eppendorf

APPLICATION NOTE No. 468

Rapid Separation of Lipoprotein Fractions From Human Serum by Ultracentrifugation

Wiâme Ben El Mostapha[1], Vincent Dufey[1], Silvia Tejerina[1], Jan Knop[2], Pascal Rowart[1]*, Françoise De Longueville[1]
[1] Eppendorf Application Technologies S.A., Namur, Belgium
[2] Eppendorf SE, Hamburg, Germany
* Corresponding author: rowart.p@eppendorf-eat.be

Abstract

The rapid and efficient isolation and characterization of lipoproteins from human serum has become an essential part in the identification and study of coronary diseases. Density gradient ultracentrifugation is being used as the standard method for the separation of the different lipoprotein fractions. In the present application note we demonstrate the suitability of the Centrifuge CS150NX and rotor S140AT with its high-speeds of 1,050,000 × *g* (140,000 rpm) with a maxium sample volume capacity of 10 × 2 mL to efficiently separate lipoproteins from human whole blood. This compact footprint bench top ultracentrifuge utilizes density gradient centrifugation to isolate three different fractions containing 1) chylomicrons (CLM) and very-low-density lipoproteins (VLDL), 2) intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL), and 3) high-density lipoproteins (HDL) in a reduced time (5h) compared to conventional high-speed separation protocols where it can sometimes take up to 60 h to separate fractions for single-class lipoproteins. Moreover, this procedure minimizes and simplifies consumable and reagent usage as the same type of gradient solutions and type of tubes can be used for all steps.

Introduction

Lipids are naturally occurring molecules that include fats, cholesterol, triglycerides, and phospholipids, among others. They were previously characterized as sources of energy storage and the building blocks of cell membranes[1]. Lipids are now known to play several key roles in intracellular signaling and membrane trafficking[2,3]. Because of their hydrophobic nature, these lipids are wrapped in spherical lipoprotein particles which serve as carriers[4]. Lipoproteins are complex particles with a central hydrophobic core consisting of non-polar lipids such as cholesterol and triglycerides. The external layer is composed of a hydrophilic membrane of phospholipids, free cholesterol, and apolipoproteins[5]. The primary function of lipoprotein particles is to transport hydrophobic molecules within the extracellular water and plasma of the body to distribute to all the cells and tissues[6]. Plasma lipoproteins can be divided into seven classes based on their size, lipid composition, and apolipoproteins. Specific apolipoproteins contained in the outer

shell determines their functional identity[7]. Lipoproteins can then be separated by their density as it increases from chylomicrons (CLM) to chylomicrons remnants (CLM Rem), very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL-I, II and III), and high-density lipoproteins (HDL2 and HDL3) and lipoprotein (a) (Lp (a))[5,8,9]. The characteristics of lipoproteins are reviewed in Table 1[5,8,9].

Table 1: Classification of lipoproteins

Family name	Density range (g/mL)	Particle diameter (nm)	Majors apolipoproteins	Others	Major function
Chylomicrons (CLM)	< 0.93	> 75	ApoB-48	ApoA-I, -IV ApoC-I, -II, -III ApoE	Transport of exogenous triglycerides
Chylomicron Remnants (CLM Rem)	0.93-1.006	30-80	ApoB-48	ApoE	Transport of triglycerides and cholesterol
Very low-density lipoproteins (VLDL)	0.93-1.006	30-80	ApoB-100	ApoA-I, -II, -V ApoC-I, -II, -III ApoE	Transport of endogenous triglycerides
Intermediate density lipoproteins (IDL)	1.006-1.019	25-35	ApoB-100	ApoC-I, -II, -III, ApoE	Precursor of LDL
Low density lipoproteins (LDL)	1.019-1.063	18-25	ApoB-100		Transport of cholesterol and phospholipids to peripheral cells
High-density lipoproteins (HDL)	1.063-1.121	5-12	ApoA-I	ApoA-II, -IV, -V, ApoC-III, ApoE	Transport of cholesterol and other lipids from plasma to the tissues
Lipoprotein (a)	1.055-1.120	25	Apo B-100	Apo (a)	Transport of cholesterol

Plasma elevation of LDL (also known as the "bad" cholesterol), VLDL, and Lp (a), and reduced levels of HDL (the "good" cholesterol) are risk factors for coronary artery disease[1,4,10,11]. Due to the close relationship between the lipoprotein levels and the development of diseases, these lipoproteins are now considered good biomarkers for coronary disease research[12].

In this regard, an increasing number of lipoprotein detection and isolation methods are being investigated. Lipoproteins can be separated by different methods including ultracentrifugation[13], size exclusion chromatography[14], gel electrophoresis[15], precipitation with polyanions[16], and immune-specific adsorber[17].

Density gradient ultracentrifugation involves the separation of lipoproteins by density, depending on the lipoprotein lipid/protein ratio. For example, CLM is almost entirely lipid-containing and is very light and less dense than water. On the other hand, small lipoproteins such as HDL are only 50% fat and the other half is composed of higher density proteins, making the HDL density between 1.063 and 1.25 g/mL. Density is the underlying basis for the nomenclature of lipids, and density gradient ultracentrifugation is the reference method for measuring lipid density[18,19].

Here, we demonstrate the use of the Eppendorf Centrifuge CS150NX to isolate lipoprotein fractions from human serum in any laboratory workspace using a streamlined procedure and simplified consumables and reagents, while utilizing traditional power requirements and minimal bench/ lab space.

Material and Methods

Serum preparation
Human whole blood from a healthy individual was collected and allowed to clot at room temperature (RT) for 30 min. The clot was removed by centrifugation at 2,000 × *g* for 10 min using Eppendorf Centrifuge 5810 R and the collected supernatant (serum) was immediately transferred to a new tube, aliquoted, and stored at -20°C for further analysis.

Separation of serum lipoproteins by ultracentrifugation
Based on their hydrated density, lipoproteins were separated by sequential flotation ultracentrifugation[20] using the compact benchtop Eppendorf Centrifuge CS150NX with a fixed-angle Rotor S140AT (Figure 1).
This refrigerated benchtop ultracentrifuge with a speed of up to 1,050,000 × *g* (140,000 rpm), coupled with fast acceleration and deceleration, ensures speedy separation of samples.

Fig. 1. A Eppendorf Centrifuge CS150NX **B** Easy rotor loading and access Rotor S140AT **C** Top view Eppendorf Centrifuge CS150NX

Fractionations of serum lipoproteins
The density solutions were prepared as follows:
- Density solution A (ρ: 1.006 g/mL): 11.4 g of NaCl (0.195 M), 0,1 g of EDTA-2Na (0.001%) and 1 mL of NaOH (1N) were mixed and dissolved with 500 mL of distilled water. Distilled water was added to achieve a total volume of 1 L and then an additional 3 mL of distilled water was added. Density of the solution was checked using an analytical balance. Water was added until the solution reached the desired density[21].
- Density solution B (ρ: 1.182 g/mL): 24.98 g of NaBr (2.44 M) was dissolved into 100 mL of solution A.
- Density solution C (ρ: 1.478 g/mL): 78.32 g of NaBr (7.65 M) was dissolved into 100 mL of solution A.

A total of 600 µL of serum was transferred to a 1 mL 1 PC Tube (Eppendorf, order no: 5720 411 000). For a better visualization of the lipoproteins fractions after centrifugation, an equivalent tube was prepared by pre-staining the serum with Sudan Fat 7B (SFT - Sigma, order no: 201618). The dye solution was composed of 2 mg of Sudan Fat 7B in 1 mL of dimethylformamide (Carl Roth, order no: T921.1) activated before use with 1 mL of 0.1 M NaOH (Sigma, order no: 72068) and 50 µL of Triton X-100 (Merck, order no: 1.12298.0101). For a volume of 600 µL of serum, 12 µL of the dye solution Sudan FAT 7B was added to the tube and mixed by inversion. To both tubes, 300 µL of the density solution A was added and mixed gently by inversion. After mixing, the tubes were loaded into 10 × 2 mL fixed-angle Rotor S140AT in a CS150NX and centrifuged at 140,000 rpm (1,050,000 × *g*) at 16°C (Acceleration: 9; Deceleration: 7) for 50 min. The deceleration is set up to 7 to avoid disrupting the phases. After centrifugation, the top layer (maximum 300 µL) containing the CLM and the VLDL fraction was removed by pipetting (Figure 2A). To the remaining (appr. 600 µL) fraction containing IDL, LDL, HDL, albumin, and serum proteins, 300 µL of the density solution B was added. The tubes were centrifuged at 140,000 rpm (1,050,000 × *g*) at 16°C
(Acceleration: 9; Deceleration: 7) for 80 min. Following centrifugation, the top layer (maximum 300 µL) containing the IDL and the LDL fraction was removed by pipetting (Figure 2B). For the last step of separation, 300 µL of the density solution C was added to the remaining fraction. The tubes were centrifuged at 140,000 rpm (1,050,000 × *g*) at 16°C (Acceleration: 9; Deceleration: 7) for 140 min. At the

end of the centrifugation, the top layer containing only the HDL fraction was aspirated by pipetting (Figure 2C). All fractions were stored at -20°C for further usage.

Analysis of apolipoproteins in fractions by western blot
Sample and buffers were prepared for gel electrophoresis in reducing and denaturation conditions following the Novex™ Tris-Glycine SDS technical Guide (Thermo Fisher Scientific, order no: LC2677). Briefly, 10 µL of Tris-Glycine SDS buffer and 2 µL of NuPAGE™ reducing agent were added to 8 µL of sample from each fraction. The samples were mixed and then heated at 85°C for 2 min before loading each sample (20 µL) into the wells of a Novex WedgeWell 4-20% Tris-Glycine protein gel (Thermo Fisher Scientific, order no: XP04205BOX). A protein molecular weight marker (WesternSure Pre-stained chemiluminescent protein lad-

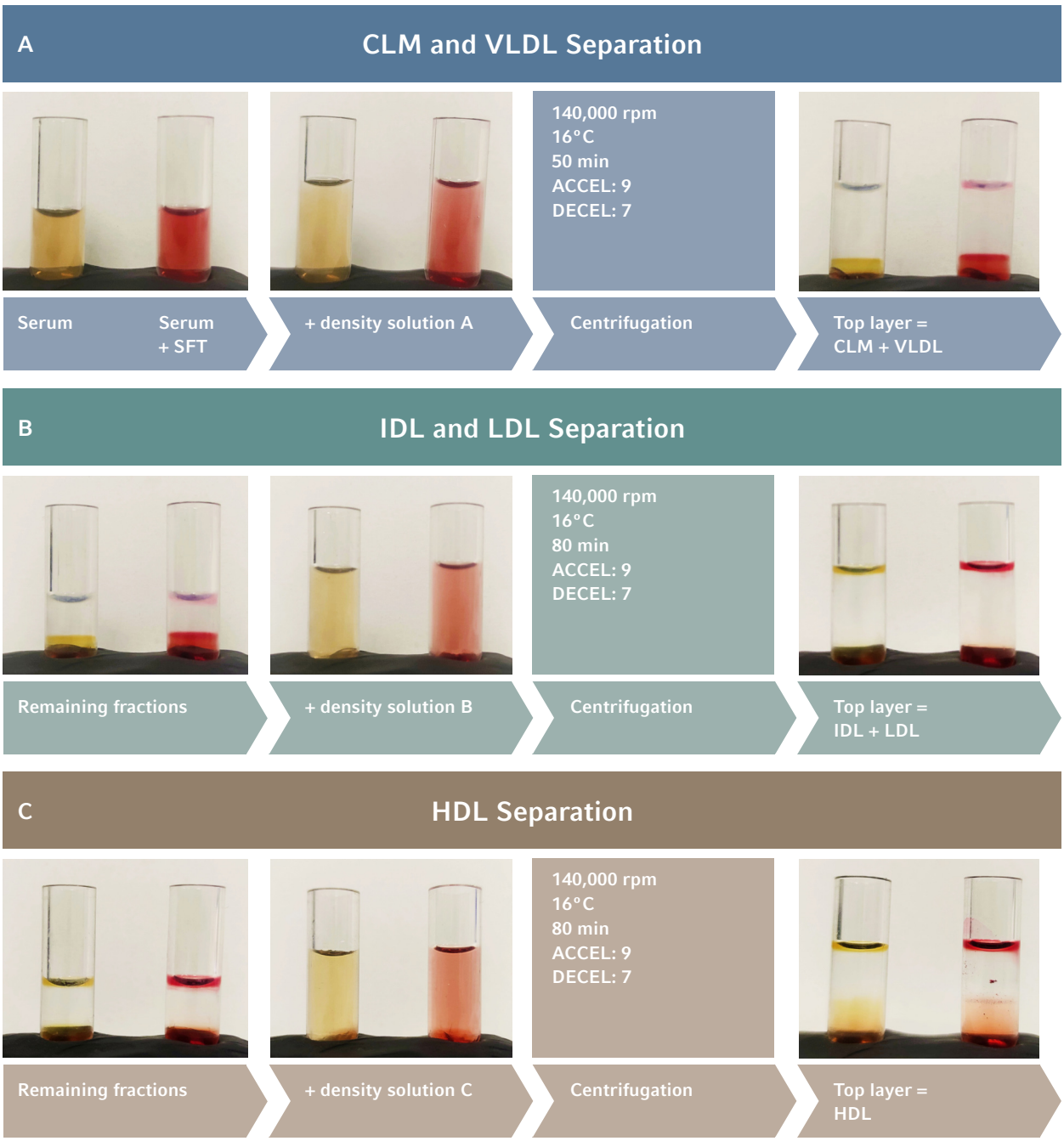

Fig. 2: Schematic representation of the separation of lipoproteins. A) CLM and the VLDL fraction, B) IDL and the LDL fraction, C) HDL fraction. SFT= Sudan Fat 7B

der, Li-cor, order no: 926-98000) loaded into each gel was used to determine the approximative weight of the proteins resolved by gel electrophoresis. Each gel was run as follows: voltage: 225 V constant, current: Start: 125 mA/gel, End: 70-80 mA/gel. Once the electrophoresis was completed, the proteins were transferred onto a nitrocellulose transfer membrane (Thermo Fisher Scientific, order no: 88025). The membranes were blocked with the Intercept (TBS) blocking buffer (Li-Cor, order no: 927-60001) for 1 h at RT and probed with primary antibodies (Table 2) diluted in Intercept (TBS) antibody diluent (Li-Cor, order no: 927-65001) overnight at 4°C. Following incubation, membranes were incubated with the secondary antibodies diluted in the Intercept (TBS) antibody diluent for 1 h at RT. Visualization of the bands was performed using the WesternSure PREMIUM chemiluminescent substrate (Li-Cor, order no: 926-95000) and blots were scanned on a C-DiGit blot scanner (Li-Cor, order no: 3600-00).

Table 2: List of primary and secondary antibodies

Primary antibody	Dilution	Secondary antibody	Dilution
ApoA1 monoclonal mouse antibody (Thermo Fisher Scientific, order no: MIA1404)	1:1,000	WesternSure goat anti-mouse secondary antibody (Li-cor, order no: 926-80010)	1:100,000
ApoB (B-100) monoclonal mouse antibody (R&D systems, order no: MAB4124)	1:250	WesternSure goat anti-mouse secondary antibody (Li-cor, order no: 926-80010)	1:100,000
ApoE monoclonal rabbit antibody (Thermo Fisher Scientific, order no: 701241)	1:200	WesternSure goat anti-rabbit secondary antibody (Li-cor, order no: 926-80011)	1:100,000

Result and Discussion

Lipoproteins can be assessed by measuring either lipid quantity or apolipoproteins. After the successful isolation of the different lipoproteins by ultracentrifugation using Centrifuge CS150NX, three of the major apolipoproteins were identified by western blot in the different fractions (Figure 3). As mentioned previously ApoA1 constitutes the main protein component of HDL. This protein with a molecular weight of 28 kDa is an exchangeable protein that is transferred between different lipoprotein particles. As shown in figure 3A, ApoA1 was mainly identified in the HDL fraction. ApoB (B-100) is a 550 kDa protein that is part of the VLDL and LDL lipoproteins. ApoB is defined as a non-exchangeable component anchored in the lipoprotein particle. It is mainly present in the LDL fraction as observed in figure 3B. ApoE (34 kDa), an exchangeable component, is mainly present in VLDL and HDL; however, this apolipoprotein can also be associated with other lipoproteins such as IDL. In the present fractions, it has been identified mainly in the LDL + IDL fractions and with less intensity in the other two fractions (CLM+VLDL and HDL) (Figure 3C).

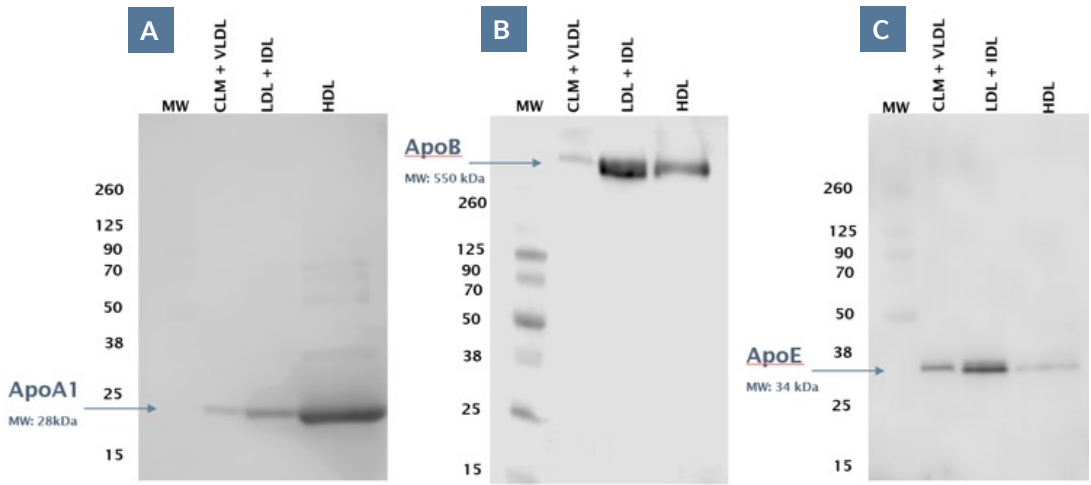

Figure 3. Western blot illustrating three of the major apolipoproteins: A) ApoA1, B) ApoB, and C) ApoC detected in each fraction to characterize them. Consecutive fractions obtained after ultracentrifugation were loaded and electrophoresed on 4-20% SDS-PAGE gels. Chylomicrons (CLM), very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

Conclusion

The present centrifugation procedure allows a rapid separation of serum lipoprotein fractions by density gradient using a micro-ultracentrifuge at a single speed. While some of the fractions contain two classes of lipoproteins, more sophisticated density gradient ultracentrifugation methods can be employed with the Centrifuge CS150NX to isolate single-class lipoproteins. Through using serial centrifugation steps with a maximum speed of 513,000 × *g*[22], single-class lipoproteins and further resolution of subcomponents can be achieved. The easy-to-use separation approach described in this work highlights the isolation of lipoproteins into three fractions, notably achievable in less than five hours.
This versatile and compact Eppendorf micro-ultracentrifuge reaches the 1,050,000 × *g* (140,000 rpm) required for the most efficient isolation of lipoproteins in only 90 seconds. Alternative protocols for the isolation of single-class lipoproteins can take up to 60 hours[23].
There is a significant time advantage of targeting three fractions and using the maximum g-force rotor and centrifuge setup achieved with the Centrifuge CS150NX and S140AT rotor. Furthermore, this approach coupled with the capabilities of the Centrifuge CS150NX can also be used for other applications such as virus and macromolecule isolation, as well as the purification of ribosomes. In conclusion, the combination of the benchtop CS150NX or floor standing CS150FNX micro-Ultracentrifuge coupled with the S140AT rotor allows for faster isolation of lipoproteins fractions in streamlined and simplified steps.

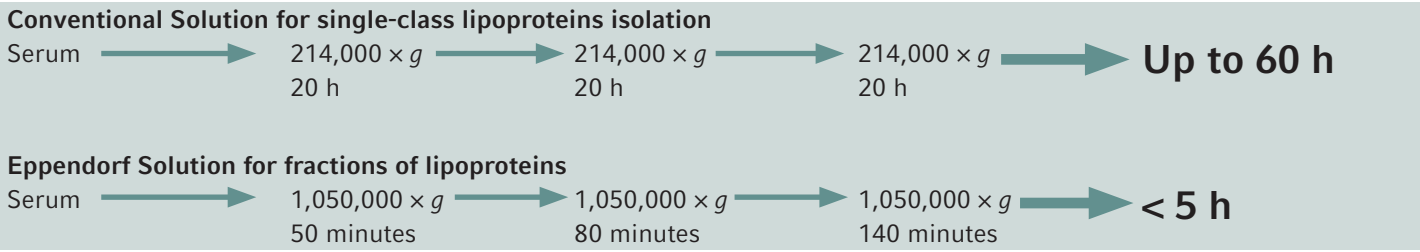

Figure 4: Conventional high-speed separation[23] vs Eppendorf protocol for isolation of lipoproteins.

Summary

> Lipoproteins are an important topic in biomarker research for coronary diseases

> Established methods can take up to sixty hours to isolate single-class lipoproteins

> This Application Note shows how, in less than five hours, lipoprotein fractions can be isolated using centrifuge CS150NX or CS150FNX and rotor S140AT

Literature

[1] Saba, A. & Oridupa, O. Lipoproteins and Cardiovascular Diseases. Lipoproteins - Role in Health and Diseases (2012) doi:10.5772/48132.
[2] Vance, D. E. & Vance, J. E. Biochemistry of lipids, lipoproteins, and membranes. 607 (2002).
[3] Fahy, E. et al. Update of the LIPID MAPS comprehensive classification system for lipids. J Lipid Res 50, S9 (2009).
[4] Genest, J. Lipoprotein disorders and cardiovascular risk. J Inherit Metab Dis 26, 267–287 (2003).
[5] Feingold, K. R. & Grunfeld, C. Introduction to Lipids and Lipoproteins. Endotext (2021).
[6] Schumaker, V. N. & Adams, G. H. Circulating lipoproteins. Annu Rev Biochem 38, 113–136 (1969).
[7] Lent-Schochet, D. & Jialal, I. Biochemistry, Lipoprotein Metabolism. StatPearls (2022).
[8] Packard, C. J. Remnants, LDL, and the Quantification of Lipoprotein-Associated Risk in Atherosclerotic Cardiovascular Disease. Curr Atheroscler Rep 24, 133–142 (2022).
[9] Nordestgaard, B. G. & Langsted, A. Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology. J Lipid Res 57, 1953–1975 (2016).
[10] Barter, P. et al. HDL Cholesterol, Very Low Levels of LDL Cholesterol, and Cardiovascular Events. New England Journal of Medicine 357, 1301–1310 (2007).
[11] Basile-Borgia, A. & Abel, J. H. Lipoproteins in heart disease. Perfusion 11, 338–345 (1996).
12] Sandhu, P. K. et al. Lipoprotein Biomarkers and Risk of Cardiovascular Disease: A Laboratory Medicine Best Practices (LMBP) Systematic Review. J Appl Lab Med 1, 214 (2016).
[13] Niimi, M., Yan, H., Chen, Y., Wang, Y. & Fan, J. Isolation and analysis of plasma lipoproteins by ultracentrifugation. Journal of Visualized Experiments 2021, (2021).
[14] Cheung, M. C. & Albers, J. J. Characterization of Lipoprotein Particles Isolated by Immunoaffinity Chromatography J Biol Chem 259, 12201–12209 (1984).
[15] Mueller, P. A. et al. A method for lipoprotein (a) Isolation from a small volume of plasma with applications for clinical research. (123AD) doi:10.1038/s41598-022-13040-4, (2022).
[16] Burstein, M., Scholnick, H. R. & Morfin, R. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J Lipid Res 11, 583–595 (1970).
[17] Kostner, G. & Holasek, A. Isolation of human serum low-density lipoproteins with the aid of an immune-specific adsorber. Lipids 5, 501–504 (1970).
[18] Chapman, M. J., Goldstein, S., Lagrange, D. & Laplaud, P. M. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. J Lipid Res 22, 339–358 (1981).
[19] Kulkarni, K. R. Cholesterol Profile Measurement by Vertical Auto Profile Method. Clin Lab Med 26, 787–802 (2006).
[20] Schumaker, V. N. & Puppione, D. L. [6] Sequential flotation ultracentrifugation. Methods Enzymol 128, 155–170 (1986).
[21] Al-Mrabeh, A., Peters, C., Hollingsworth, K. G. & Taylor, R. Measurement of intraorgan fat and hepatic output of triglycerides in human type 2 diabetes by magnetic resonance and intralipid infusion techniques. STAR Protoc 2, 100355 (2021).
[22] Niimi, M., Yan, H., Chen, Y., Wang, Y. & Fan, J. Isolation and analysis of plasma lipoproteins by ultracentrifugation. Journal of Visualized Experiments 2021, (2021).
[23] Axmann, M., Karner, A., Meier, S. M., Stangl, H. & Plochberger, B. Enrichment of Native Lipoprotein Particles with microRNA and Subsequent Determination of Their Absolute/Relative microRNA Content and Their Cellular Transfer Rate. JoVE (Journal of Visualized Experiments) 2019, e59573 (2019).

Ordering information

Description	Manufacturer	Order no.
Centrifuge 5810 R	Eppendorf	*1
Centrifuge CS150NX	Eppendorf	*2
Centrifuge CS150FNX	Eppendorf	*3
Rotor S140AT	Eppendorf	5720 221 001
1PC Tube	Eppendorf	5720 411 000

*1 For ordering information, please check our local website or contact your local sales representative. **www.eppendorf.com/centrifuge-5810**

*2 For ordering information, please check our local website or contact your local sales representative. **www.eppendorf.com/centrifuge-CS150NX**

*3 For ordering information, please check our local website or contact your local sales representative. **www.eppendorf.com/centrifuge-CS150FNX**

Your local distributor: www.eppendorf.com/contact
Eppendorf SE, Barkhausenweg 1, 22339 Hamburg, Germany
eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com