ab65348
NAD/NADH Assay Kit (Colorimetric)

Instructions for use:

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.

Version 23g Last Updated 07 January 2019
INTRODUCTION

1. BACKGROUND

NAD/NADH Assay Kit (Colorimetric) (ab65348) provides a convenient tool for sensitive detection of the intracellular nucleotides: NADH, NAD and their ratio. The NAD Cycling Enzyme Mix in the kit specifically recognizes NAD/NADH in an enzyme cycling reaction. There is no requirement to purify NAD/NADH from samples.

This kit specifically detects free NAD and NADH, but not NADP nor NADPH. The enzyme cycling reaction significantly increases the detection sensitivity and specificity. NADt (total NAD and NADH) or NADH can be easily quantified by comparing with standard NADH.

Nicotinamide nucleotides are key players in the energy and oxidation-reduction reactions of a cell. Nicotinamide adenine dinucleotide (NAD) exists in two forms, an oxidized form, NAD+, and a reduced form, NADH. NAD functions as a cofactor in the vast majority of cellular redox reactions, carrying reducing equivalents from one reaction to another. Therefore, maintaining appropriate levels of NAD is essential for maintaining normal cellular respiratory function. There are two major pathways in NAD biosynthesis. The de novo pathway is maintained by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), whereas the savage pathway recycles degraded NAD products such as nicotinamide. Studies have shown that cytosolic NAD+ concentrations range from 300 nM in mammalian cells to 2 mM in yeast. Depletion of NAD in cells is a major cause of cell death.

The importance of NAD function in modulating cellular redox status and controlling signalling and transcriptional events makes NAD an important cofactor when investigating normal cellular function.
INTRODUCTION

2. ASSAY SUMMARY

Standard curve preparation

↓

Sample preparation*#

↓

Add reaction mix and incubate RT 5 minutes

↓

Add developer and incubate RT 1 – 4 hours

↓

Measure optical density (OD450nm)

*Sample requires deproteinization.
# [NADH detection only] Sample requires decomposition step.
3. PRECAUTIONS

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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. 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.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**
5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- 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.

6. 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>
GENERAL INFORMATION

7. 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) [AEBSF, aprotinin, E-64, EDTA, leupeptin] as a general use cocktail.
- DMSO
8. TECHNICAL HINTS

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

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

9.2. **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.

9.3. **NADH Developer:**
Reconstitute NADH Developer in 1.2 mL of ddH$_2$O. 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.

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

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

9.6. **Stop Solution:**
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.
10. 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.

10.1. Prepare a 10 pmol/µL (10 µM) NADH standard by diluting 5 µL NADH Standard in 495 µL NADH/NAD Extraction Buffer.

10.2. 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)
11. SAMPLE PREPARATION

General Sample Information

- 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. Alternatively, if that is not possible, we suggest that you 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.
- Add protease inhibitors to NADH/NAD Extraction Buffer immediately prior use.

11.1. Cell (adherent and suspension) samples:

11.1.1. 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.

11.1.2. Wash cells in cold PBS.

11.1.3. Pellet cells in a tube by spinning at 2,000 rpm for 5 minutes, and discard supernatant.

11.1.4. 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).

11.1.5. Vortex the extraction for 10 seconds.

11.1.6. Centrifuge 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.

11.1.7. Collect supernatant (containing extracted NAD/NADH) and transfer into a new tube

11.1.8. Keep on ice.
11.1.9. 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.*

11.2. **Tissue samples:**

11.2.1. Harvest the amount of tissue necessary for each assay (initial recommendation = 20 mg tissue).

11.2.2. Wash tissue in cold PBS.

11.2.3. Homogenize the sample using a Dounce homogenizer (30 – 50 passages) with 400 µL of NADH/NAD Extraction Buffer.

11.2.4. Centrifuge 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.

11.2.5. Collect supernatant (containing extracted NAD/NADH) into a new tube.

11.2.6. Keep on ice.

11.2.7. 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).
12. ASSAY PROCEDURE

- 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.

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

12.1. 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.

12.1.1. Aliquot 200 µL of extracted samples into microcentrifuge tubes.

12.1.2. 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.

12.1.3. Cool samples on ice. Quickly spin the samples to remove precipitate if precipitation occurs.

12.1.4. Label samples as NAD decomposed samples.

12.2. 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).
- NADt 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).
12.3. **Reaction Mix:**

12.3.1. 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:

\[ X \mu L \text{ component} \times (\text{Number reactions} + 1) \]

<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>

12.4. Add 100 µL of Reaction Mix to each standard and sample well.

12.5. Add 100 µL of Background Reaction Mix to sample background control sample wells.

12.6. Incubate plate at room temperature for 5 minutes to convert NAD to NADH.

12.7. 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.

12.8. 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.

12.9. **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.
13. 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.

13.1. Average the duplicate reading for each standard and sample.

13.2. If the sample background control is significant, then subtract the sample background control from sample reading.

13.3. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.4. Plot the corrected absorbance values for each standard as a function of the final concentration of NADt or NADH.

13.5. 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).

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

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

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

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).

13.7. NAD/NADH Ratio is calculated as:

\[
\text{NAD/NADH ratio} = \frac{\text{NAD}_t - \text{NADH}}{\text{NADH}}
\]

13.8. 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:

\[
\text{NAD}_t \text{ or } \text{NADH} = \left(\frac{\text{sample reading}}{\text{(spiked sample reading)} - \text{(sample reading)}}\right) \times \text{amount of NADH spiked (pmol)}
\]

After working out the NADt and NADH concentration from the spiked samples, the NAD/NADH ratio can be calculated as shown in step 13.7 above.
14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1: Typical NAD Standard calibration curve. Quantification of NAD+ (diamond) and NADH (open square) following product protocol and using NADH standard provided in the kit. No NADP (triangle) was detected in this reaction.
**Figure 2:** Quantification of NADH and total NAD (NADt = NAD + NADH) in Jurkat and RAW cell lysates. 5 x 10^6 cells were lysed in 1 mL buffer, filtered through a 10 kD Spin Column (ab93349) and measured neat or at 1/5 dilution (average duplicates +/- SD).
15. QUICK ASSAY PROCEDURE

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

- Prepare NADH standard, Developer and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare NADH standard dilution [20 – 100 pmol/well].
- Prepare samples (including deproteinization step by filtration) in optimal dilutions to fit standard curve readings.
- To detect NADH: decompose samples by heating 200 µL extracted samples at 60°C for 30 minutes and quickly cooling samples on ice.
- Set up plate in duplicate for standard (50µL), total NADt samples (50 µL), NADH samples – if detecting – (50 µL) and background sample wells (50 µL).
- Prepare a master mix for NADH Reaction Mix and (if appropriate) a master mix for Background Reaction Mix:

<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>

- Add 100 µL Reaction / Background Reaction Mix to respective wells.
- Incubate plate at RT 5 minutes.
- Add 10 µL NADH Developer into each well.
- Incubate plate at RT 1 – 4 hours. Measure plate at OD450 nm.
- (Optional) Add 10 µL Stop Solution into each well.
## RESOURCES

### 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<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>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use provided protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/ thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<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></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/ Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<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></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<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>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. 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

18. 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 NADt 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 NADt 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 get you started.

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