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Isolation and Analysis of Plasma Lipoproteins by Ultracentrifugation

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Abstract

Analysis of plasma lipoproteins and apolipoproteins is an essential part for the diagnosis of dyslipidemia and studies of lipid metabolism and atherosclerosis. Although there are several methods for analyzing plasma lipoproteins, ultracentrifugation is still one of the most popular and reliable methods. Because of its intact separation procedure, the lipoprotein fractions isolated by this method can be used for analysis of lipids, apolipoproteins, proteomes, and functional study of lipoproteins with cultured cells in vitro. Here, we provide a detailed protocol to isolate seven lipoprotein fractions including VLDL (d<1.006 g/mL), IDL (d=1.02 g/mL), LDLs (d=1.04 and 1.06 g/mL), HDLs (d=1.08, 1.10, and 1.21 g/mL) from rabbit plasma using sequential floating ultracentrifugation. In addition, we introduce the readers how to analyze apolipoproteins such as apoA-I, apoB, and apoE by SDS-PAGE and Western blotting and show representative results of lipoprotein and apolipoprotein profiles using hyperlipidemic rabbit models. This method can become a standard protocol for both clinicians and basic scientists to analyze lipoprotein functions.

Introduction

Dyslipidemia is the major risk factor of atherosclerotic disease in the world. High levels of low-density lipoproteins (LDLs) and low levels of high-density lipoproteins (HDLs) are closely associated with a high risk of coronary heart disease (CHD)[1,2]. In the clinical setting, both LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) are routinely measured using an automated analyzer in a clinical laboratory[3,4]. Despite this, it is essential to analyze lipoprotein profiles in details for the diagnosis of dyslipidemia and the study of lipid metabolism and atherosclerosis in human and experimental animals. Several methods have been reported to analyze plasma lipoproteins such as ultracentrifugation, size exclusion chromatography [fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC)], electrophoresis by agarose and polyacrylamide gels, nuclear magnetic resonance, and selective chemical precipitation using polyanions and divalent cations or other chemicals. In 1950's,

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Havel's group first proposed the concept of lipoproteins defined by densities using ultracentrifugation and classified them into chylomicrons (CM), very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL)[5] and later on, the method was further modified by other groups[6,7]. Until now, ultracentrifugation is the most popular and reliable method while the practical protocol is still not available. In this paper, we attempted to describe an easy-to-use protocol for isolating a small scale of plasma using sequential density floating ultracentrifugation originally described previously[8]. Isolation of seven plasma lipoprotein fractions [VLDL (d<1.006 g/mL), IDL (d=1.02 g/mL), LDLs (d=1.04 and 1.06 g/mL), HDLs (d=1.08, 1.10, and 1.21 g/mL)] enables researchers to make an extensive analysis of both lipoproteins and their compositional apolipoproteins[9,10,11]. The intact seven consecutive density lipoproteins can be used for analyzing lipoprotein functions using cell-based in vitro strategies. This protocol should be useful for both clinical diagnosis and basic research. Here we will use rabbit plasma as an example to demonstrate this technique while plasma from other species can be applied in the same way.

Protocol

All procedures for rabbit studies were performed with approval of University of Yamanashi Institutional Animal Care and Use Committee (Approved number: A28-39).

1. Plasma separation from rabbit blood

1. Prepare 1.5 mL microtubes containing 15 µL of 0.5 M EDTA (pH 8.0) for blood collection.
2. Put a rabbit in a restrainer and puncture an auricular intermediate artery using a 22-g needle and collect blood into a tube. Mix the blood with EDTA gently and put them on ice.
3. Centrifuge the blood tubes at 1,500 x *g* for 20 min at 4 °C and collect plasma to a new tube.

   NOTE: 3 mL of blood is enough for collecting 1 mL plasma. If you collect blood from mice or other small animals, you need to pool them.

2. Isolation of plasma lipoproteins

NOTE: The schematic procedure is shown in **Figure 1**. The preparation method of potassium bromide (KBr) density solutions is shown in **Table 1**.

1. Transfer 1 mL of plasma to a polycarbonate ultracentrifuge tube (**Figure 2A**).
2. Load these tubes in a fixed angle rotor and centrifuge the plasma at 356,000 x *g* for 2.5 h at 4 °C (**Figure 2B**).

   NOTE: For Beckman TLA 120.2 rotor, 356,000 x *g* (average relative centrifugal field, Av RCF) corresponds to 100,000 rpm.
3. Cut the tubes using a slicer and then collect the top fraction [VLDL (d<1.006 g/mL)], approximately 200 µL into a new microtube (**Figure 2C**). Collect the remaining bottom fraction into a new polycarbonate ultracentrifuge tube for the next separation (**Figure 2D**). Measure the volume and make the total volume to 800 µL by adding the same density solution (d=1.006 g/mL).

   1. Set up a blade to the tube slicer. Adjust the position of the blade at a level between the top fraction (200 µL) and the bottom fraction (800 µL).

      NOTE: The viscous precipitant in the bottom fraction should be collected carefully and completely by pipetting in all following steps.

2. If pause, do so after separating the top and bottom fraction in all steps. Store the sample at 4°C until the next centrifugation.

4. Adjust the bottom fraction (total 800 µL) to d=1.02 g/mL by adding 58.9 µL of d=1.21 g/mL solution and 141.1 µL of d=1.02 g/mL solution (the total volume is 1 mL).

5. Load these tubes in fixed angle rotor and centrifuge at 356,000 x *g* for 2.5 h at 4 °C.

6. Cut the tubes using a slicer and then collect the top fraction [IDL (d=1.02 g/mL)] approximately 200 µL into a new microtube. Collect the remaining bottom fraction into a new polycarbonate ultracentrifuge tube for the next separation. Measure the volume and make the total volume to 800 µL by adding the same density solution (d=1.02 g/mL).

7. Adjust the bottom fraction (total 800 µL) to d=1.04 g/mL by adding 94.1 µL of d=1.21 g/mL solution and 105.9 µL of d=1.04 g/mL solution (the total volume is 1 mL).

8. Load these tubes in a fixed angle rotor and centrifuge at 356,000 x *g* for 2.5 h at 4 °C.

9. Cut the tubes using a slicer and then collect the top fraction [LDL (d=1.04 g/mL)] approximately 200 µL into a new microtube. Collect the remaining bottom fraction into a new polycarbonate ultracentrifuge tube for the next separation. Measure the volume and make the total to 800 µL by adding the same density solution (d=1.04 g/mL).

10. Adjust the bottom fraction (total 800 µL) to d=1.06 g/mL by adding 106.7 µL of d=1.21 g/mL solution and 93.3 µL of d=1.06 g/mL solution (the total volume is 1 mL).

11. Load these tubes in a fixed angle rotor and centrifuge at 356,000 x *g* for 2.5 h at 4 °C.

12. Cut the tubes using a slicer and then collect the top fraction [LDL (d=1.06 g/mL)] approximately 200 µL into a new microtube. Collect the remaining bottom fraction into a new polycarbonate ultracentrifuge tube for the next separation. Measure the volume and make the total to 800 µL by adding the same density solution (d=1.06 g/mL).

13. Adjust the bottom fraction (total 800 µL) to d=1.08 g/mL by adding 123.1 µL of d=1.21 g/mL solution and 76.9 µL of d=1.08 g/mL solution (the total volume is 1 mL).

14. Load these tubes in a fixed angle rotor and centrifuge at 356,000 x *g* for 2.5 h at 4 °C.

15. Cut the tubes using a slicer and then collect the top fraction [HDL_2 (d=1.08 g/mL)] approximately 200 µL into a new microtube. Collect the remaining bottom fraction into a new polycarbonate ultracentrifuge tube for the next separation. Measure the volume and make the total to 800 µL by adding the same density solution (d=1.08 g/mL).

16. Adjust bottom fraction (total 800 µL) to d=1.10 g/mL by adding 145.5 µL of d=1.21 g/mL solution and 54.5 µL of d=1.10 g/mL solution (the total volume is 1 mL).

17. Load these tubes in a fixed angle rotor and centrifuge at 356,000 x *g* for 2.5 h at 4 °C.

18. Cut the tubes using a slicer and then collect the top fraction [HDL_2 (d=1.10 g/mL)] approximately 200 µL into a new microtube. Collect the remaining bottom fraction into a new polycarbonate ultracentrifuge tube for the next separation. Measure the volume and make the total to 800 µL by adding the same density solution (d=1.10 g/mL).

19. Adjust bottom fraction (total 800 µL) to d=1.21 g/mL by adding 0.140 g of potassium bromide (KBr) powder and dissolve it completely. Measure the volume and make them to 1 mL by adding d=1.21 g/mL solution.

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20. Load these tubes in fixed angle rotor and centrifuge at 513,000 x *g* for 4 h at 4 °C.
    NOTE: For Beckman 120.2 rotor, 513,000 x *g* (Av RCF) corresponds to 120,000 rpm.
21. Cut the tubes using a slicer and then collect the top fraction [HDL_3 (d=1.21 g/mL)] approximately 200 µL into a new microtube. This is the last step for ultracentrifugation.

3. Dialysis

NOTE: Because the density fractions (except d<1.006 g/mL fraction) contain high concentrations of KBr, it is necessary to remove them by dialysis.

1. Transfer the density fractions to a dialysis tubing and clip both ends with closures.
2. Dialyze against 2 L of dialysis buffer such as PBS/ 1 mM EDTA for 6 h at 4 °C with agitation using a magnetic stirrer.
3. Replace with new buffer and dialyze again for another 6 h at 4 °C.
4. Collect the density fractions and measure the volume. Adjust all of the density fractions to the same volume (e.g., 250 µL).
   NOTE: You can calculate the concentrated factor from 1 mL of original plasma (e.g., 250 µL of density fraction corresponds to four times concentration).

4. Analysis of lipoproteins

NOTE: After dialysis, these lipoproteins are ready for different analyses. Lipoproteins can be evaluated by measuring either lipids or apolipoproteins or both. For measuring lipid contents such as total cholesterol (TC), triglycerides (TG), phospholipids (PL), and free cholesterol (FC) in each fraction, we use commercial enzymatic colorimetric assay kits. For analysis of apolipoproteins, we use SDS-PAGE visualized with CBB staining or Western blotting.

1. Lipid analysis
   NOTE: Lipids such as TC, TG, PL and FC in the lipoprotein fraction can be measured using commercially available measurement kits. The procedures depend on the reagents used, so follow the instructions of each kit used. Here we show a typical microplate assay using a commercial enzymatic assay kit.
   1. Apply 8 µL of lipoprotein sample and standard calibration substance on 96-well microplate.
   2. Add 240 µL of assay reagent and mix by pipetting.
   3. Incubate at 37 °C for 10 min.
   4. Measure the OD with a microplate reader and calculate lipid concentrations.
2. Apolipoprotein analysis
   1. SDS-PAGE and CBB staining
      1. Sample preparation: Add 10 µL of 2x Sample buffer to 10 µL of lipoprotein fraction. Heat the mixture at 80 °C for 5 min using a dry heat block.
      2. Prepare 4-20% gradient SDS-polyacrylamide gel and set up the gel on the electrophoresis chamber filled with running buffer.
      3. Load lipoprotein sample (10 µL/ lane) and protein standards (5 µL/ lane) on the stacking gel.
         NOTE: If the samples are two times concentrated, an equivalent amount of 20 µL plasma will be analyzed per lane.]
      4. Run electrophoresis at 20–40 mA constant current.

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5. CBB staining: Soak the gel twice in fixing solution with gentle shaking for 10 min. Stain the gel with CBB staining solution for 30 min. Rinse the gel in distilled water to remove the excess stain. The Gel is ready for photographing.

2. Western blotting

1. Perform electrophoresis in the same procedure as described above (4.2.1.2–4.2.1.4).

NOTE: The volume of lipoproteins loaded for the Western blotting is less than for CBB staining described above (e.g., 1–5 µL).

2. Place the gel and PVDF membrane between transfer buffer-soaked filter paper and form pad. Set up the sandwich in a holder cassette and place in a tank filled with transfer buffer.

3. Perform electroblotting at 100 mA constant current for 3 h at 4 °C.

4. Blocking: Incubate the membrane in blocking buffer for 1 h at room temperature or overnight at 4 °C.

5. Primary Ab reaction: Incubate the membranes in the primary Abs diluted with blocking buffer for 1 h at room temperature or overnight at 4 °C with mild shaking.

NOTE: Suggested primary Ab dilution is shown in the **Table of Materials**. Three kinds of primary Abs can be used singly or in cocktails.

6. Wash the membrane three times in washing buffer with agitation, 5 min per wash.

7. Secondary Ab reaction: Incubate the membrane in the secondary Abs diluted with blocking buffer for 1 h at room temperature with agitation.

NOTE: Suggested secondary Ab dilution is shown in the **Table of Materials**.

8. Wash the membrane three times in washing buffer with agitation, 5 min per wash.

9. ECL detection: Place the membrane on a plastic wrap. Add ECL detection reagents and incubate for 1 min. Drain excess liquid and seal the membrane in a bag.

10. Visualize the signals using an image analyzer.

Representative Results

Using this protocol, we isolated rabbit lipoproteins using 1 mL of plasma and obtained seven density fractions. Isolated density fractions are enough for measuring lipids and apolipoproteins as described above for most research purposes. The same procedure can also be used for isolating plasma lipoproteins from human and other species. For small-sized animals such as mice, pooled plasma is required. **Figure 3** shows lipoprotein profiles of rabbits fed either a normal standard (NS) diet or high cholesterol (HC) diet. Rabbits are herbivore animals so their plasma TC, TG, and PL levels are generally lower than humans and mice. In NS diet-fed rabbits, TC is mainly distributed in HDL_3 (d=1.21 g/mL) and followed by LDL (d=1.04 g/mL) (**Figure 3A**). In wild-type (WT) rabbits on a NS diet, 39% of plasma TG are distributed in VLDLs whereas 57% of plasma PL is contained in HDLs (**Figure 3A**). When rabbits were challenged with a diet supplemented with high cholesterol, they rapidly developed into hypercholesterolemia. As shown in **Figure 3B**, the lipoprotein profiles are characterized by marked elevation of VLDLs (165-fold↑ in VLDL-TC, 1.5-fold↑ in VLDL-TG, and 30-fold↑ in VLDL-PL compared with NS-fed rabbits). Because VLDLs isolated from cholesterol-fed rabbits are rich in cholesteryl esters and move to the β position on agarose gel

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electrophoresis, they are often called β-VLDLs to distinguish them from normal VLDLs which move to pre-β position.

In addition to lipids, apolipoproteins can be simply analyzed by SDS-PAGE either by CBB staining or Western blotting (**Figure 4**). Seven lipoprotein fractions from WT rabbits fed either a NS or a HC diet were run on a 4-20% SDS-polyacrylamide gel and visualized with CBB staining (**Figure 4A**). On a HC diet, both apoB-100 and apoE contents in VLDLs (d<1.006 g/mL), IDLs (d=1.02 g/mL), and LDLs (d=1.04 g/mL) were markedly elevated compared with rabbits on a NS diet. Furthermore, we also compared apolipoprotein and lipoprotein profiles of three different hyperlipidemic rabbits: HC diet-fed WT rabbits, apoE knockout (KO) rabbits, and Watanabe heritable hyperlipidemic (WHHL) rabbits with LDL receptor deficiency by Western blotting (**Figure 4B**). Plasma lipoproteins were fractionated by 4-20% SDS-PAGE and followed by Western blotting with antibodies against apoE, apoB, and apoA-I. The apoB-containing particles (VLDLs, IDLs and LDLs) of HC diet-fed WT rabbits are characterized by increased apoB-100 and apoE contents whereas apoE KO rabbits showed marked increase of apoB-48 along with the appearance of apoA-I. WHHL rabbits are genetically deficient in LDL receptor functions so there is a marked increase of apoB-100 in LDLs accompanied by reduced apoA-I in HDLs, which is similar to human familial hypercholesterolemia.

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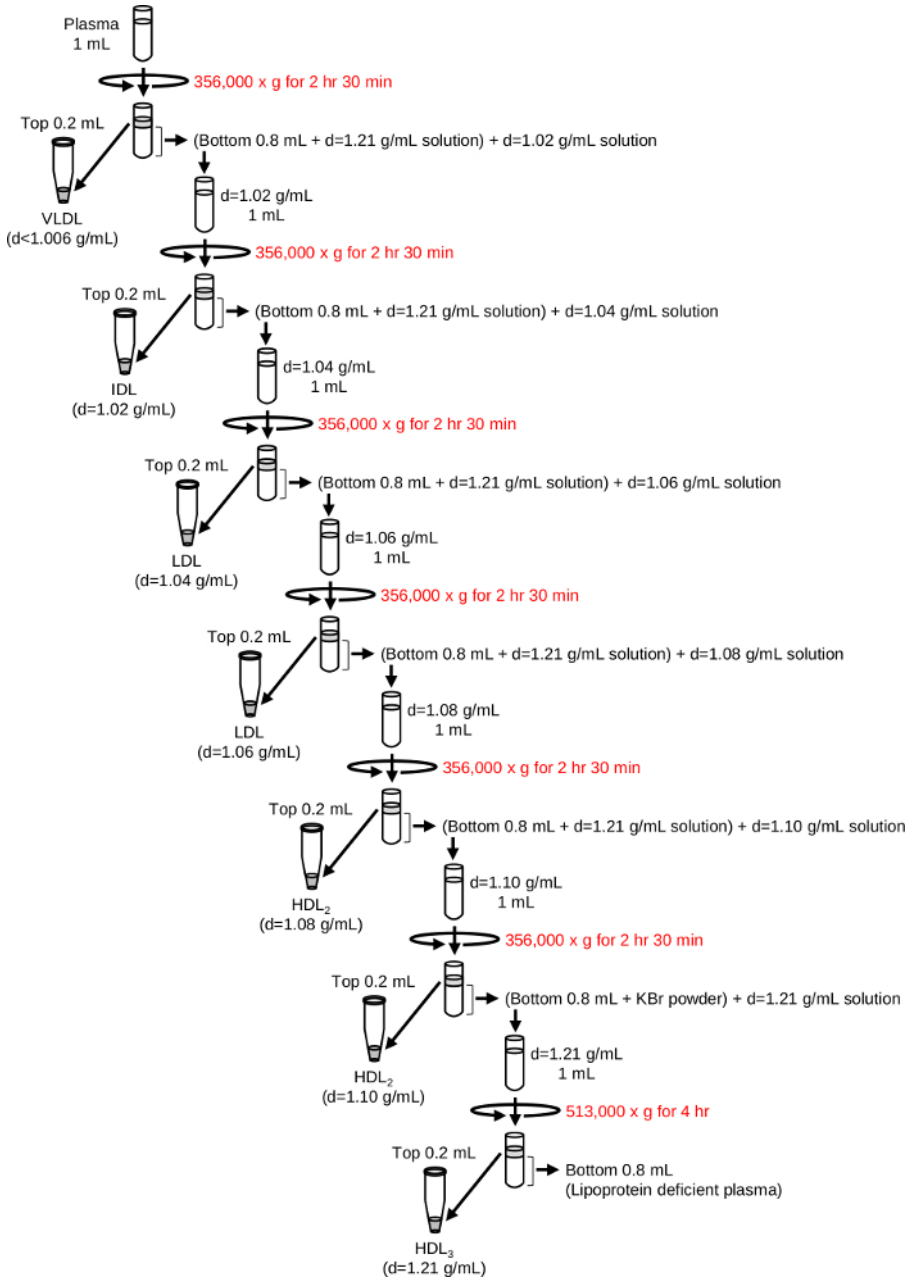

Figure 1: Schematic illustration of sequential floating ultracentrifugation. Please click here to view a larger version of this figure.

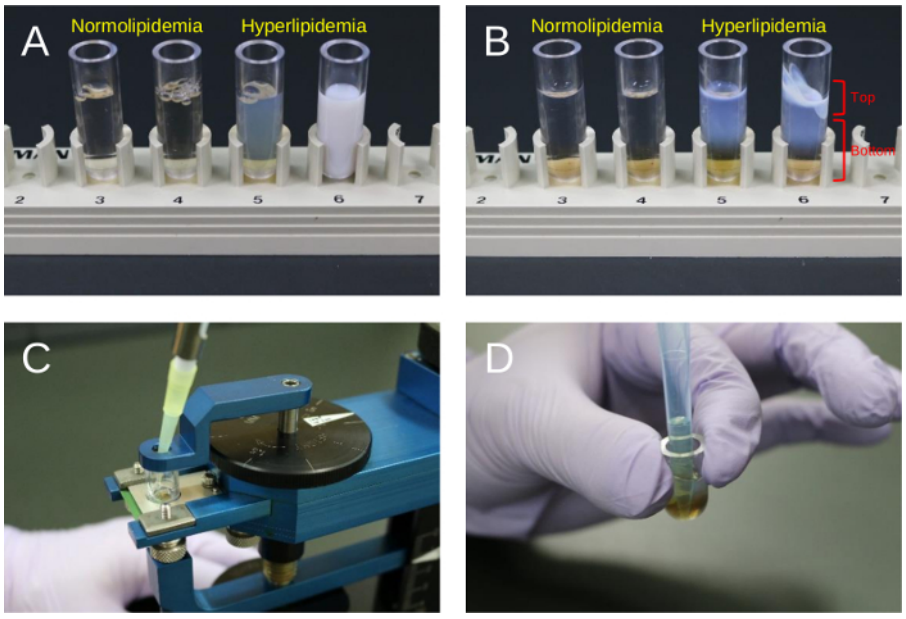

Figure 2: Representative images of plasma lipoprotein isolation. (**A**) Plasma of normolipidemic and hyperlipidemic rabbits. (**B**) Floated top VLDL (d<1.006 g/mL) and bottom fraction (d>1.006 g/mL) after ultracentrifugation. (**C**) Tube slicing and collection of the top fraction by a pipette. (**D**) Collection of the bottom fraction. Please click here to view a larger version of this figure.

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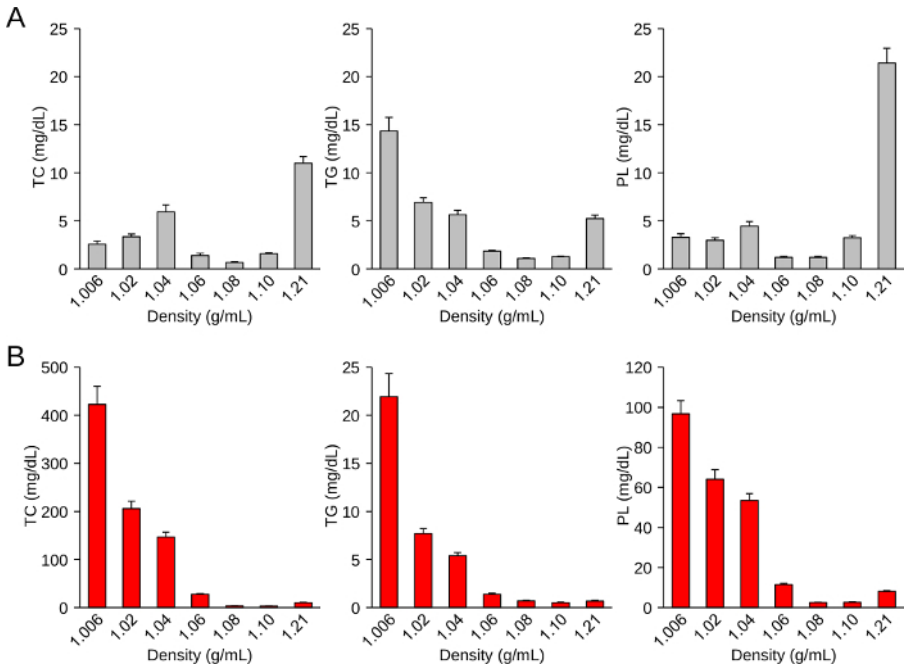

Figure 3: Quantitation of lipid contents in each lipoprotein fraction isolated from normal rabbits (A) and cholesterol-fed rabbits (B). Plasma lipoproteins were separated by sequential floating ultracentrifugation from plasma of wild-type rabbits on either a normal standard diet (**A**) or a high cholesterol diet (**B**). Total cholesterol (TC), triglycerides (TG) and phospholipids (PL) were measured. Data are expressed as mean ± SEM (n=6). This figure has been modified from Yan H et al.[12] . Please click here to view a larger version of this figure.

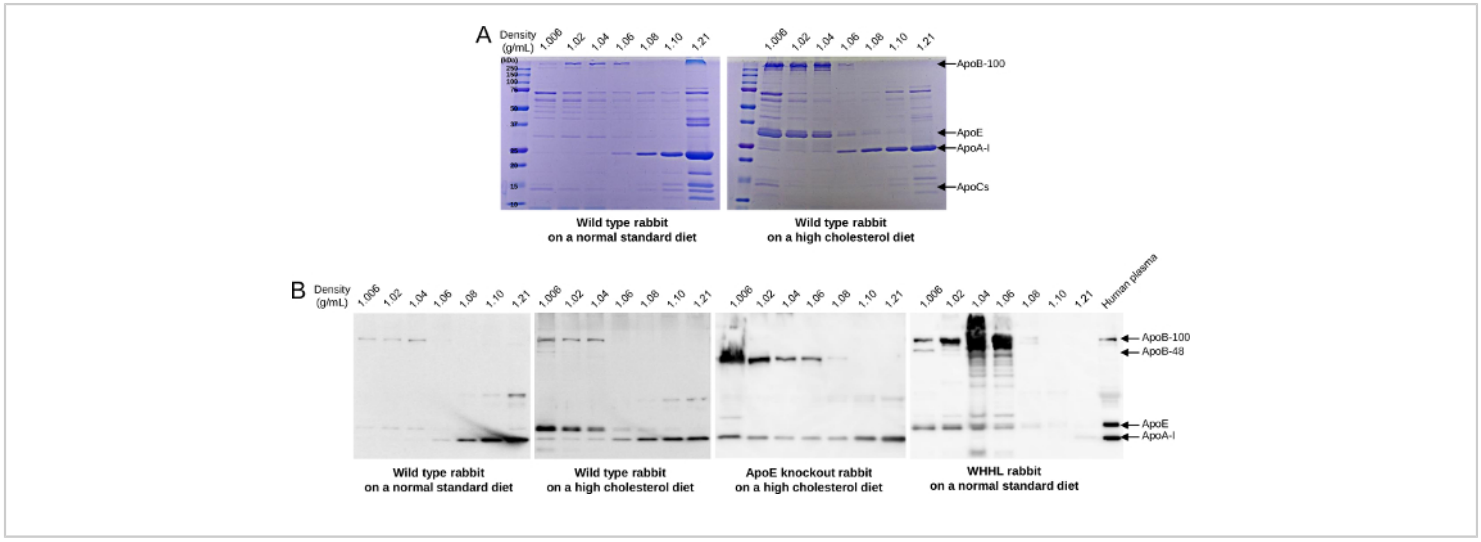

Figure 4: Comparison of apolipoprotein distribution using CBB staining (A) and Western blotting (B). Plasma was isolated by ultracentrifugation and lipoproteins were fractionated by 4-20% SDS-PAGE. (**A**) Apolipoprotein profiles of wild-type (WT) rabbit fed on a normal standard (NS) diet and a high cholesterol (HC) diet were visualized by CBB staining. Compared with NS diet-fed rabbits (left), HC diet-fed rabbits (right) showed increased apoB-100 and apoE contents in VLDLs, IDLs, and LDLs. (**B**) Comparison of apolipoprotein features of WT rabbits on a NS or a HC diet, apoE KO (knockout) rabbits on a HC diet, and WHHL rabbits on a NS diet. Western blotting was performed with “cocktail antibodies” against apoE, apoB, and apoA-I. Compared with normolipidemic rabbits (WT rabbits on a NS diet, first left), other three hyperlipidemic rabbits exhibit unique and different apolipoprotein profiles. The apoB-containing particles (VLDLs, IDLs and LDLs) of HC-fed WT rabbits are characterized by increased apoB-100 and apoE contents whereas apoE KO rabbits showed marked increase of apoB-48 along with the appearance of apoA-I. WHHL exhibited marked increase of apoB-100 in LDLs accompanied by reduced apoA-I in HDLs. This figure has been modified from Niimi M et al.[10] . Please click here to view a larger version of this figure.

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Density solution (g/mL)	KBr (g)	Distilled water (mL)
d=1.006	8.404	1000
d=1.02	28.271	1000
d=1.04	57.261	1000
d=1.06	86.958	1000
d=1.08	117.365	1000
d=1.10	148.490	1000
d=1.21	333.394	1000

Table 1: Preparation for potassium bromide (KBr) density solutions. The weight (g) of KBr add to 1000 mL distilled water is shown.

Discussion

Hyperlipidemia is one of the most important risk factors of atherosclerotic disease. Thus, analysis of plasma lipoproteins is not only essential for diagnosis of dyslipidemia patients but also important for investigation of molecular mechanisms of lipoprotein metabolism and atherosclerosis. In this study, we described the protocol of isolation and analysis of plasma lipoproteins which can be applied in the laboratories where ultracentrifugation is available. Information obtained by this method is comprehensive and straightforward therefore it is recommended for both clinical and basic research scientists. ~~Lipoproteins isolated~~ can also be used for investigating many other facets of lipoprotein functions such as negative-stain electron microscopy[13,14], oxidizability[9,15], proteomics[16], cell culture based in vitro study such as cholesterol efflux assay[9] and lipoprotein uptake assay[17].

It should be pointed out; however, there are several weak points that need to take considerations. First, this method is relatively time-consuming (three days at least) compared with other methods. If using the latest tabletop ultracentrifuge, high-speed spin (150,000 rpm) can shorten separating time 50–140 min for each lipoprotein fraction[18]. Second, sample loss may happen during the isolation. To minimize sample loss, the viscous precipitant in the bottom fraction should be dissolved and collected carefully by pipetting. The centrifuge tubes need to fix tightly with a slicer to avoid leaking of the top fraction. In addition, the samples should be recovered carefully from dialysis bags. The recovery can be calculated by comparing the total lipoprotein cholesterol with the original plasma cholesterol. According to our experience, general recovery rate will be ≈ 80%[19]. Third, this protocol is limited for small number of the samples because one rotor can only run twelve tubes a time. To perform a large number of samples, it may be suitable to use other methods such as precipitation method for HDL preparation[4] and automated HPLC lipoprotein analysis[20].

In summary, this protocol provides a guide for researchers to isolate plasma lipoproteins using sequential density ultracentrifugation and the analysis of lipids and apolipoproteins.

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Disclosures

The authors have nothing to disclose.

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