+ standards range is from 0 to 5 µM.
10. Stop the reactions by adding 10 µL of Stop Solution to each well in the same order as Master Mix using a multichannel pipette. Avoid foaming, gently shake the plate by hand on a table surface, remove bubbles.
11. Measure light absorbance at 573 nm immediately after addition of Stop Solution. If possible, shake the plate inside the microplate reader for 5 sec before the measurement. Note: after addition of stop solution color intensity can slowly increase uniformly in all the wells. This is expected due to non-enzymatic background process in the Master Mix.

CALCULATION OF RESULTS

1. Calculate average of 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 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 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 the details on page 16). If NAD+ stabilized extracts have been additionally diluted, the concentration must be multiplied by the additional dilution factor.

POSITIVE CONTROL

The purpose of Positive Control is to monitor efficiency of the stabilization step when one of the metabolites is removed from the mixture. Concentration of measured NAD+ in Positive Control is expected to be within the range of 22 - 28 µM, concentration of NADH is expected to be within the range of 1.7 - 2.3 µM.

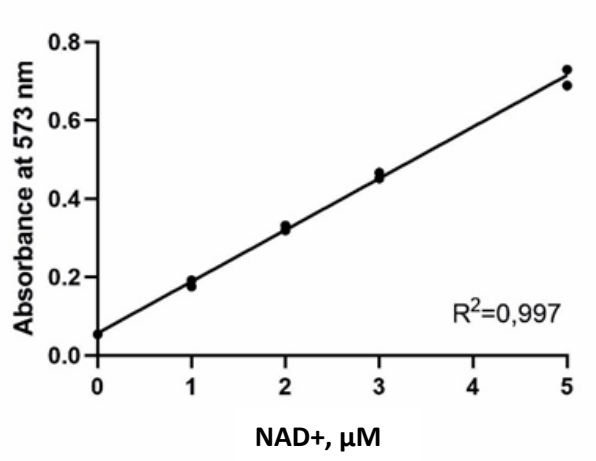
- Absorbance values of stabilized Positive Control on the NAD+ plate is expected to be between the absorbance of ST3 and ST4.
- Absorbance values of stabilized Positive Control on the NADH plate is expected to be equal to ST2 with variation 0.05 optical units.

PERFORMANCE AND LIMITATIONS

A. TYPICAL DATA

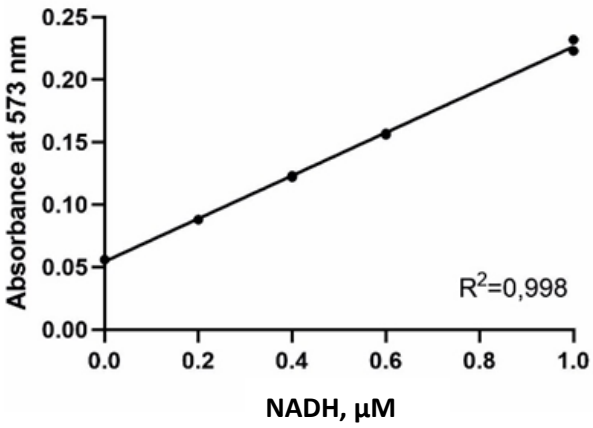
The standard curve and the concentrations in the stabilized sample extracts are provided for demonstration only and should never be used instead of the real-time calibration curve.

STANDARD CURVE FOR NAD+



Standard	NAD+ (µM)	Absorbance (573 nm) Assay time - 4min
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 Blank (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 Blank (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-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).

Low detection limit	
	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

Precision of the assay performance was determined by Intra-assay variation in measurement. Intra-Assay precision is presented in the table below (CV = coefficient of variation).

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

Results of the assay reproducibility are summarized in the table below (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 amount of pure NAD+ and NADH. The results are summarized in the table below (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 the highest concentration observed in 5 - 7% individuals of given population extract. The cut-off values are summarized in the table below.

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

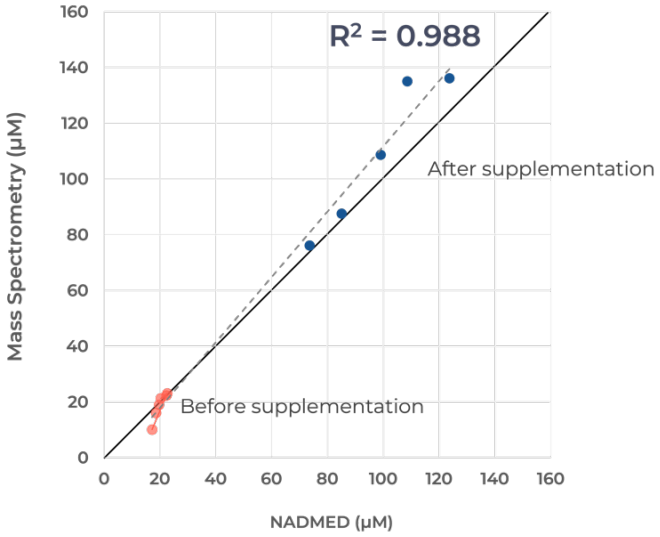
F. PERFORMANCE CHARACTERISTICS

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

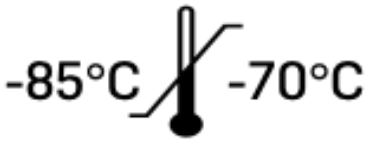
Warning: presence of potassium sorbate, borate, pyridine, bismuth in a sample can cause enzyme inhibition, and thus causing underestimation of the results.

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.



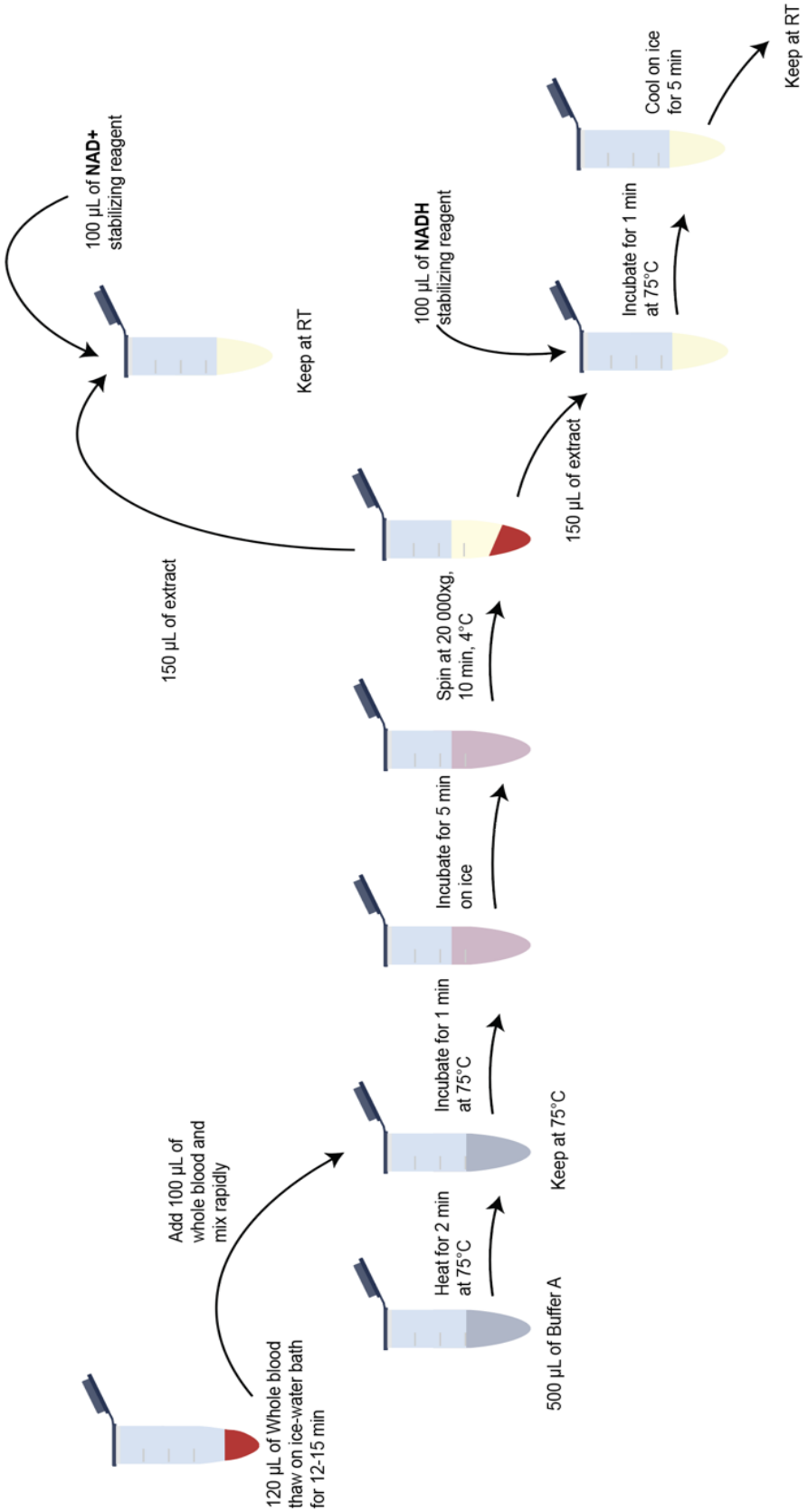
SYMBOLS

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

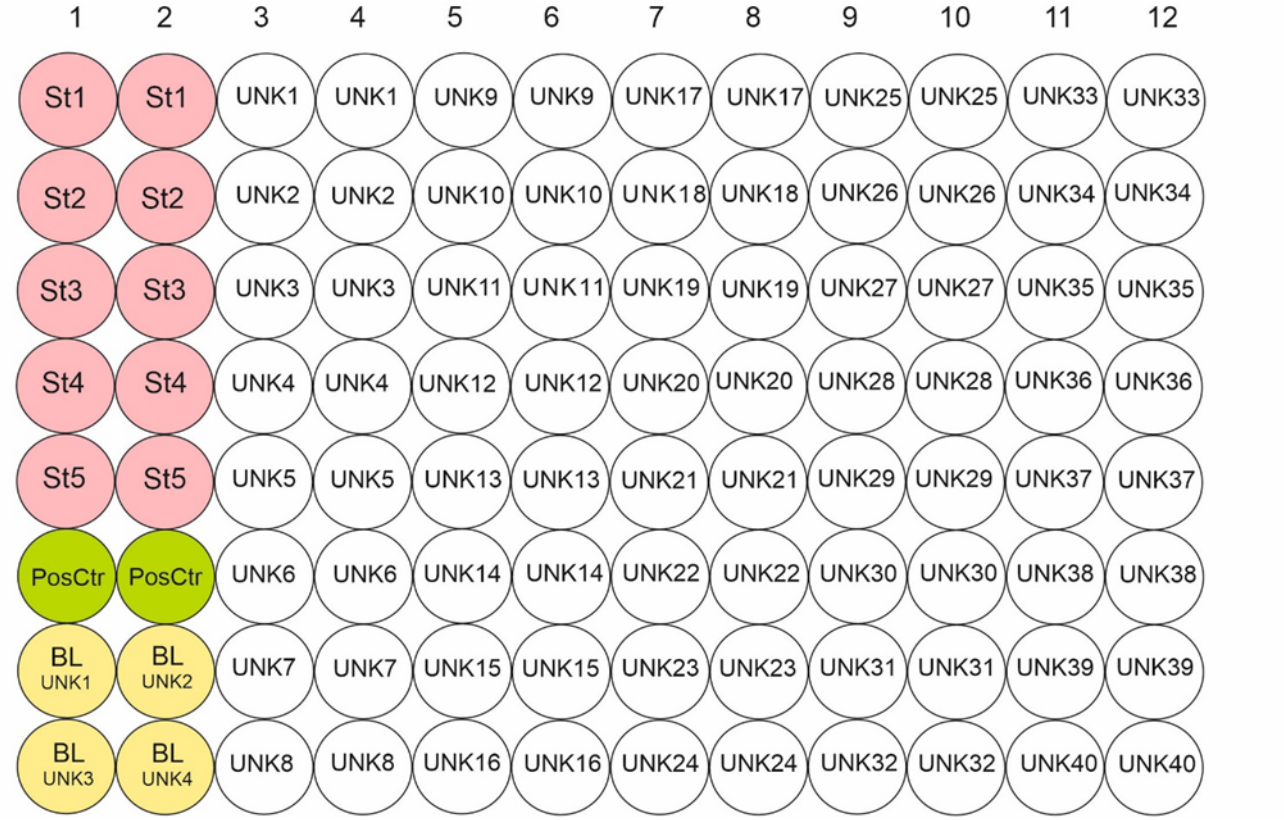


Plate layout for NAD+ or NADH assay: St – standard, BL - blanks with indicated samples used for the blank reading, UNK – unknown samples, stabilized extracts from the samples with unknown metabolite concentration, PosCtr – stabilized Positive Control. Use 20 µL of standards, Positive Control and stabilized 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.