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ab242293 Diacylglycerol Assay Kit

View Diacylglycerol Assay Kit datasheet: www.abcam.com/ab242293

For the detection of Diacylglycerol in cell lysates.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

ab242293 measures diacylglycerol (DAG) 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).

ab242293 is a simple, fluorometric assay that quantitatively measures total DAG 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.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed.

Add 20 µL standard, blank or sample to wells of microtitre plate.

Add 20 µL of Kinase Mixture to the standards and to one half of the paired sample wells, and mix the well contents thoroughly. Add 20 µL of 1X Assay Buffer to the other half of the paired sample wells and mix thoroughly.

Incubate at 37°C for 2 hours.

Transfer 20 µL of the mixture to a 96-well plate suitable for fluorescence measurement. Add 40 µL of Lipase Solution to each well.

Incubate at 37°C for 30 minutes.

Prepare Detection Enzyme Mixture. Add 50 µL to each well.

Incubate for 10 minutes at room temperature protected from light.

Read fluorescence at Ex/Em = 530-560 nm / 585-595 nm

3. General guidelines, precautions, and troubleshooting

- Please observe safe laboratory practice and consult the safety datasheet.
- For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide: www.abcam.com/assaykitguidelines
- For typical data produced using the assay, please see the assay kit datasheet on our website.

4. Materials Supplied, and Storage and Stability

- Store kit at -80°C immediately upon receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt, if components have not been reconstituted.
- Aliquot components in working volumes before storing at the recommended temperature.
- Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage condition
10X Assay Buffer	1.5 mL	-80°C
DAG Standard	1 mL	-80°C
Enzyme Mixture	3 x 1.75 mL	-80°C
Fluorometric Probe	110 µL	-80°C
Kinase Mixture	2 x 1 mL	-80°C
Lipase Solution	3 x 1.4 mL	-80°C

5. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Standard 96-well fluorescence black microtiter plate
- PBS (containing Magnesium and Calcium)
- Extraction reagents for cellular lipids (methanol, chloroform, 1M NaCl) – required to prepare PEU; see section 6.7.
- Fluorescence microplate reader capable of reading excitation in the 530-560 nm range and emission in the 585-595 nm range

6. Reagent Preparation

- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Prepare only as much reagent as is needed on the day of the experiment.
- Any components not listed here are ready to use as supplied.

6.1 1X Assay Buffer

6.1.1 10X Assay Buffer should be thawed/maintained at 4°C during assay preparation. Dilute the 10X Assay Buffer with deionized water. Stir to homogeneity.

6.1.2 The 1X solution is stable for 1 month at 4°C. For longer term storage, any unused 10X stock material should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

6.2 DAG Standard

6.2.1 Thaw at 37°C for 1-2 hours. Once homogeneous and vortexed well, maintain the standard at 37°C during assay preparation.

6.2.2 The solution is stable for 1 week at room temperature; however, the solution must be warmed to 37°C to ensure homogeneity. For longer term storage, the solution should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

6.3 Enzyme Mixture

6.3.1 Thaw at 4°C. Once homogeneous and mixed well, maintain the solution at 4°C during assay preparation.

6.3.2 The solution is stable for 1 week at 4°C. For longer term storage, the solution should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

6.4 Fluorometric Probe

6.4.1 Thaw and maintain at room temperature during assay preparation. Any unused material should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

6.5 Kinase Mixture

6.5.1 Thaw at 4°C. Once homogeneous and mixed well, maintain the solution at 4°C during assay preparation.

6.5.2 The solution is stable for 1 week at 4°C. For longer term storage, the solution should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

6.6 Lipase Solution

6.6.1 Thaw and maintain at room temperature during assay preparation.

6.6.2 Any unused material should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

6.7 PEU (pre-equilibrated upper phase) Solution

6.7.1 Mix 50 mL of chloroform, 50 mL of methanol, and 45 mL of 1M NaCl in a glass container.

6.7.2 Shake or mix the solution well, then allow it to separate into 2 phases. Use the upper phase for washing during the extraction.

ΔNote: Enzyme Mixture, Kinase Mixture and Lipase Solution are provided in multiple tubes to minimize multiple freeze/thaws.

7. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

7.1 Preparation of the DAG Standard Curve

7.1.1 Freshly prepare a dilution series of the DAG Standard in the concentration range of 0 – 2 mM by diluting the standard stock solution (provided at 2 mM) in 1X Assay Buffer.

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

8. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples for the most reproducible assay.

ΔNote: The kit is <u>not</u> recommended for urine, plasma or serum samples.

8.1 Cell lysates (adherent cells):

8.1.1 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.

8.1.2 Centrifuge at 1500 x *g* for 10 minutes. Carefully remove the supernatant and resuspend in 1 mL of cold PBS.

8.1.3 Proceed to the first step of the extraction procedure below.

8.2 Cell lysates (suspension cells)

8.2.1 For suspension cells, collect ~1 x 107 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.

8.2.2 Carefully discard the supernatant and resuspend in 1 mL of cold PBS.

8.2.3 Proceed to the first step the extraction procedure below.

8.3 Extraction procedure:

8.3.1 Sonicate the 1 mL of cell suspension on ice.

8.3.2 Add 1.5 mL of methanol to the sonicated sample.

8.3.3 Add 2.25 mL of 1 M NaCl and 2.5 mL of chloroform to the sample. Vortex well.

8.3.4 Centrifuge at 1500 x g for 10 minutes at 4°C to separate the phases.

8.3.5 Carefully remove the upper aqueous phase and discard.

8.3.6 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 4°C. Carefully remove the upper phase and discard each time.

8.3.7 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.

8.3.8 Dry the lower phase in a speedvac or under a gentle stream of nitrogen.

8.3.9 Resuspend the dried sample with 50 µL of 1X Assay Buffer (1:20 of the original volume). Samples may be stored at -80°C for up to a month.

ΔNote: 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.

9. Assay Procedure

– We recommend that you assay all standards, controls and samples in duplicate.

ΔNote: Maintain the Kinase Mixture, Lipase Solution, and Enzyme Mixture at 4°C during assay preparation.

ΔNote: 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).

9.1 Add 20 µL of the DAG Standards, samples or blanks to the 96-well microtiter plate.

9.2 Add 20 µL of Kinase Mixture to the standards and to one half of the paired sample wells, and mix the well contents thoroughly.

9.3 Add 20 µL of 1X Assay Buffer to the other half of the paired sample wells and mix thoroughly.

9.4 Incubate at 37°C for 2 hours.

9.5 Transfer 20 µL of the mixture to a 96-well plate suitable for fluorescence measurement.

9.6 Add 40 µL of Lipase Solution to each well.

9.7 Incubate at 37°C for 30 minutes.

9.8 During the Step 9.7 incubation, separately prepare the desired volume of Detection Enzyme Mixture according to the table below, based on the number of tests to be performed. Maintaining all components and mixtures at 4°C throughout this step, add components in the following sequence:

9.8.1 In a tube, add the appropriate volume of Enzyme Mixture.

9.8.2 To the Enzyme Mixture, add the corresponding volume of Fluorometric Probe. Mix well and immediately use.

ΔNote: 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)	Number of tests in a 96-well plate (100 µL/test)
5	50	5.05	100
2.5	25	2.525	50
1.25	13	1.263	25

9.9 Transfer 50 µL of the above Detection Enzyme Mixture (from step 9) to each well.
9.10 Cover the plate wells to protect the reaction from light.
9.11 Incubate at room temperature for 10 minutes.
9.12 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.

10. Data Analysis

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.
4. Subtract the sample well values without Kinase Mixture (-Kin) from the sample well values containing Kinase Mixture (+Kin) to obtain the difference.

   Net RFU = (RFU_{+Kin}) - (RFU_{-Kin})

11. Typical Data

One should use the data below for reference only. This data should not be used to interpret actual results.

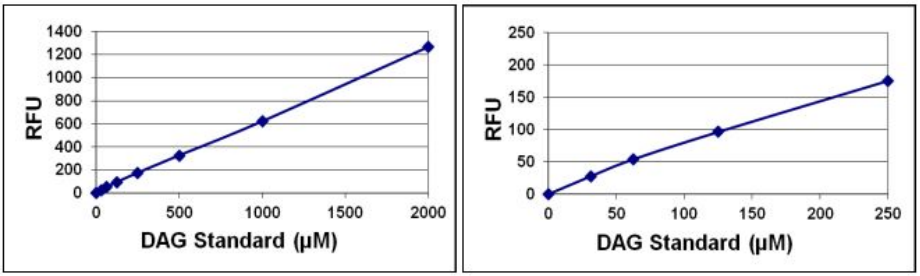

Figure 1. DAG Assay Standard Curve. DAG standard curve was performed according to the Assay Protocol. Background has been subtracted.

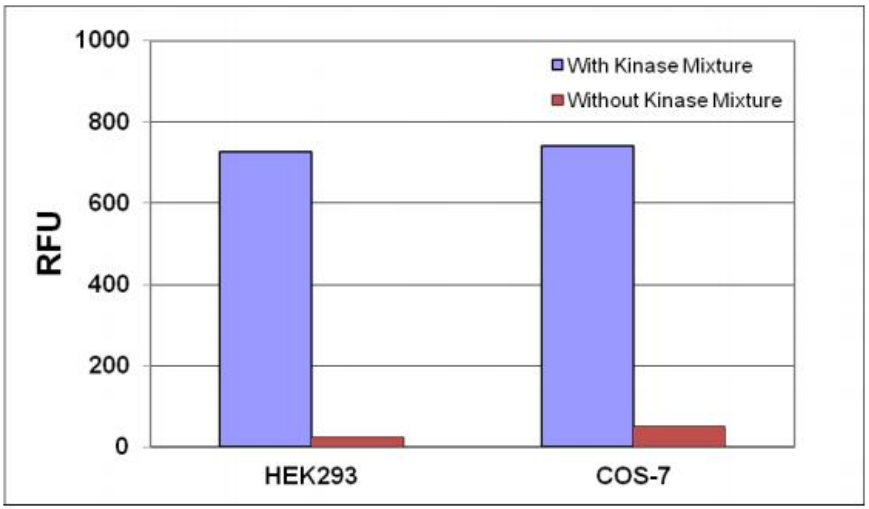

Figure 2. DAG Detection of Lipid Extracts. HEK293 and COS-7 lipid extracts were prepared according to the extraction procedure above. DAG samples were tested according to the Assay Protocol (phosphatidic acid background was determined +/- Kinase Mixture). Negative control values (without DAG) have been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

12. Notes

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