Signosis, Inc.
Innovative Plate Assay Solutions

Mitochondrial Metabolite Assay Kit

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

Introduction

Signosis' **Cellular Metabolism Combo Assay Kit** provides a comprehensive solution for measuring the key components in cellular metabolism in blood and other biological liquids, cells, or tissue samples in a streamlined workflow. This kit enables the detection of **glucose, lactate, ATP,** and **NAD^+/NADH** by integrating multiple assays into a single kit.

Principle

Pyruvate Assay

The Pyruvate Assay Kit utilizes an enzyme catalyzed oxidation reaction to measure pyruvate levels in samples. First, pyruvate is oxidized by pyruvate oxidase to form a pyruvate byproduct and hydrogen peroxide. Then, the pyruvate 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.

Lactate Assay

The Lactate Assay utilizes an enzyme oxidation reaction to measure lactate levels in samples. First, lactate is oxidized by lactate oxidase into pyruvate and hydrogen peroxide. Then, the lactate concentration 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.

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.

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.

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

- 10mM FAD (-80ºC)
- 10mM FMN (-20ºC)
- Probe Reagent (-20ºC)
- HRP Reagent (4ºC)
- 20mM Pyruvate Standard (-20°C)
- 1x POX Enzyme Stock (-80ºC)
- 5mM Lactate Standard (-20ºC)
- 1x LOX Enzyme Stock (-80ºC)
- 1mM ATP Standard (-80ºC)
- ATP Buffer (RT)
- 1x GK Enzyme Stock (-80ºC)
- 1x GPO Enzyme Stock (-80ºC)
- 1mM NADH Standard (-20ºC)
- ADH Buffer (RT)
- 1x ADH Enzyme Stock (-80ºC)
- Mediator Reagent (-20ºC)
- WST Reagent (-80ºC)
- Caspase-9 Substrate (-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.

Glucose Measurement

1. Standard curve preparation: First, take eight new tubes and label them 1-8. In tube 1, prepare a 2000 µM glucose standard by mixing 10 µL of the provided 20 mM glucose standard with 90 µL of PBS. Then, for tubes 2-8, add 50 µL of PBS to each tube. Transfer 50 µL of the 2000 µM glucose standard from tube 1 to the PBS in tube 2 and mix to make a 1000 µM glucose standard. Then, transfer 50 µL of the 1000 µM glucose standard from tube 2 to the PBS in tube 3 and mix to make an 500 µM glucose standard. Continue the serial dilutions until tube 7 is done. Leave tube 8 untouched as the PBS negative control.

Standard#	Glucose Concentration (µM)
1	2000
2	1000
3	500
4	250
5	125
6	62.5
7	31.25
8	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)
10mM FAD	0.05 µL
1x GOX	0.05 µL
PBS	49.9 µL
Total	50 µL

3. Any unused enzyme stock can be stored at -80°C for future use.
4. Add 50 µL of reaction mix to each well of the plate.
5. Add 5 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	0.5 µL
HRP Reagent	1 µL
PBS	48.5 µL
Total	50 µL

8. Add 50 µL of detection mix to each reaction 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 10-20 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.

Lactate Measurement

1. Standard curve preparation: First, take eight new tubes and label them 1-8. In tube 1, prepare a 2000µM lactate standard by mixing 40 µL of the provided 5mM lactate standard with 60 µL of PBS. Then, for tubes 2-8, add 50 µL of PBS to each tube. Transfer 50 µL of the 2000µM lactate standard from tube 1 to the PBS in tube 2 and mix to make a 1000µM lactate standard. Then, transfer 50 µL of the 1000µM lactate standard from tube 2 to the PBS in tube 3 and mix to make an 500µM lactate standard. Continue the serial dilutions until tube 7 is done. Leave tube 8 untouched as the PBS negative control.

Standard#	Lactate Concentration (µM)
1	2000
2	1000
3	500
4	250
5	125
6	62.5
7	31.25
8	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)
10mM FMN	0.05 µL
1x LOX Enzyme	0.4 µL
PBS	49.55 µL
Total	50 µL

3. Any unused enzyme stock can be stored at -80°C for future use.
4. Add 50 µL of reaction mix to each well of the plate.
5. Add 5 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	0.5 µL
HRP Reagent	1 µL
PBS	48.5 µL
Total	50 µL

8. Add 50 µL of detection mix to each reaction 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 10-20 minutes.
   Exposure to light will produce background signal in wells
10. For a stronger signal, the plate can be incubated for an additional 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.

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 GK Enzyme	0.04 µL
1x GPO 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	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

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

2. Any unused enzyme stock can be stored at -80°C for future use.
3. Add 50 µL of reaction mix to each well of the plate.
4. Add 5 µL of sample or standard 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 µL
Mediator Reagent	0.5 µL
PBS	44.5 µL
Total	50 µL

7. Add 50 µL of detection mix to each sample or standard 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.

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	5 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 37°C for 1-2 hours.
5. Measure the fluorescence of the plate in a fluorescence plate reader at Ex/Em 345nm/445nm.