Quantitative Analysis of Cellular Diacylglycerol Content

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[Abstract] Diacylglycerol (DAG) is a bioactive lipid with diverse biological roles. DAG transiently accumulates in a membrane upon receipt of an appropriate stimulus that activates phospholipase C to cleave phospholipids. The resulting hydrolysis product DAG binds to proteins such as protein kinase C to initiate a variety of downstream cellular processes. DAG kinases attenuate such responses by converting DAG to phosphatidic acid.

This protocol describes an assay designed to quantify cellular DAG levels. The assay exploits the enzymatic conversion of DAG (*sn*-1,2-diacylglycerol) to phosphatidic acid (1,2-diacyl- *sn*-glycerol-3-phosphate) in conjunction with the incorporation of a radiolabeled phosphate group by DAG kinase (Figure 1). This assay was described in (Strijbis *et al.*, 2013).

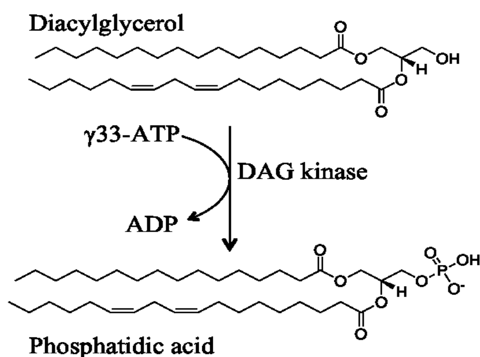

Figure 1. The enzymatic conversion of DAG to phosphatidic acid by DAG kinase

Materials and Reagents

1. Cells (~2 x 10^6 cells)
2. Octyl-β-D-glucoside (Sigma-Aldrich, catalog number: O8001)
3. Cardiolipin (Sigma-Aldrich, catalog number: C5646)
4. Diethylenetriaminepenta acetic acid (DETAPAC) (Sigma-Aldrich, catalog number: D6518)
5. Imidazole (Sigma-Aldrich, catalog number: I5513)
6. NaCl (Sigma-Aldrich, catalog number: S7653)
7. $MgCl_2$ (Sigma-Aldrich, catalog number: M8266)
8. EGTA (Boston Bio Products, catalog number: BM-151)

9. DTT (Sigma-Aldrich, catalog number: D0632)
10. DAG kinase (Sigma-Aldrich, catalog number: D3065)
11. γ^{33}-ATP (PerkinElmer, catalog number: NEG302H001MC)
12. DAG (Avanti Polar Lipids)
13. Phosphatidic acid (Sigma-Aldrich, catalog number: P9511)
14. Chloroform (Thermo Fisher Scientific, catalog number: C298-50)
15. Methanol (Thermo Fisher Scientific, catalog number: BP1105)
16. Acetic acid (Sigma-Aldrich, catalog number: 695092)
17. N_2 gas (Middlesex Gases & Technologies)
18. Acetone (Thermo Fisher Scientific, catalog number: S70090)

Equipment

1. Vortex
2. Centrifuge
3. Thin layer chromatography (TLC) (Whatman, catalog number: 4860-820) equipment (plates, developing tank)
4. Phospho-scanner (*e.g.* Fujifilm Corporation, model: BAS-2500)
5. Phospho-imaging screens (*e.g.* Fujifilm Corporation, model: BAS-MS)

Procedure

A. Prepare the following solutions before starting the assay
   1. Solubilizing buffer: 7.5% (w/v) octyl-β-D-glucoside and 5 mM cardiolipin in 1 mM diethylenetriaminepenta acetic acid (1 mM DETAPAC, pH 7.0; adjust the pH with NaOH).
   2. 1 mM diethylenetriaminepenta acetic acid (1 mM DETAPAC, pH 6.6; adjust the pH with NaOH).
   3. 2x reaction buffer: 100 mM imidazole HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA.
   4. 100 mM DTT in H_2O (prepare fresh).

B. Sample preparation and total lipid extraction
   1. Wash cells (about 2 x 10^6) with phosphate-buffered saline (PBS) and extract lipids according to Bligh and Dyer (Bligh *et al.*, 1959).
   2. Dry the chloroform/methanol phase under a gentle flow of N_2.

C. DAG kinase assay

This assay was adapted from Preiss *et al.* (1986). Steps from 4 onwards need to be performed in lab areas certified for radioactivity work and the proper lab practice should be followed.

1. Dissolve the dried lipids in 40 µl of solubilizing buffer by vigorously vortexing for 20 sec.
2. Incubate the dissolved lipid at room temperature for 10 min.
3. Keep the samples on ice for 5 min and add 100 µl of 2x reaction buffer, 4 µl of 100 mM DTT and 20 µl of *E. coli* DAG kinase while keeping the samples on ice.
4. Initiate the reaction by addition of 3 µCi [γ^{33}]-ATP prepared by dilution in 20 µl of 1 mM DETAPAC (pH 6.6).
5. After vortexing briefly, incubate the reaction at 25 °C for 30 min.
6. Stop the reaction by keeping the tubes on ice, and re-extract and dry lipids as described above.
7. Dissolve the dried lipids in ~50 µl of chloroform/methanol (2/1, v/v).
8. Apply the lipid extracts on a TLC plate along with lipid standards (Phospatidic acid and DAG).
9. Run the TLC plate for 30 min by placing it in a TLC chamber that contains about 75 ml of acetone.
10. Dry the TLC plate and further develop the TLC plate in a chloroform/methanol/acetic acid (65/15/5, v/v/v) solution.
11. Dry the TLC plate using hair dryer and expose it to phospho-imaging screens.
12. Detect radiolabeled lipids by scanning the phospho-imaging screen using BAS-2500 scanner.

Acknowledgments

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