ab65348 – NAD/NADH Assay Kit (Colorimetric)

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

For overview, typical data and additional information please visit: http://www.abcam.com/ab65348 (use http://www.abcam.cn/ab65348 for China, or http://www.abcam.co.jp/ab65348 for Japan)

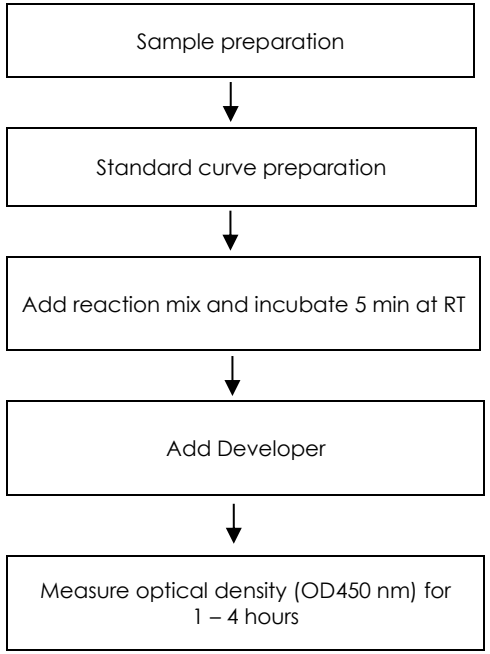
Background:

NAD/NADH Assay Kit (Colorimetric) ab65348 provides a convenient and sensitive tool to quantify NAD+, NADH, and measure their ratio, in samples from mammals and other species, such as serum, urine, cell and tissue lysates.

The NAD cycling enzyme mix in the kit specifically acts on NADH/NAD, but not NADP nor NADPH, in a cycling reaction which significantly increases sensitivity and specificity. There is no requirement to purify NADH/NAD from samples. The levels of both NADt (total NAD^+ and NADH) and NADH can be easily measured; the level of NAD^+ can be calculated by subtracting NADH from NADt. The assay is read by absorbance at 450 nm.

Assay Summary:

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.



QUICK ASSAY PROCEDURE

- Set heat block or water bath to 60°C
- Solubilize NADH Standard II, NAD Cycling Enzyme Mix and Developer Solution II. Thaw Stop Solution II, Extraction Buffer II and Cycling Buffer I (aliquot if necessary); get equipment ready.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Prepare standard curve.
- Set up plate for standard (50 µL) and samples (50 µL).
- Prepare Reaction Mix and add 100 µL to each well.
- Incubate plate at RT for 5 mins.
- Add 10 µL of Developer Solution II each well.
- Measure plate at OD 450 nm in kinetics mode during 1 – 4 hours.
- OPTIONAL: Stop the reaction by adding 10 µL

Precautions & Limitations:

Please read these instructions carefully prior to beginning the assay.
All kit components have been formulated and quality control tested to function successfully as a kit.

- Modifications to the kit components or procedures may result in loss of performance.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

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. Reconstituted components are stable for 2 months. Do not use kit or components if they have exceeded the expiry date.

Materials Supplied:

Item	Quantity	Storage Temperature (on receipt)	Storage temperature (reconstituted)
Extraction Buffer II	50 mL	-20°C	4°C /-20°C
Cycling Buffer I	15 mL	-20°C	4°C /-20°C
NAD Cycling Enzyme Mix	1 vial	-20°C	-80°C
Developer Solution II	1 vial	-20°C	-20°C
NADH Standard II	1 vial	-20°C	-20°C
Stop Solution II	1.2 mL	-20°C	-20°C

PLEASE NOTE: Extraction Buffer II was previously labeled as NADH/NAD Extraction Buffer, and Cycling Buffer I as NAD Cycling Buffer. Also, Developer Solution II was previously labeled as NADH Developer, and NADH Standard II as NADH Standard, and Stop Solution II as Stop Solution. The composition has not changed.

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 (OD) at 450 nm (colorimetric)
- 96 well clear plate with clear flat bottom (colorimetric assay)
- Orbital shaker
- DMSO
- Assorted glassware for the preparation of reagents and buffer solutions
- 10 kDa Spin Column (ab93349): for deproteinization step
- (Optional) 0.5 M 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.
- Microcentrifuge
- Dounce homogenizer (if using tissues or cells)

- 1 x PBS pH 7.4
- MilliQ water or other type of double distilled/deionized water (ddH_2O)

Reagent Preparation:

- Equilibrate reagents to room temperature before use.
- Briefly centrifuge small vials at low speed prior to opening.
- Aliquot reagents so that you have enough volume to perform the desired number of assays.

NADH Standard II: Reconstitute NADH Standard II with 200 µL of pure DMSO to generate a 1 nmol/µL (1 mM) NADH Standard II solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store aliquots at -20°C. Use within two months.

NAD Cycling Enzyme Mix: Reconstitute NAD Cycling Enzyme Mix in 220 µL Cycling Buffer I. 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: Reconstitute Developer Solution II in 1.2 mL of ddH_2O. 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 - 20°C.

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

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

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, we suggest that you complete the Sample Preparation step before storing the samples at -20°C. Alternatively, snap freeze your samples in liquid nitrogen upon extraction and store them 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.
- 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 (e.g. 60 pmol, 6 µl NADH standard at 10 pmoles/µL).

1. Add protease inhibitors to Extraction Buffer II immediately prior to use.
2. NADH quantification can be compromised after exposure to very acidic pH and therefore, we do not recommend TCA or PCA precipitation for this assay.

Serum samples:

1. Serum sample can be used directly for the assay. However, NADH consuming enzymes, such as LDH can be present in serum, interfering with assay results.
2. Remove potential interfering enzymes by filtering the samples through a 10 kDa Spin Column (ab93349) before performing the assay.
   - Add sample to the spin column, centrifuge at 10,000 x g for 10 minutes at 4°C.
   - Collect the filtrate.

Cells (adherent or suspension) samples:

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

Tissue Samples:

1. Harvest the amount of tissue necessary for each assay (initial recommendation = 20 mg).
2. Wash tissue in cold PBS.
3. Homogenize in 400 µL Extraction Buffer II using a Dounce homogenizer (30 – 50 passages).
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 tub and keep on ice.
6. Tissues may contain enzymes that consume NADH rapidly. Remove enzymes by filtering the samples through a 10 kDa Spin Column (ab93349) before performing the assay.
   - Add sample to the spin column, centrifuge at 10,000 x g for 10 minutes at 4°C.
   - Collect the filtrate. We recommend testing the tissue samples neat or at 1/5 dilution.

Standard Preparation:

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.
- Each dilution has enough standard to set up duplicate readings (2 x 50 µL).

Prepare 10 µM dilution (10 pmoles/µL) NADH Standard by adding 5 µL of NADH Standard II to 495 µL Extraction Buffer II. Gently pipette up and down a few times to ensure all standard is removed from tip. Mix well by inversion. Using 10 pmoles/µL NADH standard, add 0, 2, 4, 6, 8, 10 µl NADH Standard into a series of wells, generating 0, 20, 40, 60, 80, 100 pmoles/well of NADH Standard. Adjust the volume to 50 µl/well with Extraction Buffer II. Or prepare duplicate standard curve dilutions as described in the table below in a microplate or microcentrifuge tubes:

Standard #	Volume of 10 µM Standard (µL)	Extraction Buffer II (µL)	Final volume standard in well (µL)	End NADH Amount (pmole/well) Colorimetric Assay
1	0	125	50	0
2	5	120	50	20
3	10	115	50	40
4	15	110	50	60
5	20	105	50	80
6	25	100	50	100

Assay Procedure:

- Keep enzymes and heat labile components and samples on ice during the assay.
- Equilibrate all other materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls, and samples in duplicate.
- To only measure Total NADt (total NAD & NADH): leave your sample as it is.
- To measure NADH, NAD^+ needs to be decomposed before the reaction.

Decomposition Step for NADH detection in samples:

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.
4. If precipitation occurs, centrifuge for 5 minutes at 4°C at top speed in a microcentrifuge to remove any insoluble material.
5. Label samples as NAD decomposed samples.

Set up Reaction wells:

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

6. Each well (standards, samples, and controls) requires 100 µL of Reaction Mix as shown in the table below. To ensure consistency, use the table below to prepare a Master Mix of the appropriate Reaction Mix for your assay using the following calculation:

X µL component x (Number reactions +1)

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

7. Mix Master Reaction Mix by inversion. Add 100 µL of the Reaction Mix to each standard and sample well. Use a clean tip for each well.
8. Add 100 µL of Background Reaction Mix to sample background control sample wells.
9. Incubate plate at room temperature for 5 minutes to convert NAD to NADH.
10. Add 10 µL of Developer Solution II into each well and mix. Let the reaction cycle at room temperature for 1-4 hours or longer depending on the reading.
11. Measure plate at OD 450 nm multiple times or in kinetics mode during the 1 – 4 hours. The plate can be read multiple times while the color is still developing. Longer incubation times maybe needed depending on the OD reading.

OPTIONAL: The reaction can be stopped by adding 10 µL of Stop Solution II 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.

Calculations:

- For samples producing signals greater than that of the highest standard: dilute further in appropriate buffer and reanalyze. Multiply the concentration found by the appropriate dilution factor.

Unspiked Samples:

1. Average the duplicate reading for each standard, sample and sample background control (if required).
2. If the absorbance of the sample background control is significant, then subtract the sample background control from the sample reading.
3. Subtract the mean absorbance value of the blank (Standard #1) from all standards. This is the corrected absorbance.
4. Plot the corrected absorbance values for each standard as a function of the final amount of NADH, and calculate the equation based on the corrected standard curve data using a linear regression.
5. Interpolate the amount of NADt or NADH in the sample wells based upon the linear equation, using the sample corrected readings for NADt and NADH sample wells.
6. Concentration of NADt or NADH in the test samples is calculated as:

$$NADt\ concentration = \frac{NADt}{V} \times D = pmol/\mu L = \mu M$$

$$NADH\ concentration = \frac{NADH}{V} \times D = pmol/\mu L = \mu M$$

Where:

NADt = amount of NADt in the sample well calculated from standard curve (in pmoles).

NADH = amount of NADH in the sample well calculated from standard curve (in pmoles).

V = sample volume added to the reaction well (in µL).

D = sample dilution factor (before addition to the well).

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:

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

Spiked samples:

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:

$$NADt\ or\ NADH = \frac{Sample\ reading}{Spiked\ Sample\ reading - Sample\ reading}\ x\ amount\ of NADH\ spiked\ (pmol)$$

2. After working out the NADt and NADH concentrations from the spiked samples, the NAD/NADH ratio can be calculated as shown in step 7 above.

Technical Hints

For additional helpful hints and tips on using our assay kits please visit:
https://www.abcam.com/en-us/support/product-support

Technical Support

For technical or commercial enquiries please go to:
www.abcam.com/contactus
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