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TITLE:

Isolation and Analysis of Plasma Lipoproteins by Ultracentrifugation

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lipoprotein, apolipoprotein, lipid metabolism, dyslipidemia, ultracentrifugation, animal model

SUMMARY:

Several methods have been used for analyzing plasma lipoproteins; however, ultracentrifugation is still one of the most popular and reliable methods. Here, we describe a method regarding how to isolate lipoproteins from plasma using sequential density ultracentrifugation and how to analyze the apolipoproteins for both diagnostic and research purposes.

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, 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[9–11]. 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

1.1. Prepare 1.5 mL microtubes containing 15 µL of 0.5 M EDTA (pH 8.0) for blood collection.

1.2. 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.

1.3. 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**.

2.1. Transfer 1 mL of plasma to a polycarbonate ultracentrifuge tube (**Figure 2A**).

2.2. 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.

2.3. 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).

2.3.1. 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.

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

2.4. 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).

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

2.6. 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).

2.7. 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).

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

2.9. 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).

2.10. 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).

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

2.12. 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).

2.13. 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).

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

2.15. 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).

2.16. 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).

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

2.18. 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).

2.19. 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.

2.20. 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.

2.21. 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.

3.1. Transfer the density fractions to a dialysis tubing and clip both ends with closures.

3.2. 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.

3.3. Replace with new buffer and dialyze again for another 6 h at 4 °C.

3.4. 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.

4.1 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.

4.1.1. Apply 8 µL of lipoprotein sample and standard calibration substance on 96-well microplate.

4.1.2. Add 240 µL of assay reagent and mix by pipetting.

4.1.3. Incubate at 37 °C for 10 min.

4.1.4. Measure the OD with a microplate reader and calculate lipid concentrations.

4.2 Apolipoprotein analysis

4.2.1 SDS-PAGE and CBB staining

4.2.1.1 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.

4.2.1.2 Prepare 4-20% gradient SDS-polyacrylamide gel and set up the gel on the electrophoresis chamber filled with running buffer.

4.2.1.3 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.]

4.2.1.4 Run electrophoresis at 20–40 mA constant current.

4.2.1.5 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.

4.2.2 Western blotting

4.2.2.1 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).

4.2.2.2 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.

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

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

4.2.2.5 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.

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

4.2.2.7 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**.

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

4.2.2.9 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.

4.2.2.10 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 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.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic illustration of sequential floating ultracentrifugation.

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.

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].

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].

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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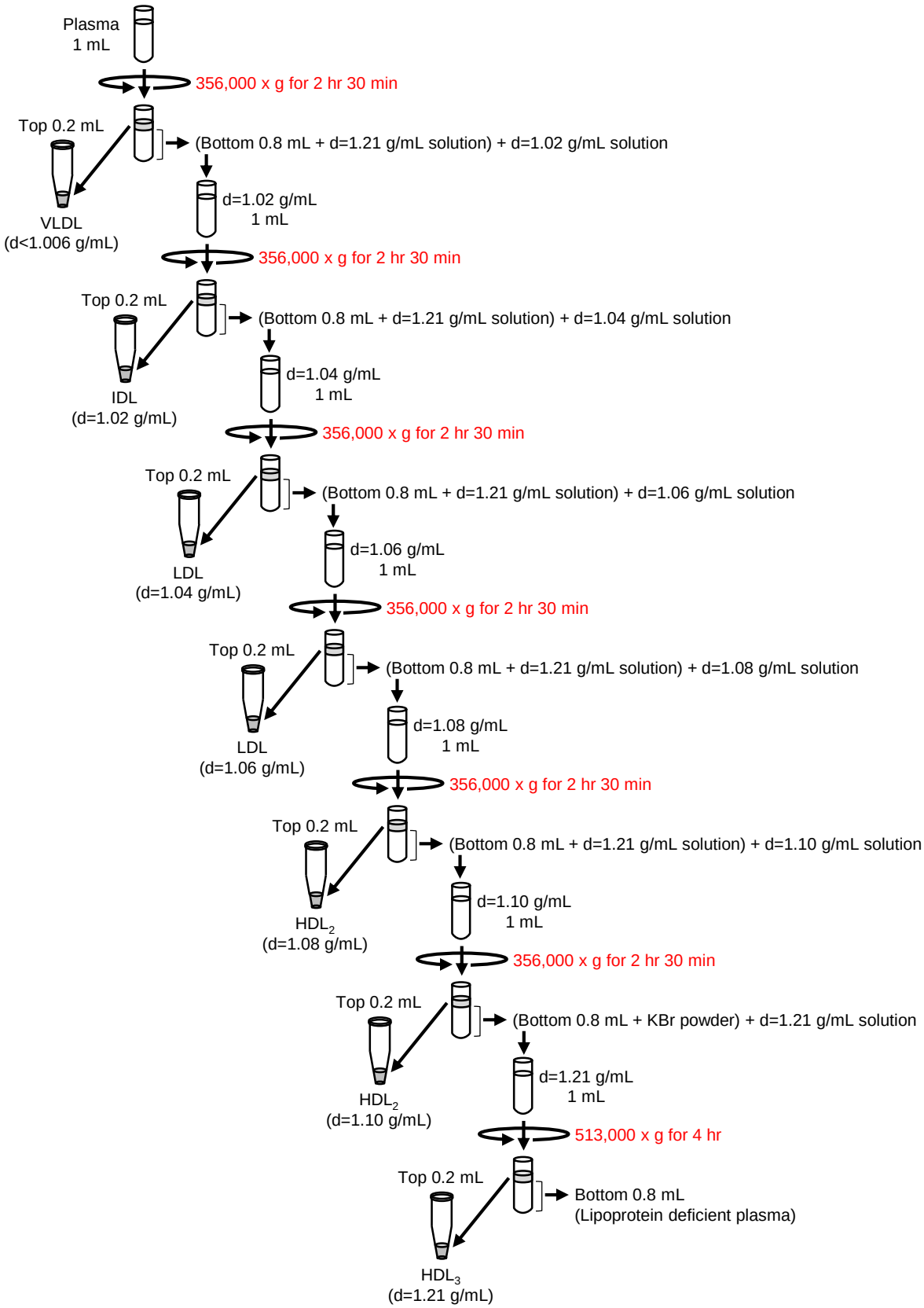


Figure 1

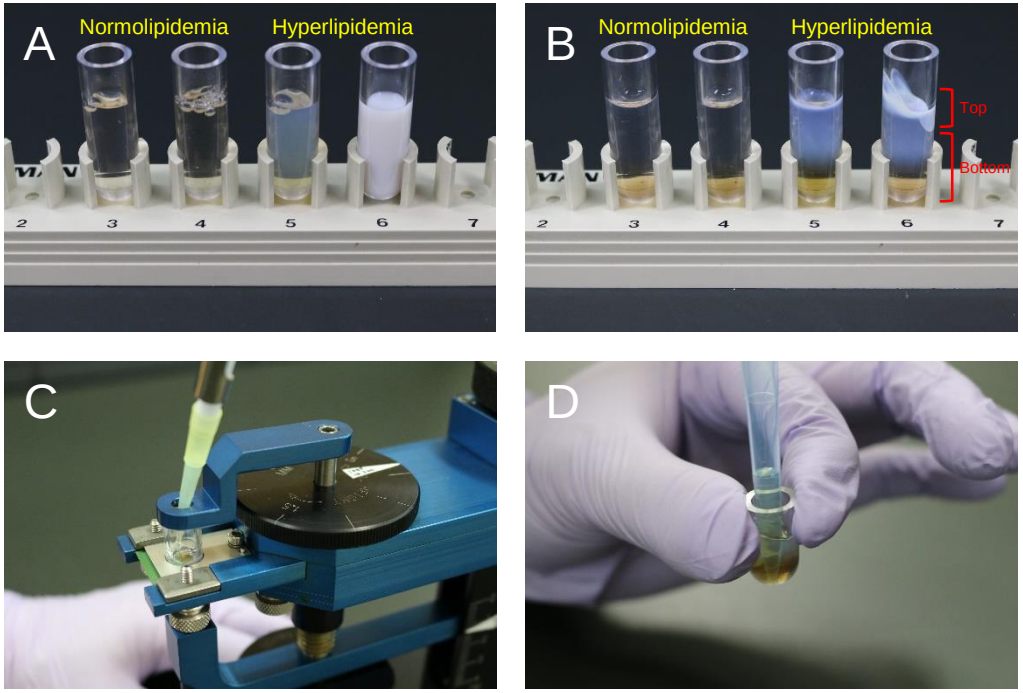
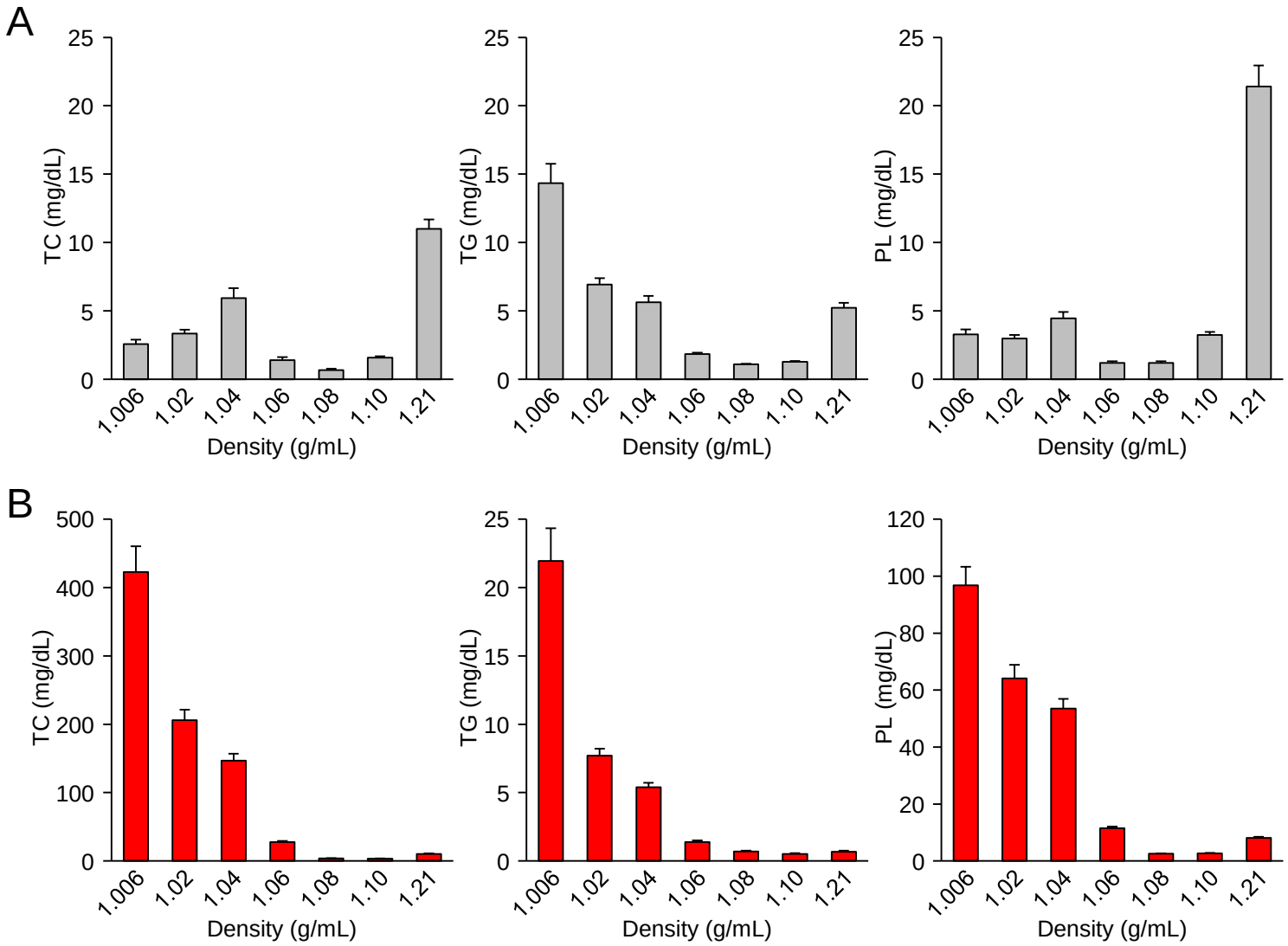


Figure 2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TC (mg/dL)
TG (mg/dL)
PL (mg/dL)
0
5
10
15
20
25
1.006
1.02
1.04
1.06
1.08
1.10
1.21
Density (g/mL)
B
TC (mg/dL)
TG (mg/dL)
PL (mg/dL)
0
100
200
300
400
500
0
5
10
15
20
25
0
20
40
60
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100
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1.08
1.10
1.21
Density (g/mL)

Figure 3

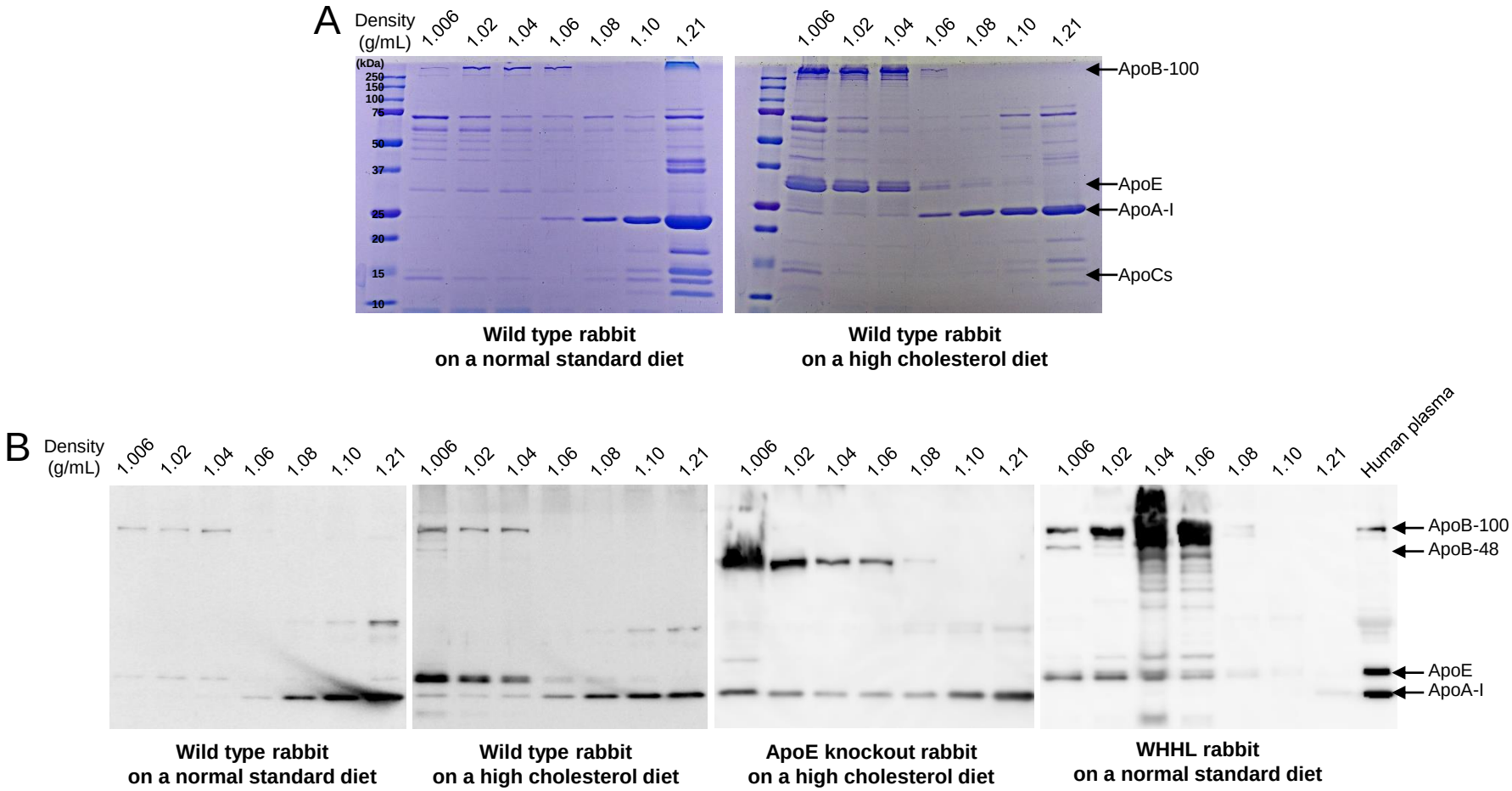
A
Density (g/mL)
1.006
1.02
1.04
1.06
1.08
1.10
1.21
(kDa)
250
150
100
75
50
37
25
20
15
10
ApoB-100
ApoE
ApoA-I
ApoCs
Wild type rabbit on a normal standard diet
Wild type rabbit on a high cholesterol diet
B
Density (g/mL)
Human plasma
ApoB-100
ApoB-48
ApoE
ApoA-I
Wild type rabbit on a normal standard diet
Wild type rabbit on a high cholesterol diet
ApoE knockout rabbit on a high cholesterol diet
WHHL rabbit on a normal standard diet

Figure 4

Table

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 of Materials

Name	Company	Catalog Number	Comments
22-gauge needle	Terumo	NN-2232S	For blood collection
96-well microplate	greiner bio-one	655101	For lipids measurment
Anti-apolipoprotein A-I antibody	LifeSpan BioSciences	LS-C314186	For Western blottng, use 1:1,000
Anti-apolipoprotein B antibody	ROCKLAND	600-101-111	For Western blottng, use 1:1,000
Anti-apolipoprotein E antibody	Merck Millipore	AB947	For Western blottng, use 1:1,000
CBB staining kit	FUJIFILM Wako Pure Chemical	299-50101	For apolipoprotein analysis
Centrifuge	HITACHI		himac CF15RN
Closure	Spectrum	132736	For lipoprotein dialysis
Dialysis tubing	FUJIFILM Wako Pure Chemical	043-30921	For lipoprotein dialysis, MWCO 14,000
Dry heat block	Major Science	MD-01N	For SDS-PAGE sample preparation
ECL Western blotting detection reagents	GE Healthcare	RPN2209	For Western blotting
Electrophoresis Chamber	BIO-RAD		Mini-PROTEAN Tetra Cell
Ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) disodium salt dihydrate	FUJIFILM Wako Pure Chemical	345-01865	For anticoagulant (0.5 M), for dialysis (1 mM)
Filter paper	ADVANTEC	590	For Western blotting
Fixed angle ultracentrifuge rotor	BECKMAN COULTER	357656	TLA-120.2
Fixing solution			For SDS-PAGE (50% Methanol/ 10% Acetic acid)
Immun-Blot PVDF menbrane	BIO-RAD	1620177	For Western blotting
Lumino image analyzer	GE Healthcare		For Western blotting, ImageQuant LAS 400
Magnetic stirrer	ADVANTEC	SR-304	For lipoprotein dialysis
Microplate reader iMARK	BIO-RAD		For lipids measurment
Microtube	INA-OPTIKA	SC-0150	
Orbital agitator USBDbo	Stovall Life Science		
Peroxidase congugated anti goat IgG antibody	Jackson ImmunoResearch	705-035-003	For Western blotting, use 1:2,000
Peroxidase congugated anti mouse IgG antibody	Jackson ImmunoResearch	715-035-150	For Western blotting, use 1:2,000
Phospholipids assay kit	FUJIFILM Wako Pure Chemical	433-36201	For lipids measurment
Polycarbonate ultracentrifuge Tubes	BECKMAN COULTER	343778	
Potassium Bromide	FUJIFILM Wako Pure Chemical	168-03475	For density solution
Power Supply	BIO-RAD		For SDD-PAGE and Western blotting, PowerPac 300, PowerPac HC
Protein standards Precidion Plus Protein Dual Xtra	BIO-RAD	161-0377	For SDS-PAGE and Western blotting
Rabbit restrainer	Natsume Seisakusho	KN-318	For blood collection
Rotor	HITACHI		T15A43
SDS-PAGE running buffer			25 mM Tris/ 192 mM Glycine/ 0.1% SDS
SDS-PAGE sample buffer (2x)			0.1M Tris-HCl (pH 6.8)/ 4% SDS/ 20% glycerol/ 0.01% BPB/12% 2-merpaptoethanol
SDS-polyacrylamide gel			4-20% gradient polyacrylamide gel
Skim milk powder	FUJIFILM Wako Pure Chemical	190-12865	For Western blotting blocking buffer (5% skim milk/ 0.1% Tween 20/ PBS)
Total cholesterol assay kit	FUJIFILM Wako Pure Chemical	439-17501	For lipids measurment
Triglyicerides assay kit	FUJIFILM Wako Pure Chemical	432-40201	For lipids measurment
Tube slicer for thick-walled tube	BECKMAN COULTER	347960	For lipoprotein isolation
Tween 20	SIGMA-ALDRICH	P1379	For Western blotting washing buffer (0.1% Tween 20/ PBS)
Ultracentrifuge	BECKMAN COULTER	A95761	Optima MAX-TL
Western blotting wet transfer system	BIO-RAD		Mini Trans-Blot Cell

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Confirmed.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Confirmed.

3. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Confirmed. The current abstract contains 161 words.

4. Please ensure the Introduction include all of the following with citations:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

Confirmed.

5. All materials and equipment should be moved to the table of materials. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have moved them to the excel file.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be”

throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
Confirmed.

7. The Protocol should contain only action items that direct the reader to do something.
Confirmed.

8. Please ensure you answer the “how” question, i.e., how is the step performed?
Confirmed.

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have highlighted in yellow.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
We have done.

11. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Confirmed.

12. Please sort the materials table in alphabetical order.
Confirmed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Plasma lipoprotein fractionation and the study of their properties and functionality are relevant in the context of lipid disorders, atherosclerosis and cardiovascular disease. Fan et al. describe a classical ultracentrifugation method based on floatability that allows recovering the fractions intact and functional, thus permit a wide range of downstream analysis and applications. Particularly, the authors show how to fractionate a small amount of plasma from rabbit in seven density layers containing the main lipoprotein types and subtypes and further i) removing the KBr to make them able for functional studies; ii) applying commercial kits to identify lipids in the sample; iii) using the common lab technique SDS-PAGE and western-blotting to identify and estimate its lipoprotein content and purity.

The title and abstract are appropriate to the protocol. This is a long protocol; in the counterpart the authors obtain high quality and purity of a wide range of plasma lipid fractions able for any functional study. The authors apply the method into plasma from rabbit but foresee its use in human and mouse plasma as well. The scheme and images are clarifying and an appropriate support to the text. The bibliography is adequate and denotes expertise in the field

Major Concerns:

The described protocol is long (i.e. to centrifuge in a 6-round-in-a-row for 2h30 min) and requires fungible material like quite expensive plastic (i.e. to carefully cut tubs at each step). Thus, the "practical" adjective used along the manuscript is misleading and should be reconsidered.

Thank you for your comment. We have modified the manuscript (Lines 25, 34, 42, 61, 376).

In line with this comment, I suggest to mention in the protocol in which steps the protocol can be paused (i.e. by storing the sample at 4°C) for several hours, if this applies to the protocol. If not, mention as well that all the procedure should be performed with the fresh sample and briefly justify.

We greatly appreciate your comment. It is possible to pause and store the samples at 4°C after a round of ultracentrifugation. We have included the note to indicate the steps and time for the stop and storage in the text (Lines 118-9).

Line 40 and 64: I suggest to including clinical applications of the protocol, as it is pointed out as promising (e.g. to perform a functional study of cholesterol efflux, lipidomics of the fractions to determine deficiencies?). Is there any advantage of this method in front of conventional automated lab determination of lipids and lipoproteins (in clinics) that are worth the time required to achieve those fractions?
Thank you for your suggestion. We have added clinical applications in the text (Lines 359-63).

Figure 4B:
- Regarding the ApoA-I (first blot) image, there is ApoA-I, thus HDL content, in all the fractions. Is that a contamination? Can you justify? Otherwise, the purity of the fractions is in doubt.
Thank you for your question. It is true that very small amount of apoA-I is contained in apoB-containing particles in cholesterol-fed rabbits. Without apoE, apoA-I is distributed in all lipoproteins in both apoE KO rabbits (Niimi M *et al*. Atherosclerosis, 2016; 245:187-193) and mice (Zhang SH *et al*. Science. 1992;258:468-71).

- In general, the gradient profile of wild type rabbit with high cholesterol diet from 4A do not match with 4B, particularly the ApoA-I blot should be improved (loading error?)
Thank you for your comment. We have replaced the Western blotting image of a wild type rabbit fed a cholesterol diet in the current version (Figure 4B).

Minor Concerns:
Introduction: Other methods to isolate certain lipoproteins are not mentioned i.e. PEG-precipitation of ApoB to generate ApoB-depleted serum.
Thank you for your comment. We have included other methods in the text (Line 55-6).

Line 114: Is there any alternative to 2h30min centrifugation that you may consider to reduce time for the protocol?
The floatation time of lipoprotein is defined by rpm and radius of the rotor. High-speed centrifugation can shorten the time as described in the revised version (Lines 364-7).

Lines 224-241: The lipid analysis chapter does not include which analyses are relevant to perform (TC, TG…). It is clarifying for the reader to mention which lipids are analysed with the commercial kits, although the details of the brand come later in the materials.
We have mentioned the lipid analysis methods in the text (Lines 204-7, 211-3).

Line 401: An 80% recovery yield of lipid particles is very acceptable for such a small volume of plasma. Is there any trick you could point out in order not to lose sample during the cutting of the tube and the dialysis (lipids normally stick to the dialysis bags very hard in my experience)? I suggest to highlight in which steps the yield may be reduced and how to improve yield in general.

Thank you for your comment. In our experience, there are three critical points that will affect the recovery rate, including dissolving the viscous precipitate left in the bottom fraction completely, slicing tubes, and collecting the samples from a dialysis bag. We have described these points in the text (Lines 367-70).

Figure 1: The scheme only shows 4 fractions. It would be clarifying for the reader to identify in the figure how from the small dots two other lipoprotein fractions are produced to reach the 7 fractions you mention along the manuscript.

Thanks for your suggestion. We have modified the scheme in Figure 1.

Figure 4B: It would be clarifying to include the antibody used for each blot below the images. Can you explain the presence (small but visible) of fraction 1.08 with ApoB?

We used a "cocktail antibodies" containing anti apoA-I, apoB, and apoE for the Western blotting which has been described in the Figure 4 legend.

We speculate that very small amount of apoB-48 in 1.08 fraction was either HDL_1 remnant or contamination caused by ultracentrifugation.

Application: It is very relevant that you proceed to eliminate KBr from the samples. Is there any other downstream analysis you may suggest to perform?

In addition to analyzing lipids and proteins in the fractions, we can also these fractions for other analyses. Using apoB-containing particles, we can compare their oxdizability, uptake by macrophages or hepatocyte *in vitro*, or label them with isotope or fluorescence to perform kinetics study *in vivo*. We can also use different HDLs to investigate their anti-inflammatory activity, cholesterol efflux from macrophages and proteomics (Lines 359-63).

Reviewer #2:

Manuscript Summary:

The protocol described in the current manuscript is a laborious technique to obtain various lipoprotein fractions and subfractions. The obtained fractions were characterized for their lipid and apolipoprotein A-I, E, and B content.

Major Concerns:

It is not mentioned the advantages of this long and laborious method consisting in 7 successive ultracentrifugations (a total of 19 h of ultracentrifugation) in comparison with the classical method of gradient ultracentrifugation/precipitation method with heparin/Mn/dextran sulfate to obtain the two major subfractions, large buoyant HDL2 (d = 1.063-1.125 g/ml) and small dense HDL3 (d = 1.125-1.210 g/ml).

Thank you for your comment. The main advantage of the current method is that we can make more detailed analyses of lipoproteins by fractionated them into seven individual fractions. For example, we can divide apoB-containing particles into VLDL, IDL, LDL and small LDL whereas we can divide HDLs into HDL_1, HDL_2 and HDL_3. In addition, we can use these samples to perform many *in vitro* and *in vivo* studies as we described.

The fractions obtained are not identified by other methods, such as electron microscopy.

The density lipoprotein fractions can also apply for particle size analysis using negatively-stained electron microscopy as we showed before (Fan J *et al*. J Biol Chem. 2001;276:40071-9, Koike T *et al*. Arterioscler Thromb Vasc Biol 2009, 29:2047-2053) (Line 361-2).

The necessary time of this method (3 days) and expensive equipment required for these techniques are disadvantageous for the processing of the large numbers of samples used in epidemiological studies, by clinicians. Thus, only researchers could use this protocol, but they probably would prefer a simple alternative method, if no major advantage is explained (e.g. various oxidative or enzymatic potential, specific miRNAs, etc).

We agree with you that the current study is not suitable for analysis of a large number of samples such as epidemiological studies because the current method is designed for the mechanism studies. Of course, any methods would have advantages and disadvantages and researchers can choose each method for their purposes. In the

revised manuscript, we have described the advantages and disadvantages of this method (Lines 359-67, 372-5).

Mention if all this successive centrifugations should be done in one day, or if one can keep the fractions and stop the protocol at a specific time. 19 centrifugation hours plus 4-7 hours for starting and stopping the centrifuge is a very long working day.
The same concern was also raised by the Reviewer 1. It is possible for one to perform all procedures by one day if you work efficiently. It is also possible to pause and store the sample at 4ºC after a round of ultracentrifugation. We have described this point in the text (Lines 118-9).

Minor Concerns:
R38: the procedure to analyze apolipoproteins is done to verify the fractions, and is not a direct aim of the paper.
Thank you for your comment. We agree with you that it is not a direct aim to analyze these apolipoproteins but it may be useful for those who are interested in analyzing apolipoproteins using these fractions.

R62: Which is the advantage to obtain two fractions of LDL (at 1.04 and 1.06 g/ml)? In Fig 4 they have the same pattern.
Thank you for your question. The size of these two LDLs is different as they are named as large LDLs and small dense LDLs. In general, small and dense LDLs are more atherogenic. In human plasma, these small LDLs are contained Lp(a) as well.

R231: mention the lipid analyzed.
We have added the note of lipid analysis in the text (Lines 204-7, 211-3).

R346: mention Fig 3B in the text.
We have mentioned Figure 3B in the text (Lines 296-300).

Fig 1. Fill in all the centrifugation steps and the fractions obtained.
The same comment was also raised by the Reviewer 1. We have modified Figure 1 and included all the steps.

Fig 2. Why the last two tubes in 2A are different? Explain how the top fraction is delimitated.

Thank you for your question. The last two tubes are plasma collected from hyperlipidemic rabbits. The blade position of the tube slicer defines the borderline of the top and bottom fractions. In the method, the blade is set at the line separating 200 µL of the top fraction and 800 µL of the bottom fraction. We have added the note in the text (Lines 112-3).

Fig 3. Name the fractions in the graphs. Mentions the diet in the figure (NS diet - upper HC diet - lower.)
We have added the diet info in the Figure 3 legend.

Fig 4. Add the gel electrophoresis for apoE deficient rabbit. ApoA-I is not present in WT rabbit with a normal standard diet?
ApoA-I is not detectable in d<1.006 fraction of WT rabbit fed a normal standard diet. We added the Western blotting of a WT rabbit fed a normal standard diet (Figure 4B).