OptiPrep™ Application Sheet M08

Fractionation of plasma lipoprotein sub-classes

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water; density = 1.32 g/ml
- The basic methodology was developed for human plasma but it has subsequently been extended to other species and also used for human Coomassie blue-stained lipoproteins
- The **Axis-Shield Mini-Review "Fractionation of plasma lipoproteins" (MM01)** provides a comprehensive bibliography of all the published papers (nearly 100) reporting the use of OptiPrep™: to access **MM01** return to the initial list of Folders and select "**Mini-Reviews**"
- To access other Application Sheets referred to in the text return to the Macromolecules and Macromolecular Complex Index; key Ctrl "F" and type the M-Number in the Find Box

1. Background

In the routine method, chylomicron-free plasma is adjusted to 12% (w/v) iodixanol and the sample, essentially fills an approx 3 ml tube for a near-vertical rotor. During the centrifugation VLDL, LDL and HDL particles and also plasma proteins migrate from all parts of the sample to their final buoyant density banding position in the self-forming density gradient. This method was developed with the Beckman TLN100 near-vertical rotor - **see Application Sheet M07**.

By modulating the experimental conditions in a variety of ways it is possible to influence the profile of the density gradient that is formed during the centrifugation and thus alter the linear resolution of the various lipoprotein fractions within the tube. The profile is modulated by:

- Small changes to the centrifugation time and iodixanol concentration.
- Choice of rotor; the recommended Beckman rotors (TLN100, VTi65.1, NVT65, NVT65.2) have similar **but not identical** sedimentation path lengths; the density gradient profile generated from the same concentration of iodixanol at the same g_{av} and centrifugation time, is consequently sufficiently different in each rotor type to affect the resolution of the lipoproteins.
- Tube loading format; unique density profile modulations can be achieved by using a two layer format (e.g. equal volumes of 12% (w/v) and 6% (w/v) iodixanol) rather than a single uniform concentration.

The effect of iodixanol concentration and tube-loading format, in the NVT65.2 is given in Figure 1. Panel 1 shows the banding of the major lipoproteins using the same tube format, centrifugation time and RCF as described in **Application Sheet M07**; the only difference is the rotor type (results from a TLN100 are shown in Figure 3 of **Application Sheet M07**). In the NVT65.2 the gradient density profile causes the HDL to band more broadly (and the LDL more sharply) across the gradient fractions than in the TLN100. There is even some indication that the use of a uniform concentration of 12% iodixanol at 365,000g for 3 h in the NVT65.2 rotor may permit the resolution of the HDL into distinct subfractions; the three boxed fractions in Panel 1 clearly indicate a minor population of HDL particles denser than the major population. Only by reducing the uniform iodixanol concentration to 9% in the NVT65.2 (Panel 2), does the relative linear distribution of the HDL and LDL approach that obtained with 12% iodixanol in the TLN100.

Panel 3 shows the distribution of lipoproteins in a two layer tube-loading format of 12% and 9% iodixanol (equal volumes of each) with the plasma confined to the denser layer. It might be expected that the gradient generated from the 12% iodixanol in the bottom half of the tube would have caused the HDL to band further up the tube when compared to the banding in a gradient generated from a uniform 9% iodixanol format (Panel 2). The important consideration here is the amount of plasma proteins in the system; plasma proteins contribute significantly to the total density of the gradient, particularly in the lower half of the gradient. The 12%/9% format contains less than half of the plasma proteins present

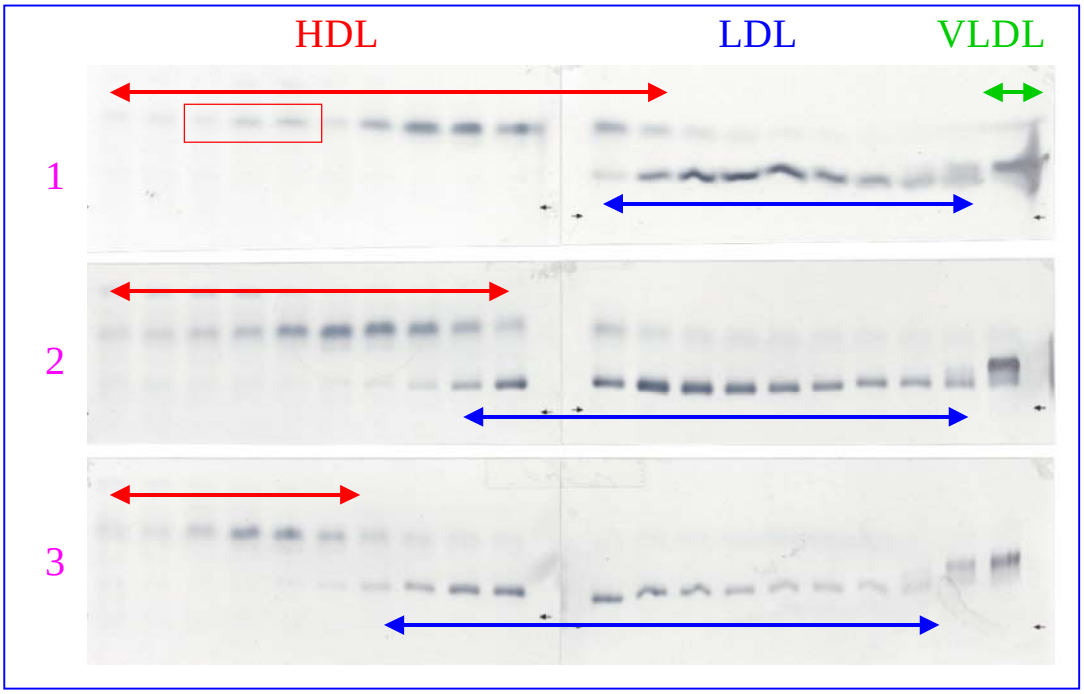

Figure 1 Sudan black stained agarose gel electrophoresis profiles of human plasma lipoproteins. Panel 1, 12% iodixanol; panel 2, 9% iodixanol; panels 3, 12%/9% iodixanol. All gradients were centrifuged in 5.1 ml tubes in the Beckman NVT65.2 near-vertical rotor at 350,000 *g* for 3h. All gradients were unloaded dense-end first. See text for more details

in the 9% format; moreover in the two layer format, the proteins molecules, already restricted to the lower half of the tube, also sediment more effectively towards the bottom of the tube.

2. Fractionation of LDL subclasses

Subfractionation requires an increase in the resolving power of the gradient, not only to aid identification and quantitation of LDL subclasses in a reproducible manner, but also to improve the linear separation of dense LDL from lighter HDL. This can be achieved in a number of ways (see above and ref 1 for more details).

- By confining the plasma sample to a dense load zone (12% iodixanol) at the bottom of the tube and layering a solution of 9% (or sometimes 6%) iodixanol in saline on top, the HDL and plasma proteins sediment through the gradient formed within the 12% layer while the LDL (and VLDL) particles float (and band) in the "clean" gradient formed by the 9% layer.
- The profile of the low density resolving gradient can be adjusted by changing the relative volumes of the high and low density layers and by changing centrifugation time.
- The use of a larger volume tube of sedimentation path length similar, but not identical, to that of the TLN100 (i.e. a taller tube) may enhance the effect of these modulations.
- Harvesting the gradient in a larger number of small volume fractions will also better maintain the fractionation achieved by the gradient.

The following protocol provides a basic strategy (adapted from refs 1 and 2). In the Notes are some recommendations from published papers for optimizing the resolving power and/or for alternative analytical procedures.

2a. Solutions required

A. OptiPrep™

B. Hepes-buffered saline: 0.85% (w/v) NaCl, 10 mM Hepes-NaOH, pH 7.4

C. Cholesterol analysis kit

Keep Hepes (free acid) as 100 mM stock solution at 4°C:
2.38 g Hepes per 100 ml water

Solution B: Dissolve 0.85 g NaCl in 50 ml water, add 10 ml of buffer stock solution; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml.

2b. Ultracentrifuge rotor and gradient harvesting requirements

Beckman near-vertical rotor; TLN100, NVT65.2 or NVT65 (see Note 1)

Beckman Fraction Recovery System (for tube puncture) or Labconco Auto Densi-Flow™ gradient collector (for collection from the meniscus) (see Note 2)

Peristaltic pump and fraction collector (to take 96 well microtitre plate)

2c. Protocol (for TLN100 rotor)

1. Using freshly drawn blood (1 mM EDTA as anti-coagulant), pellet the cells at 2000 *g* for 15 min.

2. Remove chylomicrons from the plasma by centrifugation at 100,000 *g* for 10 min (see Note 3).

3. Mix 4.25 vol. of Solution B with 0.75 vol. of OptiPrep™ (9% iodixanol final concentration) and transfer 1.4 ml to an OptiSeal™ tube for a Beckman TLN100 rotor (see Notes 4-6).

4. Mix 4 vol. of plasma with 1 vol. of OptiPrep™ (12% iodixanol final concentration) and use 1.4 ml to underlayer the 9% iodixanol solution (see Notes 4 and 5).

5. Layer Solution B on top to fill the tube (see Note 6).

6. After sealing the tube, centrifuge at approx 350,000 g_{av} for 2.5-3.0 h at 16°C, using slow acceleration to and deceleration from 2000 rpm (see Note 7).

7. Collect the gradient in 0.07-0.1 ml fractions by tube puncture into a 96 well microtitre plate and analyze the fractions for cholesterol (see Notes 8-13).

2d. Notes

1. Near-vertical rotors are the preferred type since the soluble proteins sediment towards the bottom of the tube (like a pellet in a fixed-angle rotor). In a vertical rotor the proteins will sediment on to the wall of the tube, down the length of the tube, unless a small volume (0.2 ml) of 20% iodixanol is included. See ref 1 for more information on the use of other rotors.
2. Collection from the meniscus is the preferred method because in tube puncture the first few fractions containing soluble plasma proteins are very viscous and tend to contaminate the succeeding densest HDL fractions.
3. Lower speeds for longer times may be used.
4. Alternatively overlayer the plasma with the 9% (w/v) iodixanol; sometimes 6% iodixanol is used.
5. The relative volumes of the plasma in 12% and the 9% iodixanol can be adjusted to suit the tube volume. Davies and Griffin [3] for example used 3 ml of plasma and 8 ml of 9% iodixanol in an NVT65 rotor. Larger volume tubes, in which the volume of the resolving gradient can be increased, potentially provide an improved resolution over the smaller volume tubes.
6. The saline on top of the sample not only conveniently fills the tube, it minimizes the tendency of the VLDL to adhere to the wall of the tube. This is particularly important with vertical rotors. It also enhances the separation of the VLDL from the lightest LDL in all rotors.
7. Sawle et al [2] found that 2.5 h (in the TLN100) gave a better resolution of the denser LDL from the lighter HDL; this is due to the slightly steeper gradient (in the top 2/3rds of the tube) at the shorter time. It is advisable to test the optimum time (in the 2.5-3.0 h range) for other rotor types.
8. Because iodixanol absorbs strongly in the UV it is not feasible to monitor the lipoprotein distribution in the gradient by direct spectrophotometric measurement. As an alternative to monitoring the cholesterol content of the collected gradient fractions, Davies and Griffin [3] pre-stained the plasma with Coomassie blue (see Note 12).
9. An example of the resolution achievable with the TLN100 is given in Figure 2. The three plasma samples exhibited distinctive LDL distributions with peak LDL densities in fractions 5, 6 or 8 (in a 19 fraction gradient harvest). By reducing the centrifugation time to 2.5 h and taking smaller fractions (44 in total) it is possible to detect differences in LDL banding more easily [2]; under these conditions the peak positions can differ by as many as 10 fractions.

10. The methodology for determining the LDL density banding profile using iodixanol gradients has now been validated against other techniques [2].
11. Sawle et al [2] improved the resolution significantly by decreasing the fraction volume size and collected a TLN100 gradient in 44 fractions.
12. If the lipoproteins are pre-stained with Coomassie blue [3,4], it is possible to short-cut the need to unload the gradient by taking a digital photograph of the tube. After downloading on to a PC, the image is analyzed by computerized gel scanning technology to obtain a profile of the Coomassie blue staining. The method has been validated against the established KBr gradient technology.

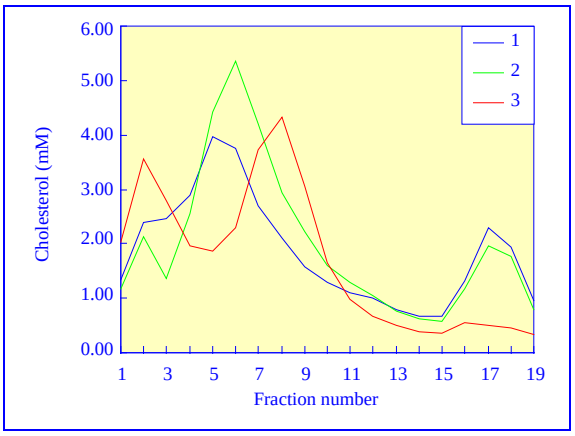

Figure 2 Cholesterol profiles of 3 plasma samples in gradients generated from 12%/9% iodixanol: Tubes (3.5 ml) centrifuged at 350,000 *g* for 3h in a TLN100 rotor. Gradients were unloaded dense-end first (fraction 1)

13. Any lipid analysis or gel electrophoresis can be carried out directly on the gradient fractions. If it is necessary to remove contamination of HDL fractions by plasma proteins, centrifuge the gradient sample through an ultrafiltration cone with a 100 kD mol wt cut-off (e.g. Whatman Vectaspin 3™ polysulphone centrifuge tube filter).

3. Fractionation of HDL subclasses

The use of iodixanol gradients to fractionate HDL subclasses from pre-stained plasma has been reported by Harman et al [5] in a meetings abstract and more recently a detailed methodology has been published [6].

♦ There are also a couple of published reviews on the gradient technology used for LDL subclass fractionation and these are listed below in Section 4 [7,8]; they also provide an overview of other methodologies required.

4. References

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8. Hirayama, S. and Miida, T. (2012) *Small dense LDL: An emerging risk factor for cardiovascular disease* Clin. Chim. Acta, **414**, 215–224

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