NADMED

TROUBLESHOOTING

Q-NADMED BLOOD & Q-NAD BLOOD ASSAY KITS

Cat numbers: IVD_001, IVD_001_01_40, RUO_001 and RUO_002

General comments: Always check the expiration date of your kit. Do not use the kit after the expiration date, and do not mix kit components between different kits. If the seal of the kit is not intact, do not use the kit. Follow the instructions for reagent preparation and storage, do not re-use/re-freeze reagents that are for single use only. For more details, see Q-NADMED Blood or Q-NAD Blood IFU. Always use the latest version of IFU from www.nadmed.com/products/.

Frequently reported problems:

The sample does not melt within 12-15 min.

Possible source	Solution
A layer of ice has formed on the outside of the sample tube	Remove the layer of ice by hand as soon as it formed and immediately immerse the tube with the sample back to ice-water bath
Ice-water bath is too dry	Add enough tap water into the ice to create slush, immerse the part of the tube containing the actual sample so that it is surrounded by icy water
Sample volume is large (> 0.5 mL)	Use quick warming cycles by hand to facilitate thawing, time outside the ice-water bath should be short, immediately immerse the sample tube into ice-water bath. For large volumes, it might take longer to thaw the samples. In this case, make sure the handling time of the samples is consistent.
Double layered/skirted or thick plastic sample tubes	Tube design and plastic thickness effect efficacy of thawing process. Facilitate thawing by repetitive quick warming cycles by hand and every time immediately immerse the tube with the sample back to ice-water bath

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The sample volume is less than 100 µL.

Possible source	Solution
Not possible to collect enough of blood, e.g. from mouse tail	Reduce the volume of BUFFER A proportionally to the volume of blood, e.g. 80 µL of blood is extracted in 400 µL BUFFER A. This will result in same target 6x dilution as in case 100µl of blood is extracted in 500 µL of BUFFER A.
Bubbles have been forming while mixing the sample	Reduce the volume of BUFFER A proportionally to volume of blood, e.g. 80 µL of blood is extracted in 400 µL BUFFER A. This will result in same target 6x dilution as in case 100µl of blood is extracted in 500 µL of BUFFER A.

The sample is not polymerized properly - liquid sample with protein clots

Possible source	Solution
Aliquot of BUFFER A was not sufficiently heated prior sample injection (temperature of buffer A prior extraction should be within range of 65-80°C)	Make sure that BUFFER A is equilibrated to room temperature before use. Set up your dry bath at 80°C for heating aliquots of BUFFER A before extraction and incubate for 3-4 min before injecting the sample.
Ratio of blood and BUFFER A is wrong	Make sure to pipette in the correct ratio: 100 µL blood into 500 µl pre-heated BUFFER A.
Mixing of blood and BUFFER A has been insufficient during extraction	Inject the sample into the bulk of BUFFER A (not to the bottom) with simultaneous rotation of the pipette tip and a couple of up-and-down pipetting cycles to efficiently mix the cold blood with the hot BUFFER A. The entire injection procedure of the sample takes about 10-15 sec.

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There is a gradient of reddish pigment in the supernatant close to the pellet observed after centrifugation of polymerized sample.

Possible source	Solution
Mixing of blood and BUFFER A has been insufficient during extraction	Inject the sample into the bulk of BUFFER A (not to the bottom) with simultaneous rotation of the pipette tip and couple of up-and-down pipetting cycles to efficiently mix the cold blood with the hot BUFFER A. The entire injection procedure of the sample takes about 10-15 sec.
Aliquot of BUFFER A was not sufficiently heated prior sample injection (temperature of BUFFER A prior extraction should be within range of 65°- 80°C)	Make sure that BUFFER A is equilibrated to room temperature before use. Set up your dry bath at 80°C for heating aliquots of BUFFER A before extraction and incubate for 3-4 min before injecting the sample.

High background in sample blanks. Normal Absorbance in Sample Blanks is in the range between first and second standard.

Possible source	Solution
Exposure of sample blanks with added Master Mix without enzyme to light during the time of adding enzyme to the rest of Master Mix.	Protect the wells with Sample Blanks with added Master mix with aluminum foil during adding the enzyme to the rest of Master Mix and preparation for pipetting to the plate.
Master Mix was not sunflower yellow upon addition to the wells due to direct light exposure at the stage of preparation for pipetting.	Reagent has been compromised if color turns to green (happens during exposure to light). Switch off artificial indoor lights and protect the Master Mix in the reservoir with the aluminum foil lid
Unsuitable type of used 96-well plate (for example, for microscopy application or sterile for cell culture).	Use non-sterile 96-well plate intended for colorimetric assays (transparent, polystyrene flat-bottom plates with medium protein binding).
Interference originating from material of reservoir for multichannel pipette.	Use non-sterile polystyrene reagent reservoirs designed for multi-channel pipette you are using.

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Absorbances in assay standards are not matching the typical data.

Possible source	Solution
Absorbances in all assay standards starting form ST1 with 0µM is much higher than the typical values. This indicates interference from either excessive light exposure of Master Mix during handling, or unsuitable type of used 96-well plate (for example, for microscopy application or sterile for cell culture).	All steps of the protocol involving handling and pipetting Master Mix have to be done in dim conditions, when indoor light is turned off and in a distance from a window. Light in the working place when you handle and pipette Master Mix should be as in a situation when you are under a bridge on a cloudy day. Use non-sterile 96-well plate intended for colorimetric assays (transparent, polystyrene flat-bottom plates with medium protein binding)
Incubation time was longer than 4 min in NAD+ assay and longer than 6 min in NADH assay	If you see a distinct color gradient in the assay standards and a color difference between samples and sample blanks, then everything was done correctly. Next time you can try to keep the time of the assays within recommended ranges for NAD+ and NADH to see Absorbance values in standards matching those in IFU.
Response in the standards is linear, but absorbances are lower than typical range, although the assay time was 4 min for NAD+ and 6 min for NADH plate. This problem occurs more often in NADH assay and indicates presence of interfering factors in the used equipment. These could be: 1) water used for standards preparation is not of correct purity grade; 2) microtubes used for preparation of the assay standards were chemically sterilized or intended for molecular biology applications or were colored tubes; 3) unsuitable type of 96-well plate (for example, for microscopy or sterile, treated plate for cell culture).	1) Use deionized water for assay standards preparation 2) Use non-sterile basic microtubes made from transparent/natural polypropylene (PP). Avoid using chemically sterilized equipment for Q-NADMED assays (e.g. microtubes, plates, tips). 3) Use non-sterile 96-well plate intended for colorimetric assays (transparent, polystyrene flat-bottom plates with medium protein binding).

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Obtained NADH values in all the samples are high/close to NAD+ values.

Possible source	Solution
NAD+ stabilized extracts were used for NADH assay	Use NADH stabilized extracts for NADH assay
Microtubes used for NADH stabilization step are not optimal. NAD+ was not removed during NADH stabilization step.	Use non-sterile basic microtubes made from transparent/natural polypropylene (PP) in all steps of sample preparation.
Heating step during NADH stabilization was insufficient. NAD+ was not removed during NADH stabilization step.	Test your dry bath as described in the IFU. For efficient NAD+ removal from NADH stabilized part of the extract, the temperature of the solution should reach 70°C. You must determine time in which your dry bath set at 80°C heat 250 µL of water to 70°C.

High variation of absorbance between replicates of same sample.

Possible source	Solution
Uncalibrated pipettes	Use calibrated pipettes for the assay.
Tips are not optimal	Beveled tips with less retention will improve the precision.
Bubbles are in the wells	Pipette Master Mix and Stop Solution by pressing pipette only to the first stop. Burst any generated bubbles with a small needle before measuring. Remember to protect the plate from the light during bubble removal (work in dim conditions).
Insoluble particle from the pellet was accidentally aspired together with the extract and ended up in one of the wells. Upon careful examination one can see the spot in the well surrounded by a circle of dark colored solution.	Be careful during pipetting of the extract from the microtube after centrifugation of polymerized sample. Do not aspire insoluble material, which can detach from the microtube walls.
Scratch on the bottom of the 96-well plate.	Inspect the bottom of the 96-well plate before use.

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NAD+ levels in all the samples are low - in range of 1-3 µM (calculated for whole blood).

Possible source	Solution
NADH stabilized extracts were used for NAD+ assay	Use NAD+ stabilized extracts for NAD+ assay
Frozen blood went through at least one thaw-freeze cycle before extraction. This caused NAD+ degradation.	Frozen blood must be always stored frozen. Blood sample should be thawed once and just before the extraction.
Time interval between the point when sample was melted and then used for the extraction has exceeded 15 min, although it was kept in ice-water bath.	Blood should be extracted right after complete melting was reached.
Frozen blood samples were left unattended during melting for a long time.	We recommend the following steps to efficiently thaw 120 - 200 µL frozen blood aliquots in 1.5 mL microtubes **within 10 - 12 min using ice-water bath**. Remember to keep time intervals, when sample is taken out from ice-water bath, as short as possible. 1) place tubes with frozen blood in ice-water bath. After 1 min remove formed ice coat formed around the tube. 2) after next 1 min, repeat removal of the ice coat. 3) after another 2 min, do a quick cycle of warming by fingers. 4) after another 2 min, take the pipette with the tip and by hummer motion quickly mix frozen and already melted layer of the sample. 5) after the next 2 min, do quick cycle of warming by fingers. 6) after the next 2 min (in total 10 min after removal from the freezer), take the pipette and by a rotation move check the status of the sample. The aim is to reach the state when most of the volume is liquid with few granules of ice (it looks like small thin plates of icy blood). When you see this state of the sample it means that after additional 2-3 min on ice-water bath the sample will be completely thawed and ready for extraction. So, this moment is also a time when one can place aliquots of BUFFER A for heating. 7) After the next 2-3 min, sample is ready for injection into hot BUFFER A.

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NAD+ levels in samples of healthy non-supplemented individuals are about 50% of the lower cut off value (see IFU for details).

Possible source	Solution
K3 EDTA collection tube was used for the blood collection	Use blood collection tubes with spray-dried either K2 EDTA or Lithium Heparin as anticoagulants. Collect target volume of blood per tube to keep correct concentration of the coagulant per mL of blood.