NADMED

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Q-NADMED BLOOD

Quantitative assay kit for NAD+ and NADH in blood

FOR SINGLE USE ONLY

These instructions must be read in their entirety before using this product.

CE IVD FOR IN VITRO DIAGNOSTIC USE

GENERAL INFORMATION

A. Proprietary name: Q-NADMED Blood: quantitative assay kit for NAD+ and NADH in blood
B. Catalog number: IVD_001, 40 samples (96-well format)
C. Storage: -80˚C
D. IFU issued: October 2022

Manufacturer:

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INTENDED USE

Q-NADMED Blood, an in-vitro diagnostic medical device, is an analytical assay kit for measurement of concentrations of NAD+ and NADH metabolites in human whole blood. The assay is quantitative. The intended users of the Q-NADMED assay kit are trained laboratory personnel. The first intended purpose is to detect systemic changes of NAD+ and NADH. The primary intended users of the assay results are healthcare professionals who interpret the obtained results in the context of disease/health status. Results of the Q-NADMED assay kit can be used for decision making on treatment such as supplementation with NAD precursors. The second intended purpose of Q-NADMED assay kit is to monitor NAD+ and NADH levels in patients receiving treatment such as supplementation of NAD precursors and to adjust the dose.

CLINICAL 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-NADMED 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 vial with 40 µL of 1 mM NAD+	See preparation guide.
NADH standard stock	1 vial with 40 µL of 1 mM NADH	See preparation guide.
BUFFER C	2 vials with 19 mL of Assay Buffer	Equilibrate to room temperature (15-25°C).
Assay color reagent	2 vials 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 vials 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 vial with 45 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 Q-NADMED Safety Data Sheet (SDS).

Disposal of used kit components is described in in Q-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, BUFFER C, NAD+ and NADH Stabilizing Reagents and Stop Solution are stable for 2 weeks at room temperature
- Assay Color reagent is stable for 2h 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:

- MilliQ water
- Test tubes (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.
- Heat block with adjustable temperature (up to 80°C)
- Table-top cooling microcentrifuge (max speed 20 000 x g)
- Ice
- 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
- Microplate reader capable of measuring absorbance at 570-573 nm
- Aluminum foil to protect tubes and plates from light

REAGENT PREPARATION

Bring all components for NAD+ and/or NADH assay to room temperature before use and keep at the temperature indicated in the table above before the assay.

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

B. **NAD+ stabilizing regent** - 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** - adding 25 µL of 1 mM NAD+ standard stock (provided) into 475 µL of MilliQ water, vortex. Protect from light. (For further instructions see preparation of standards and preparation of Positive Control).

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

I. **Master Mix** – 1 vial of **Assay Color Reagent** (3 mL) should be mixed into 1 vial 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) and properly mixed. Samples can be analyzed fresh or frozen. Fresh blood can be stored for 3 hours at room temperature or at 4°C for up to 24 hours before analysis. Fresh samples can be aliquoted (e.g. 120 - 150 µL) and stored frozen at -20°C for two weeks or at -80°C for approximately a year. Frozen samples must be kept frozen at all times before the assay. Time intervals during sample handling should be consistent for all samples. For measurement of NAD+ and NADH, 100 µL of the whole blood is needed. Subsequent freeze-thaw cycles are not recommended.

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 vials should be quickly centrifuged at low speed prior to opening.

- Both fresh and frozen blood samples can be used for the assay.
- 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 in the 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 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 by 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. Bring vials with BUFFER C and Assay Color Reagent to room temperature for thawing. 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 test tubes.
4. Heat the aliquots of BUFFER A in a heating block for 1-2 min (max 5 min) at 75° - 80°C. Keep in the heating block until step 6.
5. Quickly mix blood samples by one up-and-down pipetting cycle before extraction, avoid foaming.
6. Add 100 µL of blood by one quick firm move directly into the tube with hot BUFFER A kept in the heating block. Immediately mix by a few intensive up-and-down pipetting cycles with simultaneous rotation of the tip to efficiently mix the homogenate.
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 test tube and discard the pellet. Keep obtained total extracts containing both NAD+ and NADH at 4°C, covered with the foil lid until the next step. Optionally, the supernatants can be stored at -80°C for one week. 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 test tubes - 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 1-2 min at 75° - 80°C (max. 2 min). 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 initial 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 MilliQ water before the assay. In this case, the dilution of the initial 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 tubes with 1mM 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 MilliQ 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, MilliQ water, BUFFER A and NAD+ stabilizing reagent. Final volume of each Standard is 1mL. (**Tip**: use 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)	H_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 MilliQ 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, MilliQ water, BUFFER A and NADH stabilizing reagent. Final volume of each Standard is 1 mL. (**Tip**: use 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)	H_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). Positive control volume and concentration of NAD metabolites mimic a blood sample of healthy human subject. Prepared Positive Control undergoes the same extraction and stabilization steps as whole blood samples.

1. Thaw the tube with Positive Control (buffer) for 5 min at room temperature.
2. Add 75 µL of **50 µM NAD+ stock** (for preparation, see page 10) and 30 µL of **10 µM NADH stock** (for preparation, see page 10) into Positive Control buffer and vortex to prepare Positive Control. Expected concentration of NAD+ in Positive Control is 25 µM, and of NADH is 2 µM.
3. 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 4.
4. Prepare two separate aliquots of the Positive Control "extract" (from step 3) into clean test tubes - 150 µL / tube.
5. 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 until the assay. This stabilized Positive control aliquot will be used in **NAD+** assay.
6. To the second 150 µL aliquot add 100 µL of **NADH stabilizing reagent**, vortex. Heat this solution at 75° – 80°C for 1 min, cool on ice. Then keep refrigerated 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! Protect the Master Mix from light (see practical considerations).

- Every assay contains Sample Blanks to correct for all unspecific background signals. Prepare separately four Sample Blank wells for the first four samples (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 vial 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 21.
3. Pipette 20 µL of stabilized Positive Control and stabilized extracts in duplicates (see the scheme on p.21). For the first four samples, pipette one extra replicate to the indicated well (BL UNK1-4) according to the scheme on p.21. 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. Add 190 µL of Master Mix into each of the four Sample Blank wells.
6. Add 40 µL of Enzyme into the remaining Master Mix. Mix gently, avoid foaming.
7. Add 190 µL of Master Mix with added enzyme to all remaining wells including stabilized Positive Control, using a multichannel pipette, avoid foaming. Immediately cover the ready plate with the aluminum foil lid.
8. **For NAD+ assay**: incubate for 4 - 6 min at room temperature.

   For NADH assay: incubate for 6 - 10 min at room temperature.

   NOTE: the reaction can be stopped when there is a clear color gradient in the standards and difference of color intensity between Samples 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.
9. 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.
10. Measure light absorbance at 573 nm within 5-10 min after stopping the reactions. If possible, shake the plate inside the microplate reader for 5 sec before the measurement.

CALCULATION OF RESULTS

1. Average the duplicate 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 NAD+ and NADH in stabilized extracts added into each well using formula of linear regression of the Standard curve.
3. Average results for four Sample Blanks (BL UNK1-4). The obtained value represents unspecific reactions of the extracts with Assay Color Reagent in the Master Mix.
4. Average results for the duplicate of each sample (UNK).
5. Average results for the duplicate of the Positive control and multiply on 10 to obtain the concentration (µM) of NAD+ and NADH metabolite in the Positive Control.
6. Correct obtained concentrations in Samples for Sample Blank and multiply by 10 to obtain the concentration (µM) of NAD+ or NADH metabolite in the initial blood sample. If NAD+ Stabilized extracts have been additionally diluted, the concentration must be multiplied by 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 24 - 26 µM, concentration of NADH is expected to be within the range of 1.9 - 2.1 µM.

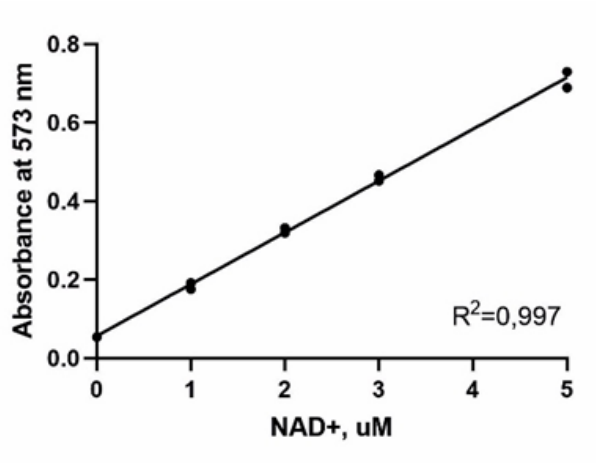
- Absorbance values of stabilized Positive control on NAD+ plate is expected to be between absorbance of ST3 and ST4.
- Absorbance values of stabilized Positive control on NADH plate is expected to be equal to ST2 with variation 0.05 optical units.

PERFORMANCE AND LIMITATIONS

A. TYPICAL DATA

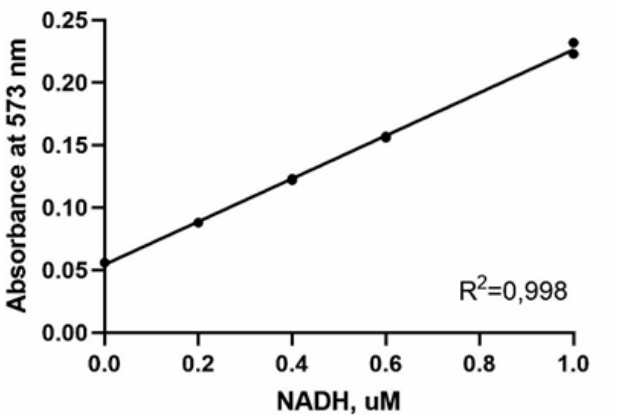
The standard curve and the unknown concentration values are provided for demonstration only and should never be used instead of the real-time calibration curve.

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 unknown samples (UNK) and Sample Blanks (BL UNK1-4) are determined from the linear fit formula of the NAD+ standard curve

Unknown	Concentration in Stabilized Extract (µM)	Concentration in Stabilized Extract 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 unknown samples (UNK) and Sample Blanks (BL UNK1-4) are determined from the linear fit formula of the NADH standard curve.

Unknown	Concentration in Stabilized Extract (µM)	Concentration in Stabilized Extract 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-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).

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

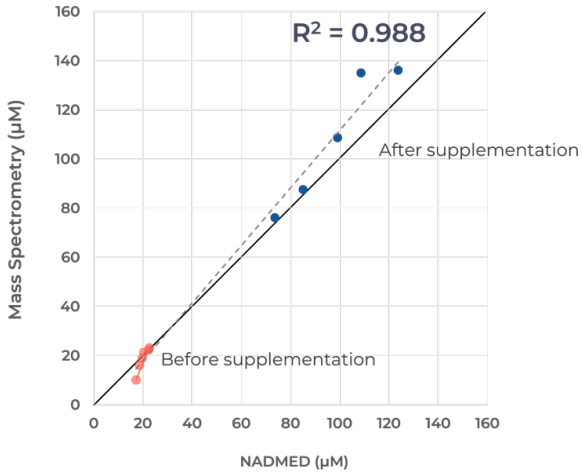
F. PERFORMANCE CHARACTERISTICS

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.

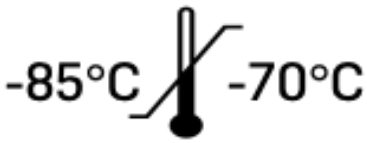
Warning: presence of potassium sorbate, borate, pyridine, bismuth in a sample can cause enzyme inhibition, and thus causing underestimation of the results.

G. METHOD COMPARISON

To validate the performance of Q-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 Q-NADMED and mass spectrometry. Results from Q-NADMED were concordant with those obtained by mass spectrometry.



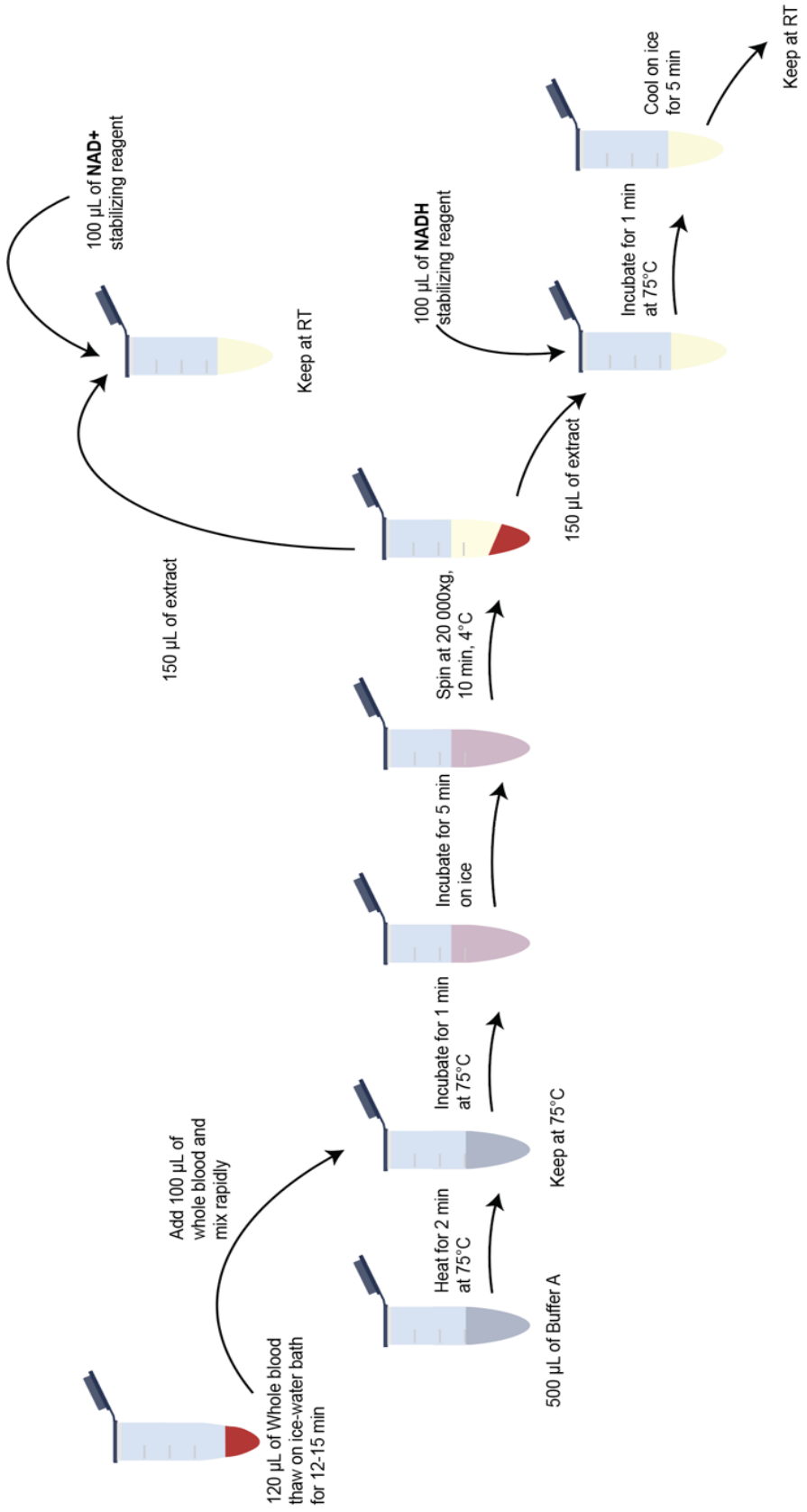
SYMBOLS

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
	In Vitro Diagnostic Medical Device
	Protect from direct light

SCHEMATIC PICTURES

A. EXTRACTION OF NAD+ AND NADH FROM BLOOD



B. RECOMMENDED PLATE LAYOUT FOR NAD+ OR NADH MEASUREMENT

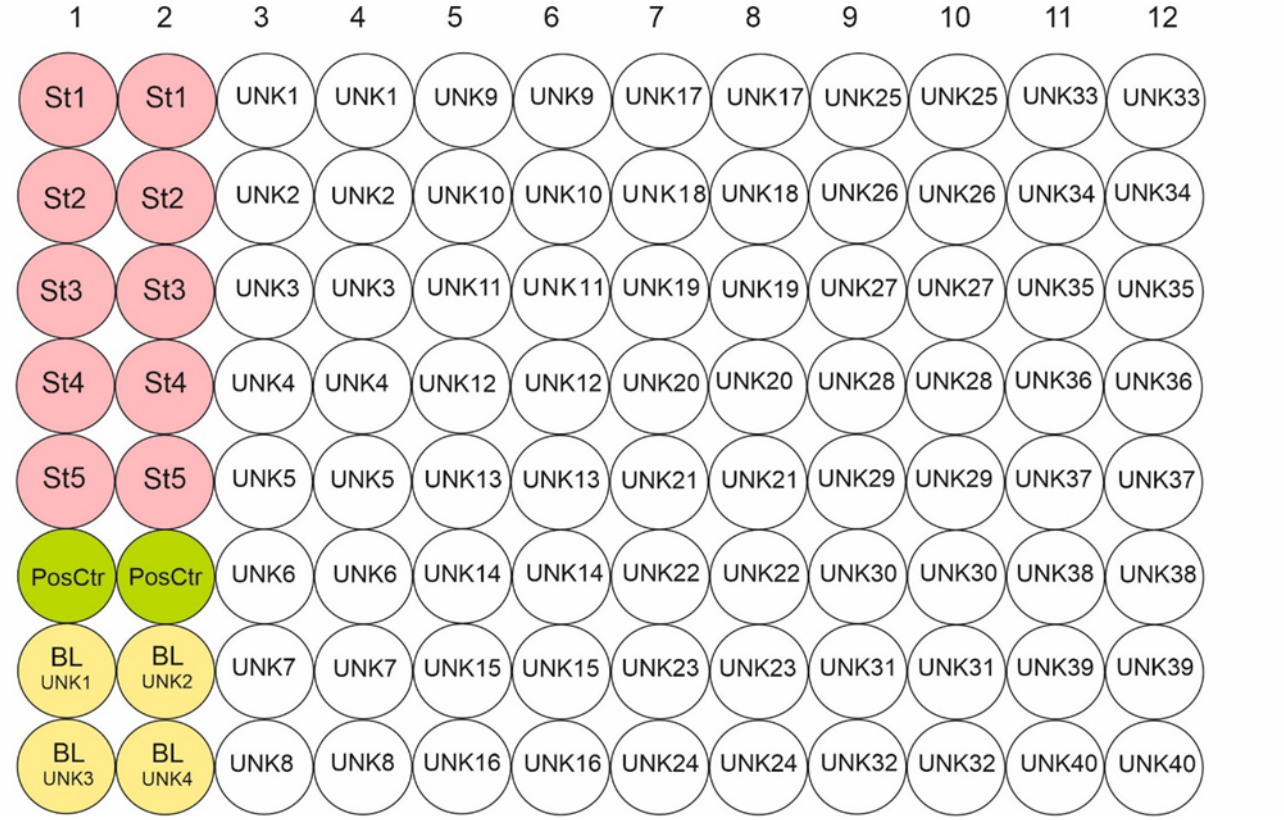


Plate layout for NAD+ or NADH assay: St – standard, BL - blanks with indicated samples used for the blank reading, UNK – extracts from the samples with unknown metabolite concentration, PosCtr – Stabilized Positive Control. Use 20 µL of Standards, Positive Control and Samples per well. Sample Blanks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