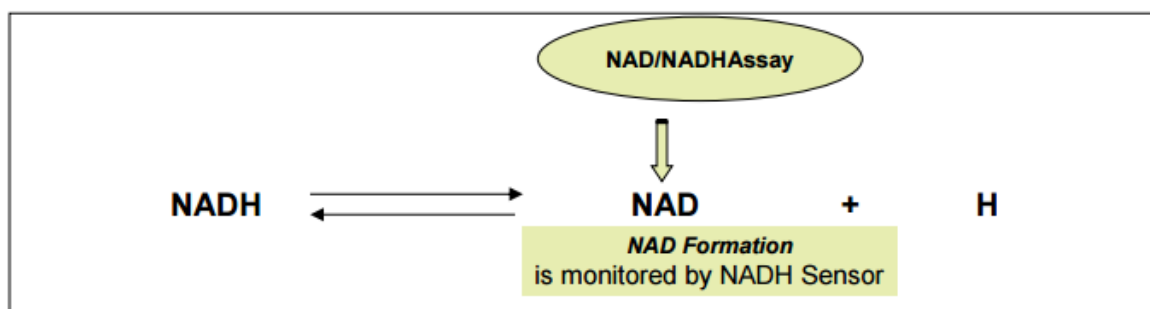


## Elite™ Fluorimetric NAD/NADH Ratio Assay Kit (Red Fluorescence)

CATALOG NUMBER: CA-N226, 250 assays

### Description

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) are two important cofactors found in cells. NADH is the reduced form of NAD<sup>+</sup>, and NAD<sup>+</sup> is the oxidized form of NADH. NAD forms NADP with the addition of a phosphate group to the 2' position of the adenyl nucleotide through an ester linkage. NADP is used in anabolic biological reactions, such as fatty acid and nucleic acid synthesis, which require NADPH as a reducing agent. In chloroplasts, NADP is an oxidizing agent important in the preliminary reactions of photosynthesis. The NADPH produced by photosynthesis is used as reducing power for the biosynthetic reactions in the Calvin cycle of photosynthesis. The traditional NAD/NADH and NADP/NADPH assays are done by monitoring the changes in NADH or NADPH absorption at 340 nm. This method suffers low sensitivity and high interference since the assay is done in the UV range that requires expensive quartz microplates.



Elite™ Fluorimetric NAD/NADH Ratio Assay Kit provides a convenient method for sensitive detection of NAD, NADH and their ratio. The enzymes in the system specifically recognize NAD/NADH in an enzyme cycling reaction that significantly increases detection sensitivity. In addition, this assay has very low background since it is run in the red visible range that considerably reduces the interference from biological samples. There is no need to purify NAD/NADH from sample mix. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = 530-570/590-600 nm (optimal Ex/Em = 540/590 nm) or an absorbance microplate reader at ~576 nm. This kit provides NAD and NADH extraction buffer, and cell lysis buffer for your convenience. It has been frequently used for determining NAD/NADH from cell lysates.

### Features

- **Broad application:** NAD/NADH detection in solution or cell extracts.
- **Sensitive:** Detect as low as 10 picomoles of NAD/NADH in solution.
- **Continuous:** Easily adapted to automation without a separation step.
- **Convenient:** Formulated to have minimal hands-on time.
- **Non-radioactive:** No special requirement for waste disposal.

### Kit Components

- |   |                                |
|---|--------------------------------|
| • <b>Component A:</b> NAD/NADH Recycling Enzyme Mixture | 2 bottles (lyophilized powder) |
| • <b>Component B:</b> NADH Sensor Buffer                | 1 bottle (20 ml)               |
| • <b>Component C:</b> NADH Standard                     | 1 vial (142 µg)                |
| • <b>Component D:</b> NADH Extraction Solution          | 1 bottle (10 ml)               |
| • <b>Component E:</b> NAD Extraction Solution           | 1 bottle (10 ml)               |
| • <b>Component F:</b> NAD/NADH Control Solution         | 1 bottle (10 ml)               |
| • <b>Component G:</b> NAD/NADH Lysis Buffer             | 1 bottle (10 ml)               |

### Storage

Keep in freezer (-20 °C) and avoid exposure to light.

### Materials Required (but not supplied)

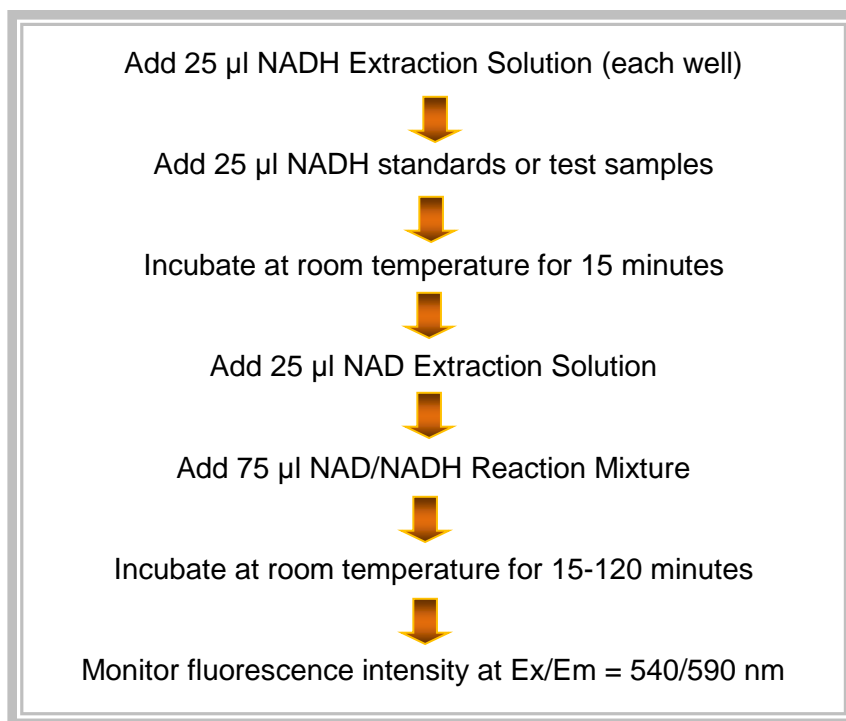
- 96-well black microplates with black bottom
- Fluorescence microplate reader

### Shelf Life

All reagents are stable for 6 months after receipt when stored properly at the recommended conditions.

## Assay Protocol (for 96-Well Plate)

### Summary of the Assay



#### 1. Prepare NADH stock solution:

Add 200 µl of PBS buffer into the vial of NADH standard (**Component C**) to have 1 mM (1 nmol/µl) NADH stock solution.

Note: The unused NADH stock solution should be divided into single use aliquots and stored at -20 °C.

#### 2. Prepare NAD/NADH reaction mixture:

Add 10 ml of NADH Sensor Buffer to the bottle of NAD/NADH Recycling Enzyme Mixture (**Component A**), and mix well.

**Note:** This NAD/NADH reaction mixture is enough for two 96-well plates. The unused NAD/NADH reaction mixture should be divided into single use aliquots and stored at -20 °C.



**3. Prepare serial dilutions of NADH standard (0 to 10 μM):**

3.1. Add 30 μl of 1 mM NADH stock solution (from Step 1) into 970 μl PBS buffer (pH 7.4) to generate 30 μM (30 pmols/μl) NADH standard solution.

**Note:** Diluted NADH standard solution is unstable, and should be used within 4 hours.

3.2. Take 200 μl of 30 μM NADH standard solution (from Step 3.1) to perform 1:3 serial dilutions to get 10, 3, 1, 0.3, 0.1, 0.03 and 0 μM of serial dilutions of NADH standard.

3.3. Add serially diluted NADH standards and/or NAD/NADH containing test samples into a solid black 96-well microplate as described in Tables 1 and 2.

**Note:** Prepare cells or tissue samples as desired or as described in **Appendix A**.

**Table 1.** Layout of NADH standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	TS (NADH)	TS (NADH)	TS (NAD)	TS (NAD)				
NS1	NS1	...	...	...	...	...	...				
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

**Note:** NS= NAD/NADH Standards; BL=Blank Control; TS=Test Samples; TS (NADH) = Test Samples treated with NADH Extraction Solution for 10 to 15 minutes, then neutralized by NAD Extraction Solution; TS (NAD) = Test Samples treated with NAD Extraction Solution for 10 to 15 minutes, then neutralized by NADH Extraction Solution.

**Table 2.** Reagent composition for each well

NADH Standard	Blank Control	Test Sample (NAD/NADH)	Test Sample (NADH Extract)	Test Sample (NAD Extract)
Serial Dilutions: 25 μl	PBS: 25 μl	Test Sample: 25 μl	Test Sample: 25 μl	Test Sample: 25 μl
<b>Component F:</b> 25 μl	<b>Component F:</b> 25 μl	<b>Component F:</b> 25 μl	<b>Component D:</b> 25 μl	<b>Component E:</b> 25 μl
Incubate at room temperature for 10-15 minutes				
<b>Component F:</b> 25 μl	<b>Component F:</b> 25 μl	<b>Component F:</b> 25 μl	<b>Component E:</b> 25 μl	<b>Component D:</b> 25 μl
Total: 75 μl	Total: 75 μl	Total: 75 μl	Total: 75 μl	Total: 75 μl

**Note:** Add the serially diluted NADH standards from 0.03 μM to 30 μM into wells from NS1 to NS7 in duplicates. High concentration of NADH (e.g. > 300 μM final concentration) may cause reduced fluorescence signal due to the over oxidation of NADH sensor (to a non-fluorescent product).

3.4. For NADH Extraction (NADH): Add 25 μl of NADH Extraction Solution (**Component D**) into the wells of NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, then add 25 μl of NAD Extraction Solution (**Component E**) to neutralize the NADH extracts as described in Tables 1 & 2.

For NAD Extraction (NAD): Add 25 μl of NAD Extraction Solution (**Component E**) into the wells of NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, then add 25 μl of NADH Extraction Solution (**Component D**) to neutralize the NAD extracts as described in Tables 1 & 2.

For Total NAD and NADH: Add 25 μl of NAD/NADH Control Solution (**Component F**) into the wells of NADH standards and NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, and then add 25 μl of Control Solution (**Component F**) as described in Tables 1 and 2.

**Note1:** Prepare cells or tissue samples as described in Appendix A. NAD/NADH Lysis Buffer (**Component G**) can be used for lysing the cells.

**Note2:** In healthy mammalian cells, there is way more NAD compare to NADH, so one could use total (NAD and NADH) minus the measured NAD to obtain the accurate amount of NADH.

**4. Run NAD/NADH assay in supernatants reaction:**

4.1. Add 75 µl of NADH reaction mixture (from Step 2) into each well of NADH standard, blank control, and test samples (from Step 3.4) to make the total NADH assay volume of 150 µl/well.

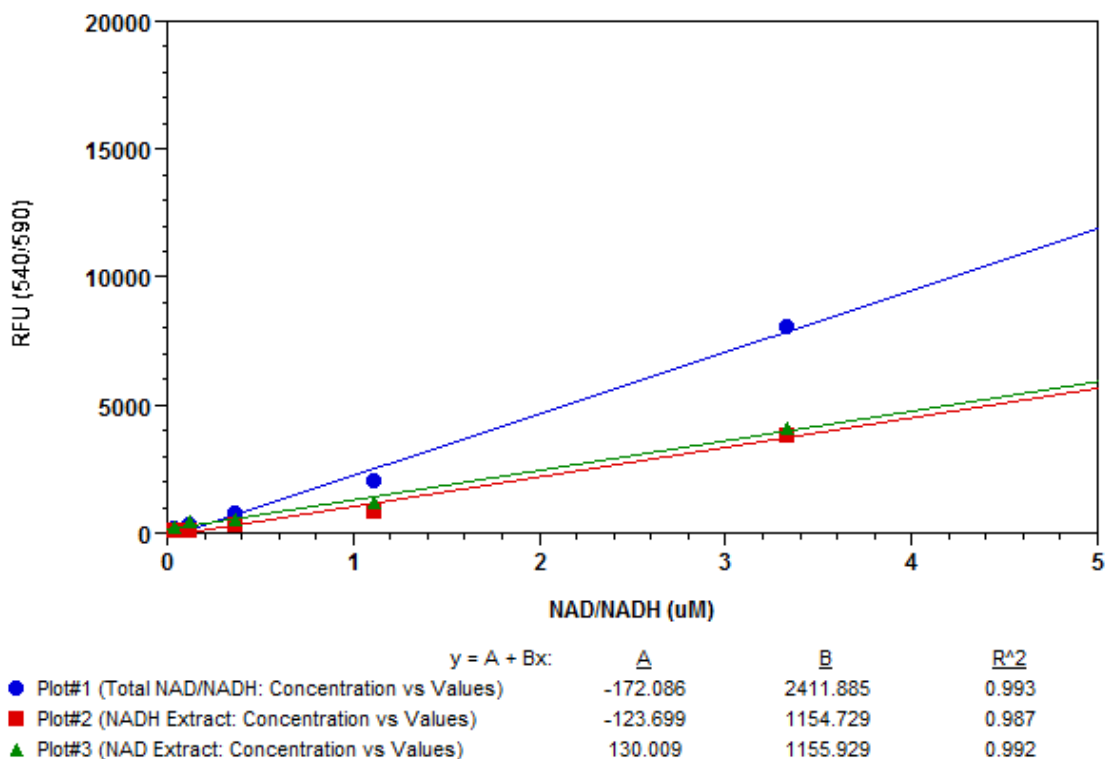
4.2. Incubate the reaction at room temperature for 15 minutes to 2 hours, protected from light.

4.3. Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540/590 nm (cutoff 570 nm).

**Note:** The contents of the plate can also be transferred to a white clear bottom plate and read by an absorbance microplate reader at the wavelength of 576 ± 5 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

**Data Analysis**

The fluorescence in blank wells (with the PBS buffer only) is used as a control, and is subtracted from the values for those wells with the NADH reactions. The typical data are shown in Figure 1 (Total NAD and NADH vs. NAD or NADH Extract).



**Figure 1.** Total NADH and NAD, and their extract dose response were measured with Elite™ NAD/NADH Ratio Assay Kit in a 96-well black plate using a Gemini microplate reader (Molecular Devices). 25 µl of equal amount of NAD and NADH was treated with or without NADH or NAD extraction solution for 15 minutes, and then neutralized with extraction solutions at room temperature. The signal was acquired at Ex/Em = 540/590 nm (cut off at 570 nm) 30 minutes after adding 75 µl of NADH reaction mixture. The blank signal was subtracted from the values for those wells with the NADH reactions (Note: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point).

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## Appendix A

### Sample cell lysis preparation for the assay kits

This protocol serves as a general guide for preparing samples for the assays. For the best results, users need to choose their own method for cell lysis preparation. ***A method of freeze-thaw (or other lysis methods using physical disruption in PBS) is strongly recommended for the preparation of cell lysate, especially when the background noise becomes an issue for this assay.***

#### 1. Lysis of plant cells

Homogenize the leaves with the Cell/Tissue Lysis Buffer (**Component G**) at 200 mg/ml, and centrifuge at 2500 rpm for 5-10 minutes. Use the supernatant for the assay.

#### 2. Lysis of bacterial cells

Collect bacterial cells by centrifugation (i.e. 10,000 x g, 0°C, 15 min). Add Cell/Tissue Lysis Buffer (**Component G**) to the pellet (1 mL per 100 to 10,000,000 cells), and leave at room temperature for 15 minutes. Centrifuge at 2500 rpm for 5 minutes, and use the supernatant for the assay.

#### 3. Lysis of mammalian cells

Remove the medium from the culture plate (wells). Use about 100 µL Cell/Tissue Lysis Buffer (**Component G**) per 1-5 million cells (or add lysis buffer 100 µL lysis buffer per well in a 96-well cell culture plate), and leave at room temperature for 15 minutes. For the assay, use the lysate directly or centrifuge at 1500 rpm for 5min then use the supernatant.

#### 4. Lysis of tissues

Weigh ~ 20 mg tissue, and then wash with cold PBS buffer. Homogenize the tissue with 400 µl of Cell/Tissue Lysis Buffer (**Component G**) in a micro-centrifuge tube, and centrifuge at 2500 rpm for 5-10 minutes. Use the supernatant for the assay.

**Note:** use Cat# CA-N015g for ordering the Cell/Tissue Lysis Buffer (Component G) separately.

