OptiPrep™ Application Sheet M02

Preparation of discontinuous and continuous gradients (macromolecules and macromolecular complexes)

- **To access other Application Sheets referred to in the text return to the Macromolecules and Macromolecular Complexes Index; key Ctrl "F" and type the M-Number in the Find Box.**

1. Discontinuous gradients

1a Overlayering technique

The most widely used method for producing discontinuous gradients is to start with the densest solution and layer solutions of successively lower densities on top using some form of pipette or syringe. Tilt the centrifuge tube (approx. 45°); place the tip of the pipette or syringe against the wall of the tube, about 1 cm above the meniscus of the denser solution, and gently deliver a slow and steady stream of liquid. This allows the liquid to spread over the tube surface and minimizes any mixing due to a sudden increase in liquid flow. Once a steady flow is established keep the tip of the pipette or syringe just above the meniscus of the liquid and against the wall of the tube.

From a pipette

Use a rubber two- or three-valve pipette filler to aliquot and dispense the gradient solutions. Check that the release valve when pressed gently, allows the delivery of a slow and steady flow of liquid. Do not use a pipette filler that uses positive pressure to deliver the liquid, as a slow even flow is often difficult to attain. Always take up more of the gradient medium than is required as it is easier and more accurate to empty the pipette to a graduation mark than to try to empty it completely.

From an automatic pipette

For small volume gradients an automatic pipette may be used. Always cut off the end of the plastic pipette tip to reduce the flow velocity of the liquid.

From a Pasteur pipette

Plastic Pasteur pipettes can be used conveniently for larger volume gradients, particularly those in calibrated centrifuge tubes. It requires some practise however to maintain a steady liquid flow by depressing the bulb of the pipette.

From a syringe

A syringe with a wide-bore metal filling cannula (i.d. approx 0.8 mm) is suitable for most gradient volumes, but make sure that the barrel can move easily and smoothly when a small pressure is applied. Placing the index finger around the bottom of the plunger, rather than around the barrel, restricts the movement of the plunger when it is depressed and thus achieves a more controlled liquid flow. Always take up more of the gradient medium than is required for the step as it is more accurate to empty the syringe to a graduation mark than to try to empty it completely.

- Metal filling cannulas can be purchased from most surgical instrument suppliers.

1b Underlayering technique

Although the overlayering technique is probably the most widely used, the easier method is to underlayer successively denser solutions beneath the lighter solutions. The only important requirement is that no air bubbles are introduced which may disturb the lower density layers above; for this reason a syringe with a metal filling cannula is the best tool for this procedure. Generally the existing steps are disturbed less as the outflowing liquid spreads upwards through the hemispherical section of the bottom of the tube.

1. To underlayer 4 ml of liquid, take up 5 ml into the syringe and expel to the 4.5 ml mark to ensure that the cannula is full of liquid.

2. Dry the outside of the cannula.

3. Move the tip of the cannula to the bottom of the tube, sliding it slowly down the wall of the tube (Figure 1A-B)

4. Depress the plunger to the 0.5 ml mark (Figure 1C).

5. After a few seconds (to allow all of the liquid to be delivered into the tube) slowly withdraw the cannula, again against the wall of the tube (Figure 1D).

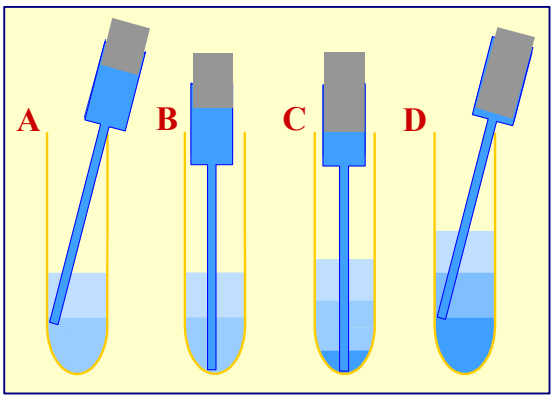


Figure 1: Formation of a continuous gradient by underlayering (see text for details)

6. Dry the outside of the cannula and repeat the procedure with successively denser solutions.

2. Continuous gradients

Continuous gradients may be made by allowing discontinuous gradients to diffuse or by using a gradient maker specifically designed for this purpose.

<u>2a By diffusion of discontinuous gradients</u>

Once a discontinuous gradient is formed, the sharp boundaries between the layers, which are observed as a sudden change in refractive index, start to disappear as the solute molecules diffuse down the concentration gradient from each denser layer to each lighter layer. Thus the density discontinuities between each layer will slowly even out and the gradient will eventually become linear (Figure 2), and given sufficient time the density will become completely uniform.

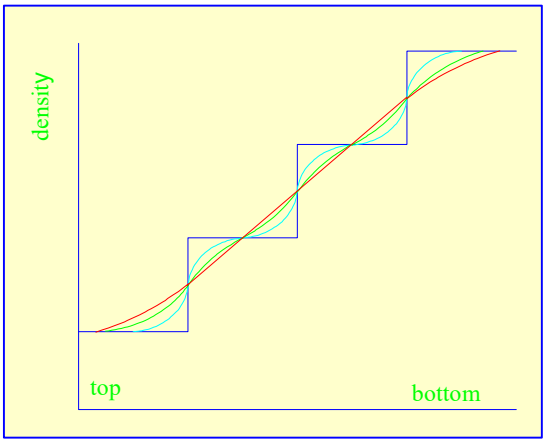


Figure 2: Formation of a continuous gradient by diffusion of a discontinuous gradient. The initial stepped density profile (dark blue) will gradually become smoothed out (→ light blue → green → red).

For a particular medium, the rate of diffusion across an interface is dependent on temperature and the cross-sectional area of the interface. In addition the rate at which the gradient becomes linear will also be a function of the distance between the interfaces. Thus a linear gradient will form more rapidly at room temperature than at 4°C and if the distance between interfaces is reduced and the cross-sectional area increased. This can be achieved as follows (refer to Figure 3).

1. Produce a discontinuous gradient by the underlayering (Section 1b) or overlayering (Section1a) method. Unless the gradient is to be very shallow use 3 or 4 layers that increase in steps of about 5-10% (w/v) iodixanol.

2. Seal the tube well with Parafilm® and carefully rotate the tube to a horizontal position and leave for 45-60 min.

3. Return the tube to the vertical, cool to 4°C if required and apply the sample to the gradient (either over- or under-layered).

The precise timing for the formation of a continuous linear gradient will depend on the dimensions of the tube, the number of layers and the concentrations of iodixanol. A series of trial experiments should be carried out in which the time is varied and the density profile of the formed gradient checked by fractionation and refractive index measurement.

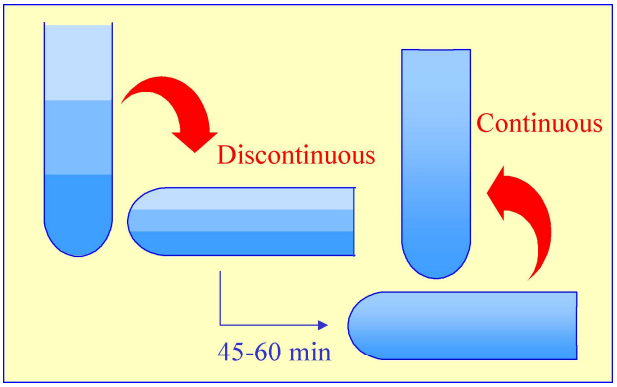


Figure 3: Rapid formation of a continuous gradient by diffusion of a discontinuous gradient

Because the continuous gradient is formed by a physical process, so long as the temperature and time are well controlled, the shape of the gradient is highly reproducible. If the diffusion is allowed to occur in a vertically maintained tube the process will take longer and at 4°C it may take more than 10 h. If however the gradients are prepared the day before the experiment and left in the refrigerator overnight this can be a convenient approach. Gradients prepared rapidly at room temperature need to be equilibrated at 4°C prior to loading of the sample. Incorporation of the sample into one or more of the layers eliminates interfaces and can improve resolution, but this useful strategy (used with cells) is unlikely to suit macromolecules or macromolecular complexes that need maintaining at 4°C.

2b Using a two-chamber gradient maker

The traditional way of constructing a continuous gradient is to use a standard two-chamber gradient maker (Figure 4). It consists of two identical chambers connected close to their bases by a tapped channel (T). One of the chambers (the mixing chamber – B in Figure 4) has an outlet directly opposite the inlet from the tapped channel.

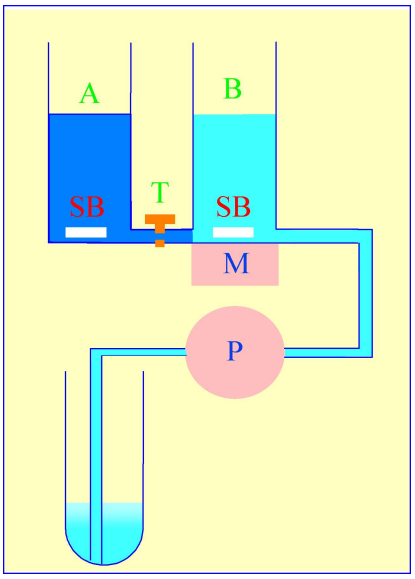


Figure 4: Two-chamber gradient maker (for details see text)

1. Set up the device as shown in Figure 4 with the mixing chamber (B) resting on a magnetic stirrer (M) and the outlet tube leading via a peristaltic pump (P) to the bottom of the centrifuge tube.

2. Place the chosen high-density solution in the non-mixing chamber (A) and then momentarily open the tap (T) to allow dense liquid to fill the connecting tube.

3. Pour an equal volume of the low-density solution in the mixing chamber (B).

4. Place two identical stirring bars (SB) in the two chambers (this ensures that the height of the two solutions is the same).

5. In rapid sequence, switch on the pump (P) and the magnetic stirrer (M) and then open the connecting tap (T). As the levels in the two chambers fall synchronously, reduce the speed of the stirrer to avoid generating air bubbles that may enter the gradient and disturb it.

6. Make sure that the pump is turned off before any air bubbles reach the bottom of the delivery tube at the end of the operation.

- The larger the density difference between the two gradient solutions the more vigorous must be the stirring to ensure good mixing. If the stirring bar in chamber B is too close to the inlet from the connecting tube, it is possible in the initial stages for the low-density medium to back flow into the high-density medium.
- The correct pumping speed depends on the volume of the gradient and the quality of the pump (ideally the outflow from the pump should not pulsate), but for a standard 10-30% (w/v) iodixanol gradient (of 12-15 ml total volume) a flow rate of approx 2 ml/min is satisfactory. Pumps that impart little or no pulsation to the liquid flow are commonly available from many sources.
- The gradient can alternatively be produced high density end-first, in which case the location of the two solutions needs to be reversed and the delivery tube to the centrifuge tube must be placed against the wall of the centrifuge tube near to its top, so the gradient flows down the tube smoothly. This is can pose some problems of mixing in the centrifuge tube if the flow down the tube wall is in the form of large drops rather than a continuous stream (this may be minimized by tilting the tube), on the other hand the tendency of the low density medium to float to the surface of the high density medium in the mixing chamber aids mixing. The Auto Densi-Flow gradient unloader can be used to deposit a gradient high-density end first with no disturbance. Although this device is no longer commercially available, it will be found in many laboratories. For details of this device **see Application Sheet M04**.
- To guard against air bubbles entering the delivery tube, a bubble trap could be included between mixer and pump. Although air bubbles are a major problem if they reach the bottom of the centrifuge tube (low density first delivery) they are no less a problem for high-density first delivery as they interfere with the smooth flow of liquid down the tube wall.
- It is possible to produce up to three gradients at a time; some gradient mixers have a three-outlet manifold. However such a device requires three tubes to pass through the peristaltic pump. It is the only reliable configuration of the delivery tube; simply splitting the liquid flow from a single tube through the pump cannot guarantee precisely equal delivery to all three tubes.

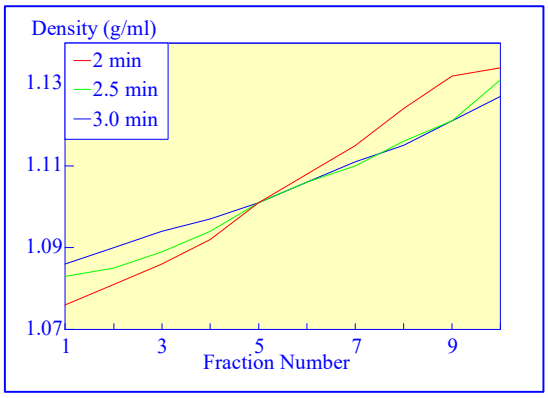
Figure 5: Biocomp Gradient Master™

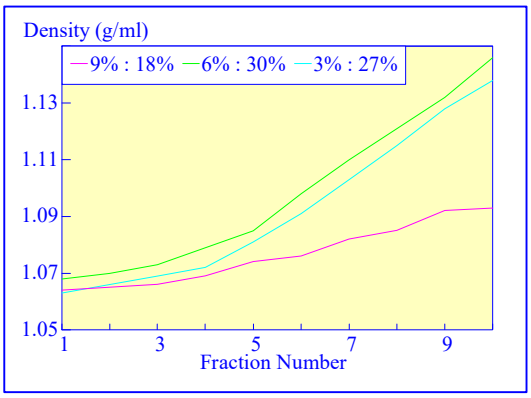
2c Gradient Master™

An alternative device for the generation of continuous density gradients - the Gradient Master™ - produces the gradient by controlled mixing of the low and high-density solutions layered in the centrifuge tube. The tubes are rotated at a pre-set angle (in the drum to the left of the control panel) - usually 80° - to increase the cross-sectional area of the interface - and speed (usually 20 rpm) for about 2 min (Figure 5). The density profile of the gradient generally becomes more shallow with time. The simplicity of the technique and the highly reproducible nature of the gradients make this a very attractive method; up to 6 gradients (17 ml tubes) can be formed at once. Some examples with iodixanol solutions are given in Figures 6 and 7.

- A very important advantage of this technique over the use of a two-chamber gradient mixer is that if it is necessary to make the sample part of the gradient, any potentially hazardous biological sample is contained within the centrifuge tube and does not contaminate the gradient forming device and ancillary tubing.
- For more information on the Gradient Master™ and other similar instruments contact the manufacturers at www.biocompinstruments.com

Figures 6 (left) and 7 (right) Figure 6: Effect of time on gradients formed from 10% and 30% (w/v) iodixanol; Figure 7: Effect of iodixanol concentration of gradients formed after 1 min 50 sec.





2d Freeze-thawing

The final manner in which continuous gradients can be produced is by freezing a solution of uniform density for at least 30 min at -20°C and then thawing at room temperature for 30-60 min. These times are for tubes of approximately 5 ml volume. The freeze-thaw cycles can then be repeated; this modulates the density profile of the gradient. Generally as the number of freeze-thaw cycles increases, the gradient becomes markedly less dense at the top. The method can produce gradients that are more or less linear. Because the shape of the gradient depends on the rate of freezing and thawing, as well as the number of freeze-thaw cycles (and the volume of the tube), the precise conditions required need to be worked out for a particular laboratory. Under well-controlled conditions however, the profiles are highly reproducible. An example of the procedure with an iodixanol solution is given in Figure 8 (data kindly supplied by Dr C A Borneque, CNRS, Centre de Génétique Moléculaire, 91198 Gif sur Yvette, France).

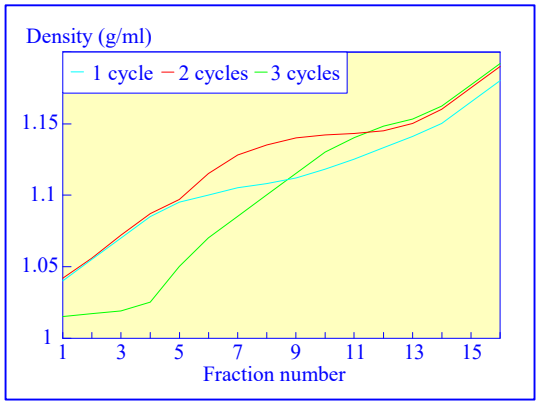


Figure 8: Gradients formed by freeze-thawing of 20% (w/v) iodixanol in 5 ml tubes

2e Non-linear gradients

It is not always desirable to use a linear gradient and either convex, concave, S-shaped or more complex gradient density profiles may be required to effect a particular resolution of particles. Convex gradients are sometimes particularly useful for the resolution of a sample containing a high concentration of particles of a wide range of densities. The steep density profile at the top of the gradient provides stable conditions for high capacity and the shallower high-density region provides high resolution.

From discontinuous gradients by diffusion

If each of the layers of the initially discontinuous gradient is of the same volume then diffusion will produce a linear gradient. The diffusion process however is also a very convenient way of producing a gradient that is not linear with volume. Convex or concave gradients or gradients containing a shallow median section can be produced by increasing the volume of the denser, lighter or median density layers respectively. The shape of the gradient may also be altered by changing the density interval between adjacent layers. Clearly reducing the density interval will make the gradient more shallow. It is important to test the density profile that is formed from such discontinuous gradients, but once satisfactory conditions are established the profile will be highly reproducible.

Using a gradient mixer or Gradient Master

Convex and concave gradients cannot be produced with the standard two-chamber gradient mixer (see Figure 4). However if the non-mixing chamber is made twice the diameter of the mixing chamber, then with low-density solution in the mixing chamber a convex gradient is produced; if the locations of the low density and high-density solutions are reversed, a concave gradient is produced. In a Gradient Master™ (see Section 2c) non-equal volumes of the two density solutions will change gradient shape.

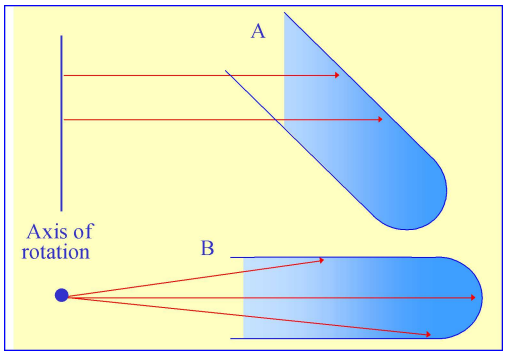


Figure 9: Sedimentation paths of (A) fixed-angle rotor and (B) swinging-bucket rotor

3. Types of rotor used with preformed gradients

Traditionally, preformed gradients of sucrose or glycerol are run in a swinging-bucket rotor and today this remains the most popular choice of rotor for any density gradient centrifugation. Sedimentation path lengths tend to be long, but because of the relatively low viscosity of iodixanol solutions, centrifugation times need not be excessive. Separation of proteins on the basis of sedimentation velocity in swinging-bucket rotors is commonly carried out overnight. However, because iodixanol is able to form its own gradient by self-generation in the centrifugal field, it is not good practice to carry out buoyant density banding through pre-formed gradients at RCFs in excess of 250,000 g_{av} for more than 3-4 h. Iodixanol molecules towards the bottom of the tube may start to form a self-generated gradient and thus deform the pre-formed density profile in the high-density region. At lower RCFs (e.g. 100,000 g) there will essentially be no density profile modulation.

Fixed-angle rotors are generally less frequently used for pre-formed gradients. Because of the angle at which the tube is held, particles tend to sediment to the wall of the tube due to the radial centrifugal field (Figure 9A); this does not occur in a swinging-bucket rotor, although even in this type of rotor, only those particles in the middle of the sample move in a plane parallel to the walls of the tube (Figure 9B). Swinging-bucket rotors were also often perceived as having an advantage over fixed-angle rotors for gradient work since the gradient always maintains the same orientation with respect to the long axis of the tube. However, so long as the particles do not adhere to the wall of the tube, a fixed-angle rotor can provide a useful alternative and there are many successful examples, particularly now that slow acceleration and deceleration facilities are widely available on centrifuges to permit smooth reorientations of the gradient.

If a fixed-angle rotor is satisfactory for a particular gradient separation, then the shorter sedimentation path length of such a rotor compared to that of a swinging-bucket rotor of the same capacity permits a shorter centrifugation time. This situation is taken to its logical conclusion with a vertical rotor which will have the shortest path length, i.e. the width of the tube and, like the swinging-bucket rotor, the sedimentation or flotation of particles is relatively unaffected by the tube wall (Figure 10). In these rotors therefore, centrifugation times are reduced to a minimum. Analysis of the oligomerization of the β-amyloid Aβ peptide for example needs only 3 h in an iodixanol gradient in a vertical rotor, **see Application Sheet M09**. In a rotor such as the Beckman VTi65.1 or VTi50 the long axis of the tubes is much larger than their diameter (Figure 10), so the steep gradient formed during centrifugation reorients to a relatively shallow one at rest. Therefore, so long as there is no mixing of the tube contents during deceleration, small volume fractionation of the gradient will provide very high resolution.

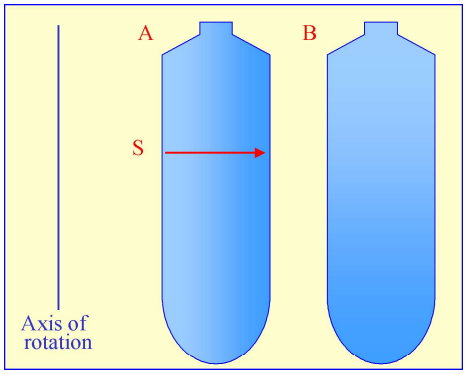


Figure 10: Sedimentation path (S) and gradient orientation in a vertical rotor, (A) at speed, (B) at rest

- It is important that the gradient is so designed to prevent particles from sedimenting to the wall of the tube, as these will tend to fall back into the medium during reorientation and unloading and thus contaminate the rest of the gradient.
- Near-vertical rotors, which hold the tube at approx. 8° to the vertical, overcome this problem.
- Vertical and near-vertical rotors can provide the most efficient form of centrifugation in gradients. They can be particularly effective for rate-zonal separations, since any sample placed on top of a gradient achieves a very small radial thickness after reorientation.
- Because the surface area of any banded material is much higher in a vertical or near-vertical rotor than in a swinging-bucket rotor during centrifugation, particles that have a significant rate of diffusion (M_r<$5x10^5$) may exhibit band broadening due to this diffusion.

The use of large volume zonal rotors for gradient centrifugation is beyond the scope of this text; for information the reader is referred to relevant review articles [1,2].

4. Types of tube for gradient centrifugation

Choice of tube material (polyallomer, polycarbonate etc) is usually governed by considerations of optical transparency, resistance to chemicals or sterilizing (autoclaving) procedures (see manufacturers specifications for more information); generally speaking there is no specific advantage or disadvantage of using one particular type of tube material for gradient centrifugation from a fractionation point of view. The tube material may also restrict the maximum permitted RCF.

Choice of tube type (open-topped, screw-capped, sealed etc) is dictated by the selection of rotor type, the RCF that is required (many tube types cannot be run at the maximum speed of the rotor); the degree of containment that is required and a consideration of the type of gradient harvesting that is to be carried out. For details on gradient harvesting see Section 4i of **Application Sheet M04**.

Gradient centrifugation in low-speed and high-speed centrifuges is not generally carried out in special tubes, unless special containment is required; the standard thick walled polycarbonate, polyallomer, polypropylene or polystyrene tubes employed for all low- and high-speed centrifugation are satisfactory.

In ultracentrifugation a wide range of tube styles are available and the reader is directed to the appropriate technical manuals published by the centrifuge companies. Principally polyallomer and polycarbonate or Ultra-Clear™ (Beckman trade-name) are used. For simplicity and convenience only those tubes manufactured by Beckman Instruments will be described although other companies supply an essentially similar range of tubes although there may be some differences in the mode of sealing.

- **Swinging-bucket rotors** Tubes for swinging bucket rotors are traditionally open topped (the seal being provided the screw-cap on the bucket), thin-walled and made from polyallomer or Ultra-Clear™. Occasionally thick-walled polycarbonate is available. See also “Vertical rotors” below.
- **Fixed-angle rotors** The types of material used for tubes for fixed-angle rotors are broadly similar to those for swinging-bucket rotors. Some of the thick walled tubes are open-topped and do not require caps, others have a variety of capping devices. Thin-walled tubes always require caps. The thick walled variety with a simple screw cap is not ideally suited to some forms of gradient harvesting. For details on gradient harvesting see Section 4i of **Application Sheet M04**. See also “Vertical rotors”, below.
- **Vertical rotors** The only types of tube recommended for vertical rotors are thin-walled sealed tubes made either from polyallomer or Ultraclear™; these are also available for many swinging-bucket and fixed-angle rotors. In swinging-bucket rotors however there is usually a variable reduction in tube volume compared to the standard thin-walled open-topped tubes. Beckman manufacture two types: Quick-Seal™ and Optiseal™, the former are sealed by a heat and the latter by a central plastic plug.

- Through the use of adaptors and spacers most rotors accommodate a range of tubes of a volume considerably smaller than that of the rotor tube pocket, many of which may have a restricted maximum RCF (compared to the standard thin walled tube). Traditionally the smaller volume tubes for swinging-bucket and fixed-angle had a much-reduced diameter but the length was only slightly less than that of the full-volume tube (Figure 11).

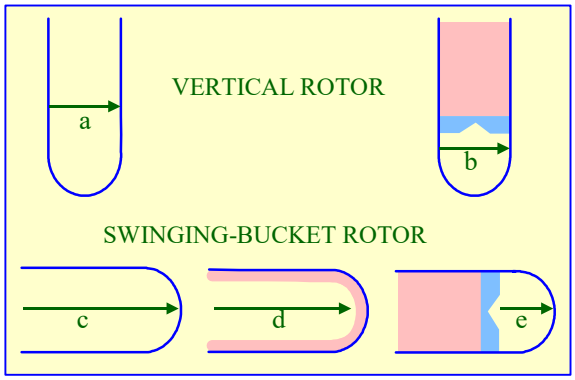


Figure 11: Effect of tube adaptors on sedimentation path length of vertical and swinging-bucket rotors. Unadapted (a and c); "old-style" adaptor (d); Beckman g-Max™ adaptor (b and e)

- **g-Max™ tubes:** Because of the advantage of a short sedimentation path length, some of the swinging-bucket and fixed-angle rotors have been adapted to take shorter sealed tubes so that the path length is reduced. They are also available for vertical rotors but in these rotors the sedimentation path length is unchanged, only the volume is altered (Figure 11).

5. References

1. Graham, J. M. (1978) *In Centrifugal separations in molecular and cell biology* (ed. G. D. Birnie and D. Rickwood). Butterworths, London, pp 63-85.
2. Graham, J. M. (1992) *In Preparative centrifugation - a practical approach* (ed. D. Rickwood) IRL Press at Oxford University Press, Oxford, UK, pp 315-350.

Application Sheet M02; 8th edition January 2018

Alere Technologies AS

Axis-Shield Density Gradient Media
is a brand of Alere Technologies AS