NADMED

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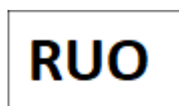
Q-NAD BLOOD
NADP+ and NADPH assay kit

Quantitative assay kit for whole blood

Version 1.0

FOR SINGLE USE ONLY

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



For Research Use Only.
Not for use in diagnostic procedures.

GENERAL INFORMATION

Proprietary name:
Q-NAD Blood NADP+ and NADPH assay kit: quantitative assay kit for whole blood
Catalog numbers:
RUO_004
Storage:
-85°- -70°C upon arrival
IFU issued:
August 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

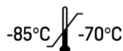
Consult instructions for usage

Use-by date

Catalogue number

Batch code

Manufacturer



Upper limit of storage temperature

Do not use if package is damaged

Number of reactions

Protect from direct light

Keep dry

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INTRODUCTION

Q-NAD BLOOD NADP+ and NADPH assay kit is intended for quantitative measurement of NADP+ and NADPH in whole blood samples. NADP+ is the phosphorylated form of NAD+ and serves as a carrier of redox equivalents supporting anabolic reactions in healthy metabolism. Upon acceptance of two electrons and one proton, NADP+ is converted into the reduced NADPH form. In healthy metabolism, NADPH concentration is higher than NADP+ to efficiently support biosynthetic reactions requiring electrons and protons, including biosynthesis of fatty acids, cholesterol, steroids, nucleotides, and amino acids; reduction of oxidized glutathione and thioredoxin, which are essential for the reductive environment in the cell; detoxification reactions and xenobiotic metabolism. There are more than 250 metabolic reactions dependent on NADP+ and NADPH as cofactors. The concentrations of $NADP^+$ and NADPH vary between different tissues and are largely determined by the specific metabolic pathways active in each tissue. They are also species-specific, meaning that the levels in a given tissue can vary between animal species. In addition, these concentrations can fluctuate with age.

PRINCIPLE OF THE ASSAY

The kit measures intracellular NADP+ and NADPH content. The principle of the assay is a cyclic enzymatic reaction with colorimetric endpoint detection. First, NADP+ and NADPH metabolites are extracted together from a whole blood sample in a single step. In the analytical process, the sample extract undergoes separate measurements for NADP+ and NADPH. In the first segment, the procedure focuses on stabilizing NADP+ while actively eliminating NADPH. Conversely, in the second segment, the emphasis shifts to stabilizing NADPH, concurrently ensuring the removal of NADP+. The NADP+ and NADPH stabilized extracts are analyzed on two separate plates by an enzymatic reaction coupled with a color change. The intensity of the color change in the assay is linearly proportional to the concentration of NADP+ or NADPH in the reaction mixture.

SAMPLE HANDLING AND STORAGE

Requirements and Limitations:

- This kit is designed for NADP+ and NADPH measurement in whole blood. This assay is NOT suitable for measuring in plasma or serum, cultured cells, or tissues.
- For measuring NADP+ and NADPH, 100 µL of whole blood is needed. However, a volume of 150–200 µL is optimal to perform the assay reliably.
- Samples can be analyzed either fresh or frozen.
  a) Fresh blood can be analyzed within 72 hours after collection. After withdrawal, store at 4°– 8°C until analysis.
  b) Frozen samples must be kept frozen before the assay. Subsequent freeze-thaw cycles are not allowed. Storage time of aliquots is one month at -20°C, or approximately one year at -80 – -70°C.
- Levels of NADP+ and NADPH in blood are affected by the freeze-thaw process, while the pool of metabolites (NADP+ plus NADPH) remains the same. NADPH undergoes oxidation to NADP+ upon freeze-thaw cycle, resulting in higher measured NADP+ and lower NADPH levels compared to fresh blood. About 50% of NADP+ measured in frozen blood originated from NADPH oxidized during the freeze-thaw cycle. This phenomenon determines the choice of NADPH Assay Standards depending on whether fresh or frozen blood samples are used.
- In clinical trials and longitudinal studies, it is highly 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 two 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 and freeze.

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-frozen sample containers in temperatures from -80°C to -20°C for freezing the samples.

Practices to avoid: Do not freeze large (more than 2 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– -70°C. Avoid temperature fluctuations in the freezer.

REAGENT	DESCRIPTION (*)	PREPARATION (**)	STORAGE and STABILITY (**)
EXTRACTION BUFFER A	28 mL Sufficient for 40 samples	Equilibrate to room temperature. Ready for use.	Stable for two weeks at room temperature after thawing.
NADP+ STABILIZER	8 mL Sufficient for 40 samples		
NADPH STABILIZER	8 mL Sufficient for 40 samples		
POSITIVE CONTROL (BUFFER)	200 µL Sufficient for two plates		
DEIONIZED WATER	10 mL Sufficient for two plates		
STOP SOLUTION	3 mL Sufficient for two plates	Equilibrate to room temperature. Ready for use. (If precipitates have formed, warm in 37°C, then cool to room temperature before the assay.)	
ASSAY BUFFER P	2x 19 mL One aliquot per 96-well plate	Equilibrate to room temperature. ASSAY BUFFER P + ASSAY COLOR REAGENT = Assay Master Mix. See preparation guide on page 18.	Stable for 12 hours at room temperature after thawing. Keep in the amber bottle.
ASSAY COLOR REAGENT	2x 3 mL One aliquot per 96-well plate		Stable for 3 hours at room temperature after thawing. Keep in the amber bottle.
NADP+ STANDARD STOCK	40 µL (1mM) Sufficient for Standards and Positive control	See preparation guide on page 15–16.	**Should be used immediately after thawing**. Should be protected from light.
NADPH STANDARD STOCK	40 µL (1mM) Sufficient for Standards and Positive control		
NADP ENZYME	2x 40 µL One aliquot per 96-well plate	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 research 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 may cause eye and respiratory irritation. Handle with care; use goggles.

NADP+ STABILIZER may cause skin and eye irritation. Handle with care; use gloves and goggles.

NADPH STABILIZER may cause skin, eye, and respiratory irritation. Avoid inhaling fumes.

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

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

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

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

TROUBLESHOOTING

If you encounter any issues during the extraction of frozen samples 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). NOT compatible with NADMED assay: a) molecular biology grade sterile microtubes that are free of endotoxin, pyrogen, human DNA, and low retention (chemically sterilized) b) microtubes intended for protein work marked "LoBind"
	96-well plates (2 pieces)	Use non-sterile, transparent, polystyrene flatbottom plates with medium protein binding intended for colorimetric assays (e.g. Revvity, 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 liquid part of the sample is immersed in water, but the ice firmly holds the inserted tubes upright (avoid samples floating in the water).
	Aluminium foil	Use foil covers to protect samples, standards, and the plates from light during assay performance as indicated in the instructions.
Equipment and Machinery	Calibrated Pipettes	Single channel for volumes of e.g. 5–50 µL, 20–200 µL, and 100–1000 µL. Multichannel pipettes for volumes of e.g. 5–50 µL and 30–300 µL.
	Microcentrifuge	Use centrifuge with cooling to 4°C and speed of 20,000 x g
	Spectrophotometric Microplate Reader	a) Measuring absorbance at 570–573 nm wavelength b) Adjustable scanning light brightness/intensity. Select "low", or alternatively, adjust the brightness based on the number of flashes per measurement 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 time needed to reach target temperature:** 1. Set your heat block at 80°C and wait until it reaches temperature within the range of 75–80°C. Use conventional lab thermometer inserted into an empty slot to determine the actual temperature corresponding to 80°C setting. If necessary, increase the temperature setting in order to reach the actual temperature within 75–80°C. 2. Add 500 µL of water into a microtube and place the tube on your heat block. Make sure the microtube fits tightly to the block. 3. Insert a conventional lab thermometer into the microtube with water. 4. Measure the time needed to reach steady-state temperature of the heat block in the range of 75–80°C. Refer to this time for equilibration of extraction buffer A prior to extraction. The heat transfer is considered efficient if this temperature is reached within 5 minutes.
Special	Possibility to work in dim light conditions for the ASSAY part of the measurement. Refer to PRACTICAL CONSIDERATIONS and WORKFLOW OF Q-NAD BLOOD NADP+ AND NADPH.	

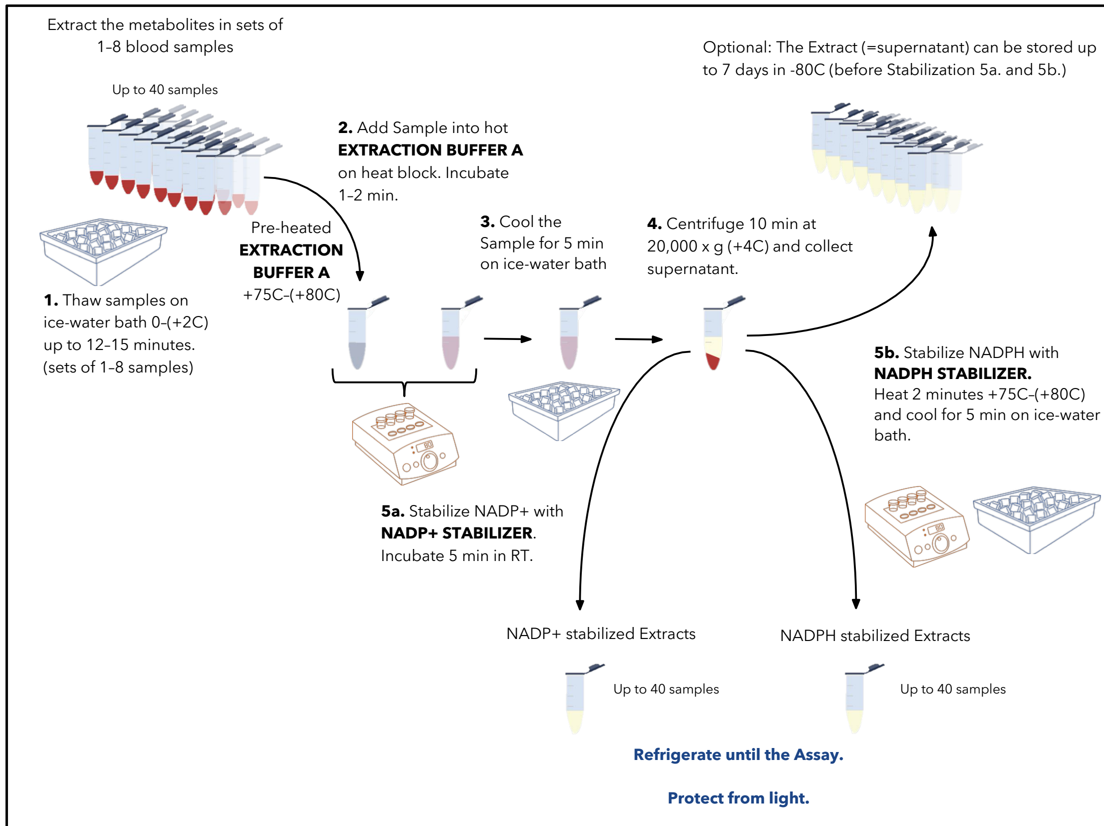
PRACTICAL CONSIDERATIONS

Please refer to contents in this link for visual instructions.

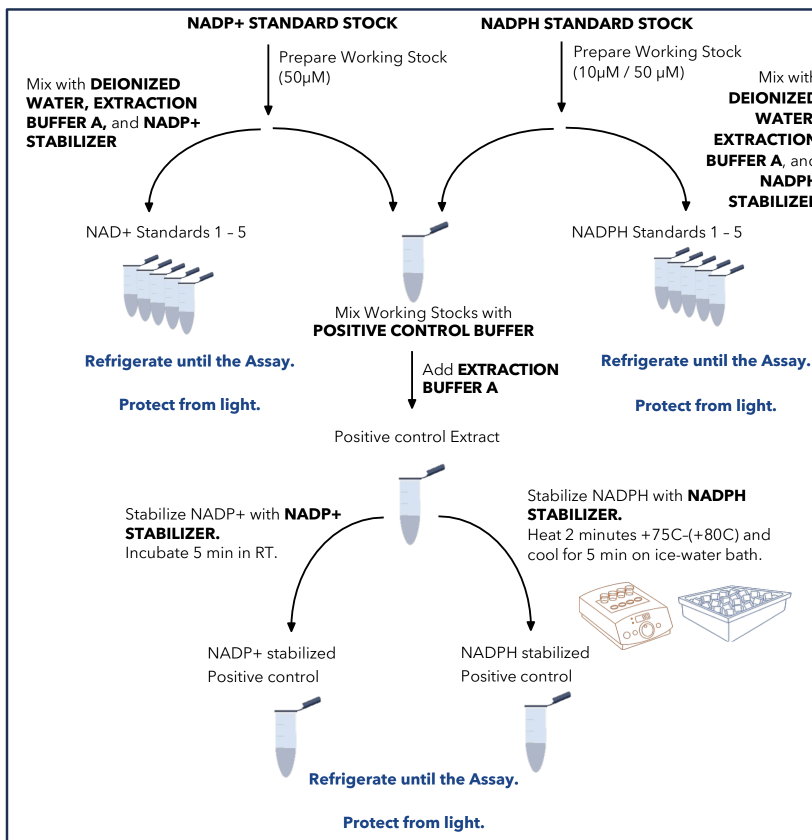
CATEGORY	INSTRUCTIONS
Limitations	Read the SAMPLE HANDLING AND STORAGE carefully. This assay is designed for whole blood and is NOT suitable for measuring NADP+ and NADPH in plasma or serum, cultured cells, or tissues. Do not use kit components beyond the expiry date. Do not mix materials from different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

WORKFLOW OF Q-NAD BLOOD NADP+ AND NADPH ASSAY

1. Extraction of Metabolites and NADP+/NADPH stabilization



2. Preparation of NADP+/NADPH Standards and NADP+/NADPH Positive Controls



3. NADP+ and NADPH Assays

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

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

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

3. Prepare the Assay buffer **(ASSAY BUFFER C + ASSAY COLOR REAGENT).** Add Assay buffer to Blank wells.

4. Add **NADP ENZYME** to the Assay buffer **(ASSAY BUFFER C + ASSAY COLOR REAGENT).** Add (+ NADP Enzyme) Assay buffer to the Standard, Positive Control and Sample wells.

NADP+ 2 – 4 min

NADPH 2 – 4 min or 5 - 8 min

5. Add **STOP SOLUTION** and measure absorbance at 573 nm.

6. Calculate the results and confirm assay quality (Positive Control readings).

EXTRACTION AND STABILIZATION OF NADP+ AND NADPH

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

TIP: Please refer to the video guidance following this link.

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

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
NADP+ STABILIZER	Room temperature
NADPH 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 if necessary: hold the sample for 2–3 seconds with fingers 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 for the time needed to reach the target temperature within 75–80°C (see section MATERIALS REQUIRED).
4. Mix the thawed whole blood sample with a few up-and-down pipetting cycles; 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, the 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– -70°C for one week. In this case, thaw the frozen extracts at room temperature before proceeding to the stabilization steps described below.

Stabilization:

10. Equilibrate the extract to room temperature and prepare two 150 µL aliquots into clean microtubes.
11. To the first 150 µL aliquot, add 100 µL of **NADP+ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
12. To the second 150 µL aliquot, add 100 µL of **NADPH STABILIZER**. Vortex and incubate for 2 min in a dry-bath set at 80°C, e.g., time needed to reach 75–80°C in the solution. Cool down on ice for 5 min.
13. Protect Stabilized sample extracts from light and if not used immediately keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

PREPARATION OF STANDARDS

Prepare standards on the day of the assay. Prepare one standard set at a time, starting with NADP+. NADP+ Assay Standards are the same for analysis of fresh and frozen blood samples, whereas NADPH Assay Standards are different in analyses of fresh and frozen blood. Preparation of NADPH working stock requires the addition of EXTRACTION BUFFER A to support the stability of the diluted NADPH standard. The working standard stocks are also used to prepare the Positive control mix. It is recommended to use the suggested NADP+ and NADPH Standard concentrations as described for the first trial. Only after obtaining and evaluating the results from this initial test should any adjustments to the concentration range be made, based on the specific response of your samples.

TIP: Use the same pipette for DEIONIZED WATER and Standard working stocks to improve accuracy.

Materials:

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

Protocol:

1. Thaw microtubes with 1 mM NADP+ STANDARD for 5 min at room temperature. Protect from light with a foil lid during thawing.
2. Prepare **50 μM NADP+ working stock** by adding 25 μL of 1 mM NADP+ STANDARD STOCK into 475 μL of DEIONIZED WATER, vortex. Proceed to the preparation of NADP+ Assay standards according to the table below, and pipette the reagents in the indicated order.

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

For analysis of fresh blood samples, prepare NADPH standards as follows:

3. Thaw microtubes with 1 mM NADPH STANDARD for 5 min at room temperature. Protect from light with a foil lid during thawing.
4. In the clean microtube, mix 250 μL of EXTRACTION BUFFER A and 225 μL of DEIONIZED WATER to obtain a 475 μL final volume.
5. Prepare **50 μM NADPH working stock** by adding 25 μL of 1 mM NADPH STANDARD STOCK into 475 μL of prepared mix of EXTRACTION BUFFER A and DEIONIZED WATER, vortex. Proceed to the preparation of NADPH Assay standards according to the table below, and pipette the reagents in the indicated order.

NADPH STANDARD PREPARATION for analysis of fresh blood					
STANDARD ID	NADPH CONCENTRATION (µM)	DEIONIZED WATER (µL)	**50 µM NADPH working stock (µL)**	EXTRACTION BUFFER A (µL)	NADPH STABILIZER (µL)
NADPH ST1	0	100	**0**	500	400
NADPH ST2	1	80	**20**	500	400
NADPH ST3	2	60	**40**	500	400
NADPH ST4	3	40	**60**	500	400
NADPH ST5	5	0	**100**	500	400

For analysis of frozen blood samples, prepare NADPH standards as follows:

6. Thaw microtubes with 1 mM NADPH STANDARD for 5 min at room temperature. Protect from light with a foil lid during thawing.
7. In the clean microtube, mix 500 µL of EXTRACTION BUFFER A and 490 µL of DEIONIZED WATER to obtain a 990 µL final volume.
8. Prepare **10 µM NADPH working stock** by adding 10 µL of 1 mM NADPH STANDARD STOCK into 990 µL of prepared mix of EXTRACTION BUFFER A and DEIONIZED WATER, vortex. Proceed to the preparation of NADPH Assay standards according to the table below, and pipette the reagents in the indicated order.

NADPH STANDARD PREPARATION for analysis of frozen blood					
STANDARD ID	NADPH CONCENTRATION (µM)	DEIONIZED WATER (µL)	**10 µM NADPH working stock (µL)**	EXTRACTION BUFFER A (µL)	NADPH STABILIZER (µL)
NADPH ST1	0.0	100	**0**	500	400
NADPH ST2	0.2	80	**20**	500	400
NADPH ST3	0.4	60	**40**	500	400
NADPH ST4	0.6	40	**60**	500	400
NADPH ST5	1.0	0	**100**	500	400

9. Vortex ready Assay Standards. Protect Standards from light, if not used immediately after preparation keep refrigerated (4°–8°C) before pipetting on the Assay plates.

PREPARATION OF POSITIVE CONTROL

The Positive control is prepared on the day of the assay, right after the preparation of the Standards, due to the limited stability of both **50 µM and 10 µM NADPH working stock**. The purpose of the Positive control is to monitor the efficiency of elimination of NADP+ from the NADPH aliquot of the extract and NADPH from the NADP+ aliquot, respectively. Positive control undergoes extraction and stabilization like whole blood samples. The expected concentration of NADP+ in the Positive control is 10 ± 1 µM, and NADPH is 2 ± 0.5 µM after calculation of results.

NOTE: Preparation of the Positive control requires 10 µM NADPH working stock. If a 50 µM NADPH working stock was prepared for analysis of fresh blood, it needs a 5-fold dilution with DEIONIZED WATER. For example, in the clean microtube, mix 100 µL of 50 µM NADPH working stock with 400 µL of DEIONIZED WATER, vortex.

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 NADP+ working stock	From Preparation of standards, room temperature
10 µM NADPH working stock	From Preparation of standards, room temperature
POSITIVE CONTROL (BUFFER)	Room temperature
EXTRACTION BUFFER A	Room temperature
NADP+ STABILIZER	Room temperature
NADPH STABILIZER	Room temperature

Protocol:

1. Prepare the 100 µL of Positive control mix in a microtube.

   60 µL of **POSITIVE CONTROL (BUFFER)**
   20 µL of **50 µM NADP+ working stock**
   20 µL of **10 µM NADPH working stock**

2. Add 500 µL of **EXTRACTION BUFFER A** to the Positive control mix. Vortex.

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

3. Prepare two 150 µL aliquots of the Positive control extract into clean microtubes.
4. To the first 150 µL aliquot, add 100 µL of **NADP+ STABILIZER**. Vortex, and incubate at room temperature for 5 min.
5. To the second 150 µL aliquot, add 100 µL of **NADPH STABILIZER**. Vortex, and incubate for 2 min in a dry bath set at 80°C. Cool down on ice for 5 min.
6. Protect the stabilized NADP+ and NADPH Positive control extracts from light, and if not used immediately, keep them refrigerated (4°–8°C) before pipetting on the Assay plates.

ASSAY PROCEDURE

The Assay procedure is the same for both NADP+ and NADPH measurements. Blanks are used to correct for unspecific background signals from unspecific interaction between the extract components and ASSAY COLOR REAGENT in the Master mix. Sample blanks are incubated with the Master mix without added NADP ENZYME. Positive control does not require a separate blank. Sample blanks are to be prepared at a minimum of four representative stabilized sample extracts if 40 samples are analyzed in one plate. For a smaller sample number, the use of individual blanks is possible. For example, for 24 samples, the stabilized extract of each sample can be analyzed in duplicate with its own blank.

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: The colour intensity in the NADPH assay for frozen blood is generally lower than in NADP+ due to a lower concentration of NADPH in the thawed sample and the used NADPH in the Standards. Follow the suggested incubation times for NADP+ and NADPH assays.

NOTE: Use separate reservoirs for Master Mix and STOP SOLUTION.

Materials:

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

Protocol (Perform the NADP+ and NADPH assays on separate plates. Work on one Assay at a time):

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 for forty samples (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 pipetting **ASSAY COLOR REAGENT** into **ASSAY BUFFER P**; 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 NADP ENZYME into each of the four sample blank wells (BL UNK1–4).
5. Add 40 µL of **NADP 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 NADP ENZYME to all remaining wells using a multichannel pipette. Avoid foaming and light. Immediately cover the ready plate with the aluminum foil lid.
7. **NADP+ assay**: incubate the covered plate for 2–4 min at room temperature.

   NADPH assay for fresh blood: incubate the covered plate for 2–4 min at room temperature.

NADPH assay for frozen blood: incubate the covered plate for 5–8 min at room temperature.

8. **NOTE:** The reaction can be stopped when there is a distinct color gradient in the standards and differences in the color intensity between samples with added enzyme and sample blanks. The longer the reaction time, the more intensive the signal observed. The warmer the environment where the assay is done, the faster the reaction and color development will be. Check the color development on the plate every 30 sec to determine the most suitable moment to stop the assay. 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.

RECOMMENDED PLATE LAYOUT FOR NADP+ OR NADPH MEASUREMENTS

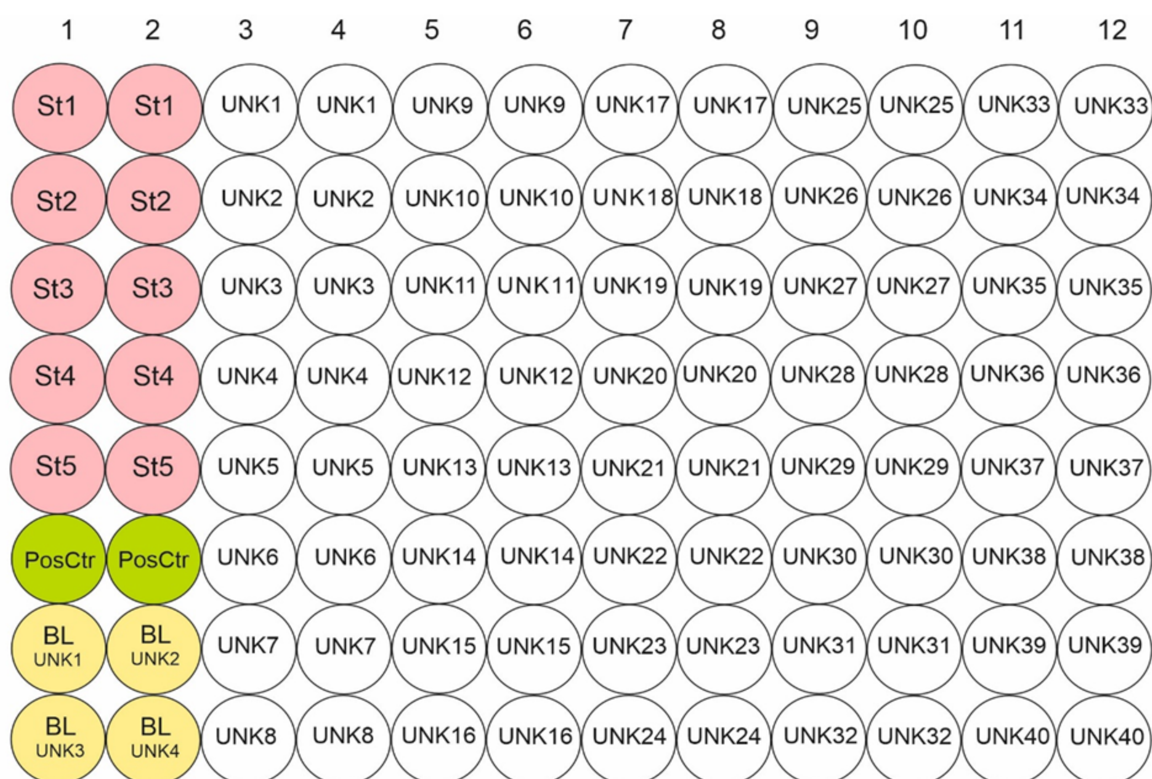


Plate layout for NADP+ or NADPH assays: St = standard, BL = blank, PosCtr – stabilized Positive control, UNK = stabilized samples with unknown metabolite concentration. Sample blanks of the selected samples are analyzed in the Master mix without added NADP ENZYME.

CALCULATION OF RESULTS

POSITIVE CONTROL (ASSAY QUALITY CONTROL)

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

NADP+:
In the NADP+ assay, the amount of light absorbed by the stabilized NADP+ Positive control should be close to the standard ST1. This absorbance corresponds to the NADP+ concentration of 9–11 µM in the original 100 µL of Positive control (after correction of 10x dilution).

NADPH:
In the NADPH assay, the amount of light absorbed by the stabilized NADPH Positive control should equal ST2 (+/-0.05 optical units) in the Standards range used for frozen blood. In the NADPH Standard range used for fresh blood, the NADPH Positive control signal is expected between ST1 and ST2. This absorbance corresponds to an NADPH concentration of 1.5–2.5 µM in the original 100 µL of Positive control (after correction of 10x dilution).

SAMPLE RESULTS

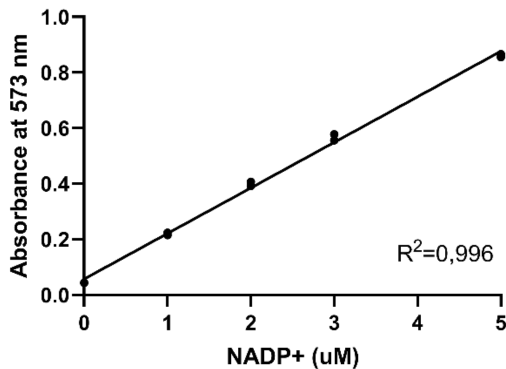
Calculate results from each plate separately as instructed below. The TYPICAL DATA section below presents examples of standard curves and the calculation of results measured in frozen control human blood.

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. Fit the response in the Standards with a simple linear regression function.
3. Using the formula of linear regression fit 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 NADP+ and NADPH in blood.

TYPICAL DATA

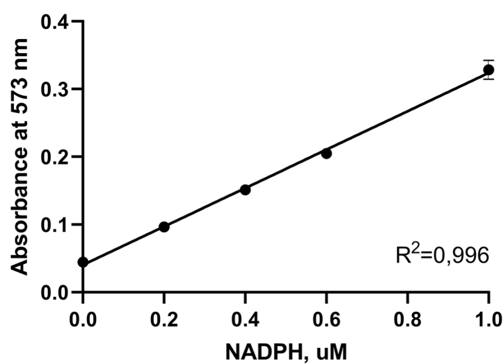
The standard curves and the concentrations in the stabilized extracts of thawed blood samples are provided for demonstration only and should never be used instead of the real-time calibration curve.

A) STANDARD CURVE FOR NADP+



Standard	NADP+ (µM)	Absorbance (573 nm) Assay time: 2 min
ST1	0	0.044
		0.042
ST2	1	0.224
		0.215
ST3	2	0.407
		0.392
ST4	3	0.578
		0.556
ST5	5	0.866
		0.855

B) STANDARD CURVE FOR NADPH



Standard	NADPH (µM)	Absorbance (573 nm) Assay time: 6 min
ST1	0	0.044
		0.045
ST2	0.2	0.097
		0.096
ST3	0.4	0.153
		0.149
ST4	0.6	0.207
		0.202
ST5	1	0.338
		0.319

C) CALCULATION OF RESULTS FOR NADP+

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

Unknown	Concentration in stabilized extracts (μM)	Concentration in stabilized extracts corrected by average of sample blanks (BL UNK 1-4, μM)	Final NADP+ concentration in the original sample (μM)*
UNK 1	1.615	1.444	14.44
	1.598		
UNK 2	1.550	1.355	13.55
	1.484		
UNK 3	1.296	1.141	11.41
	1.310		
UNK 4	1.493	1.336	13.36
	1.503		
BL UNK 1	0.162	-	
BL UNK 2	0.156		
BL UNK 3	0.152		
BL UNK 4	0.176		

*Corrected by dilution factor x10

D) CALCULATION OF RESULTS FOR NADPH

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

Unknown	Concentration in stabilized extracts (μM)	Concentration in stabilized extracts corrected by average of sample blanks (BL UNK 1-4, μM)	Final NADPH concentration in the original sample (μM)*
UNK 1	0.380	0.227	2.27
	0.392		
UNK 2	0.395	0.236	2.36
	0.395		
UNK 3	0.424	0.259	2.59
	0.411		
UNK 4	0.466	0.310	3.10
	0.472		
BL UNK 1	0.151	-	
BL UNK 2	0.145		
BL UNK 3	0.164		
BL UNK 4	0.179		

*Corrected by dilution factor x10

PERFORMANCE CHARACTERISTICS

LIMITS OF DETECTION

The Limit of Blank (LoB) reflects the maximum background signal due to non-analyte-related contributions present in the sample. It is presented in Absorbance units measured in the Blank samples without added enzyme by a plate reader at 573 nm and calculated using the formula $LoB = mean_{blank} + 1,645 \times (SD_{blank})$ (n=20).

Limit of Blank (Absorbance Units)	
NADP+	0.085
NADPH	0.120

The Limit of Detection (LoD) in whole blood was calculated based on NADP+ and NADPH standard curves and SD in the Standard with zero metabolite (n=24) using formula $LoD = (3,3*SD_{blank}/S)*10$, where S is the slope of the linear fit of the standard curve and multiplication factor to correct for 10-fold dilution of blood sample during extraction and stabilization. Calculated value is an Absorbance signal which is then converted into concentration using a Standard curve, followed by multiplication by 10 to obtain the theoretically lowest detectable NADP+ concentration in whole blood.

Limit of Detection (µM in whole blood)	
NADP+	0.22
NADPH	0.12

The Limit of Quantitation (LoQ) in whole blood was calculated using the formula $LoQ = (10*SD_{blank}/S)*10$ using the same input values as described above.

Limit of Quantitation (µM in whole blood)	
NADP+	0.67
NADPH	0.38

PRECISION

Inter-assay precision of NADP+ and NADPH assays is ≥90%. The table below summarizes measurements of six frozen aliquots from a single blood sample analyzed across six plates over two days. Each day, three aliquots were extracted and stabilized for NADP+ and NADPH measurement. Each stabilized NADP+ and NADPH extract was analyzed in triplicate on three separate assays each day.

Inter-assay precision	**NADP+**	**NADPH**
Mean (µM)	10.91	2.35
S.D. (µM)	0.94	0.14
CV (%)	8.59	5.91

Intra-assay precision of NADP+ and NADPH measurements is ≥ 90%. The Table below summarizes measurements of NADP+ and NADPH in frozen blood aliquots from three healthy human subjects. Three aliquots per individual were analyzed in three technical replicates on the same plate.

Intra-assay precision

	NADP+			**NADPH**		
Sample	Ctr1	Ctr2	Ctr3	Ctr1	Ctr2	Ctr3
N of measurements	9	9	9	9	9	9
Mean (µM)	10.03	11.59	11.01	2.39	2.57	2.62
S.D.(µM)	0.83	0,58	0.54	0.16	0.09	0.1
CV (%)	8.28	4.98	4.90	6.50	3.67	3.81

ACCURACY

The accuracy of the assay was assessed by measurement of NADP+ and NADPH in the extract of the control blood sample without and with added known amounts of pure NADP+ and NADPH compounds. The accuracy of the measurement in the spiked sample was calculated using the formula Accuracy (%) = (measured/expected)*100T.

Accuracy (%)	
NADP+	97 ± 6
NADPH	95 ± 4

NORMAL CUT-OFF LEVELS OF NADP+ AND NADPH IN FROZEN AND FRESH HUMAN CONTROL BLOOD

The normal cut-off levels of NADP+ and NADPH were measured in frozen blood samples from 337 healthy human subjects and in fresh blood samples from 12 healthy human subjects.

Normal Cut-off levels of NADP+ and NADPH in human blood		
	Frozen blood	Fresh blood
NADP+ (µM)	9,8 – 15,8	4,9 – 6,9
NADPH (µM)	1,0- 3,0	7,1 – 8,4

REFERENCES

1. https://doi.org/10.1101/2025.02.24.639825

NOTES

PLATE LAYOUT

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