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Depleting Lipoproteins from Serum

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For decades, fetal bovine serum (FBS) has been used as a supplement for cell-culture media, providing the growth factors that cultured cells require. The availability of FBS as a by-product of the food industry made it plentiful and inexpensive, but the rise of bovine spongiform encephalopathy has led to increased control in the production of FBS. Traceability of the source and uses of the livestock are commonplace, and increased restrictions on international import/export of materials from livestock have been implemented. These events have increased the cost of FBS and limited the sources available for further manufacture, specifically of lipoprotein-depleted FBS (LDFBS).

Researchers can prepare LDFBS themselves. The following procedure is adapted from Havel's method of adjusting the density of the serum (with a salt) and ultracentrifugation to float up the lipoproteins [1]. After removal of the lipoproteins, excess salt is removed by dialysis.

Procedural considerations

Lipoprotein flotation rate (based on hydrated density)

Flotation Svedberg units (S_f) describe the time required to float particles using an ultracentrifuge and a density gradient at a given temperature. The following values were determined by analytical centrifugation at 26^{o}C in an NaCl solution at a density of 1.063 g/mL [2]: LDL S_f = 0 – 20; VLDL–IDL S_f = 20 – 400. (HDL S_f could not be determined, as HDL particles do not float at 1.063 g/mL.)

The S_f of all lipoproteins at a density of 1.21 g/mL at 10^{o}C is 1.8 as estimated from published methods.

Selection of density gradient

Plasma density may be adjusted using solid salt; add it carefully to avoid protein precipitation. To calculate the amount of potassium bromide (KBr) to add to a given volume of plasma, the Radding and Steinberg formula [3] often is used:

$$X = V (d_f - d_i) / (1 - (0.312 \times d_f))$$

X = g of KBr d_f *= final density*
V = mL of plasma d_i *= initial density*
0.312 = partial specific volume of KBr mL/g

The density of plasma generally is assumed to be 1.006 g/mL—this is the initial density (d_i). The final density used to float all the lipoproteins is 1.21 g/mL. For different salts, use the appropriate partial specific volume as found in the CRC Handbook of Chemistry and Physics.

As an example, to adjust 126 mL of plasma to 1.21 g/mL using KBr:

X = 126 (1.21 – 1.006) / (1 – (0.312 x 1.21))
X = 41.2 g KBr

Ultracentrifuge and rotor selection

Clearing factor (*k*) of the rotor: Manufacturers provide a clearing factor (*k*) that is specific for the rotor and speed (rpm) of centrifugation and used to calculate the time required for separation. A lower *k*-factor separates more efficiently than a higher *k*-factor [4].

To calculate the difference in run time (*t*) required to reproduce a separation on different rotors (1 and 2), a ratio of the *k*-factors is used:

$$t_2 = (k_2 / k_1) \times t_1$$

Speed of centrifugation: Determined by the maximum speed at which the rotor may be used safely.

Temperature of centrifugation: Lower temperatures increase the viscosity of the density gradients and prolong the time required to obtain a clean separation [2]. Many references use 20°C to 25°C. For each 5°C decrease in temperature (T), the run time may need to be increased 9% to 12%.

To estimate the percent increase in time using a 9% increase per 5°C decrease in temperature:

$$\text{Percent increase in time} = ((T_1 - T_2) / 5) \times 0.09$$

Time of centrifugation (run time): Calculated (in hours) using the *k*-factor and S_f:

$$t \text{ (hr)} = k\text{-factor} / S_f$$

Example 1: Suppose you have two rotors, a Ti 70.1 (max speed 60,000 rpm, *k*-factor 49.6) and a Ti 55.2 (50,000 rpm, *k*-factor 77.2). Run time is represented by $t = k\text{-factor}/S_f$. Therefore, given an estimated $S_f = 1.8$ for total lipoproteins at a density of 1.21 g/mL at 10°C, the run time for the Ti 70.1 is 49.6/1.8 = 27.6 hr. Using the ratio of the *k*-factors, running conditions for the Ti 55.2 are (77.2/49.6) x 27.6 = 43.0 hr.

Conditions	1	2
Rotor	Ti 70.1	Ti 55.2
Speed	60,000	50,000
***k*-factor**	49.6	77.2
Run time (hr)	27.6	43.0

Example 2: Calculating the conditions to remove total lipoproteins using a Beckman type Ti 70.1 rotor (*k*-factor 49.6) at 60,000 rpm at 5°C, assuming $S_f = 1.8$ at a density of 1.21 g/mL at 10°C:

***t* (hr)**	= *k*-factor / S_f = 49.6 / 1.8 = 27.6 hr at 10°C
Extra time	=27.6 hr x ((10 – 5)/5) x 0.09 = 2.5 hrs at 5°C
Total time	=27.6 + 2.5 = 30.1 hrs (a 9% increase)

Harvest method

The choice of harvesting method can affect the purity of the recovered material. There are several options for harvesting the LDFBS located below the lipoproteins, including:

Tube slicing: Tubes that are heat-sealed may be sliced, and the desired fraction collected (usually with a syringe). The determination of where to slice the tube should be determined experimentally.

Bottom piercing: Commercially available and in-house devices use gravity to collect fractions starting at the bottom of the tubes.

Final adjustments

After the lipoproteins are removed, adjust the density back to 1.006 g/mL, the assumed density of plasma. Salt may be removed through dialysis in a tight-pored membrane while retaining small serum components. An additional dilution or concentration step may be required before using as a cell-culture media supplement. Verification of sterility also is recommended.

Example Protocol

1. In a biosafety cabinet, adjust 126 mL of FBS to a density of 1.21 g/mL.
    a. Add 41.2 g of KBr, mixing well.
2. Fill the centrifuge tubes using a sterile syringe and large bore needle.
    a. Seal all tubes according to manufacturer's instructions.
3. Ultracentrifuge for the calculated time at the desired temperature.
    a. Using a Beckman Optima L90 and a Type 70.1 Ti rotor at 60,000 rpm at 5°C, the calculated time is 30.1 hrs.
    b. For different equipment, use the calculations above to determine the time.
4. Harvest the bottom fraction (LDFBS) of each tube, working smoothly so as not to disturb the gradient and quickly to prevent a change in the gradient caused by diffusion.
5. Transfer the LDFBS to a dialysis membrane (e.g., cutoff value of 6 kDa to 8 kDa) that has been secured, tested for leaks and washed with sterile buffer. Allow extra room for expansion during dialysis because of the high initial salt concentration.
6. Dialyze the LDFBS against the buffer of choice (e.g., Dulbecco's Phosphate Buffered Saline (DPBS)) with sufficient changes to remove the KBr.
    a. Dialyze using 1 L DPBS for every 10 mL of LDFBS for 4 hours at 2°C to 8°C.
    b. Repeat two more times for complete removal of KBr.
7. Filter the LDFBS though a 0.2-µm filter into a sterile container.
8. Analyze for protein content and adjust to required concentration by dilution or concentration.
9. Lipoprotein depletion may be verified by agarose gel electrophoresis using a lipid stain (e.g., Sudan Black), with the starting serum as a reference.
10. Verify sterility by your preferred assay.

References

[1] Havel, RJ, et al., "The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum," *J Clin Invest*, 34:1345-53, 1955. [PubMed ID: 13252080]

[2] Mills, GL, Lane, PA, Weech, PK, A Guidebook to Lipoprotein Technique, Laboratory Techniques in Biochemistry and Molecular Biology, vol. 14, Elsevier Science Publishing, New York, 1984.

[3] Radding, CM, Steinberg, D, "Studies on the synthesis and secretion of serum lipoproteins by rat liver slices," *J Clin Invest*, 39:1560-9, 1960. [PubMed ID: 13738935]

[4] "Ultracentrifuge methods for lipoprotein research," Beckman Instruments Application Data DS-514B, 1989. (PDF)