EnzyFluo™ NAD+/NADH Assay Kit (EFND-100)

PROCEDURE FOR IN-PLATE NAD/NADH DETERMINATION IN 96-WELL PLATE

Note: One EFND-100 kit is sufficient for 48 wells using this protocol. To measure a full 96-well plate, ***two*** *EFND-100 kits are required.*

1. *Cell preparation*. Plate (10 to 50 × 10^3) and culture adherent cells in a clear-bottom black 96-well cell culture plate. Cells should be incubated for at least 6 hrs to ensure that cells are not labile to washes. Quantification of NAD and NADH requires cells from two separate wells. Replicates are recommended to account for cell growth variation. Eight wells should be left empty for the standard curves.
2. *Standard Curves*. Prepare 1 mL of 10 µM NAD Premix by mixing 10 µL 1 mM Standard and 990 µL distilled water. Dilute standard as follows.

   NAD-quantification Standard wells:

No	Premix + H_2O	[NAD] (pmol/well)
1	200 µL + 0 µL	100
2	120 µL + 80 µL	60
3	60 µL + 140 µL	30
4	0 µL + 200 µL	0

   NADH-quantification Standard wells:

No	Premix + H_2O	[Equivalent NADH] (pmol/well)
1	20 µL + 180 µL	10
2	12 µL + 188 µL	6
3	6 µL + 194 µL	3
4	0 µL + 200 µL	0

3. *Sample preparation*. Remove media and gently wash cells with room temperature PBS. Remove residual PBS.

   Transfer 10 µL of each standard to the designated standard wells.

   Add 50 µL of NAD extraction buffer to sample wells designated for NAD quantification plus both the NAD and NADH quantification standard wells. Add 50 µL of NADH extraction buffer to sample wells designated for NADH quantification. Heat plate at **37**°C for 5 minutes. Add 50 µL of the opposite extraction buffer to neutralize the solution. Incubate plate at room temperature away from light for 10 minutes to equilibrate the temperature of the wells.
4. *Reagent Preparation*. Prepare sufficient Working Reagent by mixing for each reaction well, 14 µL Assay Buffer, 1 µL Enzyme A, 1 µL Enzyme B, 30 µL Lactate and 8 µL Probe. Fresh reconstitution is recommended.
5. *Reaction*. Add 50 µL Working Reagent per well quickly. Tap plate immediately to mix.
6. Read fluorescence at $\lambda_{ex/em}$ = 530/585 nm for time "zero" (F_0). For NAD quantification, read F_{10} after a 10-min incubation at room temperature. For NADH quantification, read F_{60} after a 60-min incubation at room temperature. Protect plate from light during this incubation.

CALCULATION

For NAD quantification, calculate the ΔF's for standard and sample by subtracting F_0 from F_{10}. Plot the ΔF's for NAD-quantification standards to determine the slope of the NAD standard curve. For NADH quantification, calculate the ΔF's for standard and sample by subtracting F_0 from F_{60}. Plot the ΔF's for NADH-quantification standards to determine the slope of the NADH standard curve.

The NAD and NADH concentrations of the samples are computed as follows:

$$[\text{NAD(H)}] = \frac{\Delta F_{SAMPLE}}{\text{Slope (pmol/well)}^{-1}} \quad \textit{(pmol/well)}$$

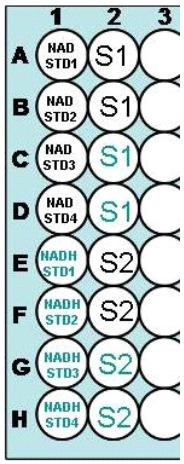
where ΔF_{SAMPLE} is the change in fluorescence intensity values of the Sample (10 min – 0 min for NAD or 60 min – 0 min for NADH). Slope is the slope of the appropriate standard curve.

Note: If the sample ΔF values are higher than the ΔF value for the 100 pmol/well for NAD or 10 pmol/well NADH, reduce the cell count per well and repeat this assay. The detection limit of this assay is 0.05 pmol/well for either NAD or NADH.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipetting (multi-channel) devices, cell culture incubator, clear-bottom black 96-well cell culture plate and fluorescent plate reader capable of reading at $\lambda_{ex/em}$ = 530/585 nm.

Example of Standards and Samples Layout in 96-Well Plate



Add NAD Extraction Buffer to Wells for
NAD/STD1, NAD/STD2, NAD/STD3, NAD/STD4,
NADH/STD1, NADH/STD2, NADH/STD3, NADH/STD4,
S1, S1, S2, S2,

Add NADH Extraction Buffer to Wells for
S1, S1, S2, S2