**ab65348 NAD/NADH Assay Kit (Colorimetric)**

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

### 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.

### Materials supplied

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH/NAD Extraction Buffer</td>
<td>50 mL</td>
<td>-20°C</td>
<td>4°C/-20°C</td>
</tr>
<tr>
<td>NAD Cycling Buffer</td>
<td>15 mL</td>
<td>-20°C</td>
<td>4°C/-20°C</td>
</tr>
<tr>
<td>NAD Cycling Enzyme Mix</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>NADH Developer</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>NADH Standard</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>1.2 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

### Materials required, not supplied

These materials are not included in the kit, but will be required to successfully perform 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) [AEGSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail.
- DMSO

### Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening

**NADH/NAD Standard:** Reconstitute NADH/NAD Standard with 200 µL of pure DMSO to generate a 1 nmol/µL (1mM) NADH/NAD 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 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.

**NADH Developer:** Reconstitute 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 so that you have enough volume to perform the desired number of assays. Store at -80°C.

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

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

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

Each dilution has enough amount of 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 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 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. Add sample to the spin column, centrifuge at 10,000 x g for 10 minutes at 4°C. Collect the filtrate. We recommend to test the cell samples neat or at 1/5 dilution.

**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 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. Add sample to the spin column, centrifuge at 10,000 x g for 10 minutes at 4°C. Collect the filtrate.

**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.

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

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 procedure

Equilibrate all materials and prepared reagents to correct temperature prior to use. We recommend to assay all standards, controls and samples in duplicate.

Prepare all reagents, working standards, and samples as directed in the previous sections.

**NOTE:** set up Sample Background Controls to discard background signal in the assay.

### Decomposition Step for NADH detection in samples:

- **Total NAD (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).
- **NAD+ Sample wells =** 1 – 50 µL samples (adjust volume to 50 µL/well with Extraction Buffer).
- **NADH Sample wells =** 1 – 50 µL decomposed samples (adjust volume to 50 µL/well with 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD Cycling Buffer</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>NAD Cycling Enzyme Mix</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

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 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 into each well and mixing thoroughly. The color should be stable for 48 hours in a sealed plate after addition of the Stop Solution.

### Calculations

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.

5. Average the duplicate reading for each standard and sample.
6. If the sample background control is significant, then subtract the sample background control from sample reading.
7. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
8. Plot the corrected absorbance values for each standard as a function of the final concentration of NADH or NADH.
9. 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).

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

    \[
    \text{NADt concentration} = \left( \frac{\text{NADt concentration}}{\text{Sample OD}} \right) \times \text{Dilution Factor}
    \]

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\[
NADH \text{ concentration} = \left( \frac{NADH}{Sv} \right) \times D
\]

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).

8. NAD/NADH Ratio is calculated as:
\[
\text{NAD/NADH ratio} = \frac{NADt}{NADH}
\]

9. 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:
\[
NADt \text{ or } NADH = \frac{\text{sample reading}}{\text{(spiked sample reading)} - \text{(sample reading)}} \times \text{amount of NADH spiked (pmol)}
\]

After working out the NADt and NADH concentration from the spiked samples, the NAD/NADH ratio can be calculated as shown in step 8 above.

Interferences

These chemicals or biological materials will cause interference in this assay causing compromised results or complete failure:
- 5 % detergents (Triton-X 100, NP-40, etc)
- Ascorbic acid

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate</td>
</tr>
</tbody>
</table>

Sample with erratic readings

<table>
<thead>
<tr>
<th>Samples not deproteinized (if indicated on protocol)</th>
<th>Use provided protocol for deproteinization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td>Samples used after multiple free/ thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
</tbody>
</table>

Lower/ Higher readings in samples and Standards

<table>
<thead>
<tr>
<th>Improperly thawed components</th>
<th>Thaw all components completely and mix gently before use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
</tbody>
</table>

Unanticipated results

<table>
<thead>
<tr>
<th>Measured at incorrect wavelength</th>
<th>Check equipment and filter setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>

FAQ

I want to deproteinize my cellular and tissue samples. Can I use PCA precipitation?

NADH quantification could be compromised after exposure to very acidic pH from the perchloric acid. Therefore, we strongly recommend using the 10 kD Spin Columns (ab93349) for sample deproteinization.

In the protocol, it is mentioned that to detect NADH, the NAD+ needs to be decomposed before the reaction. Does it mean that 1) there is no NAD+ present in the well, and 2) if the NAD cycling mix is added at this step, no reaction should be observed? Could you also summarize the way the kit works? How and when is the NAD+ measured? How and when is the NAD quantified?

This kit runs on a simple principle. You can either measure the total NAD+NADH or just the NADH. To measure the NAD+, you need to subtract NADH levels from the NAD+ levels. This kit will only measure NADH. So if NADt has to measure, all the existing NAD has to be converted to NADH prior to detection. This is what the cycling enzyme does. If the levels of NADH only are to be measured, the NAD needs to be decomposed, which is done at an elevated temp.
The protocol states that a decomposing step on 60 °C needs to be done first in order to detect NADH. The next step would be the NAD Cycling, where NAD+ is transformed into NADH. The protocol does not mention anything about leaving this step out if I only want to detect NADH. Is it useless if I decomposed NAD+ in the step before?

It seems logical to ignore the step of NAD+ conversion to NADH if NAD+ has already been decomposed, but we would still recommend to follow the protocol exactly without deleting any steps. The NAD+ conversion to NADH adds some volume to each of the samples, as well as the standards. You definitely want this volume to be consistent between the standards and samples for comparing between them. Therefore, please do all the steps even if you want to assay just for NADH.

The protocol says incubation for 1 to 4 hours, will reaction results after 1 hour differ much from results after 4 hours?

The signals from 1 – 4 hours of final incubation will ideally increase. Within that time range, whenever you are comfortable and satisfied with the signals, you can add the stop solution to terminate any more color development.

Should the Stop solution be added before or after the final measurement?

Yes, the stop solution is added before the final measurement. You just keep developing the color until it falls within the linear range of the standard curve (which you can do only when you measure the absorbance). Once this color is reached, you add the stop solution to all wells and then take the final measurement. Thus there is no measurement bias introduced.

If we choose to lyse cells by homogenizing instead of freeze-thaw, what kind of device or method is the most suitable?

Please use a Dounce homogenizer. About 30-50 passages should be good for the homogenization. You can perform a microscopic examination to ascertain the homogenization. If required, please do 10-20 more passages.

What cell dilution can I use to start with?

We would recommend you to quantify the amount of protein in your sample before any further dilution as this will provide you a reference point.

Ideally, the reading of your samples should fall within the reading of the standard curve. The highest concentration of our NADH standard is 1 µM NADH, which corresponds to 664.4 µg NAD (66.44 ng NAD/well). An initial recommendation would be 100 – 200 µg protein/well.

You should aim to have the same amount or less protein in your well. Alternatively, if you are not able to quantify the amount of protein in your sample, you could do serial dilutions from your starting material as follows = 2X – 5X – 10X – 25X – 50X – 75X – 100X.

This won’t be as accurate as when based on protein amount, but it should be enough to you get you started.

Remember that your dilutions will be further diluted when performing the assay.