ab65348 – NAD/NADH Assay Kit (Colorimetric)

For the rapid, sensitive and accurate measurement of NAD/NADH in various samples.
For research use only - not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

For overview, typical data and additional information please visit:
http://www.abcam.com/ab65348

Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Aliquot components in working volumes before storing at the recommended temperature.

Reconstituted components are stable for 2 months.

Materials Supplied

Item	Amount	Storage Condition before preparation	Storage Condition after preparation
Extraction Buffer II/NADH/NAD Extraction Buffer	50 mL	-20°C	4°C/-20°C
Cycling Buffer I/NAD Cycling Buffer	15 mL	-20°C	4°C/-20°C
NAD Cycling Enzyme Mix	1 vial	-20°C	-80°C
Developer Solution II/NADH Developer	1 vial	-20°C	-80°C
NADH Standard II/NADH Standard	1 vial	-20°C	-80°C
Stop Solution II/Stop Solution	1.2 mL	-20°C	-20°C

Materials Required, Not Supplied

- These materials are not included in the kit, but will be required to successfully utilize this assay:
- Microplate reader capable of measuring absorbance at OD 450 nm
- MilliQ water or other type of double distilled water (ddH2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- 10 kD Spin Column (ab93349): for deproteinization step
- (Optional) 0.5M Tris HCl, pH 8.0 to neutralize acidic samples
- (Optional) Protease inhibitors: we recommend Protease Inhibitor Cocktail II (ab201116) [AEBSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail.
- DMSO

Reagent Preparation

- Briefly centrifuge small vials at low speed prior to opening.

NADH Standard II/NADH Standard: Reconstitute NADH Standard II/NADH Standard with 200 µL of pure DMSO to generate a 1 nmol/µL (1mM) NADH Standard II/NADH Standard solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store aliquots at -80°C. Use within two months.

NAD Cycling Enzyme Mix: Reconstitute NAD Cycling Enzyme Mix in 220 µL Cycling Buffer I/NAD Cycling Buffer. Keep on ice protected from light during the assay. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store aliquots at -80°C. Use within two months.

Developer Solution II/NADH Developer: Reconstitute Developer Solution II/NADH Developer in 1.2 mL of ddH2O. Pipette up and down several times to ensure the pellet is completely dissolved. Do not vortex. Aliquot Developer Solution II/developer so that you have enough volume to perform the desired number of assays. Store at - 80°C.

Extraction Buffer II/NADH/NAD Extraction Buffer and Cycling Buffer I/NAD Cycling Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or at -20°C.

Stop Solution II/Stop Solution: Ready to use as supplied. Equilibrate to room temperature before use. Store at - 20°C.

Standard Preparation

Always prepare a fresh set of standards for every use. Discard the working standard dilutions after use as they do not store well.

1. Prepare a 10 pmol/µL (10 µM) NADH Standard II/NADH standard by diluting 5 µL NADH Standard II/NADH Standard in 495 µL Extraction Buffer II/NADH/NAD Extraction Buffer.
2. Using 10 pmol/µL NADH Standard II/NADH standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Extraction Buffer II/Extraction Buffer (µL)	Final volume standard in well (µL)	End Conc NADH in well (pmol/well)
1	0	150	50	0
2	6	144	50	20
3	12	138	50	40
4	18	132	50	60
5	24	126	50	80
6	30	120	50	100

Each dilution has enough standard to set up duplicate readings (2 x 50 µL)

Sample Preparation

We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

We recommend that you use fresh samples. If you cannot perform the assay at the same time, complete the Sample Preparation step before storing the samples. If that is not possible, snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

In theory, serum samples can be used directly for the assay. However, NADH consuming enzymes like LDH can be present in serum which interferes with assay measurement. So, it will be best to consider removing matrix proteins using 10kD Spin Column (ab93349).

Try multiple sample volumes to ensure readings fall within the linear range of the standard curve.

Add protease inhibitors to Extraction Buffer II/NADH/NAD Extraction Buffer immediately prior to use.

Cell (adherent and suspension) samples:

1. Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells) by scraping. Any remaining trypsin can inhibit the assay.
2. Wash cells in cold PBS.
3. Pellet cells in a tube by spinning at 2,000 rpm for 5 minutes, and discard supernatant.
4. Extract cells with 400 µL of Extraction Buffer II/NADH/NAD Extraction Buffer by two freeze/ thaw cycles (20 minutes on dry ice followed by 10 minutes at RT).
5. Vortex the extraction for 10 seconds.
6. Centrifuge 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.
7. Collect supernatant (containing extracted NAD/NADH) and transfer into a new tube.
8. Keep on ice.
9. Cells may contain enzymes that consume NADH rapidly. Remove enzymes by filtering the samples through a 10 kD Spin Column (ab93349) before performing the assay.
10. Add sample to the spin column, centrifuge at 10,000 x g for 10 minutes at 4°C.
11. Collect the filtrate. We recommend testing the cell samples neat or at 1/5 dilution.

Δ Note: NADH quantification can be compromised after exposure to very acidic pH and therefore we do not recommend TCA or PCA precipitation for this assay.

Tissue samples:

1. Harvest the amount of tissue necessary for each assay (initial recommendation = 20 mg tissue).
2. Wash tissue in cold PBS.
3. Homogenize the sample using a Dounce homogenizer (30 – 50 passages) with 400 µL of Extraction Buffer II/NADH/NAD Extraction Buffer.
4. Centrifuge 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.
5. Collect supernatant (containing extracted NAD/NADH) into a new tube
6. Keep on ice.
7. Tissues may contain enzymes that consume NADH rapidly. Remove enzymes by filtering the samples through a 10 kD Spin Column (ab93349) before performing the assay.
8. Add sample to the spin column, centrifuge at 10,000 x g for 10 minutes at 4°C.
9. Collect the filtrate.

Δ Note: NADH quantification can be compromised after exposure to very acidic pH and therefore we do not recommend TCA or PCA precipitation for this assay.

Δ Note: We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

Δ Note: Endogenous compounds in the sample may interfere with the reaction. To ensure accurate determination of NADH in the test samples, we recommend spiking samples with a known amount of Standard (60 pmol).

Assay Protocol

Equilibrate all materials and prepared reagents to correct temperature prior to use.
We recommended to assay all standards, controls and samples in duplicate.
Prepare all reagents, working standards, and samples as directed in the previous sections.
Δ Note: set up Sample Background Controls to discard background signal in the assay.

Decomposition Step for NADH detection in samples:

Total NADt (total NAD & NADH): leave your sample as it is.
NADH: NAD+ needs to be decomposed before the reaction.

1. Aliquot 200 µL of extracted samples into microcentrifuge tubes.
2. Heat samples to 60°C for 30 minutes in a water bath or heating block. Under these conditions, all NAD+ will be decomposed while the NADH will still be intact.
3. Cool samples on ice. Quickly spin the samples to remove precipitate if precipitation occurs.
4. Label samples as NAD decomposed samples.

Set up Reaction wells:

- Standard wells = 50 µL standard dilutions.
- Sample Background control wells = 1 – 50 µL samples (adjust volume to 50 µL/well with Extraction Buffer II/Extraction Buffer).
- NADt Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with Extraction Buffer II/Extraction Buffer).
- NADH Sample wells = 1 – 50 µL decomposed samples (adjust volume to 50 µL/well with Extraction Buffer II/Extraction Buffer).

Reaction Mix:

1. Prepare 100 µL Reaction Mix for each reaction. Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
Cycling Buffer I/NAD Cycling Buffer	98	100
NAD Cycling Enzyme Mix	2	0

Multiply these quantities by (number of reactions+1).

2. Add 100 µL of Reaction Mix to each standard and sample well.
3. Add 100 µL of Background Reaction Mix to sample background control sample wells.
4. Incubate plate at room temperature for 5 minutes to convert NAD to NADH.
5. Add 10 µL of Developer Solution II/NADH Developer into each well and mix. Let the reaction cycle at room temperature for 1 – 4 hours or longer depending on the reading.
6. Take multiple readings during the 1 – 4 hours at OD 450 nm. The plate can be read multiple times while the color is still developing. Longer incubation times maybe needed depending on the OD reading.
7. OPTIONAL: The reaction can be stopped by adding 10 µL of Stop Solution II/Stop Solution into each well and mixing thoroughly. The color should be stable for 48 hours in a sealed plate after addition of the Stop Solution II/Stop Solution.

Calculation:

Subtract 0 Standard reading from all readings. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

1. Average the duplicate reading for each standard and sample.
2. If the sample background control is significant, then subtract the sample background control from sample reading.
3. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
4. Plot the corrected absorbance values for each standard as a function of the final concentration of NADt or NADH.
5. Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the

trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

6. Concentration of NADt or NADH in the test samples is calculated as:

$$\text{NADt concentration} = \frac{NADt}{Sv} \times D$$

$$\text{NADH concentration} = \frac{NADH}{Sv} \times D$$

Where:
NADt = amount of NADt in the sample well calculated from standard curve (pmol).
NADH = amount of NADH in the sample well calculated from standard curve (pmol).
Sv = sample volume added to the reaction well (µL).
D = sample dilution factor.

Alternatively, NADt or NADH values can be expressed in ng/mg protein if a protein quantification assay has been previously performed (NADH MW = 664.4 g/mol).

7. NAD/NADH Ratio is calculated as:

$$\text{NAD/NADH ratio} = \frac{NADt - NADH}{NADH}$$

Calculation for spiked samples (if applicable):

1. For spiked samples, any sample interference is corrected by subtracting the sample reading from spike sample reading. So, the concentration of NADt or NADH in sample well is calculated as:

$$\text{NADt or NADH} = \frac{sample\ reading}{spiked\ sample\ reading - sample\ reading} * amount\ of\ NADH\ spiked\ (pmol)$$

2. After working out the NADt and NADH concentration from the spiked samples, the NAD/NADH ratio can be calculated as shown in step 1 above.

Notes:

a. For Unspiked Samples, if the Sample Background Control reading is significantly high, subtract the Sample Background reading from the sample reading to get the Sample corrected reading. Apply the Sample corrected reading to the NADH Standard Curve.
b. For Spiked Samples, subtract the Sample Background reading from the Spiked Sample reading to get the Spiked Sample corrected reading.