SensoLyte® NADP/NADPH Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72206**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect NADP/NADPH activity.
- ***Enhanced Value:*** Ample reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in less than an hour.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Reagent A	4 mL
Component B	Reagent B	1 mL
Component C	Enzyme Cycling Mix	70 µL
Component D	Assay Buffer	50 mL
Component E	NADP Standard	100 µM, 20 µL
Component F	NADP Extraction Buffer	25 mL
Component G	NADPH Extraction Buffer	25 mL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 590 nm with excitation at 570 nm.

Storage and Handling

- Store kit Components A, B and E at -20°C, protect from light.
- Store Component C at -80°C. Aliquot as needed to avoid multiple freeze-thaw cycles.
- Components D, F and G can be stored at room temperature for convenience.

Introduction

Pyridine nucleotides are involved in a number of critical catabolic and anabolic reactions in living organisms. Nicotinamide adenine dinucleotide phosphate (NADP) is a natural coenzyme present in all animal and plant organisms. Cellular NADPH is important for tolerance to ROS and maintenance of cellular redox homeostasis.[1-5] The reducing power of NADPH is a required cofactor for enzymes that are prooxidant such as nitric oxide synthase and NADPH oxidase.[6] Furthermore the NADP+/NADPH ratio has been found to change in the erythrocytes of subjects affected by hemolytic disorder.[7, 8] Thus an easy and accurate measurement of NADPH is very desirable.

The SensoLyte® NADP/NADPH Assay Kit is a sensitive fluorimetric assay that detects NADP and NADPH without interfering with NAD/NADH. NADP is converted to NADPH in an enzyme-cycling reaction. The kit contains a fluorogenic reagent, producing fluorescence when oxidized by NADPH. The resulting red fluorescence can be monitored at excitation /emission= 560/590 nm. The intensity of the fluorescence produced is proportional to NADP/NADPH concentration. This assay detects as low as 1.5 nM of the analyte.

Protocol

Note: Avoid reducing agents (e.g. dithiothreitol, DTT; β-mercaptoethanol) in test samples. If samples contain reducing agent, it should be neutralized by N-ethylmaleimide (NEM) using 2:1 NEM to DTT molar ratio. This assay tolerates the presence of sodium azide (up to 1 mM) and provides uncompromised signal in the presence of nonionic detergents such as Tween-20 (up to 10%), NP-40 (up to 1%) and Triton X-100 (up to 10%). Strong ionic detergents (e.g. SDS) completely inhibit assay.

1. Prepare biological samples.

1.1 Prepare cell extracts:

- Collect cells by centrifugation at 2,500 rpm for 5 min and wash cell pellets by cold PBS.
- Extract cells with either 200 µl of NADP extraction buffer (Component F) for NADP detection or NADPH extraction buffer (Component G) for NADPH detection.
- Add 200 µl of assay buffer (Component D)
- Incubate cell suspensions at 60^{o}C for 30 min. and then chill the samples on ice.
- Add 200 µl of opposite extraction buffer for neutralization.
- Quickly spin samples at 14,000 rpm for 5 min.
- Use supernatant for NADP/NADPH assay.

Note: Typically, extract from 1x 10^5 – 5x10^5 cells is used for one assay. Use several dilutions of sample to obtain readings that fit in the assay linear range.

2. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

2.1 NADP/NADPH Detection Reagent: To prepare NADP/NADPH Detection Reagent mix Component A and Component B. This amount of Detection Reagent is enough for a full 96-well plate. If not using the entire plate, adjust the amount of Detection Reagent to be prepared accordingly. The Detection Reagent is ready to use and no further dilution is necessary.

2.2 Enzyme Cycling Mix Diluent: Dilute Enzyme Mix (Component C) in assay buffer (Component D). Refer to Table 1. For each experiment, prepare fresh diluent mix. If not using the entire plate, adjust the amount of Enzyme Mix to be diluted accordingly.

Note: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

Table 1. Enzyme Cycling Mix Diluent for one 96-well plate (100 assays)

Components	**Volume**
Enzyme Cycling Mix (Component C)	70 µL
Assay Buffer (Component D)	4.930 mL
Total Volume	5 mL

2.3 Prepare NADP standard solution: Dilute NADP standard (Component E) 1:333 in assay buffer (Component D) to obtain a 300 nM solution. Do 2-fold serial dilutions to get concentrations of 150, 75, 37.5, 18.8, 9.4 and 4.7 nM. Include an assay buffer blank.

3. Set up the reaction.

3.1 Add test samples to the microplate wells. The suggested volume of test sample is 5-50 µL/well.

Note 1: Use assay buffer (Component D) to dilute test samples.

Note 2: If the samples are diluted in buffers containing substances that may affect assay performance, test the same amount of that buffer with standards.

3.2 Set up the NADP standard: Add 50 µL of serially diluted NADP standard solutions (from Step 2.3) to the wells.

3.3 Bring the total volume of all controls and samples to 50 µL.

4. Run the reaction.

4.1 Add 50 µL of Enzyme Cycling Mix Diluent (from Step 2.2) to the microplate wells.

4.2 Add 50 µL of NADP/NADPH Detection Reagent (from Step 2.1) into each well. Mix the reagents completely by shaking the plate gently for 30 sec.

4.3 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=570/590 nm continuously and record data every 5 min. for 30 min.
- For end-point reading: Incubate the reaction for 30 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=570/590 nm.

5. Data analysis.

5.1 The fluorescence reading from the blank control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing NADP/NADPH detection reagent. All fluorescence readings are expressed as relative fluorescence units (RFU).

5.2 Plot NADP standard curve as RFU versus NADP concentration. The final concentrations of NADP standards are 100, 50, 25, 12.5, 6, 3, 1.5, and 0 nM. Determine the linear

regression.

5.3 Use the standard curve for calculation of NADP/NADPH level in test samples.

Note: For direct, quantitative comparison of data obtained in independent experiments, prepare a separate calibration curve for each test series.

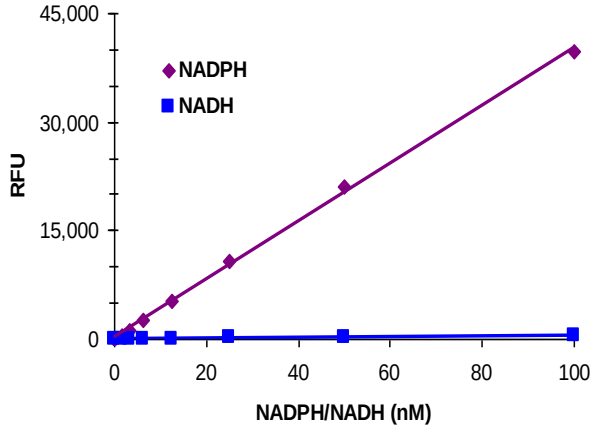

Fig 1. NADPH and NADH were serially diluted in assay buffer containing Enzyme Cycling Mix and Detection Reagent, and the fluorescence was recorded at Ex/Em=570 /590 nm. (Flexstation 384II, Molecular Devices).

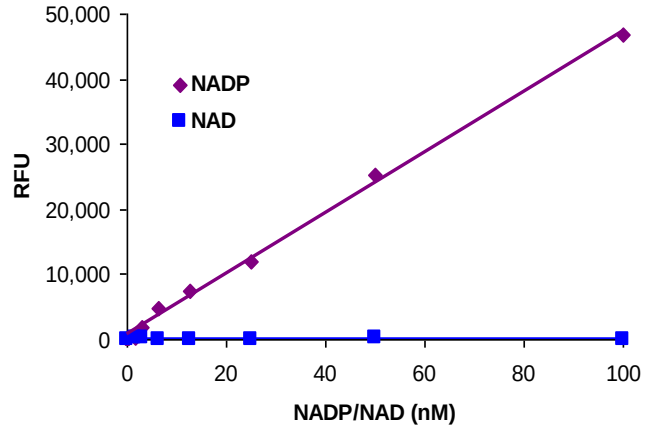

Fig 2. NADP and NAD were serially diluted in assay buffer containing Enzyme Cycling Mix and Detection Reagent, and the fluorescence was recorded at Ex/Em=570/590 nm. (Flexstation 384II, Molecular Devices).

References

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