



NAD⁺ Enzyme Combo Assay Kit

Catalog # EA-7064

(For Research Use Only)

Introduction

The Signosis NAD⁺ Enzyme Combo Assay Kit enables researchers to simultaneously quantify NAD⁺ levels and the activities of three major NAD⁺-dependent enzymes—SIRT (sirtuins), NAMPT, and PARP (poly(ADP-ribose) polymerase)—in cell and tissue samples. This integrated approach provides a comprehensive tool for studying NAD⁺ biology and its regulatory pathways.

Principle

SIRT Assay

The SIRT activity assay kit can measure the activity of SIRT using a FRET-based peptide substrate. The peptide has a fluorophore on the N-terminus, quencher on the C-terminus, and an acetylated lysine side chain. Before the peptide is cleaved, the quencher prevents fluorescence from being emitted. When a reaction mixture containing the SIRT enzyme and a Lys-protease is added to the peptide substrate, SIRT first deacetylates the acetyl group on the lysine. Then, once the lysine is exposed, the Lys-protease will cut the peptide on the carboxyl side of the lysine, splitting the peptide in half and separating the fluorophore from the quencher. Once the fluorophore is separated from the quencher, it will emit fluorescence that can be measured at Ex/Em: 350/460 nm.

NAMPT Assay

The NAMPT activity assay kit can measure the activity of NAMPT for compound screening and profiling experiments. This assay detects NAMPT activity through a series of enzyme reactions. First, NAMPT converts nicotinamide (NAM) and PRPP into nicotinamide mononucleotide (NMN). Then NMN is converted by NMNAT into NAD⁺. Lastly, NAD⁺ is reduced to NADH by alcohol dehydrogenase (ADH) in the presence of ethanol. The NADH is detected with a WST solution which reacts with NADH to product a yellow color that can be measured at an absorbance of 450 nm with a plate reader.

PARP Assay

The PARP activity assay kit measures the activity of PARP by detecting its ability to perform ADP-ribosylation on histones. First, a plate is coated with histone. Then, a mixture of activated DNA and a biotin-tagged PARP substrate (NAD⁺) is added to the wells. When samples containing PARP are added to the mixture, the PARP will attach the biotin-tagged substrate to the histone through ADP-ribosylation. Once the wells are washed, the biotin-tagged substrate, which is bound to the histones attached to the plate, can be detected using streptavidin-HRP. After the addition of HRP substrate and stop solution, a colorimetric signal is generated, which can be measured at an absorbance of 450 nm with a plate reader.

NAD⁺/NADH Assay

The NADH Assay utilizes WST-8, a water-soluble dye, to measure NADH levels in samples. WST-8 reacts with NADH to produce a yellow color, which can be measured at an absorbance of 450 nm with a plate reader. Total NAD⁺ and NADH levels can be detected by converting NAD⁺ to NADH with alcohol dehydrogenase.

Materials Required but Not Provided

- PBS
- 96-well clear microplate for absorbance reading or 96-well black microplate with clear bottom for fluorescence reading
- Microplate reader capable of measuring absorbance at 450nm and 560 nm or fluorescence at 530nm/590nm

Materials Provided

- SIRT Substrate (-80°C)
- 1x Protease Stock (-80°C)
- NAM (-20°C)
- PRPP (-20°C)
- 1x NMNAT Enzyme Stock (-80°C)
- ADH Buffer (RT)
- 1x ADH Enzyme Stock (-80°C)
- Mediator Reagent (-20°C)
- WST Reagent (-80°C)
- 1x Diluent Buffer (4°C)
- 5x Assay Wash Buffer (4°C)
- Histone Stock (-80°C)
- Activated DNA (-80°C)
- PARP Substrate (-80°C)
- HRP Reagent (4°C)
- TMB Substrate (4°C)
- Stop Solution (4°C)
- 1mM NADH Standard (-20°C)
- ADH Buffer (RT)
- 1x ADH Enzyme Stock (-80°C)
- Mediator Reagent (-20°C)
- WST Reagent 2 (-80°C)

Cell Sample Preparation

1. Wash the cells once with PBS before lysing the cells.
2. For a 96-well culture plate, add 40 µL of lysis buffer to each well and incubate at room temperature for 10 minutes.
3. Pipette the lysis buffer up and down to detach the cells and transfer the cell lysates into a new tube.
4. If necessary, homogenize the cell lysates with a sonicator.
5. The cell lysates may be assayed directly or stored at -80°C.
6. Use PBS to dilute the cell sample to the appropriate concentration for each assay, if necessary.

Tissue Sample Preparation

1. Weigh tissue sample and add 1 mL of lysis buffer per 100mg of tissue.
2. Homogenize the tissue samples with a tissue grinder.
3. If necessary, further homogenize the tissue samples with a sonicator.
4. Centrifuge the sample at 10,000 RPM for 5 minutes to pellet the tissue debris.
5. Collect the supernatant and measure the protein concentration of the supernatant. The tissue sample can be assayed directly or stored at -80°C.
6. Use PBS to dilute the tissue sample to the appropriate concentration for each assay, if necessary.

****Spin down small tubes before starting experiment. ****

SIRT Measurement

1. Reaction mix preparation: calculate the amount of each reagent needed to make the reaction mix according to the table below.

Component	Reaction Mix (per well/sample)
SIRT substrate	1 μ L
1x Protease stock	1 μ L
PBS	48 μ L
Total	50 μ L

2. Add 50 μ L of reaction mix to each well of the plate.
3. Add 50 μ L of sample to each well with reaction mix and mix thoroughly.
4. Measure the fluorescence of the plate at Ex/Em: 350/460 nm. Obtain kinetic data for SIRT activity by making multiple readings of the plate. Start at one-minute intervals and increase the interval time as the signal begins to stabilize.

NAMPT Measurement

1. Reaction mix preparation: calculate the amount of each reagent needed to make the reaction mix according to the table below.

Component	Reaction Mix (per well/sample)
NAM	1 μ L
PRPP	1 μ L
1x NMNAT Enzyme	0.05 μ L
1x ADH Enzyme	0.05 μ L
ADH Buffer	49.95 μ L
Total	50 μ L

2. Any unused enzyme stock can be stored at -80°C for future use.
3. Add 50 μ L of reaction mix to each well of the plate.
4. Add 10 μ L of sample to each well with reaction mix and mix thoroughly.
5. Cover the plate and incubate at 37°C for 10 minutes.
6. Detection mix preparation: calculate the amount of each reagent needed to make the detection mix according to the table below.

Component	Detection Mix (per well/sample)
WST Reagent	5 μ L
Mediator Reagent	0.5 μ L
PBS	44.5 μ L
Total	50 μ L

7. Add 50 μ L of detection mix to each well in the plate.
8. Cover the plate and incubate at 37°C away from light for 30-60 minutes.
Exposure to light will produce background signal in wells
9. For a stronger signal, the plate can be incubated for an additional 1-2 hours at 37°C away from light.
10. Measure the absorbance of the plate at 450 nm using a plate reader.

PARP Measurement

1. Make Histone solution by diluting the Histone stock 1:1000 in PBS.
2. Coat the plate by adding 100 μ L of Histone solution to each well.
3. Incubate the plate at room temperature for at least 2 hours or at 4°C overnight for optimal results.
4. Discard the histone solution in the plate and wash the plate by aspirating and washing each well with 200 μ l of 1x Assay Wash Buffer. Repeat the process three times for a total of three washes.
5. Reaction mix preparation: calculate the amount of each reagent needed to make the reaction mix according to the table below.

Component	Reaction Mix (per well/sample)
Activated DNA	0.05 μ L
PARP Substrate	0.5 μ L
PBS	49.45 μ L
Total	50 μ L

6. Add 50 μ L of reaction mix to each well of the plate.
7. Add 50 μ L of sample to each well with reaction mix and mix thoroughly.
8. Incubate the plate at room temperature for 1 hour.
9. Make HRP solution by diluting the HRP reagent 1:200 in 1x Diluent Buffer.
10. Discard the contents in the plate and wash the plate by aspirating and washing each well with 200 μ l of 1x Assay Wash Buffer. Repeat the process three times for a total of three washes.
11. Add 100 μ L of HRP solution to each well of the plate.
12. Incubate the plate at room temperature for 1 hour.
13. Discard the HRP solution in the plate and wash the plate by aspirating and washing each well with 200 μ l of 1x Assay Wash Buffer. Repeat the process three times for a total of three washes.
14. Add 100 μ L of TMB substrate to each well of the plate.
15. Incubate the plate at room temperature for 30-60 minutes until the wells begin to turn blue.
16. Once the desired color is reached, add 50 μ L of Stop solution to each well of the plate to terminate the HRP reaction and form a yellow color.
17. Using a microplate reader, measure the absorbance of the plate at 450 nm.

NADH Measurement

1. **Standard curve preparation:** First, take eight new tubes and label them 1-8. In tube 1, add 120 μL of the provided 1mM NADH standard. Then, for tubes 2-8, add 60 μL of PBS to each tube. Transfer 60 μL of the 1mM NADH standard from tube 1 to the PBS in tube 2 and mix to make a 500 μM NADH standard. Then, transfer 60 μL of the 500 μM NADH standard from tube 2 to the PBS in tube 3 and mix to make an 250 μM NADH standard. Continue the serial dilutions until tube 7 is done. Leave tube 8 untouched as the PBS negative control.

Standard#	NADH Concentration (μM)
1	1000
2	500
3	250
4	125
5	62.5
6	31.25
7	15.625
8	0

2. Add 50 μL of sample or standard to each well.
3. **Detection mix preparation:** calculate the amount of each reagent needed to make the detection mix according to the table below.

Component	Detection Mix (per well/sample)
WST Reagent	5 μL
Mediator Reagent	0.5 μL
PBS	44.5 μL
Total	50 μL

4. Add 50 μL of detection mix to each sample or standard well in the plate. **Be sure to add the detection mix quickly, since the signal begins to develop when the reagents are added. Use a multichannel pipette if possible.**
5. Cover the plate and incubate at 37 $^{\circ}\text{C}$ away from light for 15-30 minutes.
Exposure to light will produce background signal in wells
6. For a stronger signal, the plate can be incubated for another 30-60 minutes at 37 $^{\circ}\text{C}$ away from light.
7. Measure the absorbance of the plate at 450 nm using a plate reader.

NAD⁺ and NADH Total Measurement

18. **Reaction mix preparation:** calculate the amount of each reagent needed to make the reaction mix according to the table below.

Component	Reaction Mix (per well/sample)
1x ADH Enzyme	0.05 μL
ADH Buffer	49.95 μL
Total	50 μL

19. Any unused enzyme stock can be stored at -80 $^{\circ}\text{C}$ for future use.
20. Add 50 μL of reaction mix to each well of the plate.
21. Add 5 μL of sample or standard to each well with reaction mix and mix thoroughly.
22. Cover the plate and incubate at 37 $^{\circ}\text{C}$ for 10 minutes.
23. **Detection mix preparation:** calculate the amount of each reagent needed to make the detection mix according to the table below.

Component	Detection Mix (per well/sample)
WST Reagent	5 μL
Mediator Reagent	0.5 μL
PBS	44.5 μL
Total	50 μL

24. Add 50 μL of detection mix to each sample or standard well in the plate.
25. Cover the plate and incubate at 37 $^{\circ}\text{C}$ away from light for 30-60 minutes.
Exposure to light will produce background signal in wells
26. For a stronger signal, the plate can be incubated for an additional 1-2 hours at 37 $^{\circ}\text{C}$ away from light.
27. Measure the absorbance of the plate at 450 nm using a plate reader.