Product Manual

DAG (Diacylglycerol) Assay Kit

Catalog Number

MET-5028 **100 assays**

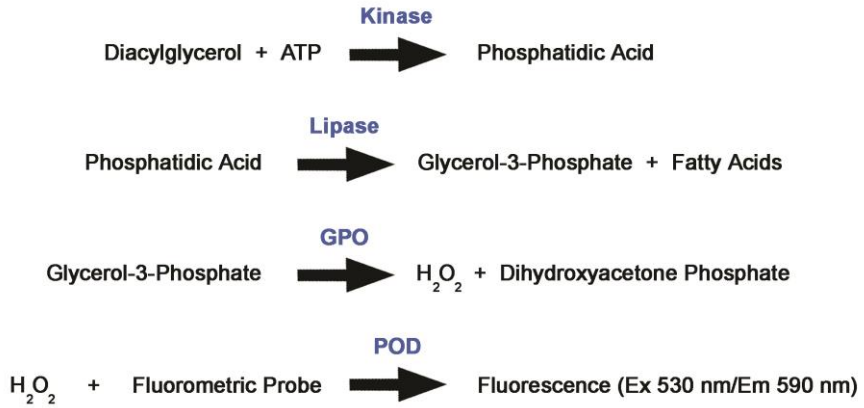
FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Diacylglycerols (DAG) are key intermediates in the biosynthesis of many cellular lipids and play a fundamental role in biochemical signaling. Structurally, diacylglycerol is a glyceride consisting of 2 fatty acid chains linked by a central glycerol backbone. Diacylglycerol acts as a precursor to many lipids (e.g., triglycerides, phospholipids); however, DAG also functions as a second messenger signaling lipid, produced through hydrolysis of PIP2 by phospholipase C, which initiates intracellular Ca^{2+} release and PKC activation. Ultimately, the PKC enzymes are involved with many modifications to normal cellular physiology, affecting hundreds of substrates.

Cell Biolabs' DAG Assay Kit measures diacylglycerol content in samples by a coupled enzymatic reaction system. First, kinase is used to phosphorylate DAG samples, yielding phosphatidic acid. Next, a lipase is used to hydrolyze phosphatidic acid to glycerol-3-phosphate. Finally, the glycerol-3-phosphate product is oxidized by glycerol-3-phosphate oxidase (GPO), producing hydrogen peroxide which reacts with the kit's Fluorometric Probe (Ex. 530-560 nm/Em. 585-595 nm).



The DAG Assay Kit is a simple, fluorometric assay that quantitatively measures total DAG (diacylglycerol) in cell lysates using a 96-well microtiter plate format. Each kit provides sufficient reagents to perform up to 100 assays*, including blanks, standards and unknown samples. The kit contains a DAG Standard and has a detection sensitivity limit of ~15 µM.

Note: Each sample replicate requires 2 assays, one treated with Kinase Mixture (+Kin) and one without (-Kin). DAG is calculated from the difference in fluorescence readings from the 2 wells.

Related Products

1. STA-390: Total Cholesterol Assay Kit
2. STA-394: HDL Cholesterol Assay Kit
3. STA-397: Serum Triglyceride Quantification Kit (Fluorometric)
4. STA-399: Free Glycerol Assay Kit (Fluorometric)
5. STA-619: Free Fatty Acid Assay Kit (Fluorometric)

Kit Components (shipped on dry ice)

1. DAG Standard (Part No. 50281D): One 1 mL vial of 2 mM 1-2-dioleoyl-*sn*-glycerol.
2. 10X Assay Buffer (Part No. 50192D): One 1.5 mL vial.

3. Kinase Mixture (Part No. 50282D): Two 1 mL vials containing ATP.
4. Lipase Solution (Part No. 50193D): Three 1.4 mL vials.
5. Enzyme Mixture (Part No. 50194D): Three 1.75 mL vials.
6. Fluorometric Probe (Part No. 261901): One 110 µL amber vial.

Materials Not Supplied

1. Standard 96-well fluorescence black microtiter plate
2. PBS (containing Magnesium and Calcium)
3. Extraction reagents for cellular lipids (methanol, chloroform, 1M NaCl)

Storage

Store the entire kit at -80ºC. Avoid multiple freeze/thaws by aliquoting. The Fluorometric Probe is light sensitive and should be maintained in amber tubes.

Preparation of Reagents

- DAG Standard: Thaw at 37ºC for 1-2 hours. Once homogeneous and vortexed well, maintain the standard at 37ºC during assay preparation. The solution is stable for 1 week at room temperature; however, the solution must be warmed to 37ºC to ensure homogeneity. For longer term storage, the solution should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.
- 1X Assay Buffer: 10X Assay Buffer should be thawed/maintained at 4ºC during assay preparation. Dilute the 10X Assay Buffer with deionized water. Stir to homogeneity. The 1X solution is stable for 1 month at 4ºC. For longer term storage, any unused 10X stock material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.
- Kinase Mixture, Lipase Solution, and Enzyme Mixture: Thaw at 4ºC. Once homogeneous and mixed well, maintain the solution at 4ºC during assay preparation. The solution should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.

  Note: These components are provided in multiple tubes to minimize multiple freeze/thaws.
- Fluorometric Probe: Thaw and maintain at room temperature during assay preparation. Any unused material should be aliquoted and frozen at -80ºC to avoid multiple freeze/thaws.
- PEU (pre-equilibrated upper phase) Solution: Mix 50 mL of chloroform, 50 mL of methanol, and 45 mL of 1M NaCl in a glass container. Shake or mix the solution well, then allow it to separate into 2 phases. Use the upper phase for washing during the extraction.

Preparation of DAG Standard

Thaw the DAG Standard at 37ºC (see Preparation of Reagents above). Mix well by vortexing to ensure the solution is homogeneous. Freshly prepare a dilution series of the standard in the concentration range of 0 µM – 2 mM by diluting the standard stock solution (provided at 2 mM) in 1X Assay Buffer (see Table 1). Standards should be prepared fresh, vortexed well and used immediately.

Standard Tubes	2 mM DAG Standard (µL)	1X Assay Buffer (µL)	Final DAG Standard (µM)
1	100	0	2000
2	50 of Tube #1	50	1000
3	50 of Tube #2	50	500
4	50 of Tube #3	50	250
5	50 of Tube #4	50	125
6	50 of Tube #5	50	62.5
7	50 of Tube #6	50	31.2
8	0	50	0

Table 1. Preparation of DAG Standards

Preparation of Samples

- Urine, plasma and serum: This kit is not recommended for these samples**.**
- Cell lysates:

  For adherent cells, remove media and wash cells twice with cold PBS. Harvest ~1x 10^7 cells by using a rubber policeman. Do not use proteolytic enzymes. Centrifuge at 1500 x g for 10 minutes. Carefully remove the supernatant and resuspend in 1 mL of cold PBS. Proceed to step 1 of the extraction procedure below.

  For suspension cells, collect ~1 x 10^7 cells by centrifugation at 1500 x g for 10 minutes. Carefully remove the supernatant and wash the cell pellet with cold PBS. Repeat PBS wash/centrifugation once more. Carefully discard the supernatant and resuspend in 1 mL of cold PBS. Proceed to step 1 of the extraction procedure below:

  Extraction Procedure

  1. Sonicate the 1 mL of cell suspension on ice.
  2. Add 1.5 mL of methanol to the sonicated sample.
  3. Add 2.25 mL of 1 M NaCl and 2.5 mL of chloroform to the sample. Vortex well.
  4. Centrifuge at 1500 x g for 10 minutes at 4ºC to separate the phases.
  5. Carefully remove the upper aqueous phase and discard.
  6. Wash the lower chloroform phase 2 times with 2 mL of pre-equilibrated upper phase (PEU) (see Preparation of Reagents Section). Separate the phases each time by centrifuging at 1500 x g for 10 minutes at 4ºC. Carefully remove the upper phase and discard each time.
  7. After the final wash, carefully transfer the lower organic phase to a glass vial or tube (a syringe works well). Avoid transferring any remaining upper, aqueous phase.
  8. Dry the lower phase in a speedvac or under a gentle stream of nitrogen.
  9. Resuspend the dried sample with 50 µL of 1X Assay Buffer (1:20 of the original volume). Samples may be stored at -80ºC for up to a month.

     Note: Not all dried samples will be solubilized completely, but the Assay Buffer will solubilize the DAG in the samples. The supernatant may be removed for use in the assay.

Assay Protocol

Note: A freshly prepared standard curve should be used each time the assay is performed. Maintain the Kinase Mixture, Lipase Solution, and Enzyme Mixture at 4ºC during assay preparation.

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknowns and standards, should be assayed in duplicate or triplicate.

Note: Each unknown sample replicate requires two paired wells, one to be treated with Kinase Mixture (+Kin) and one without (-Kin) to determine phosphatidic acid background (1X Assay Buffer will be added in place of the Kinase Mixture).

2. Add 20 µL of the DAG standards, samples or blanks to the 96-well microtiter plate.
3. Add 20 µL of Kinase Mixture to the standards and to one half of the paired sample wells, and mix the well contents throughly.
4. Add 20 µL of 1X Assay Buffer to the other half of the paired sample wells and mix thoroughly.
5. Incubate at 37ºC for 2 hours.
6. Transfer 20 µL of the mixture to a 96-well plate suitable for fluorescence measurement.
7. Add 40 µL of Lipase Solution to each well.
8. Incubate at 37ºC for 30 minutes.
9. During the step 8 incubation, separately prepare the desired volume of Detection Enzyme Mixture according to Table 2 below, based on the number of tests to be performed. Maintaining all components and mixtures at 4ºC throughout this step, add components in the following sequence:
   a. In a tube, add the appropriate volume of Enzyme Mixture.
   b. To the Enzyme Mixture, add the corresponding volume of Fluorometric Probe. Mix well and immediately use.

Note: Detection Enzyme Mixture may appear slightly pink in color. This is normal background and should be subtracted from all absorbance values

Enzyme Mixture (mL)	Fluorometric Probe (µL)	Total Volume of Detection Enzyme Mixture (mL)	**# of Tests in 96-well Plate (100 µL/test)**
5	50	5.05	**100**
2.5	25	2.525	**50**
1.25	13	1.263	**25**

Table 2. Preparation of Detection Enzyme Mixture

10. Transfer 50 µL of the above Detection Enzyme Mixture (from step 9) to each well.
11. Cover the plate wells to protect the reaction from light.
12. Incubate at room temperature for 10 minutes.
13. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-560 nm range and for emission in the 585-595 nm range.

Example of Results

The following figures demonstrate typical DAG Assay Kit results. One should use the data below for reference only. This data should not be used to interpret actual results.

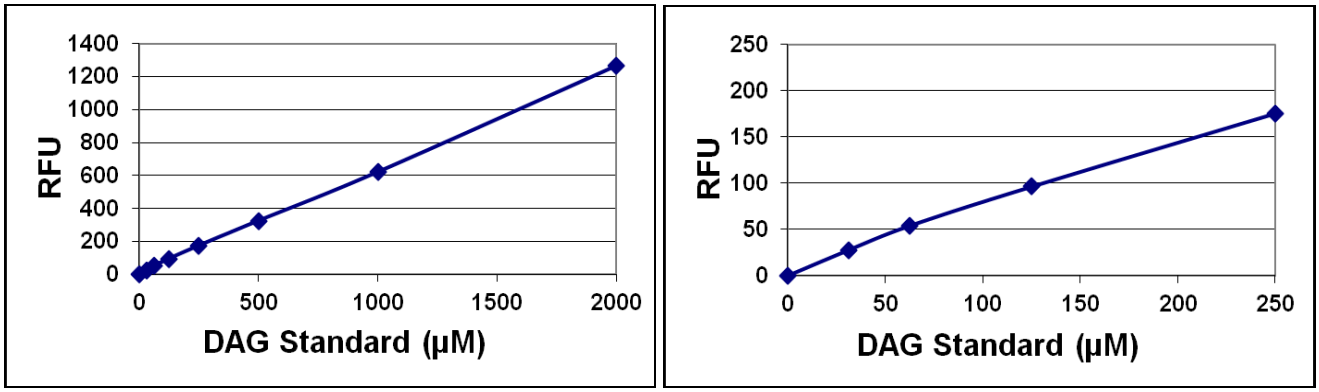


Figure 1: DAG Assay Standard Curve. DAG standard curve was performed according to the Assay Protocol. Background has been subtracted.

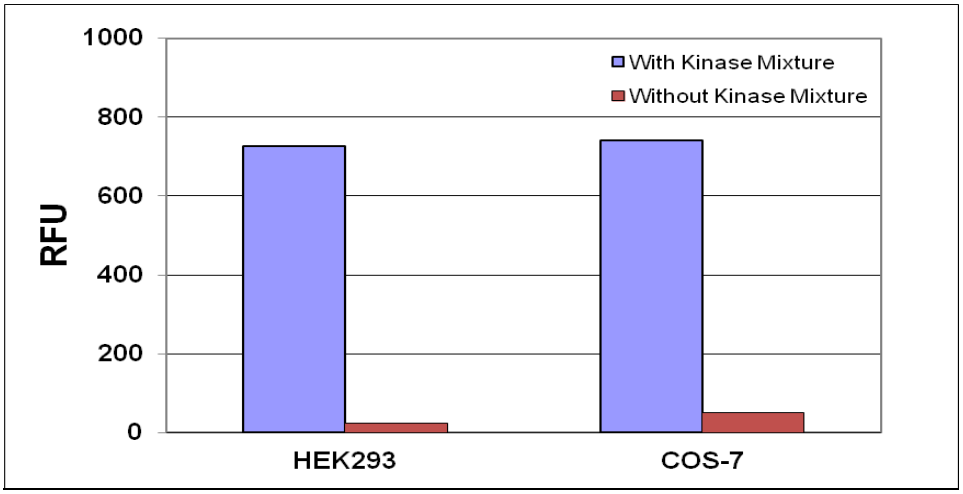


Figure 2: DAG Detection of Lipid Extracts. HEK293 and COS-7 lipid extracts were prepared according to the extraction procedure above. DAG samples were tested according to the Assay Protocol (phosphatidic acid background was determined +/- Kinase Mixture). Negative control values (without DAG) have been subtracted.

Calculation of Results

1. Determine the Average Relative Fluorescence Unit (RFU) values for each sample, control, and standard.
2. Subtract the average zero standard value from itself and all standard values.
3. Graph the standard curve (see Figure 1).
4. Subtract the sample well values without Kinase Mixture (-Kin) from the sample well values containing Kinase Mixture (+Kin) to obtain the difference.

$$\text{Net RFU} = (\text{RFU}_{+\text{Kin}}) - (\text{RFU}_{-\text{Kin}})$$

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Recent Product Citations

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