Instructions for use for NAD test kit for the analysis of dried blood spots

Cat. no.: SL2502

V 3.1

For research use only

Version Log

Version	Date	Key changes
V 3.1	2026-01	Minor revisions and fixes
V 3.0	2025-04	Revisions in protocol structure, and quality control requirements
V 2.2	2025-01	Minor fixes in clarity
V 2.0	2024-03	Revisions in specification and testing parameters
V 1.3	2023-04	Fixes for numbering order
V 1.2	2022-12	Fixes for clarity
V 1.0	2022-09	Initial protocol

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1. Manufacturer and regulation

- The NAD test kits are manufactured by SensoLife.
  Legal name: UAB “Bioanalizės sistemos”.
  Company code: 303339076.
  Address: Saulėtekio al. 15, Vilnius, LT-10224, Lithuania.
  The company is registered in Lithuania under national law and the law of the European Union.

- The composition of the NAD test kit and the instructions for use may be changed by the manufacturer without prior notice.

- The NAD test kits are intended for research use only.

- The medical professional should never take action, prescribe medication or adjust treatment based solely on the result of this test.

- All updates to the test specifications can be found at: https://sensolife.lt/nad-test/

- The manufacture could be contacted at: info@sensolife.lt

- The use of this product complies with SensoLife's terms of use, such as the confidentiality of this document and the prohibition of reverse engineering.

2. NAD test protocol

E. Equipment

The following equipment is required for analysis but is NOT included in the kit.

E1. Dry blood punch robot with a 3.0 mm diameter tip, or a 3.0 mm diameter manual punch.

Manual punching devices are less recommended. They may cause additional measurement errors and depend on the skill and experience of the laboratory personnel.

E2. Spectrophotometer (plate reader) for the analysis of 96-well plates, suitable for measurement at **450 nm.**

A kinetic measurement mode is recommended but not required. Endpoint measurements are suitable for analysis.

E3. Shaker for 96-well shaking at 25.0 °C, 500 rpm.

E4. Other standard laboratory equipment (variable volume pipettes, multichannel pipettes).

C. Consumables

Are required for the analysis but is NOT included in a kit.

C1. 96-well plates.
C2. Pipettes with adjustable volume.
C3. 10 mL reagent containers.

R. Reactants

Are required for the analysis, and included in a kit (kit composition).

R0. Extraction buffer solution.
R1. Reaction solution.
R2. Reaction solution.
R3. Reaction buffer solution.
RS. NAD standard solution (concentration=0.1 mmol/L).
RP. Positive control (concentration=0.2 µmol/L).
RN. Negative control (concentration=0.0 µmol/L).

*The solutions **R0**, **R1**, **R2**, **R3** and standard **RS** contained in the kit are sufficient for 96 measurements (see reagent labels). All reagent solutions must be stored at –20°C and must not be refrozen. Use them immediately after thawing. In the case of transportation, the integrity of a cold chain is crucial.*

M. Sample and standard preparation, colorimetric measurements

M1-M5. Pretreatment of samples, NAD extraction

M1. Positive (**RP**) and Negative (**RN**) controls are placed in two wells (usually G1 and H1) "below" the calibration line.

M2. A dried blood sample punch (**3.0 mm** diameter) is inserted into a 96-well plate.
The diameter – 3.0 mm – is critical. Any significant deviation in the diameter, e.g., 2 mm or 4.7 mm will lead to inaccurate results.

M3. The extraction buffer solution **R0** (V=**200.0 µL**) is added to the well with the inserted sample.

M4. Incubate the 96-well plate for **30.0 min** at **25.0 °C** with shaking at 500 rpm. The solution turns pink.

M5. After extraction, the pink sample (residual volume **~180 µL**) is resuspended by careful pipetting several times and is ready for analysis.

M6-M8. Preparation of NAD standard solutions

M6. Add **20.0 µL** of the NAD standard solution **RS** to **1980.0** µL of the extraction buffer solution **R0** and mix very well to obtain a **1.0 µmol/L** NAD solution.
The NAD standard RS is prepared in a high-density solvent and tends to settle to the bottom of the tube. Therefore, mix the solution very well by pipetting and vortexing.

M7. After taking **six 0.2 mL** tubes, dilute the calibration solutions with the extraction buffer solution **R0** according to the table to obtain the calibrators **Cal0**–**Cal60**:

No.	R0 V, µL	1 µM NAD V, µL	[NAD], µmol/L
Cal0	200	0	0.00
Cal5	190	10	0.05
Cal10	180	20	0.10
Cal20	160	40	0.20
Cal40	120	80	0.40
Cal60	80	120	0.60

M8. Add **50.0 µL** of the **Cal0**–**Cal60** calibrators to the wells of a column.

M9. Dispensing of the prepared samples

M9. Add **50.0 µL** of the prepared solution for analysis (the solution was obtained after step **M5**) to each well.
The prepared sample (obtained after Step M5) is pink in color. This is normal and does not interfere with the NAD determination.

M10-M15. Preparation of the reaction solution, colorimetric reaction

Important: the prepared reaction solution, consisting of the reagents R1, R2 and R3, must be used within 10 minutes.

M10. Add the respective reagents indicated on the bottle label to the reaction buffer solution **R3**:
M10a. **200 µL** of the reaction solution **R1**.
M10b. **1000 µL** of the reaction solution **R2**.

M11. After everything is well suspended, transfer the reaction solution to a 10 mL reagent reservoir.

M12. Using a multichannel pipette, add **110.0 µL** of the reaction solution (obtained in step **M11**) to each well containing the sample or calibrator. The total volume per well is 160 µL (50 µL + 110 µL).

M13. After transferring the reaction solution to all wells, measure the absorbance of the samples at a wavelength of **450 nm** to obtain a **blank** value.
Before measuring, make sure that there are no air bubbles in the wells. Otherwise, air bubbles could increase the errors in the measurements.

M14. Incubate the plate for **4.0 hours (240 min) in the dark at 25.0 °C.**

M15. After incubation (obtained in step **M14**), the absorbance of the samples is measured at a wavelength of **450 nm**.
Again, ensure that no new air bubbles have formed in the wells before measuring.

N. Calculation of the NAD concentration in the blood sample

N1. Before you create a calibration curve and calculate the sample concentrations, you must determine the change in absorbance over 4 hours. The change is determined by subtracting the absorbance at 450 nm after incubation (step **M15**) from the blank absorbance at 450 nm after delivery of the blank reaction mixture (step **M13**).

N2. The sample concentrations in the wells are determined according to the calibration curve obtained from the optical density change values of the NAD calibrators of this series. The changes in the absorbance values of the NAD calibrators of different concentrations are fitted to a linear model and the equation of the calibration curve is obtained:

$$y = a\,x + b$$

where y – change in absorbance, x – concentration of the calibrator, a – slope, b – intercept. A typical calibration curve can be found in Figure 1.

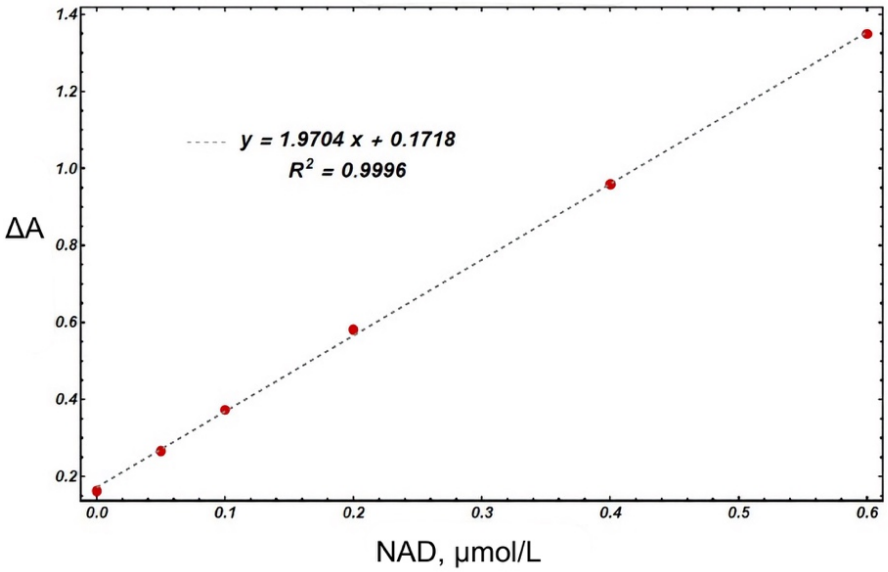

Figure 1. Typical calibration curve.

N3. The NAD concentration in the well is calculated according to:

$$c_i = \frac{(A_{240} - A_0) - b}{a}$$

Here:
c_i –the concentration of the sample in the well (**µmol/L**);
A_{240} – the absorbance of the sample 240 minutes after the start of the reaction at 450 nm; A_0 – the absorbance of the sample immediately after addition of the reaction mixture at 450 nm;
a – the slope of the calibration curve;
b – the intercept of the calibration curve.

Typical concentrations of NAD in the samples in the well range from 0.10 to 0.30 µmol/L.

N4. The NAD concentration in blood is calculated as:

$$c_{blood} = c_i * 100$$

where:
c_{blood} – is the concentration of NAD in blood sample (**µmol/L**),
c_i – is the NAD concentration of the sample in a well,
100 is the experimental extraction and compensation coefficient, relating dried blood spot measurement to actual value present in liquid blood.

Typical NAD concentrations in blood samples range from 10 to 30 µmol/L.

N5. The positive control (**RP**) should be carefully monitored for each testing set.
The desired range of **RP** is **18.7–21.3 µmol/L.**
The negative control (**RN**) should always give <**LOD**.

3. Sample acceptance and rejection criteria

3.1. Sample Acceptance Criteria

The following conditions must be met for a DBS to be accepted for analysis:

- The DBS was dried for 3 hours prior to storage or shipment and stored prior analysis for at least 24 h. **Freshly dried samples are unsuitable for analysis**.
- The DBS punch is taken from the center of the blood spot and the diameter is exactly 3.0 mm.
- The DBS card has no visible spreading, layering, or incomplete saturation.
- The DBS card has been stored and shipped according to specifications (see *Table of specifications*).
- The sample card is not exposed to direct sunlight, moisture, or temperatures >40 °C.
- The spot is free of visible contamination.

3.2. Sample Rejection Criteria

A sample must be rejected if any of the following apply. Rejected samples should not be analyzed:

- Punch diameter differs from 3.0 mm (e.g., 2 mm or 4.7 mm).
- DBS is not fully dried, shows mold, is physically damaged.
- DBS card has been stored improperly or beyond the validated storage window.
- Punch taken from the edge of the DBS spot or through an incomplete spot.
- The card contains any visible fluid contamination.

4. Critical steps (do not deviate)

These steps below are essential for the validity and accuracy of testing. Deviations may lead to incorrect NAD quantification.

- Punch diameter must be exactly 3.0 mm.
- Reagents R0–R3, RS, RP, and RN must never be refrozen.
- The reaction mixture (R1+R2+R3) must be used within 10 minutes after preparation.
- Incubation of the extraction step must be exactly 30 min at 25.0 °C with shaking at 500 rpm.
- Incubation of the colorimetric reaction must be exactly 4.0 h at 25.0 °C in the dark.
- Air bubbles must not be present during blank (A0) or final (A240) readings.
- Mix the NAD standard RS thoroughly before dilution, as RS tends to sediment.

If any of these steps are compromised, results may be invalid and the run should be repeated.

5. Quality control requirements

Each analytical run must include:

5.1. Calibration curve

- Six calibrators (Cal0–Cal60) must be prepared fresh for each run.
- The calibration curve must be linear with $\mathbf{R^2 \geq 0.98}$.
- The slope and intercept must fall within the typical range observed for the laboratory.

5.2. Quality controls

- The **Positive control (RP)** must fall within **18.7–21.3 µmol/L** after calculation.
- The **Negative control (RN)** must be <LOD.
- If either control is out of specification, the entire run must be repeated.

5.3. Acceptance of analytical run

- Calibration curve meets linearity criteria.
- RP and RN meet acceptance criteria.
- No critical procedural deviations have occurred.
- QC replicate variability is within acceptable range.

6. Recommended plate layout

To minimize plate-to-plate variability and simplify interpretation, the following plate layout is recommended:

- **Column 1:** Calibrators Cal0–Cal60 (top to bottom).
- **Well G1:** Positive Control (RP).
- **Well H1:** Negative Control (RN).
- **Remaining wells:** Samples, preferably in **duplicate**.

This layout ensures consistent positioning and minimizes edge effects.

7. Reagent handling and stability after thawing

- All reagents (R0–R3, RS, RP, RN) must be stored at **–20 °C**.
- Reagents must be **thawed only once**. Do not refreeze.
- Reagents should remain on ice during preparation and be used immediately.
- Once thawed, reagents are stable for **up to 4 hours** when kept cold.
- The prepared **reaction mixture** (R1 + R2 + R3) must be used within **10 minutes**.
- Do not expose reagents to direct light or elevated temperatures.

8. Documentation, traceability and biosafety

For each test run, we recommend the following information must be recorded:

- Date and time of analysis.
- Reagent lot numbers and expiry dates.
- Punch device identification (manual or robotic).
- Plate reader ID and wavelength setting (450 nm).
- Incubation start and end times for steps **M4** and **M14**.
- Calibration curve parameters (slope, intercept, R^2).
- Values obtained for RP and RN controls.
- Any deviations or irregularities observed during the run.

Although DBS samples present reduced biohazard risk, they must be handled as potentially infectious materials. The following biosafety practices apply:

- Wear laboratory gloves, protective eyewear, and lab coat.
- Avoid aerosol formation during punching and pipetting.
- Do not pipette by mouth.
- Dispose of used punches, plates, pipette tips, and DBS material as biological waste according to institutional regulations.
- Clean surfaces with 70% ethanol or appropriate disinfectant after handling samples.
- Wash hands thoroughly after completing the procedure.

9. Table of specifications

Parameter	Specification
Name of the method	Enzymatic cycling assay for the detection of NAD
Suitability	For the analysis of dried human blood spots. Not suitable for liquid blood, serum or tissue.
Shelf life (NAD test kit), –20 °C	6 months
Legal regulatory	For research use only
Relative standard deviation (RSD)	6.6 %
Limit of detection (LOD)	0.23 µmol/L
Limit of quantification (LOQ)	0.40 µmol/L
Linear range	5–60 µmol/L
Shipping conditions (DBS test cards)	Shipping at room temperature Ship immediately. Next day at the latest Maximum shipping time – 14 days
Storage conditions (DBS test cards)	Storage at +4 °C for up to 18 days Storage at –20 °C for up to 60 days
Conditions for sampling at home	Take the blood sample first thing in the morning before you eat or drink anything Dry the DBS test card completely for 3 hours