Fluoro NADP/NADPH

Fluorescent NADP/NADPH Detection Kit

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Notes:

I. Introduction:

Nicotinamide adenine dinucleotide phosphate (NADP+) is used in anabolic reactions, such as lipid and nucleic acid synthesis, which require NADPH as a reducing agent. NADPH is the reduced form of NADP+, and NADP+ is the oxidized form of NADPH. In cells, NADPH plays the role of a carrier of reducing power and is primarily involved in maintaining optimal redox metabolism. A simplified assay for the measurement of NAD and NADP is critical to understanding the roles of these pyridine nucleotides in normal and abnormal cells.

NADPH is produced in the oxidative phase of the pentose phosphate pathway in cells, a multifunctional pathway whose primary purpose is to generate reducing power in the form of NADPH. NADPH is a cofactor for enzymes that synthesize energy-rich molecules and provide the reducing equivalents for the oxidation-reduction involved in protecting the cell from the toxicity of reactive oxygen species (ROS) and NADPH oxidase-dependent ROS generation. Both NAD and NADP have been shown to influence hemoglobin affinity for oxygen in erythrocytes. In plant cells, NADPH is used as the reducing power for the biosynthetic reactions in the Calvin cycle of photosynthesis [1-2].

Cell Technology's Fluoro NADP/NADPH provides a reliable, sensitive fluorimetric assay for the quantification of NADP, NADPH and their ratio in biological samples.

Applications:

- Detection of NADP/NADPH activity in cells or tissue extracts.
- Study of NADP/NADPH levels antioxidation and oxidative stress.
- Detection NADP/NADPH in cell death, energy metabolism, mitochondria function.
- NADP/NADPH detection in Bacterial, fungal and plant cells.

II. Assay Principle:

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

Reaction:

1. NADPH + non-fluorescent detection reagent+ electron coupler ⟶ fluorescent analog + NADP

2. NADP + enzyme coupled reaction ⟶ NADPH (then proceeds to reaction 1).

Excitation: 530-570nm and Emission at 590-600nm

III. Storage:

1. **Upon arrival store the following components at -20^{0}C.**
   Part # 4019 NADPH Detection reagent.
   Part # 3048 3X Substrate mix.
   Part# 7014: NADPH Standard.
2. The rest of the components should be stored at 4-8^{0}C.

IV. Schematic Representation of the NADP/NADPH Fluorimetric Assay:

NADP Extraction/ NADPH decomposition

Cell pellet/Tissue sample
+
200µL NADP Extraction buffer (Green label)
+
200µL Lysis Buffer (Part#3050)

↓ Heat 60°C for 20-30 minutes

↓ Cool on Ice

100µL of Reaction Buffer (Part# 3049)
+
200µL of NADPH Extraction Buffer (Magenta label)

↓ Spin

Use supernatant in assay

NADPH Extraction/ NADP Decomposition

Cell pellet/Tissue sample
+
200µL NADPH Extraction buffer (Magenta label)
+
200µL Lysis Buffer (Part # 3050)

↓ Heat 60°C for 20-30 minutes

↓ Cool on Ice

100µL of Reaction Buffer (Part #3049)
+
200µL of NADP Extraction Buffer (Green label)

↓ Spin

Use supernatant in assay

ASSAY:

50µL of sample supernatant/NADPH standard (Part # 7014)
+
50µL of Reaction cocktail

↓ Incubate 37°C for 10 minutes

50µL of 1X Detection Reagent (Part #4019; diluted)

↓ Incubate 30-60 minutes; RT; DARK

Read on plate reader: Excitation: 530-570nm Emission: 590-600nm

V. Warnings and Precautions:

1. **For Research use only. Not for use in diagnostic procedures**.
2. Practice safe laboratory procedures by wearing gloves, protective clothing and eyewear.
3. The reaction is not stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10µM.
4. **Once the vial of Part # 4019 NADPH 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.**

VI. Catalog # NADPH 100-2 Kit contents and Storage (for 100 assays):

1. **Part # 6022. Enzyme Mix 170µL:** 1 vial. Upon arrival store at 4-8^{0}C. Ready to use; use 30µL per ml of the 3X substrate mix (Part # 3048) to prepare Reaction cocktail.

2. **Part# 3050: NADP/NADPH Lysis Solution**: 22 mL. Upon arrival store at 4-8^{0}C. Ready to use.

3. **Part# 3057: NADP Extraction Buffer : (Green label):** 1 bottle 22 mL. Upon arrival store at 4-8^{0}C. **Corrosive**. Wear gloves and safety goggles while handling.

4. **Part# 3058: NADPH Extraction Buffer : (Magenta label):** 1 bottle 22 mL. Upon arrival store at 4-8^{0}C. **Corrosive**. Wear gloves and protective eye wear while handling.

5. **Part# 3049: Reaction Buffer:** 1 bottle 14 mL. Upon arrival store at 4-8^{0}C

6. **Part # 3052: Standard curve diluent:** 14.5 ml. Upon arrival store at 4-8^{0}C

7. **Part # 4019. NADPH Detection reagent 110 µL:** 1 vial frozen. **Light sensitive.** 50X strength to be diluted in Reaction buffer just prior to use. Upon arrival aliquot into single use vials and store at -20^{0}C. 20 µL is sufficient for 20 tests. Thaw the 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. Once thawed it should not be refrozen.
   Note: Repeated freeze thaw cycles may increase background. (See: **Part V: Warnings and Precautions**). **Store in a manual defrost freezer.**

8. **Part # 3048. 3X Substrate Mix:** 1 bottle 6mL. Upon arrival store at -20^{0}C. Aliquot and freeze down as single use portions. 1mL is sufficient for 20 tests.

9. **Part# 7014: NADPH Standard:** 3 vials dried. Upon arrival store at -20^{0}C.
   Prepare 1ml of a 1:2 dilution of the NADPH extraction buffer (Part# 3058: **Magenta label**) by adding 500µL of the NADPH extraction buffer to 500µL of water. At the time of the experiment, add 30µL of this solution to the vial contents to give a 150µM solution of NADPH. Vortex and let dissolve for 1-2 minutes at room temperature.

   Note: The NADPH standard should be reconstituted fresh at the time of the experiment. We do not recommend storing the solution.

VII. 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. 0.9% saline

VIII. Tissue Preparation

See Section XI: Technical notes a, b & c.
Determination of NADP and NADPH 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 (NADP or NADPH) and rinse in ice cold PBS. Transfer the tissue into a 1.5mL eppendorf tube and add either 200µL of NADP or NADPH extraction buffer to the respective tube and next add 200µL of NADP/NADPH lysis solution (Part #3050) to all the tubes.
Then using standard techniques homogenize the tissue samples on ice.
Next heat the homogenates at 60^0C for 20 minutes.
Cool the heated homogenates on ice and add 100µL of the Reaction Buffer (Part # 3049) and then 200µL of the opposite extraction buffer to neutralize the homogenates.

Vortex the tubes and spin homogenates 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 –70^0C.

IX. Mammalian Cell Preparation.

See Section XI: Technical notes a, b & c.

Determination of NADP and NADPH requires two separate samples.

1. Spin down $2 \times 10^5 - 1 \times 10^6$ cells. Decant supernatant (media) and wash cells with 5 mL of PBS. Spin down to pellet cells.
   Immediately after the wash, decant the supernatant as completely as possible; dislodge the cell pellet by gently vortexing.
2. Add 200 µL of either NADP or NADPH extraction buffer to the respective tubes.
3. Next add 200µL of the NADP/NADPH lysis solution (Part #3050) and using standard techniques, homogenize the samples.
4. Gently vortex the samples and heat them at 60^0C for 20-30 minutes.
   Use a double layer of Parafilm to seal the tubes to reduce loss of liquid by evaporation.
5. After heating, immediately cool the samples on ice. While vortexing the tube, add 100µL of the Reaction Buffer (Part# 3049) and 200µL of the opposite extraction buffer to neutralize the samples.

Notes:

1. **It is very important to follow the order of addition of the reagents.**
2. **While adding the neutralizing buffers, it is recommended to vortex the contents of the tube to prevent formation of pH gradients.**

6. Vortex the tubes and spin down the lysates for 2-5 minutes at 5000g to clarify the supernatants.

7. 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 until ready for use. (Note that this may result in some loss of NADP/NADPH).
   Note: Each investigator should optimize the number of cells used per test.

It is recommended to titrate the cell supernatant 1:2 or 1:4 so that the concentrations of NADP and NADPH will fall within the range of the standard curve. These dilutions should be prepared in the Standard curve diluent (Part# 3052) supplied.

X. Assay Protocol:

NADPH Standard Curve.

We only supply a NADPH standard as the detection reagent only reacts with NADPH. NADP is converted to NADPH. Label suitable tubes 1-8. To tube#1 add 995 µL of Standard curve diluent (Part #3052) and 5µL of the reconstituted NADPH standard. This will make a 750nM standard. Next serially dilute (1:2) the 750nM NADPH standard in the standard curve diluent to construct a standard curve. This can be accomplished by adding 250 µL of the standard curve diluent (Part #3052) into tubes #2-8. From tube #1 remove 250µL of the 750nM NADPH standard and add it to tube #2. Gently vortex tube #2 and pipette out again 250 µL from tube#2 to tube#3. Continue this process to tube #7. Tube # 8 is the blank control. The final NADPH concentration in the well will be three times less than in the tube.

Tube #	NADPH Concentration in tubes.	Final NADPH Concentration in wells.
1	750 nM	250 nM
2	375 nM	125 nM
3	187.5 nM	62.5nM
4	93.75 nM	31.25nM
5	46.87 nM	15.625 nM
6	23.43 nM	7.8125 nM
7	11.715	3.905 nM
8	0	0

Prepare the Reaction cocktail:

To 1mL of 3X Substrate mix (Part # 3048), add 30µL of enzyme mix (Part# 6022). Vortex gently. This is enough for 20 tests. Make enough Reaction cocktail for one day's worth of experiments.

Preparation of 1X Detection Reagent:

In low lighting, thaw an aliquot of the 50X detection reagent (Part # 4019). Once thawed gently vortex the tube and add 20µL of the detection reagent to 0.98mL of the reaction buffer (Part # 3049). This is enough for 20 tests.

Note: Dilute the Detection reagent just before use. Light sensitive. Avoid direct and prolonged exposure to light, as this will increase background.

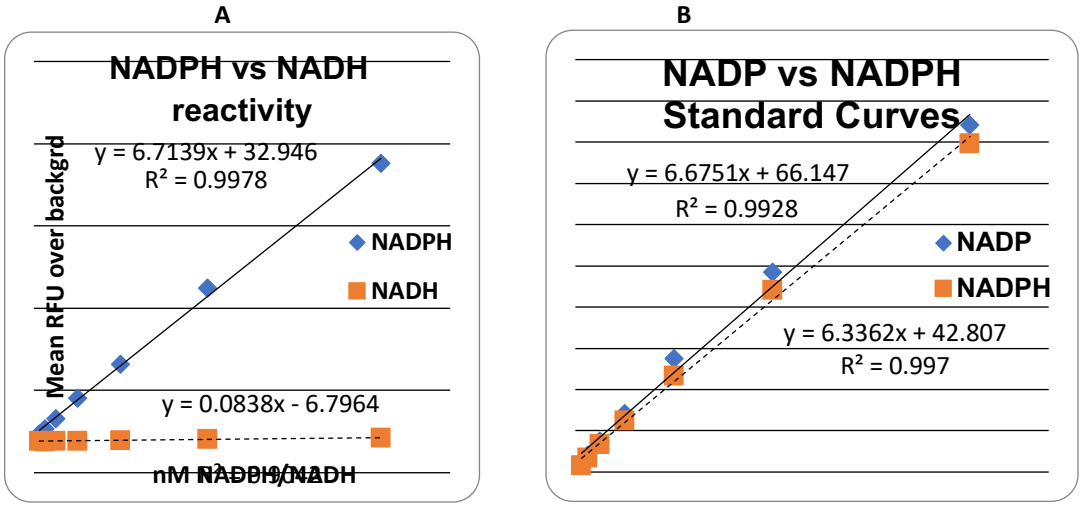

Figure 1. Comparison of NADPH vs. NADH Standard curves (graph A) and NADP vs. NADPH Standard curves (graph B) at T=30 minutes.

ASSAY:

1. Add 50µL 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 standard curve diluent (Part #3052), several fold so its values will fall within the range of the standard curve.

2. Next pipette in 50µL of the reaction cocktail (from step 1 above) to all the wells.

3. Place cover on the plate and incubate at 37^{0}C for 10 minutes.

4. After this incubation time remove the plate from the incubator.
   Pipette 50µL of the 1X detection reagent (from step 3 above) into each well of the sample and standard.

5. Incubate in the **dark** at room temperature for 30-60 minutes.

6. Measure fluorescence with excitation at 530-570 nm and emission at 590-600nm using a fluorescent plate reader.

7. The concentration of NADP or NADPH can be determined using the slope of the equation generated by the standard curve.

XI. Technical Notes:

a. **SH groups like DTT or Reduced Glutathione will interfere with the assay. Keep below 10µM.**
b. **It is important to add the NADP or NADPH extraction buffer first before adding the lysis buffer to the samples. NADP and NADPH recovery from the samples is severely compromised if the lysis buffer is added first.**
c. **It is recommended to perform all reactions on ice and use all solutions and buffers chilled, as this will reduce the rate of decomposition of NADP/H.**

XII. Spike and recovery results:

Condition	NADP recovery in NADP extraction Buffer	NADPH recovery in NADP extraction Buffer	NADP recovery in NADPH extraction Buffer.	NADPH recovery in NADPH extraction Buffer.
% Recovery	90-95%	Not detectable <1%	Not detectable <1%	85-90%

Table 1: We conducted spike and recovery experiments to estimate the decomposition and recovery of NADP and NADPH. 750nM solutions of NADP and NADPH were processed as described in step IX. Mammalian Cell Preparation and % recovery calculated from a standard curve (n=4).

	Jurkats	Jurkats
	nM NADP	nM NADPH
Calculated	22.629	74.056

Table 2. NADP and NADPH levels were assayed using the Fluoro NADP/NADPH kit.
Yield from 6 X10^5 Jurkat cells (n=3).

References:

1. Comprehensive Invited Review
WEIHAI YING. NAD /NADH and NADP /NADPH in Cellular Functions and Cell Death: Regulation and Biological Consequences. ANTIOXIDANTS &REDOX SIGNALING Volume 10,Number 2,2008.

2. Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology.*Physiol Rev* 87:245 –313,2007.