NADMED

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# Q-NADMED BLOOD
# NAD+ assay kit

## Quantitative assay kit for whole blood

Version 8.0

**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

**Proprietary name**:
Q-NADMED Blood NAD+ assay kit: quantitative assay kit for NAD+ in whole blood
**Catalog number**:
IVD_001_01_40, 40 samples (96-well format)
IVD_001_01_40/TH, 40 samples (96-well format)
**Storage**:
-85°C – -70°C upon arrival
**IFU issued**:
March 2025

**Manufacturer:**
**NADMED Ltd / Oy**
www.nadmed.com
info@nadmed.com
Haartmaninkatu 4, Building 14
00290 Helsinki
FINLAND

# SYMBOLS ON THE PACKAGING


Contains flammable liquid and vapor. Refer to PRECAUTIONS AND WARNINGS


Warning/danger. Refer to PRECAUTIONS AND WARNINGS


Consult instructions for usage


Use-by date

Catalogue number

Batch code


Manufacturer

Upper limit of storage temperature


Do not use if package is damaged

Number or reactions

In vitro diagnostic medical device


Protect from direct light


Keep dry

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# INTENDED USE

Q-NADMED Blood, an in-vitro diagnostic medical device, is an analytical assay kit for measuring concentrations of NAD+ metabolite 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 in NAD+. The primary intended users of the assay results are healthcare professionals who interpret the obtained results in the context of disease/health status. The 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 the Q-NADMED assay kit is to monitor NAD+ levels in patients receiving treatment, such as supplementing NAD precursors and adjusting the dose.

# CLINICAL BACKGROUND

NAD+ is a known central regulator of human body metabolism and energy homeostasis. 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 (Covarrubias et al. 2021 doi: 10.1038/s41580-020-00313-x). The degree of NAD+ decrease varies in different patients and different 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. The Q-NADMED assay facilitates the screening of individuals and patients for NAD+ deficiency, enabling targeted intervention to rectify this deficiency and enhance the effectiveness of therapeutic regimens.

# PRINCIPLE OF THE ASSAY

The kit measures intracellular NAD+ content. The principle of the assay is a cyclic enzymatic reaction with a colorimetric endpoint detection. First, NAD+ is extracted from a whole blood sample in a single step, followed by a stabilization step. Then, the stabilized extract is analyzed using 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+ in the reaction mixture.

This kit offers the flexibility of NAD+ analysis by allowing the measurement of two smaller sets of samples at two different time points. For example:

- The extraction of 30 samples should be followed by NAD+ analysis on one 96-well plate the same day using components of Set 1.
- On a different day, the extraction of the remaining 10 samples should be followed by NAD+ analysis on the second 96-well plate using components from Set 2.

*Optional:* All 40 samples can be analyzed at once on one 96-well plate using one set of reagents (Set 1). In this case, the second set of reagents (Set 2) will remain unused or can be utilized to repeat the first measurements on the same day.

# SAMPLE HANDLING AND STORAGE

**Requirements and Limitations:**

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

**Blood collection:**

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

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

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

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

*Mixing the Sample*: When whole blood remains stationary, it separates into different phases. During processing, a fresh sample should be thoroughly and frequently mixed.

*Timing of Analysis and Aliquoting:* If you cannot analyze the blood sample immediately after collection, make sure to divide (=aliquot) the sample within 72 hours into the preferred volume of 150–200 µL.

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

*Practices to avoid:* Do not freeze large (2–3 mL) volumes of blood directly in the collection tubes. Do not use skirted double-wall microtubes. These practices can significantly increase the time needed to freeze and thaw. Long freezing/thawing times can cause variability in assay results, affecting the accuracy and reliability of the analysis.

# REAGENT STORAGE, STABILITY, AND PREPARATION

**Before opening, all kit components should be stored at -85°C – -70°C. Avoid temperature fluctuations in the freezer.**

| REAGENT | DESCRIPTION (*) | PREPARATION (**) | STABILITY (**) |
|---|---|---|---|
| **EXTRACTION BUFFER A** | 28 mL<br>Sufficient for 40 samples | Equilibrate to room temperature. Ready for use. | Stable for two weeks at room temperature, or re-frozen at -80°C. |
| **NAD+ STABILIZER** | 2x 8 mL<br>Sufficient for 40 samples | | |
| **POSITIVE CONTROL BUFFER** | 200 µL<br>Sufficient for two plates | | |
| **DEIONIZED WATER** | 10 mL<br>Sufficient for two plates | | |
| **STOP SOLUTION** | 3 mL<br>Sufficient for two plates | Equilibrate to room temperature. Ready for use.<br>(If precipitates have formed, warm in 37°C and cool to room temperature before the assay.) | |
| **ASSAY BUFFER C** | 2x 19 mL<br>One aliquot per 96-well plate | Equilibrate to room temperature. ASSAY BUFFER C + ASSAY COLOR REAGENT = Assay Master Mix.<br>See preparation guide on page 15. | Stable for 12 hours at room temperature after thawing. Keep in the amber bottle. |
| **ASSAY COLOR REAGENT** | 2x 3 mL<br>One aliquot per 96-well plate | | Stable for 3 hours at room temperature after thawing. Keep in the amber bottle. |
| **NAD+ STANDARD STOCK** | 2x 40 µL (1mM)<br>Sufficient for Standards and Positive control | Equilibrate to room temperature. See preparation guide page 13. | Should be used immediately after thawing. Standards should be protected from light. |
| **NAD ENZYME** | 2x 40 µL<br>One aliquot per 96-well plate | Equilibrate to room temperature. Add to the Master mix after processing of plate blanks. | Should be used immediately after thawing. |

*Accepted variation of the filling volume +/-5%.

** Room temperature: 15–25°C

## PRECAUTIONS AND WARNINGS

For *in vitro diagnostic* use only. For trained personnel use only. Do not smoke, drink, eat, or apply cosmetics in the working area. Wear protective gloves, clothing, and eye protection. Wash hands thoroughly after handling.

**EXTRACTION BUFFER A** can cause eye irritation. Handle with care; use goggles.

**NAD+ STABILIZER** can cause skin and eye irritation. Handle with care; use gloves and goggles.

**STOP SOLUTION** may cause skin, eye, and respiratory irritation. Avoid inhaling fumes.

**ASSAY COLOR REAGENT** may cause skin irritation. Handle with care; use gloves.

The Q-NADMED Safety Data Sheet (SDS) presents the identified hazards of the chemicals in this kit and the appropriate warning information associated with those hazards.

The Q-NADMED Safety Data Sheet (SDS) describes the disposal of used kit components.

## TROUBLESHOOTING

If you encounter any issues during extraction or assay performance, refer to the NADMED troubleshooting guide at https://www.nadmed.com/documents/.

# MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

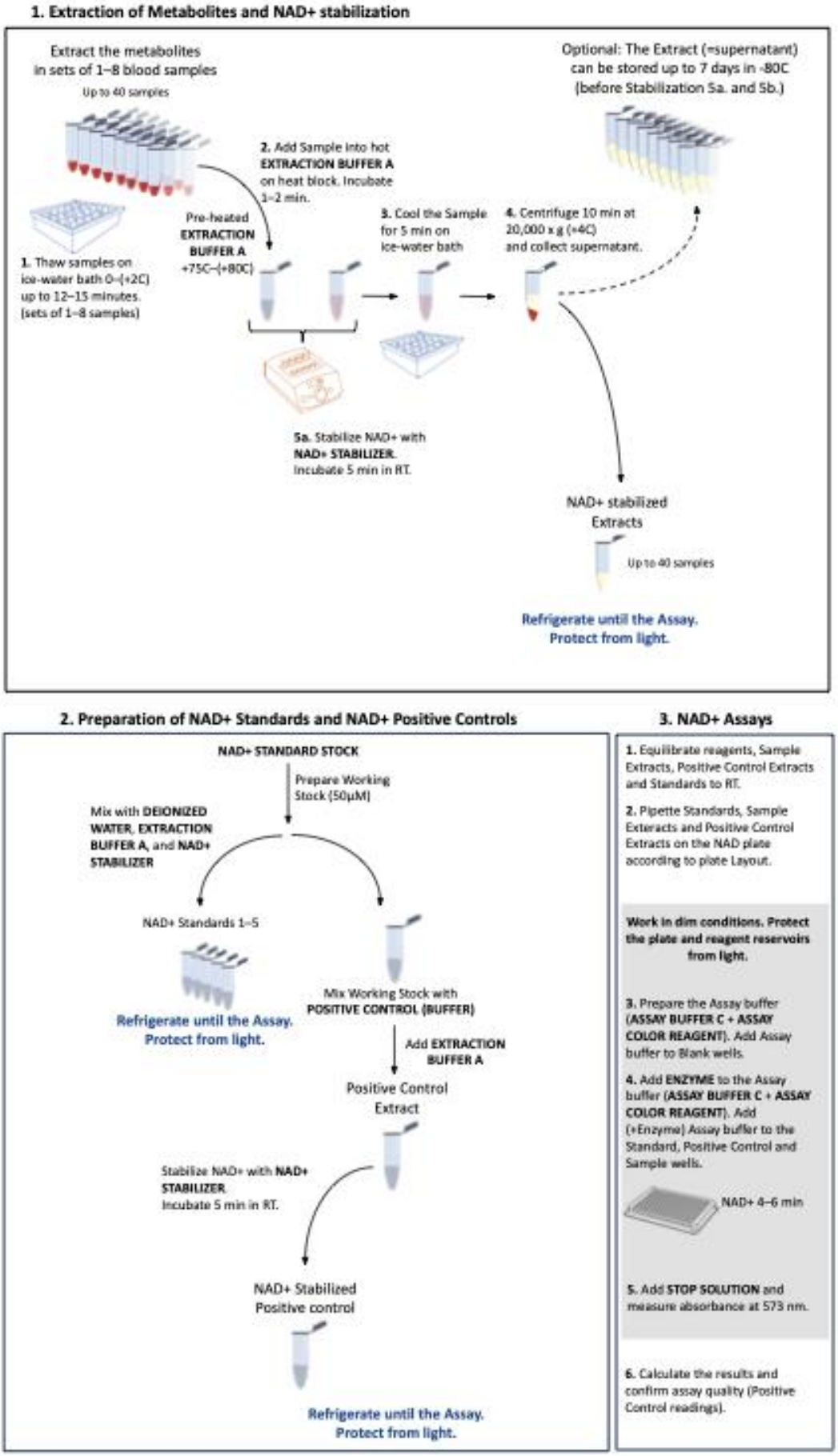
| CATEGORY | ITEM | SPECIFICATIONS/REQUIREMENTS |
|---|---|---|
| **Consumables** | Microtubes, 1.5 mL | Use non-sterile microcentrifuge tubes made from transparent/natural color polypropylene (PP) intended for *in vitro diagnostics* (e.g., Sarstedt ref. 72.690.001).<br>NOT compatible with NADMED assay:<br>a) molecular biology grade sterile microtubes that are free of endotoxin, pyrogen, human DNA, and low retention (chemically sterilized)<br>b) microtubes intended for protein work marked "LoBind". |
| | 96-well plates (2 pieces) | Use non-sterile, transparent, polystyrene flatbottom plates with medium protein binding intended for colorimetric assays (e.g. Revvity, ref. 6055640). |
| | Liquid reservoirs for multichannel pipetting (2 pieces) | Use non-sterile polystyrene plastic. Use separate reservoirs for Assay Master Mix and STOP SOLUTION. |
| | Pipette tips | Use non-sterile, bevelled pipette tips with low retention. |
| | Ice (Ice-water bath) | Fill a container with packed laboratory ice and pour cold tap water to reach to a slush-like state. The added water is sufficient when the L<br>liquid part of the sample is immersed in the water but the ice firmly holds the inserted tubes upright (avoid samples floating in the water). |
| | Aluminium foil | Use foil to protect samples, standards, and the plates from light during assay as specified in the instructions. |
| **Equipment and Machinery** | Calibrated Pipettes | Single channel for volumes of e.g. 5–50 µL, 20–200 µL, and 100–1000 µL.<br>Multichannel pipettes for volumes of e.g. 5–50 µL, and<br>30–300 µL |
| | Microcentrifuge | Use centrifuge with cooling to 4°C and speed of 20,000 x g |
| | Spectrophotometric Microplate Reader | a) Measuring absorbance at 570–573nm wavelength<br>b) Adjustable scanning light brightness/intensity to "low". Alternatively, possibility to adjust the brightness based on the number of flashes per measurement (set to 5–10 flashes). |
| | Dry bath Heat Block fitted for 1.5 mL Microtubes | Adjustable temperature up to 80°C is required. To ensure consistent and reliable results, **test the heat transfer and calibrate the temperature**:<br>1. Set your heat block at 80°C and wait until it reaches 75–80°C.<br>2. Add 500 µL of water into a microtube and place the tube on your heat block. Make sure the microtube fits tightly to the block.<br>3. Insert a conventional lab thermometer into the microtube with water.<br>4. Measure the time needed to reach 75°C. The heat transfer is considered sufficient if the temperature is reached within 5 minutes.<br><br>If the correct temperature is NOT reached with 80°C setting in 5 min:<br>a) ensure the tubes fit the block tightly<br>b) increase the target temperature of your device |
| **Special** | Possibility to work in dim light conditions for the ASSAY part of the measurement. Refer to PRACTICAL CONSIDERATIONS and WORKFLOW OF Q-NADMED BLOOD NAD+ ASSAY. | |

# PRACTICAL CONSIDERATIONS

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

| CATEGORY | INSTRUCTIONS |
|---|---|
| **Limitations** | Read the SAMPLE HANDLING AND STORAGE carefully. This assay is designed for whole blood and is NOT suitable for measuring NAD+ in plasma or serum, cultured cells, or tissues.<br><br>Do not use kit components beyond the expiry date. Do not mix materials from different kit lots. Subsequent freeze-thaw cycles of reagents are not allowed. |
| **Usability** | Thoroughly mix all reagents by gentle swirling. Small microtubes should be quickly centrifuged at low speed before opening.<br><br>We recommend taking the DEIONIZED WATER, EXTRACTION BUFFER A, NAD+ STABILIZER, and STOP SOLUTION to room temperature one day before the assay. Take ASSAY BUFFER C and ASSAY COLOR REAGENT to room temperature on the day of the assay. These bottles take about 2–3 hours to melt. |
| **Accuracy** | The analysis of NAD+ can be done twice separately, for altogether 40 samples. EXTRACTION BUFFER A, POSITIVE CONTROL (BUFFER), DEIONIZED WATER, and STOP SOLUTION are shared components, other provided components in Set 1 and Set 2 are for separate analyses.<br><br>To avoid cross-contamination, change to new pipette tips between the additions of each standard, samples, and reagents. Avoid touching the content of the wells with pipette tips when working with multi-channel pipettes.<br><br>High-precision pipettes and beveled tips with less retention will improve the precision.<br><br>ASSAY BUFFER C and STOP SOLUTION contain detergents. To avoid bubbles, pipette the Master mix and STOP SOLUTION by pressing the pipette to the first stop position only. Remove any bubbles in the wells with a small needle before inserting the plate into plate reader. |
| **Protection from light** | Protect the stabilized sample extracts, standards, and positive controls from light when they are not being actively processed. However, for convenience, extraction, preparation, and pipetting of them onto the 96-well plates can be performed under normal light conditions.<br><br>ASSAY COLOR REAGENT is a yellow, light-sensitive compound that turns brown upon enzymatic reaction of the assay. Exposure to excess natural light or direct artificial light causes unspecific color change to green.<br><br>To minimize the light interference with the assay, the protocol indicates the steps specifically requiring dim conditions. To protect the reactions from both natural and direct artificial light, we recommend the following:<br>• Switch off artificial light source directly above your bench. Close blinds or move further away from a window.<br>• Use aluminum foil covers for the plate and pipetting reservoirs whenever working with ASSAY COLOR REAGENT and Assay Master Mix.<br>• Cover the 96-well plates with aluminum foil covers during Assay incubation steps until the plate is inserted into plate reader. (Do not wrap). |

# WORKFLOW OF Q-NADMED BLOOD NAD+ ASSAY



# EXTRACTION AND STABILIZATION OF NAD+

This section provides guidance on the extraction of NAD+ from whole blood. Following the extraction, NAD+ is stabilized in preparation for colorimetric assays. Extracts (after centrifugation) can be stored at -80°C – -70°C for one week before stabilization on the day of assay.

**TIP:** Please refer to the video guidance (**https://www.nadmed.com/products/nad-only-kit/**).

**NOTE:** Final dilution of the original whole blood sample will be 10 times. In the case of supplementation with NAD-precursors, the levels of NAD+ may increase in the subject's blood. Thus, the NAD+ stabilized extract should be further diluted 1:2 using **DEIONIZED WATER** (provided) before the colorimetric assay. In this case, the dilution of the original blood sample will be 20 times for NAD+.

**Materials:**

| | |
|---|---|
| Dry bath heat block set at 75°–80°C | Refer to MATERIALS REQUIRED-Table |
| Ice-water bath | Refer to MATERIALS REQUIRED-Table |
| Microcentrifuge | Refer to MATERIALS REQUIRED-Table |
| Microtubes | Marked for all steps |
| EXTRACTION BUFFER A | Room temperature |
| NAD+ STABILIZER | Room temperature |
| DEIONIZED WATER | Room temperature |

**Extraction:**

1. Pipette 500 µL of **EXTRACTION BUFFER A** into 1.5 mL microtubes for all your samples. Close the caps.
2. a) If you work with fresh blood samples, proceed to extraction with EXTRACTION BUFFER A.

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

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

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

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

6. Incubate each reaction at 75°–80°C for 1–2 min. Keep the incubation time constant for all your samples.
7. Cool down the extract in the ice-water bath for at least 5 min. Check the sample for successful extraction. After cooling on ice, homogenate should polymerize without any free liquid.

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

8. Centrifuge the extracts at 20,000 x g at 4°C for 10 min. Transfer the supernatant into a clean microtube and discard the pellet.
9. Protect the sample extracts (supernatants) from light and keep them refrigerated (4°–8°C) for **up to 1h before proceeding to the Stabilization steps.**

Optional: The supernatants can be stored at -80°C – -70°C for one week. In this case, thaw the frozen extracts at room temperature for 10 min before proceeding to the stabilization steps described below.

**Stabilization:**

10. Equilibrate the extract to room temperature and prepare a 150 µL aliquot into a clean microtube.
11. To this 150 µL aliquot, add 100 µL of **NAD+ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
12. Protect Stabilized sample extracts from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

# PREPARATION OF STANDARDS

Prepare standards on the day of the assay. The working standard stocks prepared here are used to prepare the Positive control mix.

**NOTE:** Use the same pipette for DEIONIZED WATER and NAD+ working stock to improve accuracy.

**Materials:**

| | |
|---|---|
| 1 mM NAD+ STANDARD STOCK | Thaw upon usage. Spin down at low speed before opening |
| EXTRACTION BUFFER A | Room temperature |
| NAD+ STABILIZER | Room temperature |
| DEIONIZED WATER | Room temperature |

**Protocol:**

1. Thaw one microtube with 1 mM **NAD+ STANDARD STOCK** for 5 min at room temperature. Protect from light with a foil lid during thawing.
2. Prepare **50 µM NAD+ working stock** by adding 25 µL of 1 mM NAD+ STANDARD STOCK into 475 µL of DEIONIZED WATER, vortex.
3. Prepare NAD+ Standards according to the table below by mixing the volumes of reagents in the indicated order.
4. Vortex the Standards. Protect Standards from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

| NAD+ STANDARD PREPARATION | | | | | |
|---|---|---|---|---|---|
| STANDARD ID | NAD+ CONCENTRATION (µM) | DEIONIZED WATER (µL) | **50 µM NAD+ working stock (µL)** | EXTRACTION BUFFER A (µL) | NAD+ STABILIZER (µL) |
| **NAD+ ST1** | 0 | 100 | **0** | 500 | 400 |
| **NAD+ ST2** | 1 | 80 | **20** | 500 | 400 |
| **NAD+ ST3** | 2 | 60 | **40** | 500 | 400 |
| **NAD+ ST4** | 3 | 40 | **60** | 500 | 400 |
| **NAD+ ST5** | 5 | 0 | **100** | 500 | 400 |

# PREPARATION OF POSITIVE CONTROL

The positive control is prepared on the day of the assay right after the preparation of the NAD+ Standards. The Positive control is mimicking the level of NAD metabolites in a blood sample of a healthy human subject. Positive control undergoes extraction and stabilization like the whole blood samples.

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

**Materials:**

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

**Protocol:**

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

   75 µL of **POSITIVE CONTROL (BUFFER**)
   75 µL of **50 µM NAD+ working stock**

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

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

4. Prepare a 150 µL aliquot of the Positive control extract into a clean microtube.
5. To this 150 µL aliquot, add 100 µL of **NAD+ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
6. Protect the stabilized NAD+ Positive control extracts from light and keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

# ASSAY PROCEDURE

Blanks are used to correct for unspecific background signals from unspecific interactions between the extract components and the ASSAY COLOR REAGENT in the Master mix. Sample blanks are incubated with Master mix without added NAD ENZYME. Positive control does not require a separate blank. Sample blanks are to be prepared at a minimum from four representative stabilized sample extracts. If the analyzed subjects have records with NAD supplementation, we recommend using samples with and without supplementation as blanks(two wells per condition, at minimum).

**NOTE:** Steps 1.–2. are performed under normal light conditions. **Steps from 3. onwards are performed in dim conditions (refer to PRACTICAL CONSIDERATIONS: Protection from light).**

**NOTE:** Follow the suggested incubation times for NAD+. However, the reaction can be stopped when there is a distinct color gradient in the standards and differences in color intensity between samples with added enzyme and sample blanks. The longer the reaction time, the more intensive the signal observed.

**NOTE:** Use a separate reservoir for the Master Mix and STOP SOLUTION.

**Materials:**

| | |
|---|---|
| Spectrophotometric Reader | Refer to MATERIALS REQUIRED-Table |
| ASSAY BUFFER C | Room temperature |
| ASSAY COLOR REAGENT | Room temperature |
| NAD ENZYME | Thaw upon usage. Spin down at low speed before opening. |
| STOP SOLUTION | Room temperature |

**Protocol:**

1. Equilibrate the Standards, Stabilized sample extracts, and Stabilized Positive controls for 10 min at room temperature before pipetting onto the plate.
2. According to the recommended plate layout (see next page), pipette on the 96-well plate:

   20 µL Standards (ST1–5) in duplicates
   20 µL of stabilized Positive control and Stabilized sample extracts in duplicates (Unknowns, UNK)
   20 µL of selected blanks (BL UNK1–4) as instructed above.

NOTE: From this step onward, work in dim conditions.

3. Prepare the Master mix by adding **ASSAY COLOR REAGENT** into **ASSAY BUFFER C**; mix gently by rotation.

   **NOTE:** Protect the Master mix in the reservoir and plate during pipetting with an aluminum foil lid.
4. Add 190 µL of the Master mix WITHOUT ENZYME into each of the four sample blank wells (BL UNK1–4).
5. Add 40 µL of **NAD ENZYME** into the bottle with the remaining Master mix. Mix gently, avoid foaming. Pour the Master mix with the added enzyme into the reservoir.
6. Add 190 µL of the Master mix WITH NAD ENZYME to all remaining wells using a multichannel pipette. Avoid foaming and light. Immediately cover the ready plate with the aluminum foil lid.
7. **NAD+ assay:** Incubate the covered plate for 4–6 min at room temperature.
8. Stop the reactions by adding 10 µL of STOP SOLUTION to each well in the same order as the Master Mix using a multichannel pipette. Avoid foaming. Gently shake the plate by hand on a table surface and remove any bubbles with a needle.

9. Measure light absorbance at 573 nm immediately after adding STOP SOLUTION. If possible, shake the plate inside the microplate reader for 5 sec before the measurement.

**NOTE:** After adding STOP SOLUTION, the color intensity can uniformly increase in all the wells. This is expected due to the non-enzymatic background process in the Master mix.

## RECOMMENDED PLATE LAYOUT FOR NAD+ MEASUREMENT

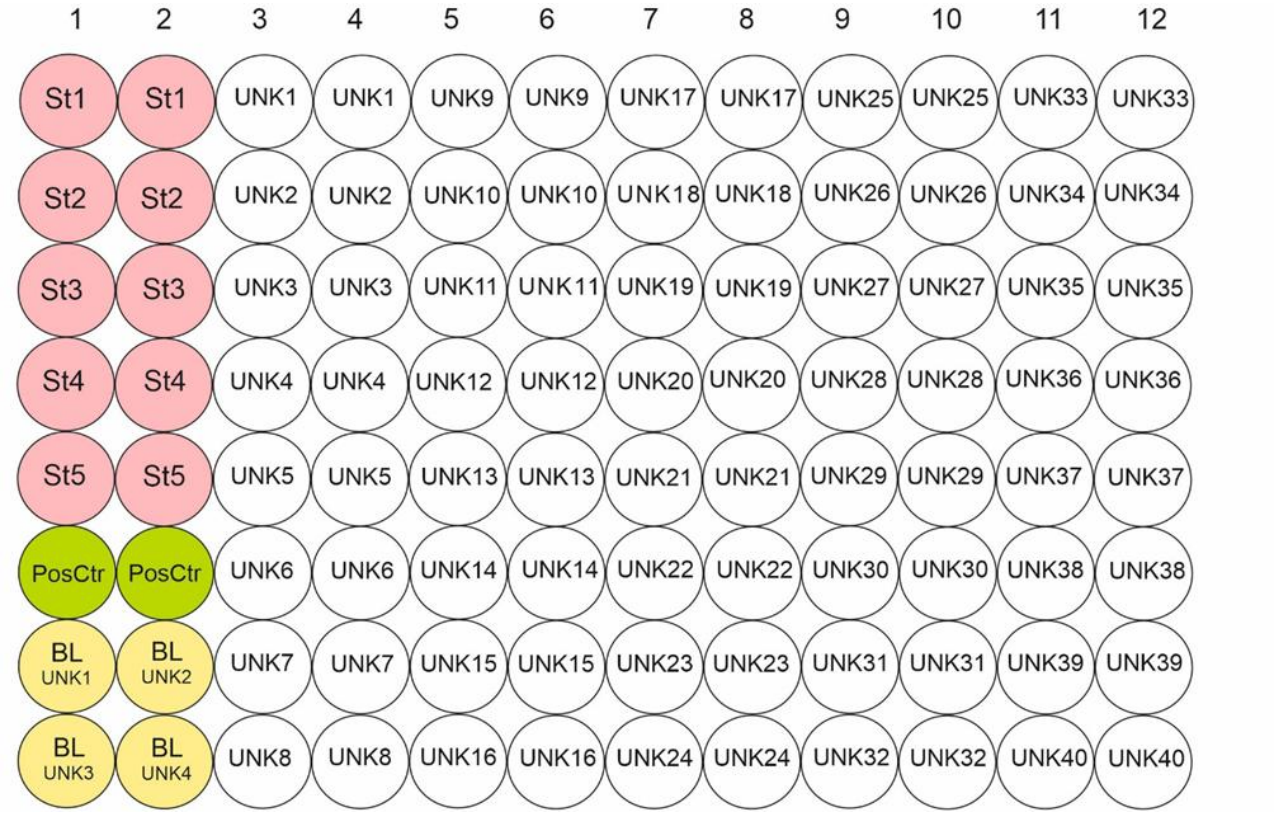



**Plate layout for NAD+ assay:** St = standard, BL = Sample blank, PosCtr = stabilized Positive control, UNK = stabilized samples with unknown metabolite concentration. Note that the BL (Sample blanks) of the selected samples are analyzed in the Master mix without added NAD ENZYME**.**

# CALCULATION OF RESULTS

**POSITIVE CONTROL (ASSAY QUALITY CONTROL)**

Positive control is not a reference, but it aims to monitor the efficiency of the NAD+ stabilization and colorimetric assay. Before calculating your sample results, confirm that your Positive controls perform as expected.

**NAD+:**
In the NAD+ assay, the amount of light absorbed by the stabilized NAD+ Positive control should be within the range observed for standards ST3 and ST4. This absorbance range corresponds to an NAD+ concentration of 23–27 µM (after correction of 10x dilution).

**SAMPLE RESULTS**

Calculate results as instructed below. The TYPICAL DATA section below presents examples of standard curves and the calculation of results for control subjects.

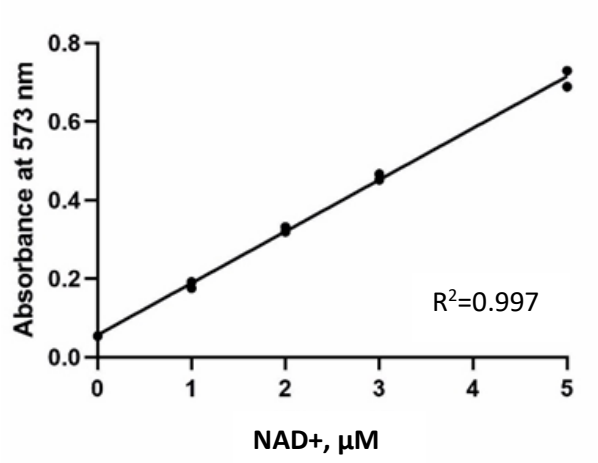
1. Calculate the average of the absorbance readings for each standard (ST1–ST5).
2. Create a standard curve by plotting the mean absorbance for each Standard on the y-axis against the known Standard concentration (in µM) on the x-axis. Calculate a simple linear regression fitting of the standard curve.
3. Using the formula of linear regression for the standard curve, calculate the concentration in each of the Sample and Blank wells (UNK and BL UNK).
4. Calculate the average of duplicates of each stabilized sample extract.
5. Calculate the average of the sample blanks (BL UNK1–4). The obtained value represents an unspecific signal of the stabilized extract used for sample normalization.
6. Correct for unspecific signals by subtracting the average of blanks from the average of sample concentrations.
7. Multiply by 10 to obtain the concentration (µM) of NAD+ in blood.

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

## TYPICAL DATA

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

### A) STANDARD CURVE FOR NAD+




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

### B) 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

# PERFORMANCE AND LIMITATIONS

## 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 |

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

| Limit of Detection | |
|---|---|
| | µM in whole blood |
| NAD+ | 0.33 ± 0.2 |

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 |

## 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 |

The table below summarized the results of the assay reproducibility.

| **Reproducibility** | | | |
|---|---|---|---|
| | NAD+ | | |
| Sample | Ctr1 | Ctr2 | Ctr3 |
| N of measurements * | 9 | 9 | 9 |
| Mean (µM) | 27.41 | 29.41 | 22.00 |
| Standard deviation | 0.62 | 1.31 | 0.87 |
| CV (%) | 2.28 | 4.45 | 3.95 |

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

## ACCURACY

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

| | | Accuracy (%) |
|---|---|---|
| NAD+ | N = 32 | 97.13 ± 7.6 |

## ASSAY CUT-OFF

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

| | Cut-off value | |
|---|---|---|
| | Low | High |
| NAD+ (µM) | 20 | 36 |

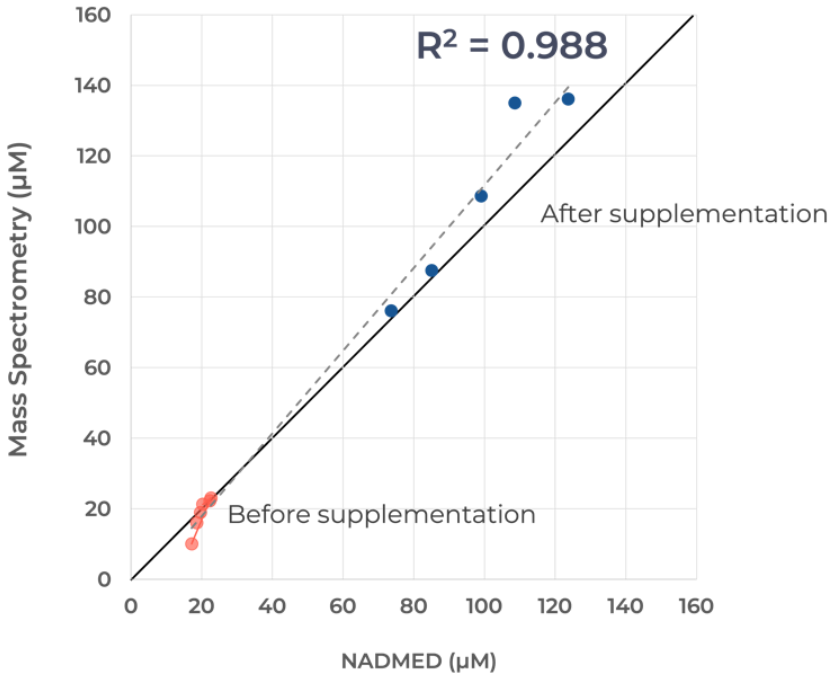
## PERFORMANCE CHARACTERISTICS

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, and thus causing underestimation of the results.

## METHOD VALIDATION

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.



## NOTES

## PLATE LAYOUT

Use this plate layout to record standards and samples assayed.