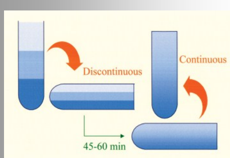


Types of centrifugal separations

Rate zonal centrifugation

The behaviour of particles in a centrifugal field is described by the following equation:

$$v = \frac{d^2(\rho_p - \rho_l)g}{18\mu}$$

v = velocity of sedimentation

d = diameter of particle

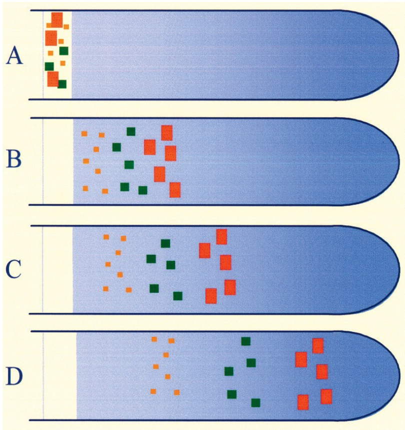
ρ_p = density of particle

ρ_l = density of liquid

g = relative centrifugal force (RCF)
μ = viscosity of liquid

One way of improving the resolution of particles by size is to minimize the difference in RCF between the top and bottom of the sample. Practically this is achieved by layering the sample as a narrow band over a continuous density gradient. This is called rate-zonal gradient (sedimentation velocity) centrifugation; the conditions are chosen such that ρ_p always remains greater than ρ_l. In a continuous gradient the density increases in a smooth but not necessarily linear fashion and can be generated by a number of methods described later. By allowing the particles to sediment through such a density gradient the resolution and recovery of particles is generally improved.

The particles move down through the gradient in the form of discrete zones at a rate that depends principally on their size (see figure below). Resolution between the



zones decreases as the radial thickness of the sample increases so the efficacy of rate zonal fractionation is limited by sample size, which is commonly no more than 10% of the total gradient volume. The aim of rate-zonal centrifugation is to devise conditions that will allow each zone of particles to move down through the gradient at a more or less constant velocity (isokinetic gradient). In a continuous gradient the particles are decelerated by the increasing viscosity of the medium and the reducing (ρ_p - ρ_l) factor; ideally the acceleration (RCF) and deceleration forces should balance one another. A discontinuous density gradient (density increases in step-wise fashion) is never used for rate -zonal separations as sudden changes in viscosity and density would lead to irregular separation of the zones of particles as they move from one step to another.

Obviously the particles must never be allowed to sediment into a region of the gradient where (ρ_p - ρ_l) approaches zero, so the density range of the gradient and the centrifugation time have to be carefully controlled.

Buoyant density centrifugation

When the centrifugation is carried out for a time that is sufficient to allow all the particles in the sample to reach that part of the gradient whose density is equivalent to that of the particles (i.e. where $\rho_p = \rho_l$, and $v = 0$) the gradient system is said to be **isopycnic** and the particles are separated according to their **buoyant or banding density**. Irrespective of the size of the particles and their starting position in the gradient, all particles of the same density will band at the same position. For the same reason, the efficacy of the separation is not limited by the size of the sample.

Buoyant density gradient centrifugation may be carried out in continuous or discontinuous gradients. A single step called a density barrier - is sometimes used to isolate one type of particle whose density is either greater or less than that of the barrier and greater or less (respectively) than that of all of the other particles.

In buoyant density fractionation there is no requirement for top loading: the sample can be placed in a dense medium at the bottom of the gradient, in which case the particles are in a medium of density greater than their own density, so $\rho_p < \rho_l$ and v will be negative: the particles will float up through the gradient rather than sediment through it. The sample can alternatively be placed in a median position in a discontinuous gradient or distributed throughout the gradient.

Discontinuous gradients

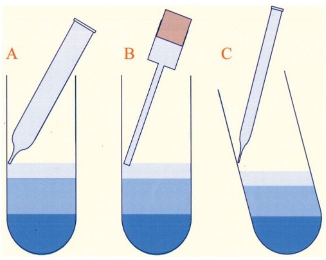
Overlayering technique

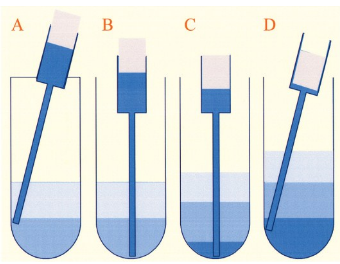
The most widely used method for producing discontinuous gradients is to start with the densest solution and layer successively lower densities on top from a pipette. The higher the viscosity of the medium, the easier it is to achieve a relatively slow and smooth flow of liquid from the pipette. Low density solutions, having very low viscosities are more difficult to layer in this manner. A syringe with a wide-bore metal filling cannula (i.d. 1-2 mm) can be used as an alternative to a pipette, but make sure that the barrel can move easily and smoothly when a small pressure is applied. If the gradient solution is pre-measured, it may be applied from a Pasteur pipette, but practice is required in achieving a slow smooth flow of liquid when the bulb is gently depressed (see figure on the left below). A is with a pipette, B with a syringe and C with a Pasteur pipette.

Underlayering technique

Although the overlayering technique is probably the most widely used, the method in which successively denser solutions are underlayered under lighter ones is certainly the easier and the recommended one. 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 less disturbance to the existing steps is produced as the outflowing liquid spreads upwards through the hemispherical section of the bottom of the tube (see figure on the right above).

To layer 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. Then dry the outside of the cannula. Place the tip of the cannula at the bottom of the tube (slide it slowly down the wall of the tube) and depress the plunger to the 0.5 ml mark (Figure A-C). 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 D).



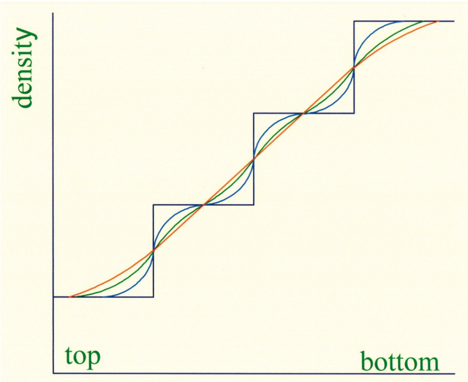


Continuous gradients

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

By diffusion of discontinuous gradients

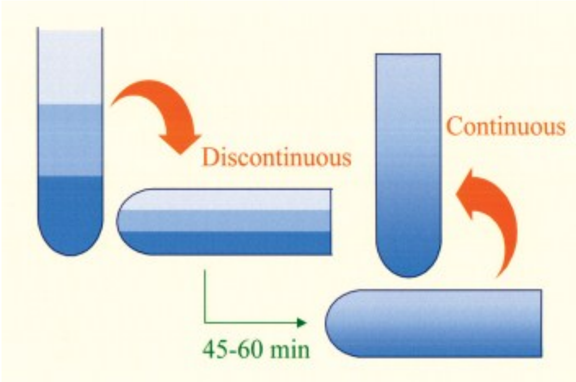
Once a discontinuous gradient is formed, the sharp boundaries between the layers which are observed as a sudden change in refractive index, start to disappear as the solute molecules diffuse down the concentration gradient from each denser layer to each lighter layer. Thus the density discontinuities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



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 their cross-sectional area increased. This can be achieved as follows (see figure below):

Produce a discontinuous gradient by the underlayering or overlayering method. Unless the gradient is to be very shallow use 3 or 4 layers which increase in steps of about 5-10% (w/v) iodixanol. Seal the tube well with Parafilm and carefully rotate the tube to a horizontal position and leave for 45-60 min. 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 will depend on the dimensions of the tube, the number of layers and the concentrations of iodixanol or Nycodenz®. A series of trial experiments should be carried out in which the time is varied and the



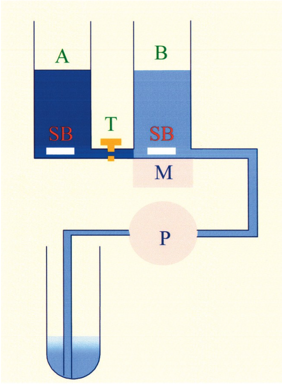
density profile of the formed gradient checked by fractionation and refractive index measurement.

Because the continuous gradient is formed by a physical process, then so long as the temperature and time are well controlled the shape of the gradient is highly reproducible. The diffusion can be allowed to occur in the vertical tube but the process will take longer and at 4°C it may take more than 10 h. If however the gradients can be prepared the day before the experiment and left in the refrigerator overnight then this can be a convenient approach. The sample may be applied to the gradient after diffusion or it may be incorporated into one or more of the layers before diffusion. If the sample is an homogenate, the diffusion process would have to be carried out at 4°C, for mammalian cells however this can be carried out at room temperature.

Using a two-chamber gradient maker

The traditional way of constructing a continuous gradient is to use a standard two-chamber gradient maker (see figure below). It consists of two identical chambers connected close to their bases by a tapped channel. One of the chambers (the mixing chamber) has an outlet directly opposite the inlet from the tapped channel. The device is set up with the mixing chamber resting on a magnetic stirrer and the outlet tube leading via a peristaltic pump to the bottom of the centrifuge tube.

The chosen high density solution is placed in the non-mixing chamber and the tap is momentarily opened to fill the connecting tube. An equal volume of the low density solution is placed in the mixing chamber. Two identical stirring bars are placed in the two chambers (this ensures that the height of the two solutions is the same). The pump is switched on, the magnetic stirrer started and the connecting tap opened. As the levels in the two chambers fall synchronously, the speed of the stirrer is reduced to avoid generating air bubbles which may enter the gradient and disturb it.



The 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 - usually 80° - to increase the cross-sectional area of the interface - and speed (usually 20 rpm) for about 2 min (see figure on the right). 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 particularly as up to 6 gradients (17 ml tubes) can be formed at once.

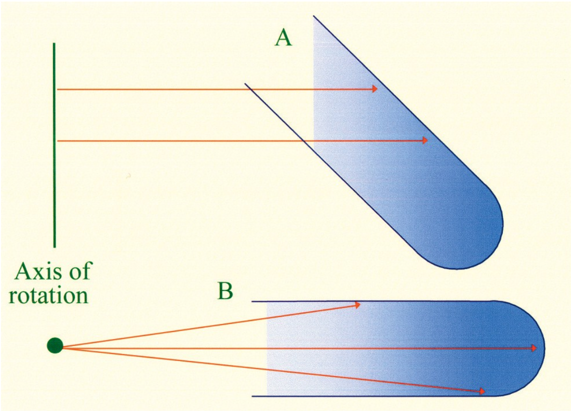
A very important advantage of this technique over the use of a two-chamber gradient mixer is that if it is necessary to make the sample part of the gradient, any potentially hazardous biological sample is contained within the centrifuge tube and does not contaminate the gradient forming device and ancillary tubing.

Types of rotor used with preformed gradients

Traditionally, preformed gradients are run in a swinging -bucket rotor and today this remains the most popular choice of rotor. Sedimentation path lengths tend to be long, but because of the low viscosity of Nycodenz® and iodixanol, centrifugation times need not be correspondingly long. In a standard 6x17 ml swinging-bucket rotor (path length approx 100 mm) for example, the major organelles (Golgi, lysosomes, mitochondria and peroxisomes) from mammalian liver can be resolved by flotation through a 10-30% (w/v) iodixanol gradient (ρ = 1.078-1.175 g/ml) at 50,000g_{av} for 90 min. Microsomes require a slightly higher RCF of 100,000g_{av}. 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 centrifugation of particles through pre-formed gradients at RCFs higher than 150,000g_{av} for more than 3-4 h because the iodixanol towards the bottom of the tube may start to form a self-generated gradient and thus deform the pre-formed gradient in the high density region.

Fixed-angle rotors are generally less frequently used for pre-formed gradients. Because of the angle at which the tube is held, some particles tend to sediment to the wall of the tube due to the radial centrifugal field (see figure A below); 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 (see figure B below). 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 now 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 (see figure above). 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 (see figure below). In these rotors therefore, centrifugation times are reduced to a minimum. In a rotor such as the Beckman VTi65.1 the long axis of the tubes is much larger than their diameter, 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.

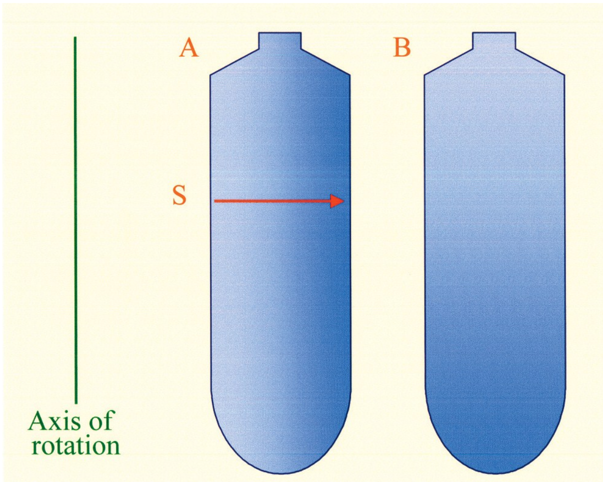
It is important that the gradient is so designed to prevent particles from sedimenting to the wall of the tube as these will tend to fall back into the medium during reorientation and unloading and thus contaminate the rest of the gradient. This is avoided in a near-vertical rotor.

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 rotor than in a swinging-bucket rotor during centrifugation, particles which have a significant rate of diffusion (M_r<5x10^5) may exhibit band broadening due to this diffusion.

Types of tube for gradient centrifugation

Apart from 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 tube type for gradient centrifugation from a fractionation point of view. Principally, choice is dictated by the selection of rotor type (see below); 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 (see “Harvesting density gradients”).



Gradient centrifugation in low-speed and high-speed centrifuges is not generally carried out in special tubes: the standard thick walled polycarbonate, polyallomer, polypropylene or polystyrene tubes employed for all low - and high-speed centrifugation are generally 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 by 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".

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 are not ideally suited to some forms of gradient unloading. See "Vertical rotors".

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 the former however there is usually a variable reduction in tube volume compared to the standard 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.

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 (see figure below).

Self-generating gradients

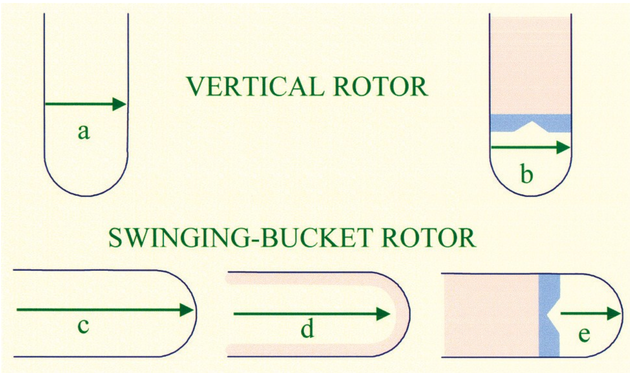
Solutions of iodixanol and Nycodenz®, like those of heavy metal salts (e.g. CsCl) can form a gradient from a solution of uniform density under the influence of the centrifugal field. Once the solute begins to sediment through the solvent a concentration gradient is formed which is opposed by back-diffusion of the solute. With a sufficiently high RCF, at equilibrium, the sedimentation of the solute is exactly balanced by the diffusion and the gradient is stable. It is possible to calculate the time for a self-generating gradient to reach equilibrium and it is described by the following equation:

$$t = k(r_b - r_t)^2$$

where t is the time in hours; r_b and r_t the distance from the centre of rotation to the bottom and top of the gradient respectively and k is a constant which depends on the diffusion coefficient and viscosity of the solute and on temperature. The slope of the gradient is given by the equation:

$$\rho_r - \rho_i = \frac{1.1x10^{-2}\, x Q^2}{2\beta^o}(r_c^2 - r^2)$$

where ρ_r is the density at a point r cm from the axis of rotation, ρ_i is the starting density of the homogeneous solution, r_c is the distance in cm from the axis of rotation where the density of the gradient = ρ_i , Q is the rotor speed in rpm and β^o is a constant depending on the properties of the solute).



The shape of the gradient that is formed for a particular solute thus depends on the following factors:

- sedimentation path length of the rotor
- time of centrifugation
- speed of centrifugation
- temperature

The big advantages of the use of any self-generated gradient is the ease of sample handling (the sample is simply adjusted to the required starting concentration of Nycodenz® or iodixanol) and the great reproducibility of the gradient density profile under a particular set of centrifugation parameters.

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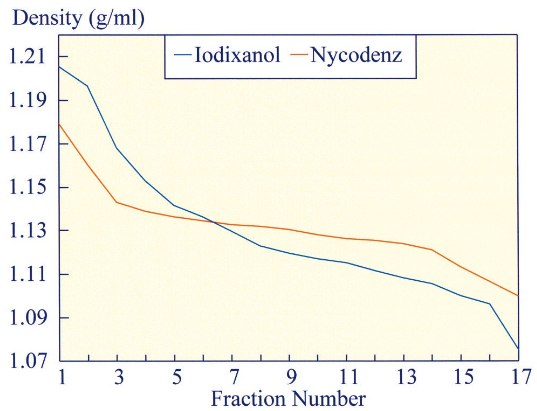
Iodixanol is able to form useful self-generating gradients in 1-4 h depending on the centrifugation speed and the rotor. The figure below compares the gradient density profile generated from 20% (w/v) iodixanol and 20% (w/v) Nycodenz® in 0.25 M sucrose in a 20° fixed-angle rotor at 270,000g_{av} for 3 h at 4°C. Clearly a steeper gradient is formed from the iodixanol and this is a function of the higher molecular mass of iodixanol (approx. twice that of Nycodenz®): it therefore sediments rather more rapidly and diffuses more slowly.



Types of rotor

Swinging-bucket rotors which have rather long sedimentation path lengths are little used for the formation of self-generating gradients. The shorter sedimentation path length rotors are much better suited to this task. Vertical and near-vertical rotors are particularly useful, although some fixed-angle rotors (preferably those with shallow angles of 20-24°) may be used.

Gradients generated in the Beckman TLN100 near-vertical rotor (for the TLX120 table-top ultracentrifuge) which accommodates tubes of 3.5-4.0 ml, the Beckman VTi65.1 vertical rotor (for an appropriate floor-standing ultracentrifuge) which accommodates tubes of approx 11.0 ml (but which can be adapted down to smaller volumes) and the Beckman NVT65 (a near vertical rotor of similar tube capacity to that of the VTi65.1) are particularly useful for iodixanol self-generated gradients. The TLN100 and VTi65.1 rotors have approximately the same sedimentation path length (about 17 mm), that of the NVT65 is marginally longer (approx. 25 mm); consequently under the same centrifugation conditions, they generate very similar gradient profiles.

Time of centrifugation

After 1 h at 15-18°C, centrifugation at approx 350,000g_{av}, gradients generated in the TLN100 are S-shaped (i.e. they contain a relatively shallow region in the middle) and span a relatively narrow density range, while after 3 h, gradients are considerably steeper and cover a much wider density range. The exponential nature of the gradient becomes more apparent with time but times greater than 4 h result in little further change in the shape of the gradient at 350,000g, indicating that an equilibrium point has been reached.

RCF

As the RCF decreases, so the gradient becomes more and more shallow in the middle of the tube; the minimum RCF that produces a useful gradient will vary with the time and the rotor type. In the VTi65.1 vertical rotor, even at 170,000g_{av}, a useful shallow gradient is produced within 3 h. In the latest very high performance rotors that can run at up to 150,000 rpm (and also have very short sedimentation path lengths, self-generated gradients can form in as little as 15 min. (see figure next page).

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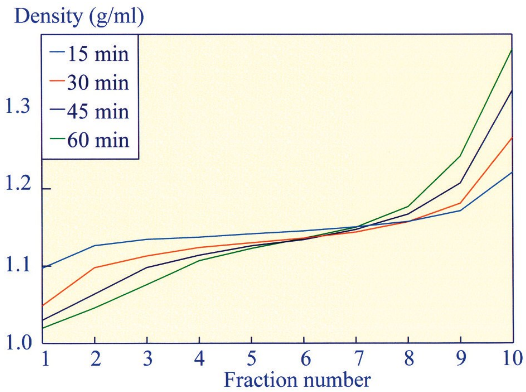
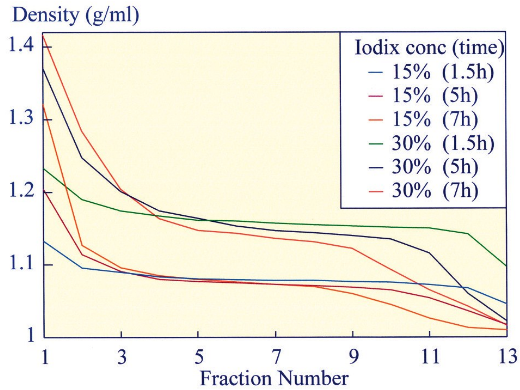
References are available on request





Sedimentation path length

The longer the sedimentation path length of the rotor, the greater the tendency to form S-shaped gradients. The figure below compares the gradient formed from 15% and 30% iodixanol using the 80Ti fixed-angle rotor with a sedimentation path length of 43 mm (13.5 ml tube volume) at a series of times. At 70,000 rpm, (equivalent to 345,000g_{av}) approx 5 h are required to produce a useful gradient.

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