SensoLyte® NADP/NADPH Assay Kit
Colorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72205**
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	180 µL
Component B	Reagent B	80 µL
Component C	Assay Buffer	50 mL
Component D	Enzyme Cycling Mix	80 µL
Component E	NADP Standard	1.5 mM, 20 µL
Component F	NADP Extraction Buffer	25 mL
Component G	NADPH Extraction Buffer	25 mL
Component H	Stop solution	20 mL

Other Materials Required (but not provided)

- 96-well microplate: Clear microplates provide better signal for absorbance reading.
- Microplate reader: Capable of detecting absorbance at 565nm.

Storage and Handling

- Store kit Components A, B and E at -20°C, protect from light.
- Store Component D at -80°C. Aliquot as needed to avoid multiple freeze-thaw cycles.
- Components C, F, G and H 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 prooxidants, 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 provides a simple and convenient colorimetric assay to detect NADP and NADPH without interfering with NAD/NADH. This kit utilizes enzyme cycling reaction to produce a blue colored product, formazan, which can be monitored at 565 nm using a microplate reader. The intensity of the color produced is proportional to NADP/NADPH concentration.

Protocol

1. Prepare biological samples.

Note: Avoid reducing agents (e.g. dithiothreitol, DTT; β-mercaptoethanol) and thiol alkylating agents (e.g. N-ethylmaleimide) in test samples.

1.1 Prepare cell extracts:

- Collect cells by centrifugation at 2500 rpm for 5 min and wash cell pellets using 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 C)
- Incubate cell suspensions at 60°C for 30 min. and then chill 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 $1x\ 10^6 – 5x10^6$ 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. dilute Components A and B in assay buffer (Component C). Refer to Table 1.

Table 1. NADP/NADPH Detection Reagent for one 96-well plate (100 assays)

Components	**Volume**
Reagent A (Component A)	166 µL
Reagent B (Component B)	76 µL
Assay buffer (Component C)	4.758 mL
Total Volume	5 mL

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 in assay buffer (Component C). Refer to Table 2. 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 2. Enzyme Cycling Mix Diluent for one 96-well plate (100 assays)

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

2.3 Prepare NADP standard solution: Dilute NADP standard (Component E) 100-fold in assay buffer (Component C) to obtain a 15 µM solution. Do 2-fold serial dilutions to get concentrations of 7.5, 3.75, 1.875, 0.94, 0.47 and 0.24 µM. 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 C) 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 Add 150 µL of stop solution (Component H) to all wells. Mix well.

4.4 Measure the signal: Read absorbance immediately at 565 nm using a microplate reader.

5. Data analysis.

5.1 The absorbance reading from the blank control well is used as the background absorbance. This background reading should be subtracted from the readings of the other wells containing detection reagent.

5.2 Plot NADP standard curve as absorbance versus NADP concentration. The final concentrations of NADP standards are 2.5, 1.25, 0.6, 0.3, 0.15, 0.075, 0.037 and 0 µM. 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.

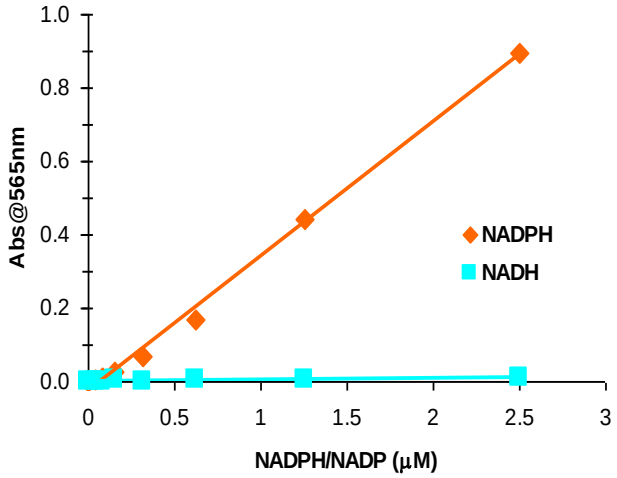

Fig 1. NADPH and NADH were serially diluted in assay buffer containing Enzyme Cycling Mix and Detection Reagent, followed by addition of stop solution. Absorbance was recorded at 565 nm. (Ultra Microplate Reader EL808, Bio-Tek Instruments, Inc).

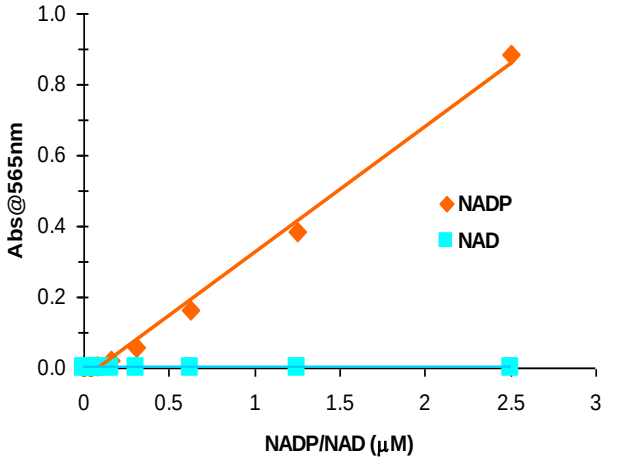

Fig 2. NADP and NAD were serially diluted in assay buffer containing Enzyme Cycling Mix and Detection Reagent, followed by addition of stop solution. Absorbance was recorded at 565 nm. (Ultra Microplate Reader EL808, Bio-Tek Instruments, Inc).

References

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