OptiPrep™ Application Sheet M04

Harvesting gradients

1. Introduction

The mode of harvesting depends very much on the type of tube used for the gradient, the distribution of particles in the gradient and the aim of the fractionation. Thick-walled tubes cannot be unloaded by any of the methods that involve piercing the tube wall with a needle and tubes with a narrow neck, such as some sealed tubes, make access with the tip of an automatic pipette impossible.

2. Tube handling prior to band or gradient recovery

The traditional open topped flexible-walled tubes for swinging-bucket rotors pose few, if any, problems for any mode of sample recovery. Heat-sealed or crimp-sealed tubes pose the biggest problems and for some modes of harvesting it may be necessary to slice off the top, to convert it to an open-topped tube.

- **Do not use a scalpel blade**
- Use a special tube cutter (Seton Scientific, Los Gatos, CA; sales@setonscientific.com) - the Beckman tube slicer is a possible alternative

3. Recovery of individual bands of material

If the position of the particles of interest has been clearly established and, if there is more than one band in the gradient, the linear separation of those bands is ≥1 cm, then the band(s) may be removed individually by aspiration.

3a. Using a Pasteur pipette or syringe (applicable to any open-topped tube)

If a syringe is used, attach it to a flat-tipped metal cannula (i.d. 0.8-1.0 mm) not to a syringe needle. Metal filling cannulas may be obtained from any surgical equipment supplies company.

- Place the tip of the pipette or cannula at the top of the band of interest and aspirate the liquid very slowly, moving it across the diameter of the tube.
- To minimise the aspiration of any liquid from below the band, the tip of a glass Pasteur pipette may be fashioned into an L-shape.
- If the band of interest is below other material in the gradient then remove the latter first.

3b. Using a syringe (flexible-walled tubes only)

It is also possible to collect a specific band within the gradient by puncturing the tube wall with a needle attached to a syringe.

- To allow easy piercing of the tube wall; the centrifuge tube is best restricted by some sort of tube clamp.
- Insert the needle just below the band and with the inlet to the needle (bevel uppermost); aspirate the band into the syringe (Figure 1).
- If a sealed tube is used, air must be allowed to displace the falling column of liquid in the tube (see Figure 1) by puncturing the tube close to its top with another syringe needle.
- Once the band has been aspirated, the syringe needle is withdrawn and the hole in the tube sealed with silicone grease.
- The procedure may be repeated to harvest a denser band.

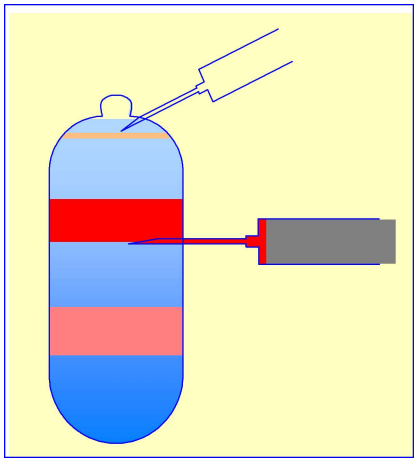


Figure 1: Collection of banded material from a sealed tube. See text for details

4. Harvesting the entire gradient into a series of equal volume fractions

The volume of each fraction collected from a gradient is determined as much by the operator's requirements as by the resolving power of the gradient. As a general rule however, the volume of each fraction should be approx 5% of the gradient volume, but this may be decreased or increased for higher or lower resolution respectively.

4a. Using a Pasteur pipette, automatic pipette or syringe (applicable to any open-topped tube)

Most Pasteur pipettes are calibrated on the stem so if the tip of the pipette or cannula (attached to a 1 or 2 ml syringe) is placed at the meniscus, the total gradient may be collected in suitably sized fractions. If an automatic pipette is used, trim the end of the tip to make the orifice diameter 0.8-1.0 mm. The method is however tedious, prone to error and difficult to obtain equal volume fractions because of the need to keep the tip of the cannula or pipette at the meniscus without occasionally aspirating some air or removing some of the gradient from below the meniscus. For a crude fractionation however into four or five gradient cuts it is quite satisfactory.

4b. Aspiration from the bottom using a peristaltic pump

Ideally the harvesting system should be devised so that the effluent from the tube should not have to pass through a pump, but as long as the dead space volume of the tubing is small compared to the volume of the gradient it is permissible to insert a narrow rigid tube to the bottom of the centrifuge tube and to aspirate the contents (dense-end first). Theoretically, mixing will occur in the vertical section of the collection tubing as the decreasingly dense medium enters the bottom of the tube. In practice however this seems not to be a serious problem, again as long as the enclosed volume of the collecting tube is small compared to that of the gradient.

- If there is a pellet, make sure that the tip of collecting tube is maintained above it.

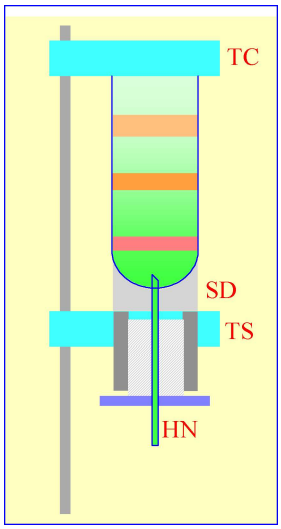


Figure 2: Gradient collection (dense-end first) by tube puncture.
The tube is clamped between the sealing disc (SD) and the tube support (TS). A hollow needle (HN) is advanced through the bottom of the tube, sometimes by a screw-device (shown by the hatched area) or, more commonly by a pivoted lever.

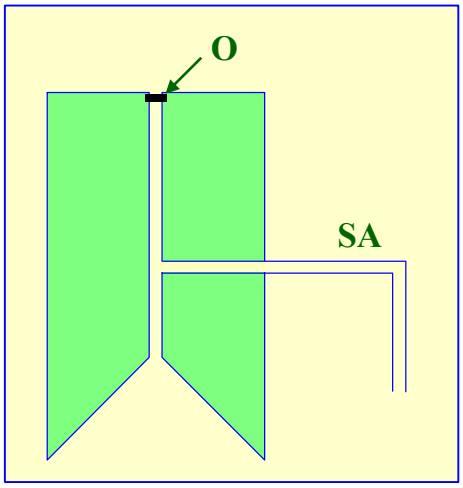
4c. Tube puncture

Practically this is best achieved by securing the tube vertically in some form of clamping device and to advance the needle through a rubber seal into the bottom of the tube by a screw or lever mechanism (Figure 2). The Beckman-Coulter Fraction Recovery System incorporates such a device. If sealed tubes are used, then either the central plug should be removed (Optiseal™) or the top punctured with a syringe needle (Quick-Seal™) to allow air to displace the liquid, which exits the tube under gravity. The system is simple and the dead space of the collecting tube is very small and the gradient is collected almost ideally, the hemispherical section of the bottom of the tube directing banded material into the collecting needle.

Because of the viscosity of the dense end of some gradients, gravitational flow will be slow at first and then speed up as the viscosity of the liquid decreases. To overcome this, the effluent from the hollow needle can be passed through a small volume peristaltic pump. So long as the dead space of the silicone tubing is small compared to the volume of the gradient, resolution is not seriously sacrificed.

- Thick-walled tubes cannot be used and it may not be a useful method if there is a large pellet, which may obstruct the hollow needle.

- Collecting equal volume fractions by this method or that described in **Section 4b** is not easy. The low-tech answer is to use calibrated collection tubes, although this requires continual attention from the operator to move on the delivery tube at the appropriate time. This problem is discussed further in **Section 4h**.



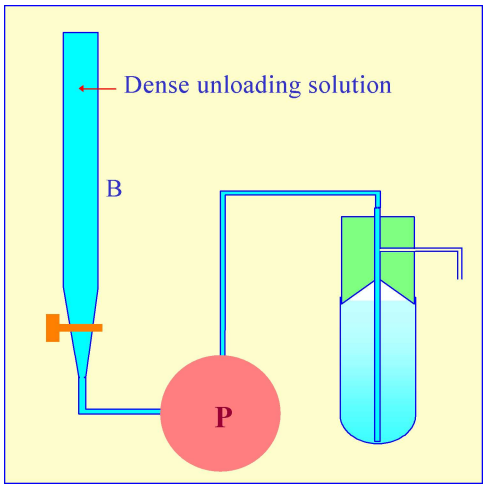


Figure 3 (left) and Figure 4 (right) Figure 3: Simple gradient unloader for upward displacement: O = O-ring, SA = side arm (see text for details). Figure 4: Gradient unloader sealed into top of centrifuge tube; dense unloading solution from a burette (B) delivered to bottom of gradient by peristaltic pump (P) and a metal cannula inserted through the central channel of the unloader. The gradient is displaced upwards through the conical section, into the side arm and from there, via a flexible delivery tube, into the collection tubes.

4d. Upward displacement

A dense liquid introduced to the bottom of the tube can displace the entire gradient upwards and with a suitable device attached to the top of the tube, the gradient can be delivered into the collection tubes. The use of a burette to contain the unloading solution does allow the collection of equal volume fractions.

4d-1. Delivery of dense liquid through a central tube inserted into the gradient

A simple device fashioned from a cylindrical block of Perspex (Lucite or acrylic) shown in Figure 3 can be produced by any laboratory workshop. To fit flexible-walled tubes the cylinder should be slightly tapered towards the bottom (not shown in figure). The block contains a central channel, which leads to a hollowed-out cone, and a side-arm, which connects with the central channel. The dense unloading solution is introduced to the bottom via a long metal cannula inserted down the central channel and through the gradient (Figure 4). The gradient is displaced upwards by the incoming dense liquid into the cone and an O-ring around the cannula diverts the flow into the collection tubes via the side-arm.

- For rigid-walled open-topped tubes the collecting device requires sealing on to the tube with a gasket, under pressure. Such a device can indeed be used for any type of tube and one is incorporated as one of the options in the Beckman-Coulter Fraction Recovery System.
- By placing the dense unloading solution in a burette and delivering it to the bottom of the centrifuge tube via a peristaltic pump (Figure 4), the unloading process can be executed at a uniform flow rate
- By using the graduations on the burette to signal the manual advancement of the delivery tube to the next collection tube, it is the only method that guarantees equal volume fractions.
- The gradient could alternatively be collected using an automated fraction collector (see **Sections 4g** and **4h**).
- The best unloading medium is a low viscosity, dense, non-water-miscible, fluorocarbon such as perfluorodecalin (ρ = 1.9 g/ml). This was previously commercially available from Axis-Shield and its distributors as Maxidens™. Perfluorodecalin can currently be purchased from F2 Chemicals Ltd, Lea Lane, Lea Town, Preston PR4 0RZ, UK (tel: +44 (0)1772 775802, fax +44 (0)1772 775808). Also available from the same company is a similar fluorocarbon containing a blue dye (Flutec-blue), which makes visual assessment of the progress of gradient unloading very easy.
- The rate of gradient unloading should be 1-2 ml/min for 10-20 ml gradients and 0.5-1.0 ml/min for smaller volume gradients.

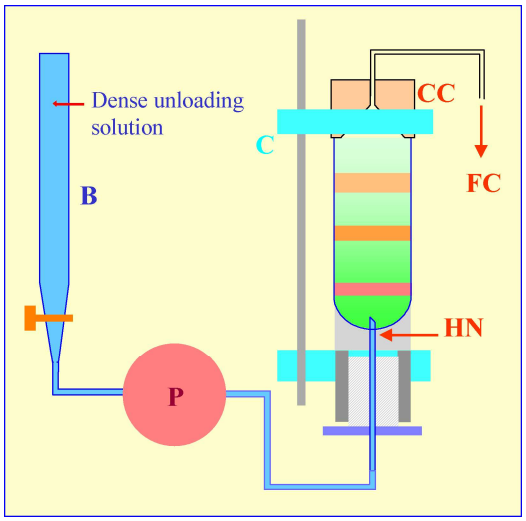
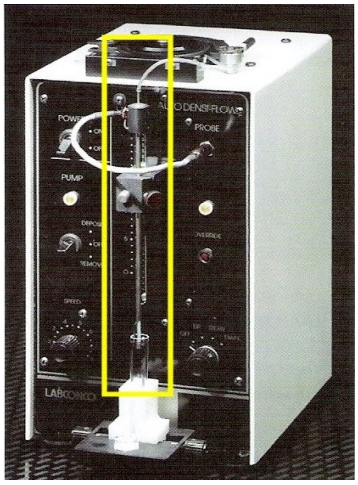
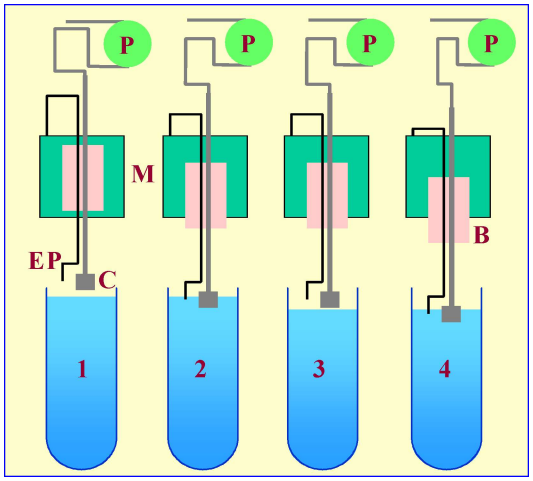


Figure 5: Gradient harvesting by upward displacement with a dense medium delivered by tube puncture. The hollow needle (HN) is completely filled with the dense unloading solution from the burette (B) using the peristaltic pump (P) before the tube is located within the clamping device of a Beckman-Coulter Fraction Recovery System. The conical collection head (CC) is located sealed on to the tube, and the tube held vertically, by the clamp (C). When the pump (P) is reactivated after puncturing the tube, the dense unloading solution displaces the gradient upwards through the conical collection head (CC) and into the fraction collection tubes (FC).

4d-2. Delivery of dense liquid by tube puncture

An alternative mode of delivering the dense unloading solution to the bottom of the tube is by tube puncture. In this case the burette is attached via the pump to the lower end of the hollow needle (Figure 5), which must be primed with the dense solution, prior to tube puncture. The hollow needle (HN) of the Beckman-Coulter Fraction Recovery System has an important design feature – the exit port is on the vertical side of the needle, thus its sharp point is solid. This not only facilitates tube puncture, fragments of tube material removed by the puncturing process or any pellet in the tube, are much less likely to impede the flow of the dense unloading solution than if the exit port was tip-located, as in a standard syringe needle.





Figures 6 (left) and 7 (right) Figure 6: Photograph of the Labconco Auto Densi-Flow™ gradient collector, the area bounded by the yellow box is shown in diagrammatic form in Figure 7. The collection head (C) and the adjacent electronic probe (EP) are amounted in the (pink) block (B), which is moved vertically by the (dark green) motor (M), (P) = peristaltic pump (see text for description of operation).

4e. Automatic aspiration from the meniscus

The Auto Densi-Flow™, produced by the Labconco Corporation comprises a hollow metal tube that terminates in a small collection head (Figures 6 and 7); the upper end of the tube is connected to a peristaltic pump, which aspirates the gradient. The motor, which is activated when the electronic probe (mounted at the side of the collection head) is in a non-conductive medium (air), advances the collection head towards the gradient until the tip of the probe reaches the meniscus of the gradient (Figure 7, 1 and 2). Now the tip of the probe is in an aqueous conductive medium, the motor stops and the gradient starts to be aspirated by the pump and the meniscus falls (Figure 7, 3). The motor is consequently re-activated as the meniscus recedes from the probe and the collection head advances further downwards until again the probe reaches the meniscus (Figure 7,4) and so on. For clarity, the procedure has been described and shown in Figure 7 in an exaggerated step-like manner. In reality, the aspiration of the gradient and the steady advance of the collection head occur almost simultaneously. In this way the entire gradient is collected in a smooth and continuous fashion.

- Note that the collection head of this device also provides an excellent means of depositing a continuous gradient, dense end first, from a two-chamber gradient maker. In this mode the motor moves the collection head upwards; the sequential activation and deactivation of the motor by the rising meniscus being the reverse of the collection mode.
- **IMPORTANT NOTE**: although this device is no longer produced by Labconco, many remain available in laboratories and second hand machines are available from instrument "recycling" companies on the internet.

4f. Biocomp Instruments piston fractionator

Rather different to the other types of fractionator, the Biocomp piston fractionator comprises a piston containing a central channel, which at its lower end expands outwards in the form of a curved conical section. As the piston advances down the tube, the gradient is displaced upwards into the central channel. The progressively decreasing volume of the conical section experienced by the displaced liquid effectively increases the linear separation of particles in the gradient and so maximises resolution. The device is available in conjunction with a detection system (and the Biocomp Gradient Master™ gradient former – **see Application Sheet M02 Section 2c**). The device is only suitable for open-topped tubes and tubes of different diameters require their own piston.

4g. Integrated automatic gradient harvesting process

In the system illustrated in Figure 8, the Labconco Auto Densi-flow gradient unloader is being used to harvest the gradient (from the meniscus) from a standard tube for a swinging-bucket rotor. The effluent from the peristaltic pump on top of the Auto Densi-flow is directed to the collection head of a Gilson FC205 fraction collector for dispensing into a 96-well polypropylene "MasterBlock Deep-Well" plate (Greiner Bio-One Inc). These MasterBlocks can easily accommodate volumes of up to 2.0 ml. The multi-well plate format for gradient collection allows simple gradient analysis if the gradient fractions are subsequently sampled using a multiple channel automatic pipette (see below); it also provides an easy means of storage. A standard 96-well plate can replace the large-volume MasterBlock for the collection of smaller gradient volumes.

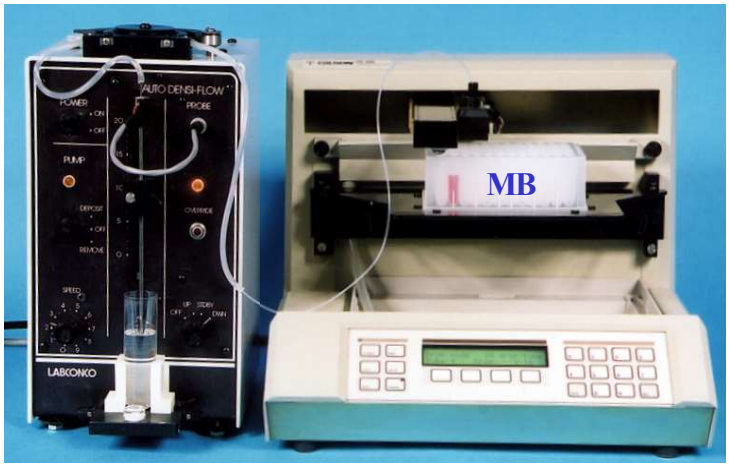


Figure 8: Integrated automatic gradient harvesting and fractionation. The gradient is shown being collected from the meniscus using a Labconco Auto Densi-flow, fractionated in a Gilson FC205 fraction collector into a 96-well Greiner Bio-One MasterBlock multiwell plate.

Any gradient unloader that incorporates a peristaltic pump to maintain a reasonably consistent flow rate can be linked up to fraction collector, but note that as the density of the liquid progressively changes so do other physical parameters such as viscosity and surface tension. Drop size will thus vary during the collection process and fraction volumes will change progressively during a drop-counting collection process. So whether the fraction advance is signaled by drop number or time, there will be a progressive change in fraction volume whose severity depends on the density-range of the gradient. Nevertheless if this change is acceptable, it will at least be reproducible from gradient to gradient. This fraction collection system has been used very successfully for analysis of lipoprotein banding in self-generated iodixanol gradients [1,2].

4h. Influence of tube type on harvesting strategy

- Open topped thin walled tubes (polyallomer, polycarbonate or Beckman Ultraclear™) for swinging-bucket or fixed-angle rotors can be unloaded by any of the above methods.

- Thick-walled open-top tubes can be unloaded by any of the methods except tube puncture (**Sections 4c and 4d-2**).
- Thick-walled tubes (screw-capped) with a wide shoulder are best unloaded from the meniscus (**Section 4e**) using the Labconco Auto Densi-flow™ or aspiration from the bottom (**Section 4b**). Upward displacement (**Section 4d-1**) may be satisfactory if the shoulder is narrow, sloped or rounded, otherwise material may get trapped at the shoulder.
- Heat-sealed or crimp-sealed tubes cannot be unloaded directly by any of the methods except tube puncture (**Section 4c**). Any other method of unloading requires the tube to be cut horizontally just below the shoulder (see **Section 2**). This is might cause disturbance to the gradient unless carried out very carefully.
- Sealed tubes that are sealed by a central plastic plug (e.g. Beckman Optiseal™ tubes) can be unloaded by any of the methods. Note however that upward displacement is best carried out using the **Section 4d-2** option with a length of Teflon tubing secured to the neck of the tube by a silicone rubber collar to carry the gradient effluent to the collection tubes. Note also that the neck of some of the smaller volume sealed tubes may be too narrow to accept the collection head of the Labconco Auto Densi-flow machine (**Section 4e**).

5. References

1. Graham, J., Higgins, J. A., Gillott, T., Taylor, T., Wilkinson, J., Ford, T. and Billington, D. (1996) *A novel method for the rapid separation of plasma lipoproteins using self-generated gradients of iodixanol* Atherosclerosis, **124**, 125-135
2. Sawle, A., Higgins, M.K., Olivant, M.P. and Higgins, J.A. (2002) *A rapid single-step centrifugation method for determination of HDL, LDL, and VLDL cholesterol, and TG, and identification of predominant LDL subclass* J. Lipid Res., **43**, 335-343

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