MA-0114

NAD/NADH Assay

(100 wells, Absorbance-based, OD 450 nm, Store at -20°C)

Background Information:

The NAD/NADH redox couple is essential for maintaining cellular redox homeostasis. NADH is the primary vehicle whereby reducing equivalents are converted to ATP in the cell. With thousands of oxidoreductases in the cell, each with its own set of optimal conditions, the assay of nicotinamide nucleotides becomes a useful very broad index of the overall health of the cell. AkrivisBio's NAD/NADH Assay provides a simple, sensitive means of detecting NAD and NADH. The ability to measure these concentrations allows for the determination of the NAD/ NADH ratio which gives important insight into the overall redox state of the cell. The assay is specific for the NAD/NADH couple (not NADP/NADPH). AkrivisBio's use of a cycling enzyme system provides you with a rate assay where the change in OD over time gives an assay with very high sensitivity.

Assay Principle:

1 – In the absence of recycling enzyme, NADH converts WST to its colored form without any contribution from NAD.
2 – In the presence of the recycling enzyme, NAD in the sample is converted to NADH
3 – NADH is utilized to reduce WST from a colorless tetrazolium to a highly colored formazan converting the NADH back to NAD.
4– The recycled NAD is again reduced to NADH and the cycle repeated giving rise to a rate of color formation proportional to [NAD + NADH]
5 – [NAD] is determined from the difference between NADH alone and [NAD + NADH]

Assay Components:

Sample Extraction Buffer	50 ml	NM	MA-0114A
Cycling Buffer	15 ml	NM	MA-0114B
Cycling Enzyme Mix	lyoph	Green	MA-0114C
WST Reagent	lyoph	Purple	MA-0114D
Stop Solution	1.2 ml	Red	MA-0114E
NADH Standard	50 nmol	Yellow	MA-0114F

Storage and Handling: Store kit at -20°C. Centrifuge all small vials briefly prior to opening. Warm all components to room temperature before using.
Sample Extraction Buffer: Ready to use as supplied. Store at 4°C.
Cycling Buffer: Ready to use as supplied. Store at 4°C.
Cycling Enzyme Mix: Add 220 µl Cycling Buffer and dissolve. Aliquot immediately into convenient portions and store at -70°C. Keep the Cycling Enzyme on ice while in use.
WST Reagent: Add 1.2 ml of DI H_2O and allow the vial contents to dissolve for a few minutes without agitation. Invert a few times and the solution should be ready. Store at -20°C
NADH Standard: Add 250 µl DMSO giving a 200 µM solution. Store at -20°C.

Assay Protocol:

1. Standard Curve: Add 10 µl of the 250 µM NADH standard to 990 µl Sample Extraction Buffer giving a 2.0 µM working solution. Add 0 – 10 – 20 – 30 – 40 – 50 µl of the diluted NADH Standard to a series of wells in a 96-well plate in duplicate, giving 0, 20, 40, 60, 80, 100 pmol per well. NADH Standard. Adjust all well volumes to 50 µl with Sample Extraction Buffer.

2. Sample Preparation:

Cells: Wash cells with cold PBS. Aliquot 1-2 X 10^5 cells to a microcentrifuge tube for each test to be run. Pellet the cells at 200 X g, 5 min then extract with 400 µl of Sample Extraction Buffer using 2 freeze/thaw cycles (freeze on dry-ice / thaw at room temperature). Vortex for 10 seconds then centrifuge at 16,000 X g for 5 minutes. Filter supernatant through a 10 kDa centrifugal filter and store filtrates in labeled tubes on ice.

Tissue: Wash 10 mg with cold PBS then homogenize in 400 µl of Sample Extraction Buffer. Centrifuge at 16,000 X g for 5 minutes. Filter clear supernatant through a 10 kDa centrifugal filter and store in labeled tubes on ice.

3. Select NAD (Total) or NADH (only) Assay:

NADH only: Decompose NAD selectively by transferring 200 µl of each extracted sample to microcentrifuge vials. Heat the samples to 60°C for 30 minutes. This treatment degrades only NAD leaving NADH intact. Chill the samples, then centrifuge at 16,000 X g for 2-3 minutes. Transfer 50 µl of each sample to wells in a 96-well plate.
Total NAD (NAD + NADH): Transfer 50 µl of extracted samples to wells in a 96-well plate without heat treatment.

Notes:
- Sample readings must be within the range of the standard curve. If the rates observed are too fast, dilute the sample and rerun.
- Endogenous compounds may interfere with the reaction. In those cases, see the addendum at the end of this protocol.
- Diluted NADH solution is unstable, must be used within 4 hours.

4. Prepare Reaction Mix: Prepare enough Reaction Mix for the total number of samples and standards to be run, containing:

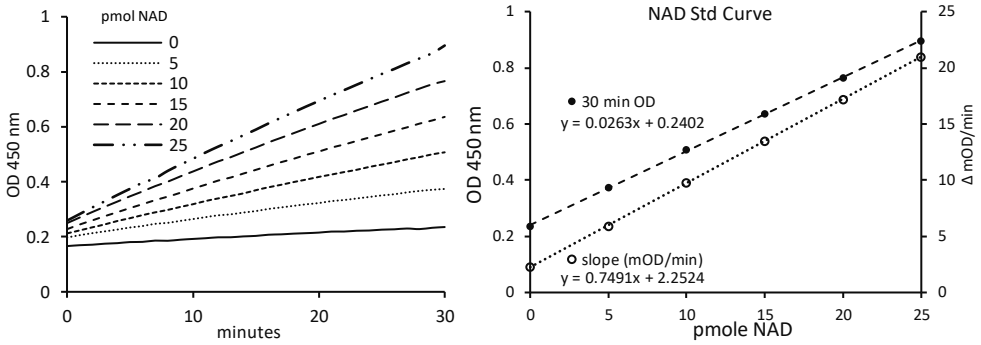
	Reaction Mix
NAD Cycling Buffer	98 µl
NAD Cycling Enzyme Mix	2 µl

Add 100 µl of Reaction Mix to all Standard and sample wells. Leave the plate undisturbed for 5 minutes at RT to allow all NAD to be converted to NADH.

5. Measurement: Add 10 µl WST Reagent to each well, mix and start monitoring the plate immediately at 450 nm. The reaction will generate color at a rate proportional to the amount of NAD present. Allow the reaction to proceed at RT until a well-defined linear rate of color development is achieved (typically 30 min – 2 hours but longer may be necessary). Any or all reactions can be stopped at any time by the addition of 10 µl of Stop Solution to each well.

6. Typical results:

pmoles	OD at 30 min Raw	OD at 30 min Corrected	Slope (OD/min)
0	0.2355	0	0.0023
5	0.3741	0.1386	0.0059
10	0.5072	0.2717	0.0098
15	0.6360	0.4005	0.0135
20	0.7660	0.5305	0.0172
25	0.8954	0.6599	0.0210



7. Calculation: Determine Endpoint OD for all Standards. Subtract 0 Standard OD value from all Standards to give corrected OD. Plot OD vs. pmol NAD. Determine the slope of the Standard Curve. The slope defines the change in OD per pmol NAD. Subtract the OD of each test sample at t = 0 from the endpoint OD to correct for any OD not due to NAD. Apply the slope of the standard curve to the corrected endpoint OD of the test samples to determine the pmoles of NAD in the test sample wells.
Alternatively, determine OD/minute slope of all standards and test samples. Subtract 0 Standard slope from all other standard slopes and test sample slopes to give corrected slopes (OD/min/pmol NAD). Plot the NADH Standard Curve (Corrected Slope vs. pmol NAD)., Apply the standard curve (Slope of slopes) to the corrected slopes of the test samples to determine pmoles of NAD in the well.

To determine the amount of NAD in the original sample, measure the total volume of the filtrates obtained during sample preparation. Then:

NAD in original sample = [pmoles in well X (total filtrate/volume transferred to wells)] / mg tissue (or # of cells).

Notes: Assays such as AkrivisBio's NAD/NADH Assay can occasionally be affected by so-called matrix effects, where the background is significantly increased, and the reaction rate inhibited by endogenous materials. To circumvent this, an alternative method can be used to accurately determine NAD content by using triplet test samples. One, the Background Control, is used to determine any background drift due to non-NAD/NADH contributors, by omitting the cycling enzyme mix. The second (Authentic Test Sample) is run normally as described above. To the third (Spiked Sample) is added a fixed, known amount of NADH. The reaction starts in the three samples normally. After sufficient time has elapsed to obtain a measurable linear slope from the samples, the slope of the Authentic test sample (with cycling enzyme mix, without added NADH) is subtracted from the slope of the (Spiked) test sample with added NADH. The difference between the two samples defines the response to the fixed amount of NADH added (ΔOD/pmol/time). The slope of the background control is subtracted from the Authentic sample to give a corrected slope due to NADH present. This slope is divided by the Δ slope just determined to give the actual NAD content in the (Authentic) test sample.

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