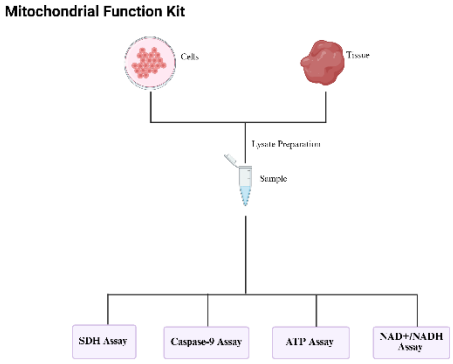
Signosis, Inc.
Innovative Plate Assay Solutions

Mitochondrial Function Combo Assay Kit

Catalog # EA-7021 **(For Research Use Only)**

Introduction

Signosis' Mitochondrial Function Combo Assay Kit provides a comprehensive solution for measuring key components of cellular metabolism in the mitochondria in cells or tissue samples. This kit enables the detection of succinate dehydrogenase activity, caspase-9 activity, ATP, and NAD^+/NADH by integrating multiple assays into a single kit.



Principle

Succinate Dehydrogenase Assay

The Succinate Dehydrogenase (SDH) Assay Kit utilizes DCPIP, a blue-colored redox dye, to measure SDH activity in samples. When SDH converts succinate to fumarate, it generates FADH2, which transfers electrons to an electron acceptor. In this assay, the electrons are transferred to DCPIP, which becomes reduced and changes color. This color change can be measured with a spectrophotometer at an absorbance of 600 nm.

Caspase-9 Assay

The Caspase-9 Assay utilizes a unique fluorogenic substrate that is specifically cleaved by caspase-9. Once cleaved, the substrate produces a luminescent signal which can be detected with a fluorescence plate reader at Ex/Em: 400nm/505nm.

ATP Assay

The ATP Assay utilizes a couple of enzymes which catalyze the metabolism of glycerol to measure ATP levels in samples. First, glycerol kinase is used to convert glycerol to glycerol 3-phosphate. This step consumes ATP so the end product of this enzyme reaction can be used to quantify the amount of ATP present in the sample. In the next step, glycerol 3-phosphate oxidase reacts with glycerol 3-phosphate to produce hydrogen peroxide. The ATP level in the sample is determined by quantifying the hydrogen peroxide generated by the enzyme reaction with a fluorogenic probe that can be measured with a spectrophotometer.

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 600 nm and fluorescence at Ex/Em: 400nm/505nm and 530nm/590nm

Materials Provided

- 10mM FAD (-80ºC)
- Probe Reagent (-20ºC)
- HRP Reagent (4ºC)
- Succinate Substrate (-20°C)
- Transport Reagent (-20ºC)
- 20mM DCPIP (-20ºC)
- Caspase-9 Substrate (-80ºC)
- 1mM ATP Standard (-80ºC)
- ATP Buffer (RT)
- 1x GKi Enzyme Stock (-80ºC)
- 1x GPOx Enzyme Stock (-80ºC)
- 1mM NADH Standard (-20ºC)
- ADH Buffer (RT)
- 1x ADH Enzyme Stock (-80ºC)
- Mediator Reagent (-20ºC)
- WST Reagent (-80ºC)

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

Plasma Sample Preparation

1. Centrifuge citrated or EDTA-collected blood at 4°C (1,000 x *g* for 10 minutes) to separate plasma from erythrocytes. Alternatively, blood collected without anticoagulant can be centrifuged to collect serum.
2. Transfer the plasma layer to a new tube without disturbing the buffy layer.
3. The plasma may be assayed directly or stored away at -80°C. About 100 µL of sample will be needed for all four assays.

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. About 100 µL of sample will be needed for all four assays.

Tissue Sample Preparation

1. Weigh tissue sample and add 1 mL of Tissue 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 the Dilution buffer to dilute the tissue sample to the appropriate concentration for each assay. About 100 µL of sample will be needed for all four assays.

SDH Activity 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)
10mM FAD	0.05 µL
Succinate Substrate	0.05 µL
Transport Reagent	0.5 µL
20mM DCPIP	0.5 µL
Total	48.9 µL

2. Add 50 µL of reaction mix to each well of the plate.
3. Add 50 µL of sample to each well with reaction mix and mix thoroughly.
4. Measure the absorbance of the plate at 600 nm. Obtain kinetic data for SDH activity by making multiple readings of the plate. Start at one-minute intervals and increase the interval time as the signal begins to stabilize.

Caspase-9 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)
Caspase 9 Substrate	1 µL
PBS	45 µL
Total	50 µL

2. Add 50 µL of reaction mix to each well of the plate.
3. Add 50 µL of sample to each well with reaction mix and mix thoroughly.
4. Cover the plate and incubate at room temperature for 10 minutes away from light.
5. Measure the fluorescence of the plate in a fluorescence plate reader at Ex/Em: 400nm/505nm. Take measurements at multiple time intervals to monitor change in fluorescence.

ATP Measurement

1. Standard curve preparation: Using the provided 1mM ATP standard, prepare a standard curve dilution as described in the table below

Standard #	1	2	3	4	5	6	7	8
ATP Standard Volume (µL)	10	8	6	4	2	1	0.5	0
PBS (µL)	40	42	44	46	48	49	49.5	50
Total (µL)	50	50	50	50	50	50	50	50
ATP Final Concentration (µM)	200	160	120	80	40	20	10	0

2. 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 GKi Enzyme	0.04 µL
1x GPOx Enzyme	0.04 µL
ATP Buffer	39.92 µL
Total	40 µL

3. Any unused enzyme stock can be stored at -80°C for future use.
4. Add 40 µL of reaction mix to each well of the plate.
5. Add 40 µL of sample or standard to each well with reaction mix and mix thoroughly.
6. Cover the plate and incubate at room temperature for 30 minutes.
7. 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)
Probe Reagent	0.8 µL
HRP Reagent	1.6 µL
PBS	77.6 µL
Total	80 µL

8. Add 80 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.**
9. Cover the plate and incubate at room temperature away from light for 15-30 minutes. **Exposure to light will produce background signal in wells**
10. For a stronger signal, the plate can be incubated for another 30-60 minutes away from light.
11. Measure the absorbance of the plate at 560 nm using a plate reader. Alternatively, measure the fluorescence of the plate in a fluorescence plate reader Ex/Em 530nm/590nm.

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 500µM NADH standard. Then, transfer 60 µL of the 500µM NADH standard from tube 2 to the PBS in tube 3 and mix to make an 250µM lactate 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°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°C away from light.
7. Measure the absorbance of the plate at 450 nm using a plate reader.

NAD+ and NADH Total Measurement

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)
1x ADH Enzyme	0.05 µL
ADH Buffer	49.95 µL
Total	50 µL

6. Any unused enzyme stock can be stored at -80°C for future use.
7. Add 50 µL of reaction mix to each well of the plate.
8. Add 5 µL of sample or standard to each well with reaction mix and mix thoroughly.
9. Cover the plate and incubate at 37°C for 10 minutes.
10. 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

11. Add 50 µL of detection mix to each sample or standard well in the plate.
12. Cover the plate and incubate at 37°C away from light for 30-60 minutes.
    Exposure to light will produce background signal in wells
13. For a stronger signal, the plate can be incubated for an additional 1-2 hours at 37°C away from light.
14. Measure the absorbance of the plate at 450 nm using a plate reader.