Fluoro NAD/NADH

Fluorescent NAD/NADH Detection Kit

Contact Information

Address	Cell Technology Inc 48820 Kato Road Suite 400B Fremont, CA 94538 USA
Telephone	650-960-2170
Toll Free	888 7ASSAYS (727-7297)
Fax	650-960-0367
General Information	info@celltechnology.com
Sales	sales@celltechnology.com
Technical Questions	techsupport@celltechnology.com
Website	www.celltechnology.com

Notes:

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Important – This protocol is for use with Lot#'s beginning with KBD. If you have a kit with the lot number beginning with KBB, please contact us at 650-960-2170 or at techsupport@celltechnology.com

Introduction

The role of pyridine nucleotides (NAD/NADH) metabolism in health is of continual and increased interest. A growing amount of evidence supports the fact that NAD metabolism regulates important biological effect including life span. NAD, through poly-ADP-ribosyl polymerase (PARP), mono-ADP-ribosyltransferase (ARTs) and recently characterized sirtuin enzymes, exerts potential biological effects. These enzymes modify proteins to regulate their function via ADP-ribosylation or deacetylation. These enzymes are involved in several pathways including apoptosis, DNA repair, senescence and endocrine signaling, which suggests that either the enzymes or NAD could be an important therapeutical target [1].

Applications:

- Detection of NAD/NADH activity in cells or tissue extracts.
- Detection of NAD/NADH levels in apoptosis, metabolism, proliferation, DNA repair. senescence, endocrine signaling and life span.
- NAD/NADH detection in Bacterial, fungal and plant cells.

I. Assay Principle:

The Fluoro NAD/NADH detection kit utilizes a non-fluorescent detection reagent, which is reduced in the presence NADH to produce its fluorescent analog and NAD. NAD is further converted to NADH via an enzyme-coupled reaction. The enzyme reaction specifically reacts with NAD/NADH and not with NADP/NADPH.

Reaction:

1. NADH + non-fluorescent detection reagent+ electron coupler $\longrightarrow$ fluorescent analog + NAD

2. NAD + enzyme coupled reaction $\longrightarrow$ NADH (then proceeds to reaction 1).

excitation: 530-570nm and emission at 590-600nm

II. Storage:

1. **Part # 6021: Enzyme Mix, Part# 7013: NADH Standard and Part # 4018: NADH Detection reagent.** Upon arrival store below –20^{0}C and away from light.
2. The rest of the components should be stored at 2-8^{0}C until first use.

III. Warnings and Precautions:

1. **For Research use only. Not for use in diagnostic procedures**.
2. Practice safe laboratory procedures by wearing protective clothing and eyewear.
3. The fluorescent product of the detection reagent is not stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10 □M.
4. **Once the vial of Part # 4018 (NADH Detection reagent) is opened, it is important that low lighting conditions be used while aliquoting as well as performing the experiment. Direct and prolonged light exposure may increase the background, resulting in compromised linearity.**

IV. Catalog # FLNADH 100-2 Kit contents (for 100 assays):

1. **Part # 6021. Enzyme Mix:** 1 vial dried. Upon arrival store at –20^{0}C.
2. **Part # 4018. NADH Detection reagent:** 1 vial liquid. Upon arrival store at –20^{0}C.
3. **Part# 7013: NADH Standard:** 1 vial dried. Upon arrival store at –20^{0}C.
4. **Part # 3044 NAD/NADH reaction Buffer:** 30mL. Upon arrival store at 2-8^{0}C.
5. **Part# 3045: NAD/NADH Lysis Buffer**: 25 mL. Upon arrival store at 2-8^{0}C.
6. **Part# 3046: NAD Extraction Buffer:** 22mL. Upon arrival store at 2-8^{0}C.
7. **Part# 3047: NADH Extraction Buffer:** 22mL. Upon arrival store at 2-8^{0}C.
8. **Part# 3051: NADH Standard Diluent:** 15mL. Upon arrival store at 2-8^{0}C.

Materials required but not supplied:

1. Black 96-well plates (clear bottom optional for bottom reading instruments).
2. Fluorescence plate reader.
3. Deionized water.
4. 0.01 M TRIZMA base (Sigma T6066, powder) pH to 8-8.5 OR dilute 1M Tris-Cl buffer at pH 7.4 (Sigma, T2663) to 0.01M and pH to 8-8.5.
   Note: It is important to resuspend the NADH standard in a solution buffered at pH 8-8.5.

V. Preparation of reagent working solutions and Storage:

1. **Part # 6021. Enzyme Mix**. The enzyme mix is dried down. Add 110 µL of distilled water. Gently vortex and allow the contents of the vial to dissolve for 15 minutes at room temperature. Once reconstituted the enzyme mix should be aliquoted into single use vials and store at –80^{0}C. The reconstituted enzyme mix is stable for 2 months**.**
   Note: Store in a manual defrost freezer.

2. **Part # 4018. NADH Detection reagent:** The reagent is ready to use. Once thawed it should be aliquoted into single use vials and store at –20^{0}C or below. **Light sensitive.** Thaw the NADH Detection reagent and spin down to remove any material that may be trapped in the cap. Some material may get trapped on the lip inside the tube.
   Note: Repeated freeze thaw cycles may increase background. (See: **Part III: Warnings and Precautions**). **Store in a manual defrost freezer.**

3. **Part # 3044 NAD/NADH reaction Buffer**. Ready to use. Store at 2-8^{0}C.
   Note: Storage at 2-8^{0}C may produce crystals. Gently warm to 37^{0}C with agitation to dissolve them

4. **Part# 3045: NAD/NADH Lysis Buffer**. Ready to use. Store at 2-8^{0}C.
   Note: Storage at 2-8^{0}C may produce crystals. Gently warm to 37^{0}C to dissolve them.

5. **Part# 7013: NADH Standard:** Add 100 µL of 0.01 M TRIZMA buffer @ pH 8- 8.5. Vortex the vial and let it dissolve for 15 minutes at room temperature. Aliquot into single use vials and store at –20^{0}C or below. The standard is more stable at –40^{0}C to –70^{0}C (stable for 4-6 months). At –20^{0}C it is stable for 2 months.

6. **Part# 3046/ Part# 3047: NAD Extraction Buffer/NADH Extraction Buffer.**

Ready to use. Store at 2-8^{0}C.

7. **Part# 3051: NADH Standard Diluent:** Ready to use. Store at 2-8^{0}C.

VI. Tissue Preparation

See technical note #1 &2

Determination of NAD and NADH requires two separate samples.

Tissue preparation: Prior to tissue extraction exsanguinate (optional) the animal to remove red blood cells from tissue. Weigh 20-40 mg of tissue for each extraction (NAD or NADH) and rinse in ice cold PBS. Transfer the tissue into a 1.5 mL eppendorf tube and add either 200 µL of NAD or NADH extraction buffer to the respective tube. Next add 200 µL of NAD/NADH lysis buffer to all the tubes. Then using standard techniques homogenize the tissue samples. Next heat the homogenates at 60 ^{0}C for 15 minutes. Cool the heated homogenates and add 100 µL of the Reaction Buffer (Part # 3044) and then 200 µL of the opposite extraction buffer to neutralize the homogenates. Vortex the tubes and spin homogenates (5000-8000 g) for 5 minutes to clarify the supernatants. Supernatants are ready for the assay (keep on ice until they are ready for the assay). If not using the supernatants immediately, freeze at –70^{0}C, however this may result in some loss of NAD/NADH.
Note: Optional: By calculating protein concentration, for example using the BCA protein assay, data can be normalized and expressed as concentration of NAD or NADH per mg/mL of protein.

VII. Mammalian Cell Preparation.

See technical note #1 &2

Determination of NAD and NADH requires two separate samples.

Wash 1 x 10^6 – 5 x 10^6 cells twice with 2 mL of PBS. After the final wash aspirate the supernatant (remove as much of the PBS as possible), gently vortex the cell pellet and add 200 µL of either NAD or NADH extraction buffer to their respective tube. Next add 200 µL of the NAD/NADH lysis buffer to all the tubes and then using standard techniques homogenize the samples. Gently vortex the samples and heat them at 60 ^{0}C for 15 minutes. After heating, immediately cool the samples on ice and add 100 µL of the Reaction Buffer (part# 3044) and 200 µL of the opposite extraction buffer to neutralize the samples. Vortex the tubes and spin lysates (5000-8000 g) for 5 minutes to clarify the supernatants. The samples are ready for the assay (keep the samples on ice until they are ready for the assay). If not using the supernatants immediately freeze at –70^{0}C, however this may result in some loss of NAD/NADH.
Note: Each investigator should optimize the number of cells used per test.

VIII. Assay Protocol:

1. **Make the Reaction cocktail**
   To each 1 mL of NAD/NADH reaction Buffer (Part # 3044) add 10 µL of the reconstituted enzyme mix (part# 6021) and 10 µL of NADH Detection reagent (part# 4018). Vortex gently. This is enough for 10 tests. Make enough Reaction cocktail for one day's worth of experiments.

   Note: Make the reaction cocktail right before use. Light Sensitive. Avoid direct laboratory light as this will increase background.

2. **NADH Standard Curve.**
   We supply only a NADH standard as the detection reagent only reacts with NADH. NAD is converted to NADH. Label suitable tubes 1-8. To tube #1 add 997 µL of NADH Standard Diluent (Part# 3051) and 3 µL of the reconstituted NADH. This will make a 3000nM solution of NADH. Next serially dilute (1:2) the 3000nM NADH standard in NADH Standard Diluent (Part# 3051) to construct a standard curve. This can be accomplished by adding 250 µL of NADH Standard Diluent into tubes #2-8. From tube #1 remove 250 µL of the 3000nM NADH standard and add it to tube #2. Gently vortex tube #2 and pipette out again 250 µL from tube#2 and add it to tube#3. Continue this process to tube #7. Tube # 8 is the blank control. The final NADH concentration in the well will be three times less than in the tube.

Tube #	NADH Concentration in tubes.	Final NADH Concentration in wells.
1	3000 nM	1000 nM
2	1500 nM	500 nM
3	750 nM	250 nM
4	375 nM	125 nM
5	187.5 nM	62.5 nM
6	93.75 nM	31.25 nM
7	48.87 nM	15.625 nM
8	0	0

Add 50 µL of standard or sample in triplicate to individual wells of a black 96 well plate. It is recommended to titrate out the sample in the NADH Standard Diluent (Part# 3051) several fold so it's values will fall within the range of the standard curve. Next, pipette in 100 µL of the reaction cocktail from step 1 above to all the wells. Incubate at room temperature in the **dark** for 1-1.5 hours. Take a reading with excitation at 530-570 nm and emission at 590-600nm.

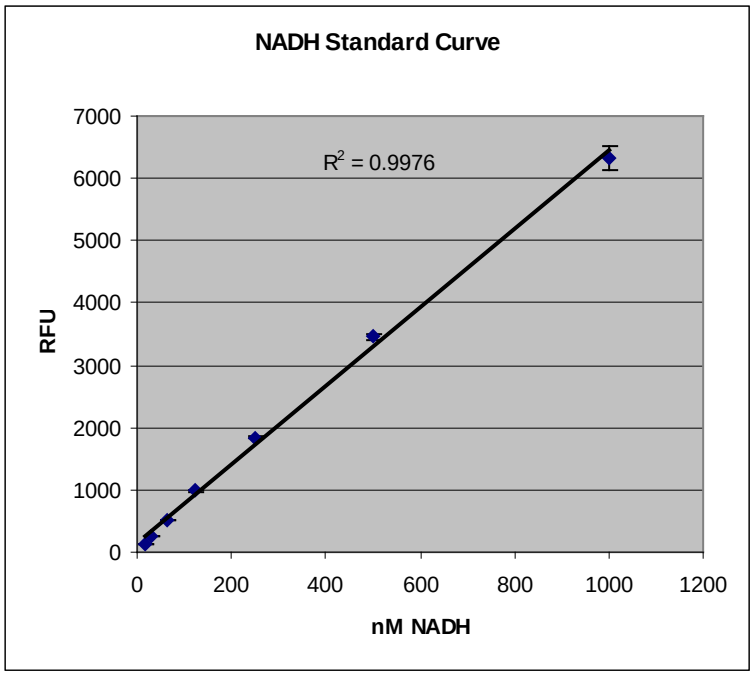

Figure 1. NADH standard curve titrated in NADH Standard Diluent. Incubation time = 1 hour.

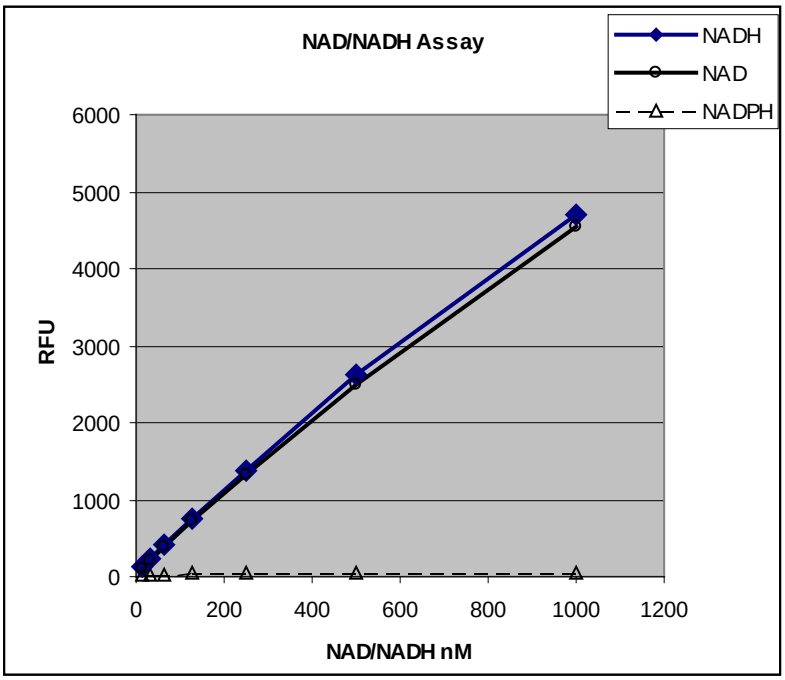

Figure 2. NAD/NADH Assay showing no cross-reactivity with NADPH.

Incubation time = 1 hour.

Condition	NAD recovery in NAD extraction Buffer	NADH recovery in NAD extraction Buffer	NAD recovery in NADH extraction Buffer.	NADH recovery in NADH extraction Buffer.
% Recovery	90-95%	8-10%	Not detectable <99%	85-90%

Table 1: We have conducted spike and recovery experiments to estimate the decomposition and recovery of NAD and NADH. Briefly 3 VIIM solutions of NAD a
Mammalian Cell Preparation and % recovery calculated from a standard curve (n=4).

	Jurkats	Jurkats	K562	K562
	nM NAD	nM NADH	nM NAD	nM NADH
Calculated	196.65	4.58	436.80	23.10

Table 2. NAD and NADH levels were assayed using the Fluoro NAD/NADH kit. Jurkat cells 6 $X10^5$ or K562 cells 7 $X10^5$ per tube (n=3).

IX. Technical Notes:

1. **SH groups like DTT or Reduced Glutathione will interfere with the assay. Keep below 10 □M**
2. **It is important to add the NAD or NADH extraction buffer first before adding the lysis buffer to the samples. NAD and NADH recovery from the samples is severely compromised if the lysis buffer is added first [(2)].**

References:

1. Anthony A. Sauve NAD^+ and Vitamin B3: From metabolism to therapies J. Pharmacol. Exp. Ther. 2007 : jpet.107.120758v1.
2. Lowry, Oliver H., Passonneau, Janet V. and Rock, Martha K. The Stability of Pyridine Nucleotides. The Journal of Biological Chemistry, 236, #10, 1961.