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Direct Measurement of Free and Esterified Cholesterol Mass in Differentiated Human Podocytes: A TLC and Enzymatic Assay-Based Method

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Abstract

Esterified cholesterol content is often lower than free cholesterol content in biological systems and thus the determination of the esterified cholesterol content of cells is often challenging. Traditional methods use enzymatic assays in which an indirect measurement of the esterified cholesterol content is obtained by subtracting the measurements of the free from the total cholesterol content. However, this approach fails in the case where the total cholesterol content of cells is unchanged while the ratio of free to esterified cholesterol changes such that total and free cholesterol content are very similar and thus the difference may fall within the background noise of the enzymatic assay. To overcome this challenge, we here describe a method that utilizes a TLC-based technique to isolate esterified cholesterol. Isolated esterified cholesterol can then be measured using traditional enzymatic methods. Therefore, this method provides a practical and more sensitive assay to measure esterified cholesterol content in cellular extracts.

Keywords

Esterified cholesterol content; Esterified cholesterol mass; Podocyte cholesterol; Cellular cholesterol

1 Introduction

In order to further our understanding of the influence of altered cholesterol metabolism on cellular function, sensitive methods are necessary for the detection and quantification of the cholesterol content of cells. The observation that the esterified cholesterol content of cells is often much lower than the free cholesterol renders the determination of the esterified cholesterol content often challenging. Currently, esterified cholesterol analysis is achieved in various ways including: (a) radiolabeled methods [1], (b) mass spectrometry-based methods [2], or (c) enzymatic assays [3, 4]. Established methods detecting esterified cholesterol were developed in the 1930s [5] and because variable and discrepant results were reported using these methods, the need for more sensitive methods remained. Progress was made that dramatically increased the sensitivity when compared to the original methods by including saponification steps. This step is still utilized in some current methods especially those requiring mass spectrometry analysis [6]. Later, the development of enzymatic methods utilizing the difference between free and total cholesterol for the determination of esterified

cholesterol was developed in tissue extracts [4]. Further enhancement of the sensitivity of these methods was achieved by the introduction of fluorescent probes such as Amplex Red Reagent [3]. While each method is not without limitations as reviewed elsewhere [7, 8], either requiring expensive specialized equipment or being hampered by the lack of sensitivity, the development of *hybrid methods*, similarly to the one described here, allows for a more sensitive detection of the esterified cholesterol content in cells where esterified cholesterol accounts for less than 10% of the total cholesterol. The method presented here requires the isolation of total cholesterol, followed by a TLC-based separation of esterified cholesterol. Esterified cholesterol is then converted to free cholesterol by an enzymatic reaction and the cholesterol content is measured using the sensitive Amplex Red-based cholesterol determination method.

2 Materials

2.1 Lipid Extraction

1. 10× PBS: weigh 800 g NaCl, 20 g KCl, 144 g Na₂HPO₄*2H₂O, 24 g KH₂PO₄ up to 8 L of deionized water. Adjust pH with HCl to 6.8. Store at room temperature.
2. 1× PBS: add 100 mL of 10× PBS to 900 mL of deionized water. Store at room temperature.
3. Extraction Solution: Hexane:Isopropanol (3:2, v/v). Add 30 mL of hexane to 20 mL of isopropanol. Store at room temperature.

2.2 Protein Assay

1. Lysis Buffer: 0.1% SDS in 0.1 M NaOH. Add 500 µL of 10% SDS to 45 mL of deionized water. Then add 5 mL of 1 M NaOH. Store at room temperature.

2.3 Isolation of Esterified Cholesterol

1. Chloroform.
2. TLC Solvent: Hexane:Ether:Acetic Acid (130:40:1.5). Add 130 mL Hexane, 40 mL Ether, and 1.5 mL Acetic Acid. Store at room temperature.
3. TLC Plate: Silica-coated glass plates.
4. Iodine.
5. Cholesteryl Oleate. Add 1 mg cholesteryl oleate to 1 mL of chloroform. Store at −20 °C.

2.4 Cholesterol Quantification

1. 5× Assay Buffer: 0.5 M Potassium Phosphate pH 7.4, 0.25 M NaCl, 25 mM Cholic Acid, 0.5% Triton X-100. Make 500 mL of 0.5 M anhydrous K₂HPO₄ (43.55 g) and 0.5 M anhydrous KH₂PO₄ (34.02 g). To 400 mL of the 0.5 M anhydrous KH₂PO₄ solution, gradually add the 0.5 M anhydrous K₂HPO₄ (43.55 g) until the pH reaches 7.4. Adjust with HCl if necessary. Store solution at 4 °C.

2. 1× Assay Buffer. Add 2.5 mL of 5× Assay Buffer to 10 mL of deionized water.
3. 20 mM Amplex Red Reagent. Add 200 µL DMSO to 1 mg Amplex Red.
4. 200 U/mL Horseradish peroxidase. Dissolve HRP to 200 U/mL in 1× Assay Buffer. Store at −20 °C.
5. 200 U/mL Cholesterol oxidase. Dissolve cholesterol oxidase to 200 U/mL in 1× Assay Buffer. Store at −20 °C.
6. 200 U/mL Cholesterol Esterase. Dissolve cholesterol esterase to 200 U/mL in 1× Assay Buffer. Store at −20 °C.
7. Working Solution: 0.3 mM Amplex Red, 10 U HRP, 10 U Cholesterol Oxidase, 1 U Cholesterol Esterase, 1× Reaction Buffer. Add 75 µL Amplex Red Reagent, 50 µL horse radish peroxidase, 50 µL Cholesterol Oxidase and 5 µL Cholesterol Esterase to 4.82 mL 1× Assay Buffer (*see Note 1*).

3 Methods

Carry out all procedures under a ventilated hood unless specified.

3.1 Lipid Extraction

1. Differentiate normal human podocytes (approximately 2×10^5 cells per 10 cm dish) for 14 days at 37 °C (*see Note 2*).
2. Rinse cells with 1× PBS and remove all liquid (*see Note 3*).
3. Add 5 mL of extraction solution to each dish and incubate for 30 min at room temperature.
4. Collect the extraction solution from the plate and transfer to a glass test tube.
5. Repeat **steps 3 and 4**.
6. Allow plates to dry for 5 min at room temperature (*see Note 4*).

3.2 Protein Assay

1. Add lysis buffer to the plate and detach the cells using a cell scraper. Transfer the cell lysate to an Eppendorf tube.
2. Samples were centrifuged at $21,000 \times g$ for 20 min.
3. Transfer supernatants to a new eppendorf tube.
4. Determine protein concentration for each supernatant using standard BCA protein quantification (*see Note 5*).

¹The Amplex Red Cholesterol Assay Kit (Invitrogen) can be used as it contains all elements necessary for quantification.

²At least 10 cm² area dish of differentiated normal human podocytes is necessary for measuring esterified cholesterol content.

³If long-term storage of samples is required, the cell-containing plates can be stored at −80 °C.

⁴If long-term storage of samples is required before proceeding to protein extraction, the cell-containing plates can be stored at −80 °C.

⁵Our experience shows no matrix interference using the Pierce BCA protein assay kit.

3.3 Isolation of Esterified Cholesterol

1. Place samples from Subheading 3.1, **step 5** in 37 °C water bath and simultaneously dry lipids under a nitrogen (N₂) gas stream (*see* Note 6).
2. Dissolve lipids in 150 µL of chloroform in a glass test tube.
3. Centrifuge samples at 3000 × *g* for 5 min at room temperature.
4. Spot the entire sample and reference sample (cholesterol oleate) onto TLC plates (*see* Note 7).
5. Develop the TLC plate in Hexane:Ether:Acetic Acid (130:40:1.5) in TLC Chamber (*see* Fig. 1) until solvent reaches approximately ¾ up plate (*see* Note 8).
6. Remove the TLC plate from the chamber and allow solvents to evaporate at room temperature.
7. Place open Iodine bottle and TLC plate into clean TLC chamber until yellow spots are visible (*see* Note 9).
8. Mark the spots gently using a pencil.
9. Leave plate in a ventilated hood until the yellow color has dissipated.
10. Scrape silica-containing spots off the plates using a razor blade and transfer into glass test tubes.
11. Add 1 mL of chloroform to each test tube and incubate at room temperature for 5 min.
12. Centrifuge samples at 3000 × *g* for 3 min at room temperature.
13. Pipette supernatant to new test tube and repeat **steps 11–13** (*see* Note 8).
14. Place samples in 37 °C water bath and simultaneously dry lipids under nitrogen (N₂) gas stream (*see* Note 10).

3.4 Measurement of Esterified Cholesterol Mass

1. Prepare a standard curve in 1× Assay Buffer with standards ranging from 8 to 0.06 µg/mL.
2. Dilute esterified cholesterol samples in 100 µL of 1× Assay Buffer.
3. Add 50 µL of sample or standard to each well.
4. Add 50 µL of working solution per well.

⁶A water bath is not absolutely necessary in this step but will significantly shorten the drying process.

⁷Use of an AIS Analytical Instrument Specialties 4A TLC Multi-Spotter will allow for higher sample throughput.

⁸The Hexane:Ether:Acetic Acid solvent solution should be added to the TLC chamber prior to the addition of the TLC plate. If necessary, line the inside of the TLC Chamber with filter paper to ensure proper equilibration of solvent solution. Additionally, upon addition of the TLC plate into the TLC chamber, make sure the Hexane:Ether:Acetic Acid fills the bottom of the chamber, but does not directly touch the samples.

⁹Pre-equilibration of the TLC chamber with Iodine can reduce the visualization time to less than 5 min.

¹⁰If long-term storage of the samples is necessary before proceeding to the quantification, samples can be stored at –20°C.

5. Incubate at 37 °C for 30 min.
6. Measure fluorescence on plate reader, Excitation 530–560 nm and Emission 590 nm.
7. Perform analysis utilizing standard protocol for the determination of analytes by standard curve.

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Fig. 1. Cholesterol ester isolation. The TLC chamber is pre-equilibrated chamber with TLC solvent. The TLC plate is placed into the chamber, with the samples above the solvent. Allow the solvent to travel up the plate until approximately $\frac{3}{4}$ to the top at room temperature