NADMED

EN

Q – NAD TISSUES/CELLS

NAD+ and NADH assay kit

Quantitative assay for TISSUES and CELLS

FOR SINGLE USE ONLY

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

RUO

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

GENERAL INFORMATION

A. Proprietary name: Q-NAD Tissues/Cells NAD+ and NADH assay kit: quantitative assay kit for TISSUES and CELLS
B. Catalog number: RUO_003, 40 samples (96-well format)
C. Storage: -80°C
D. IFU issued: April 2023

Manufactured and Distributed by:

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INTRODUCTION

NAD+ and its reduced form NADH are derivatives of vitamin B3. NAD metabolites are essential for maintenance of healthy metabolic balance in all living organisms. Their levels are dynamic and change in response to different endogenous and exogenous stimuli. This kit is designed for selective measurement of NAD+ and NADH in tissues and cells (human or animal).

PRINCIPLE OF THE ASSAY

The principle of the assay is a cyclic enzymatic reaction with a colorimetric detection. First, NAD+ and NADH metabolites are extracted together from a 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 stabilized extract. Obtained concentration values are then corrected for dilution factor and normalized on protein amount or tissue mass.

REAGENTS PROVIDED

REAGENTS	DESCRIPTION*	BEFORE THE ASSAY
BUFFER A	2 bottles with 25 mL of Extraction buffer in each	Equilibrate to room temperature (15-25˚C).
NAD+ stabilizing reagent	1 bottle with 8 mL of buffered solution for NAD+ measurement	Equilibrate to room temperature (15-25˚C).
NADH stabilizing reagent	1 bottle with 8 mL of buffered solution for NADH measurement	Equilibrate to room temperature (15-25˚C).
NAD+ standard stock	1 microtube with 40 µL of 1mM NAD+	See preparation guide.
NADH standard stock	1 microtube with 40 µL of 1mM NADH	See preparation guide.
BUFFER C	2 bottles with 19 mL of Assay Buffer in each. One per plate.	Equilibrate to room temperature (15-25˚C).
Assay color reagent	2 bottles with 3 mL of assay color reagent in each. One per plate	Equilibrate to room temperature (15-25˚C). Should be used within 3 hour after equilibration.
Enzyme	2 microtubes with 40 µL of enzyme in each. One per plate	Thaw only before adding into Master Mix.
Stop solution	1 bottle with 3 mL of Stop solution, for both plates	Equilibrate to room temperature (15-25˚C).
BUFFER D	12 mL of buffered solution to solubilize protein pellet for protein measurement.	Equilibrate to room temperature (15-25˚C) at the day of use.
Positive control buffer	1 microtube with 50 µL of buffer to prepare positive control	Equilibrate to room temperature (15-25˚C).

*Note: Accepted variation of the filling volume 5%.

PRECAUTIONS AND WARNINGS

Only for use by trained personnel

The BUFFER A, Stop Solution and BUFFER D may cause skin, eye and respiratory irritation in case of direct contact. Avoid breathing the fumes. Handle with care, where protective clothes and use gloves and googles.

Assay Color reagent may case cause skin irritation in case of direct contact. Handle with care, use gloves, wear protective clothes.

BUFFER A is toxic if swallowed. Handle with care, use goggles, wear protective clothes, use gloves.

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

Leftover of BUFFER A cannot be disposed into a drain. It can be utilized in accordance with the regulation of hazardous waste utilization or by evaporation by keeping opened bottle under the fume hood.

Disposal of hazardous components of the kit are described in the Safety Data Sheet associated with the kit.

STORAGE AND EXPIRATION OF REAGENTS

- Kit components should be stored at -80°C until use. Avoid temperature fluctuations in the freezer.
- After thawing, BUFFER A, NAD+ and NADH stabilizing reagents, BUFFER D, Positive Control buffer and Stop Solution are stable for two weeks at room temperature.
- BUFFER C should be thawed and used on the day of the assay.
- BUFFER A should be protected from direct light upon storage at room temperature.
- Assay Color reagent is stable for up to 3 hours at room temperature after thawing.
- The Enzyme should be used directly after thawing.
- Standards and the Positive Control should be prepared and used on the day of the assay.
- Standards should be protected from light.

OTHER MATERIAL REQUIRED

These materials are not included in the kit, but will be required to successfully perform the assay:

- Deionized water
- Microtubes (1.5 mL, e.g., microcentrifuge tubes)
- Two 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 suitable for colorimetric and absorbance assays
- Dry bath (heat block) with adjustable temperature (up to 80°C)
- Table-top cooling microcentrifuge (max speed 20 000 x g)
- Ice
- Dry ice
- Lysis tubes with beads and Tissue homogenizer (e.g., Precellys) OR Dounce (glass) homogenizer to homogenize tissues.
- Calibrated single channel (e.g. 5-50 µL, 20-200 µL, 100-1000 µL) and multichannel pipettes (5-50 µL, 30-300 µL) and beveled tips with low retention
- Microplate reader capable of measuring absorbance at 570-573 nm
- Aluminum foil to protect tubes and plates from light
- (Optional) Water bath with adjustable temperature to 50 °C
- (Optional) BCA Protein assay kit or similar
- (Optional) Sonicator with a tip fitting into 1.5 ml microtube
- (Optional) 100 mM PBS, pH 7.0 (61.5 mM K_2HPO_4, 38.5 mM KH_2PO_4)

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix reagents of the kit with those from other sources.
- Variation in sample collection, processing and storage may cause variation in metabolite values.
- This assay is NOT suitable for measurement of NAD+ and NADH in whole blood. For NAD+ and NADH measurement in blood, please use “Q-NAD BLOOD NAD+ and NADH assay kit”, RUO_001.

REAGENTS PREPARATION

Thaw all components for NAD+ and/or NADH assay according to instructions.

A. **BUFFER A** - Ready to use. Do not shake vigorously.

B. **NAD+ stabilizing reagent** - Ready to use

C. **NADH stabilizing reagent** - Ready to use

D. **Enzyme** - Ready to use

E. **Stop Solution** - Ready to use. If a precipitate forms in Stop Solution upon thawing, it should be re-dissolved by incubation at +37°C for 5 min, and then cooled back to room temperature before use. Do not shake Stop Solution vigorously.

F. **BUFFER D** –Ready to use. If a precipitate forms in BUFFER D upon thawing, it should be re-dissolved by incubation at +37°C for 5 min, and then cooled back to room temperature before use. Do not shake vigorously.

G. **Positive Control buffer** - Ready to use. (For further instructions see preparation of Positive Control).

H. **50 µM NAD+ standard working stock** - add 25 µL of 1 mM NAD+ standard stock (provided) into 475 µL of deionized water, vortex. Protect from light. (For further instructions see preparation of standards and preparation of Positive Control).

I. **50 µM NADH standard working stock** - add 25 µL of 1 mM NADH standard stock (provided) into 475 µL of deionized water, vortex. Protect from light. (For further instructions see preparation of standards and preparation of Positive Control).

J. **Master Mix** – 1 bottle of **Assay Color reagent** (3 mL) should be added into 1 bottle of **BUFFER C** to create 1 bottle of Master Mix required for 1 plate of either NAD+ or NADH assay. Protect from light by keeping it in the original amber bottle. Do not shake vigorously.

SAMPLE COLLECTION AND STORAGE

Tissue samples must be snap frozen in liquid nitrogen as soon as possible after collection. In case the tissue has significant blood contamination, rinse the tissue with ice-cold PBS and tap over a paper towel prior to freezing. We recommend freezing small pieces of material (10 - 20 mg per tube). **Important:** collect samples consistently from the same area of an organ to minimize variability. Weigh each piece either before freezing or in a frozen state. Sample aliquots should be stored at -80°C before analysis. Requirement for sample amount: 10 - 20 mg of tissue is sufficient (tested for mouse tissues). For extraction, use 1 mL of Extraction BUFFER A per 20 mg of tissue aliquot.

Cultured cells should be washed with an excess of PBS before collection to remove protein of the culture media (important if normalization per protein will be used). Collect cells by scraping or using enzymes, such as trypsin. Pellet the cells in a tube by centrifugation at 750 x g for 5 min and discard the supernatant. Snap freeze the pellet in liquid nitrogen and store at -80°C until analysis. Requirement for sample amount: 1.5 - 2 million cells is sufficient (tested for cultured fibroblasts and HeLa cells). Guideline for extraction: use 300 µL of extraction BUFFER A per 1.5 – 2 million cells.

PRACTICAL CONSIDERATIONS

- Content of Q-NAD tissues/cells assay kit is sufficient for extraction and analysis of NAD+ and NADH in 40 samples. The number of samples is defined by provided volume of BUFFER A calculated for tissues (e.g. 1 mL of BUFFER A for 20 mg of tissue). If cells are used, volume of provided BUFFER A is provided in excess (as for 1.5 - 2 million cells 0.3 mL of BUFFER A is used).
- Both fresh and frozen samples can be used for the assay. Frozen sample should be stored at -80°C all the time before the assay. If accidental melting occurred during storage/shipping time the sample is not suitable for the assay and should be discarded as freezing again will not save the sample.
- Do not mix materials from different kit lots. Subsequent freeze-thaw cycles are not allowed.
- We recommend homogenizing samples one at the time. Obtained homogenates can be kept on ice until all the samples are homogenized and then centrifuged all at once to separate extracts.
- For analysis of both NAD+ and NADH, 10 mg - 20 mg of tissue, 1.5 - 2 million cells are sufficient.
- 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.
- The kit allows normalization of the results on tissue mass or cell amount and optionally on protein content.
- 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.
- 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.
- Technical requirements for dry bath (heat block) – important for NADH stabilization step:
    - Test the efficiency of heat transfer in the dry bath as follows:
    1. Add 200 µL of water into a microtube that is fitting into the dry bath you are using.
    2. Insert the conventional lab thermometer into the microtube with the liquid and place into the dry bath set to 80°C.
    3. Measure the time needed to increase the temperature of the liquid to 70°C.
    4. Incubation time for heating the NADH stabilized extract is given for a dry bath equilibrated to 80°C, which heats 200 µL of water to 70°C in one minute.

- Positive Control is an artificial sample containing known amounts of pure NAD+ and NADH. Positive Control is prepared on the day of the assays using provided Positive control Buffer and 50 nmol/mL NAD+ and NADH standard solutions.
- 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.
- BUFFER A, Master Mix, Stop Solution and BUFFER D contain detergents. Do not shake vigorously.
- To avoid bubbles, pipette Master Mix and Stop Solution by pressing the pipette to the first stop position, remove all bubbles in the wells with a small needle if needed. Avoid touching the content of the wells by pipette tips.
- We recommend the following order of steps:
    1. Bring BUFFER A, BUFFER D, 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. If a precipitate forms in BUFFER D and in Stop Solution during thawing, it should be re-dissolved by incubation at 37°C for 5 min. Do not shake the buffers vigorously.
    2. On the day of the assay first prepare the Standards and Positive Control. Keep them refrigerated and light protected until the assay.
    3. On the day of the assay, thaw bottles with BUFFER C and Assay Color Reagent at room temperature. It takes about 2 - 3 hours to melt. During this time perform extraction of the samples, 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.

PREPARATION OF STANDARDS

1. Prepare standards on the day of the assay. Thaw tubes with 1 mM standard stocks for 5 - 10 min at room temperature under light protection.
2. Prepare **50 nmol/mL stock of NAD+** by adding 25 µL of 1 mM NAD+ standard stock (provided) into 475 µL of deionized water, vortex. This stock is used for preparation of NAD+ Assay standards and Positive Control.
3. Prepare **NAD+ Assay Standards** according to the scheme below by mixing indicated volumes of 50 nmol/mL NAD+ stock, deionized water, BUFFER A and NAD+ stabilizing reagent. Final volume of each Assay standard is 1 mL. (**Tip**: use the same 20 - 200 µL pipette for pipetting 50 nmol/mL NAD+ stock and water).

STANDARD NUMBER	CONCENTRATION of NAD+, nmol/mL	STANDARD PREPARATION			
		50 nmol/mL NAD+ (µL)	dH2O (µL)	BUFFER A (µL)	NAD+ stabilizing reagent (µL)
ST1	0	0	100	450	450
ST2	1	20	80	450	450
ST3	2	40	60	450	450
ST4	3	60	40	450	450
ST5	5	100	0	450	450

4. Prepare **50 nmol/mL stock of NADH** by adding 25 µL of 1 mM NADH standard stock (provided) into 475 µL of deionized water, vortex. This stock is used for preparation of NADH Assay standards and Positive Control.
5. Prepare **NADH Assay Standards** according to the scheme below by mixing indicated volumes of 50 nmol/mL NADH stock, deionized water, BUFFER A and NADH stabilizing reagent. Final volume of each Assay standard is 1 mL. (**Tip**: use the same 5 - 50 µL pipette for pipetting 50 nmol/mL NADH standard and 20 - 200 µL pipette for pipetting water).

STANDARD NUMBER	CONCENTRATION of NADH, nmol/mL	STANDARD PREPARATION			
		50 nmol/mL NADH (µL)	dH2O (µL)	BUFFER A (µL)	NADH stabilizing reagent (µL)
ST1	0	0	100	450	450
ST2	0.5	10	90	450	450
ST3	1	20	80	450	450
ST4	1.5	30	70	450	450
ST5	2	40	60	450	450

6. Cover the stand with ready Standards with an aluminum foil lid to protect from light and keep at 4˚C prior to pipetting on the plate.

PREPARATION OF POSITIVE CONTROL

Positive Control is prepared before the assay by mixing known amounts of NAD+ and NADH standards with the Positive Control Buffer (provided). The purpose for Positive Control is to monitor efficiency of the stabilization step when one of the metabolites has to be removed from the extract to allow selective measurement of either NAD+ or NADH.

1. Thaw the microtube with Positive Control buffer for 5 - 10 min at room temperature.
2. Pipette 30 µL of Positive Control buffer into a separate microtube.
3. Add 50 µL of 50 nmol/mL NAD+ stock and 20 µL of 50 nmol/mL NADH (preparation guide for 50 nmol/mL solutions are on page 10) stock into 30 µL of Positive control buffer. Total volume: 100 µL.
4. Add 200 µL of BUFFER A into this mix to mimic sample extraction process, vortex. Total volume: 300 µL of obtained Positive Control "extract".
5. Prepare two separate aliquots of the Positive Control "extract" from step 4 into clean microtubes – 100 µL/tube.
6. To the first 100 µL aliquot, add 100 µL of **NAD+ Stabilizing Reagent**, vortex, and incubate at room temperature for 5 min. Then keep refrigerated and covered with foil until the assay. This stabilized Positive Control will be used in **NAD+ assay**.
7. To the second 100 µL aliquot, add 100 µL **NADH Stabilizing Reagent**, vortex. Next, incubate this tube in the dry batch (heat block) set at 80°C for 1 min, cool on ice. Then keep refrigerated and light protected till the assay. This stabilized Positive Control will be used in **NADH assay**.

EXTRACTION OF NAD+ AND NADH FROM TISSUES

IMPORTANT:

- Tissue samples must be pre-weighted before the homogenization in order to allow normalization of obtained NAD+ and NADH values on tissue mass. Weighing can be done either before freezing of fresh sample or under liquid nitrogen for the tissue, which was already frozen.

- BUFFER A should be warmed to 50°- 55°C before the extraction process (this is optimal temperature range). In this protocol, frozen sample is added into warmed extraction buffer followed by mechanical homogenization.

1. Keep tissue samples with known mass frozen on dry ice before the extraction. We recommend homogenizing one sample at the time or maximum two, if tubes with beads and automated Homogenizer are used.
2. Calculate volume of BUFFER A needed for homogenization of each sample based on the ratio - 1 mL of BUFEER A per 20 mg of tissue.
3. Equilibrate BUFFER A to 50°- 55°C before extraction. To do so, there are two options: A) Pipette calculated volume of BUFFER A needed for homogenization into a homogenization tube, close the tube and place it for 5 min into a dry bath equilibrated to 50°- 55°C. B) Place the whole bottle with BUFFER A into a water bath equilibrated to 50°- 55°C for 10 min prior extraction and pipette calculated volumes of warmed buffer into homogenization tubes or glass homogenizer, if latter is used.
4. Add the frozen tissue piece with known weight directly into the warm buffer. Homogenize the tissue sample in an automated homogenizer OR Dounce homogenizer until solution is homogenous without visible tissue fragments. Presence of white threads of connective tissue is acceptable.
5. Cool the homogenate in an ice-water bath for at least 5 min. NAD+ and NADH metabolites are stable in obtained homogenate when kept on ice. Proceed with the next sample from step 4 to 5 and continue until all samples are homogenized. In the next step, all homogenates will be processed at the same time.
6. Mix homogenate with 1 mL pipette and transfer into clean microtube trying to collect all protein precipitate formed during homogenization/extraction process.
7. Centrifuge at 20 000 x g for 10 min at 4°C. Transfer the extracts into clean microtubes and save the pellets for protein measurement, if normalization per protein content is needed. If protein content in the pellets cannot be measured at the day of extraction freeze pellets at -80°C.
8. Prepare two separate aliquots from the obtained extracts into clean microtubes – 100 µL/tube.
9. To the first 100 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 NAD+ stabilized extracts refrigerated, and light protected until the assay.
10. To the second 100 µL aliquot, add 100 µL of **NADH stabilizing reagent** to get stabilized extract with NADH (keeps NADH and removes NAD+). Vortex and incubate in dry bath equilibrated at 80°C for 1 min. Cool on ice for 5 min. If the assay is not performed immediately, keep the NADH stabilized extracts refrigerated, and light protected until the assay.

EXTRACTION OF NAD+ AND NADH FROM CULTURED CELLS

Fresh and frozen cell pellets can be used for analysis. If fresh cell pellets are to be analyzed, they must be kept on ice prior homogenization. If frozen cells are to be analyzed, they must be kept on dry ice before procedure. We recommend processing maximum two samples at a time.

1. Equilibrate BUFFER A to 50°- 55°C before extraction. To do so, either make 1.2 mL aliquots of BUFFER A in microtubes and place them for 5 - 10 min to a dry bath equilibrated to 50°- 55°C or place the bottle with BUFFER A into a water bath equilibrated to 50°- 55°C for 10 min prior extraction.
2. Keep deeply frozen cell pellets for 2 min on ice before the extraction. This is needed to reduce temperature drop of the BUFFER A upon addition to the sample.
3. Add 300 µL of warm BUFFER A to every 1.5 - 2 million cells and resuspend by a few cycles of pipetting up-and-down until there are no cell clumps.
4. Incubate the homogenate in a dry bath equilibrated to 50°- 55°C for 2 min. Cool the homogenate in the ice-water bath for at least 5 min. NAD+ and NADH metabolites are stable in obtained homogenate when kept on ice. Proceed with the next sample from step 3 to 4 and continue until all samples are homogenized. In the next step, all homogenates will be processed at the same time.
5. Centrifuge at 20 000 x g for 10 min at 4°C. Transfer the obtained extracts into clean microtubes, save protein pellet for protein measurement if normalization per protein is needed. If protein content in the pellets cannot be measured at the day of extraction freeze pellets at -80°C.
6. Prepare two separate aliquots from the obtained extracts into clean microtubes – 100 µL/tube.
7. To the first 100 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 NAD+ stabilized extracts refrigerated, and light protected until the assay.
8. To the second 100 µL aliquot, add 100 µL of **NADH stabilizing reagent** to get stabilized extract with NADH (keeps NADH and removes NAD+). Vortex and incubate in dry bath equilibrated at 80°C for 1 min. Cool on ice for 5 min. If the assay is not performed immediately, keep the NADH stabilized extracts refrigerated, and light protected until the assay.

NOTE: Dilution of the initial extract after stabilization step is 2 times.

NOTE: Levels of NAD+ and NADH are tissue and cell-type specific. Levels also can be modulated by supplementation with NAD precursors or drugs. We recommend to first extract samples according to the provided guidelines (sample amount per volume of extraction BUFFER A). If the light absorbance in reactions with added stabilized extract is very low (close to 0 nmol/mL standard) or significantly higher than absorbance of the highest standard, then the amount of starting material needs adjustment. In case of high concentration of metabolite in the stabilized extract, the stabilized extract should be diluted with deionized water before the assay. Take the additional dilution into account in calculation of the results.

ASSAY PROCEDURE

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

- Every assay should contain Sample Blanks to correct for all unspecific background signals. Each sample type must have its own Sample Blank. If all analyzed samples are of the same type (for example all 40 samples are muscle samples or fibroblast cultures) prepare four Sample Blank wells from 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. If several tissue types are analyzed - every sample type should have at least one well with Sample Blank representing this sample type. Positive Control does not require a separate Blank.
- **All Sample Blanks are incubated with Master Mix WITHOUT added enzyme**.

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

1. Incubate ready Assay 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 nmol/mL) according to the plate scheme on page 19.
3. Pipette 20 µL of stabilized Positive Control and stabilized sample extracts in duplicates. For the first four samples, pipette one extra replicate to the indicated well (BL UNK1-4) according to the scheme on page 19. These four wells are Sample Blanks needed for the analysis without enzyme to correct for unspecific interaction between the extract and Assay Color reagent within the Master Mix.
4. Prepare Master Mix by adding Assay Color Reagent into BUFFER C, mix gently by rotation.
5. From this step onwards **turn off the direct indoor lights**. Add 190 µL of Master Mix without enzyme into four Sample Blank wells.
6. Add 40 µL of Enzyme into the remaining Master Mix in the bottle. Mix gently, avoid foaming. Pour into reservoir for multichannel pipette and protect the reservoir from light by keeping an aluminum foil lid above (please, see further instructions in our instructional video at www.nadmed.com).
7. Add 190 µL of Master Mix with added enzyme to all remaining wells, 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 - 8 min at room temperature.

   NOTE: The reaction can be stopped when there is a distinct color gradient in the standards and difference in color intensity between samples with added enzyme and Sample Blanks. The longer the reaction time the more intense signal will be observed. Color intensity in the NADH assay is lower than in the NAD+, because the physiological concentration of NADH is lower than NAD+ in tissues and cells. Therefore, the NADH standard range is from 0 to 2 nmol/mL, while

the NAD+ standard range is from 0 to 5 nmol/mL (concentration in standard solution of which 20 µL is added into the well).

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 the light absorbance at 573 nm immediately after addition of Stop Solution. If possible, shake the plate inside a microplate reader for 5 sec before the measurement. Note: after addition of Stop Solution color intensity can slowly increase uniformly in all the wells. This is expected due to non-enzymatic background process in the Master Mix.

MEASUREMENT OF PROTEIN CONTENT

Important! Remove all liquid from the tube with the pellet obtained after metabolite extraction before adding solubilization BUFFER D. Do not solubilize by pipetting up-and-down. Solubilization requires short sonication using microtip.

1. Equilibrate BUFFER D to room temperature.
2. If frozen pellets will be analyzed, they must be equilibrated to room temperature for 10 min before addition of BUFFER D.
3. Add 250 µL of BUFFER D to each tissue protein pellet.
4. Add 100 µL of BUFFER D to each cell protein pellet.
5. Sonicate the samples using a micro tip for 2 - 5 sec. Place the tip of the sonicator, so that it touches the pellet.
6. Dilute samples 10 times with 100 mM PBS, pH 7.0 (for example, mix 10 µL of the sample with 90 µL of PBS, vortex).
7. Measure protein concentration in the diluted solution using any available protein assay kits, for example a BCA Protein assay kit.
8. Using the obtained result, calculate the absolute amount of protein in the initial pellet (mg) using formula:

$$total\ protein, mg = obtained\ concentration\ \frac{mg}{mL} * 10 * V(mL)\ used\ for\ pellet\ solubilization$$

CALCULATION OF RESULTS

Perform calculations for each plate separately using the following algorithm:

1. Calculate average of Absorbance readings for duplicate of each standard (ST1-ST5). Create a Standard curve by plotting mean absorbance for each standard on the Y-axis against concentration (in nmol/mL) on the X-axis and perform a simple linear regression fitting of the Standard curve. Please, note, that concentration units are nmol/mL in 20 µL of each Standard solution added to the well.
2. Find concentration of measured metabolite as nmol/mL in 20 µL of stabilized sample extract added to each well using the formula of linear regression of the Standard curve.
3. Calculate the average of concentration values for the duplicates of each stabilized sample extract.
4. Calculate the average of concentration values for all Sample Blanks.
5. Correct mean concentration value for each stabilized sample extract for the mean Sample Blank value. Obtained concentration values are: nmol/mL in 20 µL of stabilized sample extract added into the assay.
6. Normalize obtained concentration values to tissue mass, total protein and cell amount as shown below.

Normalization per mg of tissue using formula:

$$Conc.\frac{nmol}{mg}\ tissue = \frac{Concentration\ in\ stabilized\ extract, \frac{nmol}{mL} * 2}{20mg/mL}$$

Normalization per mg of protein using formula:

$$Conc.\frac{nmol}{mg} protein = \frac{Conc.\ in\ stabilized\ extract, \frac{nmol}{mL} * 2 * V, mL\ of\ added\ BUFFER\ A}{total\ protein, mg}$$

Normalization per million of cells using formula:

$$Conc., \frac{nmol}{million} cells = \frac{Conc.\ in\ stabilized\ extract, \frac{nmol}{mL} * 2 * 0{,}3\ mL\ of\ BUFFER\ A}{(cell\ amount)}$$

7. For convenience, obtained concentration in nmol/mg can be converted to pmol/mg by multiplying on 1000.

POSITIVE CONTROL

The purpose of Positive Control is to monitor efficiency of the stabilization step when one of the metabolites is removed from the extract.

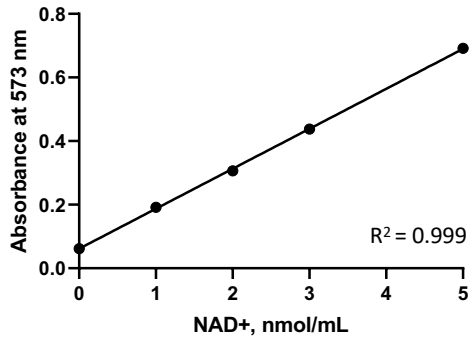
1. Find concentration of NAD+ in stabilized positive control “extract” on NAD+ plate as nmol/mL (in added 20 µL) using the formula of linear regression of the NAD+ Standard curve.
2. Find concentration of NADH in stabilized positive control “extract” on NADH plate as nmol/mL (in added 20 µL) using the formula of linear regression of the NADH Standard curve.

Concentration of NAD+ in stabilized Positive control “extract” is expected in the range of 4.0 - 4.5 nmol/mL. Concentration of NADH in stabilized Positive control “extract” is expected in the range of 1.5 - 1.9 nmol/mL.

TYPICAL DATA

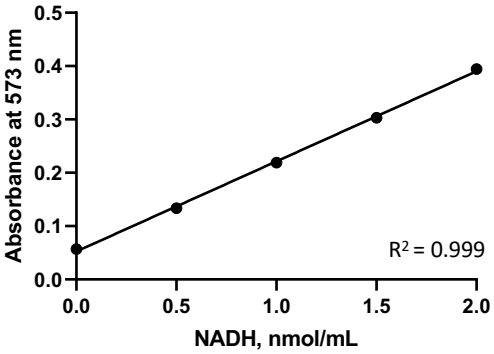
The standard curve values are provided for demonstration only. A standard curve for each NAD+ and NADH plate is generated based on the obtained absorbances on a target plate. Do not use the standard curve from one plate to calculate concentrations in samples measured on a different plate.

STANDARD CURVE FOR NAD+



Standard	NAD+ (nmol/mL)	Absorbance, 573nm Assay time – 4 min
ST1	0	0.064 0.006
ST2	1	0.192 0.192
ST3	2	0.309 0.304
ST4	3	0.432 0.444
ST5	5	0.703 0.681

STANDARD CURVE FOR NADH



Standard	NADH (nmol/mL)	Absorbance, 573nm Assay time – 6 min
ST1	0	0.057 0.057
ST2	1	0.133 0.134
ST3	2	0.224 0.214
ST4	3	0.300 0.306
ST5	5	0.395 0.394

RECOMMENDED PLATE LAYOUT FOR NAD+ OR NADH MEASUREMENT

An example plate for an experiment with 40 samples of the same type. If the experiment includes different sample types on one plate, each sample type should have at least one Sample Blank (BL UNK) well per sample type.

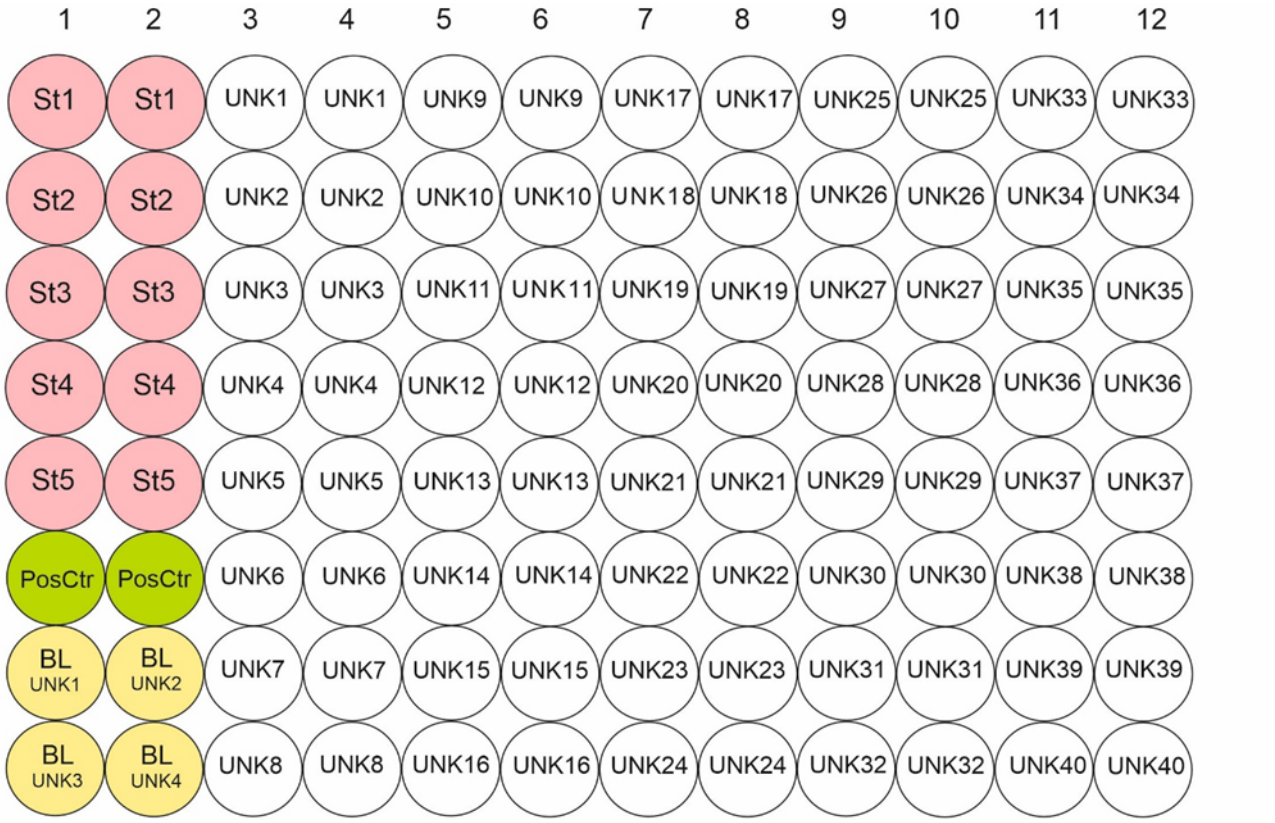
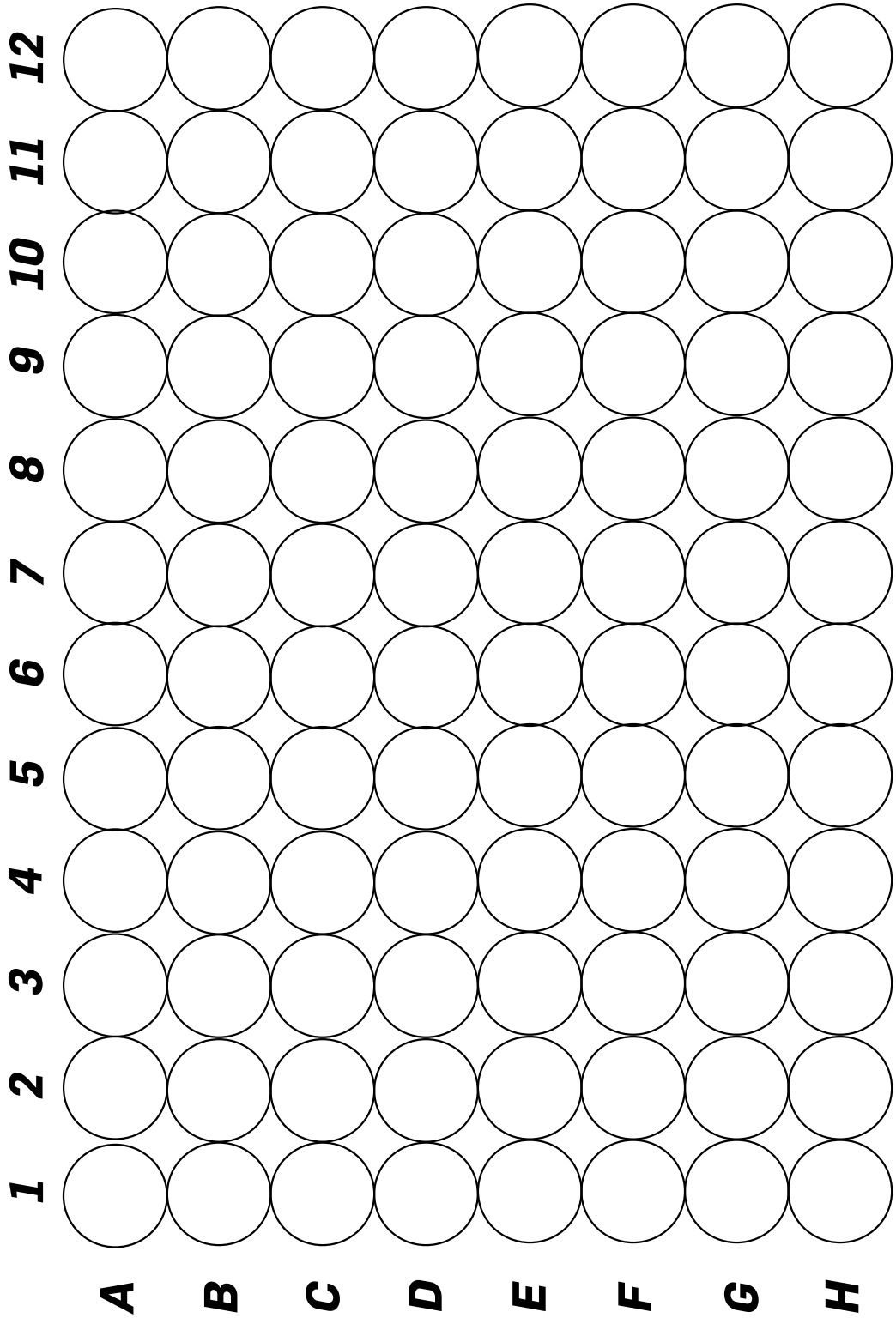


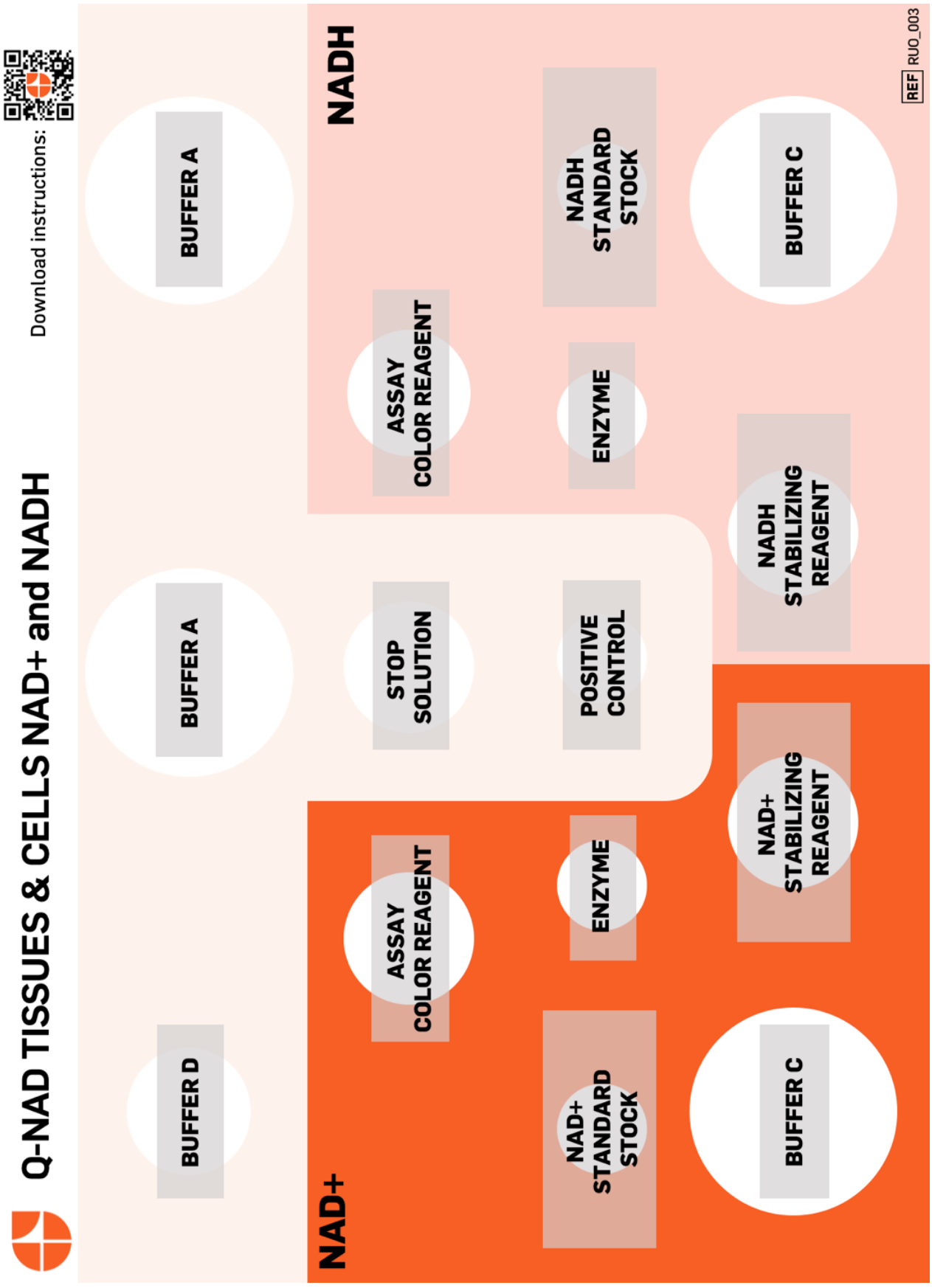
Plate layout for NAD+ or NADH assay: St – standard, BL - blanks with indicated samples used for the blank reading, UNK – unknown samples, stabilized extracts from the samples with unknown metabolite concentration, PosCtr – stabilized Positive Control. Use 20 µL of standards, stabilized Positive Control and stabilized sample extracts per well. Sample Blanks of the first four samples are analyzed in the Master Mix without added enzyme.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



INSERT CARD


Q-NAD TISSUES & CELLS NAD+ and NADH
Download instructions:
BUFFER A
BUFFER A
BUFFER D
NADH
ASSAY COLOR REAGENT
NADH STANDARD STOCK
ENZYME
BUFFER C
NADH STABILIZING REAGENT
STOP SOLUTION
POSITIVE CONTROL
NAD+
ASSAY COLOR REAGENT
ENZYME
NAD+ STANDARD STOCK
NAD+ STABILIZING REAGENT
BUFFER C
REF RUO_003