Signosis, Inc.
Innovative Plate Assay Solutions

Free Fatty Acid Assay Kit

Catalog # EA-7012 **(For Research Use Only)**

Introduction

The Free Fatty Acid (FFA) assay kit utilizes a series of enzyme reactions to measure free fatty acid levels in samples. First, Acyl-Coa synthetase attaches coenzyme A to the FFAs to form Acyl-Coa. Then, Acyl-Coa oxidase reacts with Acyl-Coa to produce hydrogen peroxide. The FFA 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.

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 560 nm or fluorescence at 530nm/590nm

Materials Provided

- KRPG buffer (RT)
- DMSO (RT)
- 100mM ATP (-20ºC)
- 10mM FAD (-80ºC)
- Probe Reagent (-20ºC)
- HRP Reagent (4ºC)
- 10mM FFA Standard (-20ºC)
- Coenzyme A (-20ºC)
- 1x ACS Enzyme Stock (-80ºC)
- 1x ACO Enzyme Stock (-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.

Cell Sample Preparation

1. From a 96-well culture plate, detach adherent cells with trypsin. For suspension cells, centrifuge at 1,000 x g for 5 minutes to pellet cells.
2. Wash the cells twice with cold PBS to remove residual media.
3. Resuspend the cells in 1 mL of PBS and homogenize using a tissue grinder or sonicator.
4. Add 2 mL of chloroform and 1 mL of methanol to the homogenized cell sample and mix thoroughly by vortexing for 30 seconds.
5. Add 0.5 mL of ddH_2O to the mixture and vortex again for 30 seconds to induce phase separation.
6. Centrifuge the sample at 1,500 x g for 10 minutes at room temperature to separate the phases.
7. Carefully collect the lower chloroform phase containing the lipids and transfer to a new tube.
8. Vacuum dry the lipid sample until all of the chloroform is evaporated.
9. Reconstitute the dry lipid sample in PBS.
10. The lipid sample may be assayed directly or stored at -80°C.

Tissue Sample Preparation

1. Weigh 100 mg of tissue and place in a tube.
2. Add 1 mL of cold methanol and homogenize using a tissue grinder.
3. Add 2 mL of chloroform to the homogenized tissue sample and mix thoroughly by vortexing for 30 seconds.
4. Add 0.5 mL of ddH_2O to the mixture and vortex again for 30 seconds to induce phase separation.
5. Centrifuge the sample at 1,500 x g for 10 minutes at room temperature to separate the phases.
6. Carefully collect the lower chloroform phase containing the lipids and transfer to a new tube.
7. Vacuum dry the lipid sample until all of the chloroform is evaporated.
8. Reconstitute the dry lipid sample in PBS.
9. The lipid sample may be assayed directly or stored at -80°C.

FFA Measurement

1. Standard curve preparation: Take five new tubes labeled 1-5 and add 10 µL of DMSO to each tube. Transfer 10 µL of the provided 10mM FFA standard to the first DMSO tube and mix to make a 5mM FFA dilution. Then, transfer 10 µL of the 5mM FFA dilution from the first tube to the second DMSO tube and mix to make a 2.5mM dilution. Continue the serial dilutions until the fourth tube is done. Leave the fifth tube untouched as the DMSO negative control.

   Take six new tubes labeled A-F and add 19 µL of PBS to each tube. Transfer 1 µL of the provided 10mM FFA standard to tube A and mix to make a 500 µM FFA standard. Transfer 1 µL from each of the FFA dilutions prepared in tubes 1-5 to tubes B-F and mix to make the rest of the FFA standards.

Standard#	FFA Concentration (µM)
A	500
B	250
C	125
D	62.5
E	31.25
F	0

2. Dissolve the provided 6mg of coenzyme A in 780 µL of ddH_2O to make a 10mM coenzyme A solution. Unused coenzyme A solution can be stored at -20°C for future use.
3. Reaction mix #1 preparation: calculate the amount of each reagent needed to make the reaction mix according to the table below.

Component	Reaction Mix (per well/sample)
100mM ATP	0.2 µL
10mM Coenzyme A	1 µL
1x ACS Enzyme	2 µL
KRPG Buffer	16.8 µL
Total	20 µL

4. Add 20 µL of reaction mix #1 to each well of the plate.
5. Add 20 µL of sample or standard to each well with reaction mix #1 and mix thoroughly.
6. Cover the plate and incubate at 37°C for 30 minutes.
7. Reaction mix #2 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	0.04 µL
1x ACO Enzyme	0.04 µL
PBS	39.92 µL
Total	40 µL

8. Add 40 µL of reaction mix #2 to each reaction well in the plate.
9. Cover the plate and incubate at 37°C for 30 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)
Probe Reagent	0.8 µL
HRP Reagent	1.6 µL
PBS	77.6 µL
Total	80 µL

11. Add 80 µL of detection mix to each sample or standard well in the plate.
12. Cover the plate and incubate at room temperature away from light for 30 minutes.
    Exposure to light will produce background signal in wells
13. For a stronger signal, the plate can be incubated for additional hours or overnight away from light.
14. 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.