Preparation of gradients for cells

- To access other Application Sheets referred to in the text return to the Cell Index; key Ctrl "F" and type the C-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 stainless-steel 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 conical section of the bottom of the tube.

1. To underlayer 3 ml of liquid, take up 4 ml into the syringe and expel to the 3.5 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 1).

4. Depress the plunger to the 0.5 ml mark.

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.

6. Repeat the procedure with successively denser solutions.

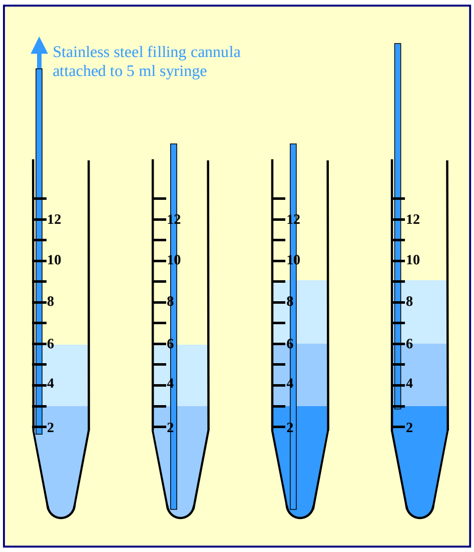


Figure 1: Formation of a discontinuous gradient by underlayering (see text for details

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.

2a 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 (Figure 2), 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, the cross-sectional area of the interface and the viscosity of the solution. 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 minimized.

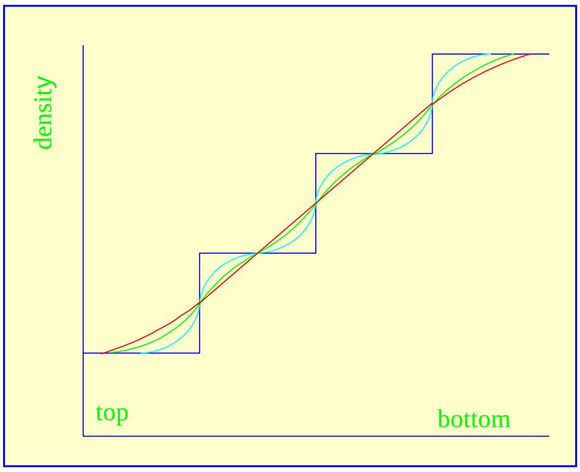


Figure 2: Formation of a continuous gradient by diffusion of a discontinuous gradient. The initial stepped density profile (dark blue) will gradually become smoothed out (→ light blue → green → red).

The precise timing for the formation of a continuous linear gradient will depend on the dimensions of the tube, the number of layers, the concentrations of iodixanol and the temperature. If the gradients are prepared the day before the experiment and left in the refrigerator overnight then this can be a convenient approach. At room temperature the time may be reduced to approx. 4 h. 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 to establish the correct conditions. In the absence of refractometer density profiles can also be determined by absorbance measurements (see Application Sheet C52)

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. The sample may be applied to the gradient after diffusion or it may be incorporated into one or more of the layers before diffusion. The latter strategy eliminates any interface between the sample and the gradient and may improve resolution. It is only useful however if the gradients can be rapidly prepared at room temperature.

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 3). 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 3) has an outlet directly opposite the inlet from the tapped channel.

1. Set up the device 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.

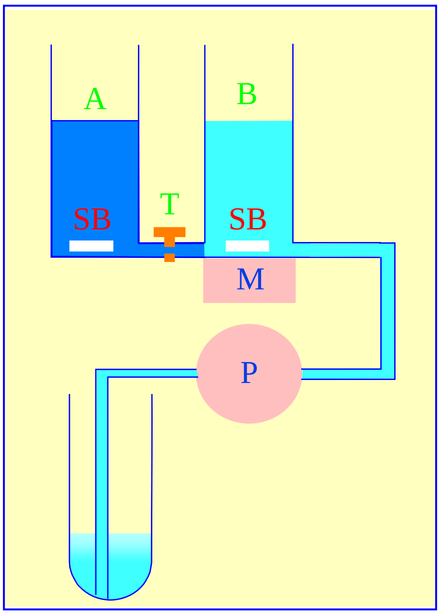


Figure 3: Two-chamber gradient maker (for details see text)

- 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) or 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 (B) aids mixing. The Labconco Auto Densi-Flow gradient unloader can be used to deposit a gradient high-density end first. Although this device is no longer commercially available, it will be found in many laboratories and often appears in laboratory equipment websites.
- 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.

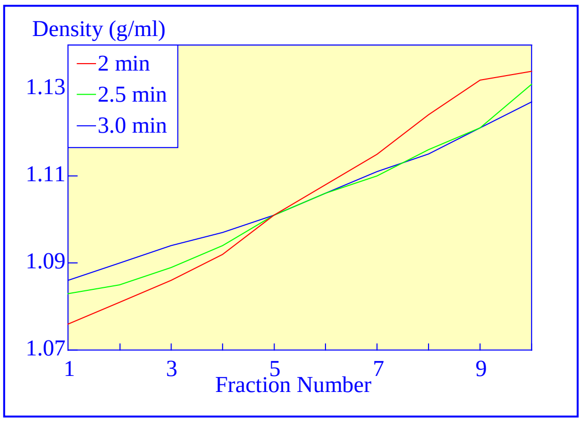
- It is possible to produce up to three gradients at a time; some gradient mixers have a three-outlet manifold. However such a device requires three tubes to pass through the peristaltic pump. It is the only reliable configuration of the delivery tube; simply splitting the liquid flow from a single tube through the pump cannot guarantee precisely equal delivery to all three tubes.

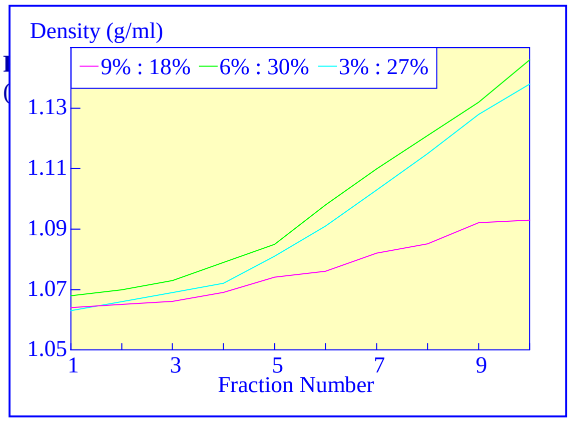
2c Gradient Master™

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 - usually 80° - to increase the cross-sectional area of the interface - and speed (usually 20 rpm) for about 2 min (Figure 4). 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 5 and 6.

Figure 4: The Gradient Master™

- 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.
- For more information on the Gradient Master™ and other similar instruments contact the manufacturers at www.biocompinstruments.com





Figures 5 (left) and 6 (right) Figure 5: Effect of time on gradients formed from 10% and 30% (w/v) iodixanol; Figure 6: 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 7 (data kindly supplied by Dr C A Borneque, CNRS, Centre de Génétique Moléculaire, 91198 Gif sur Yvette, France).

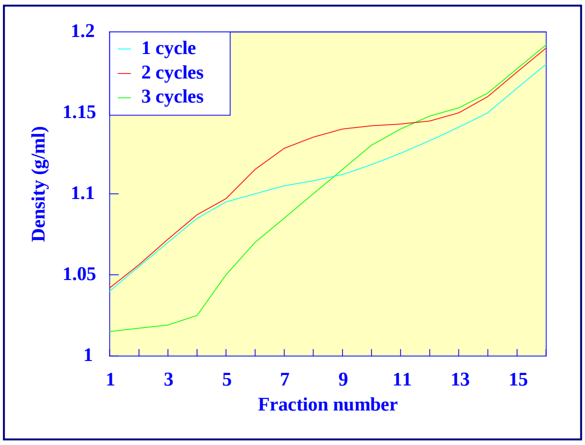


Figure 7: Gradients formed by freeze-thawing of 20% (w/v) iodixanol in 5 ml tubes

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

Convex and concave gradients cannot be produced with the standard two-chamber gradient mixer (see Figure 3). 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.

Using a Gradient Master™ (see Section 2c)

By using non-equal volumes of the two density solutions the gradient shape may also be changed.