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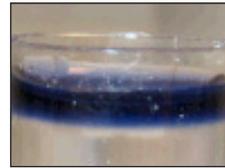
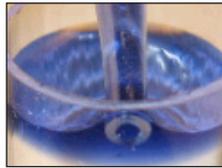
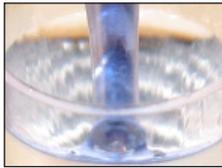
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# Gradient Primer

by David Coombs



## Section 1. Gradient Theory

### 1.0 The stable liquid column

Density gradients are marvellous creatures with unlimited potential for separation of biomolecules. They can be of any composition and volume and centrifuged in a variety of modes that can influence the separation. This primer is a modest attempt to explain the use of this time-honored technique at a time when its use has declined in the face of "higher tech" solutions. We will attempt to highlight its continued utility and the multiple advantages it has over other separation techniques.

The most important part of a density gradient is its density gradient. This may sound obvious and trite, but it is widely overlooked. The density gradient is the source of the gradient's stability as well as its capacity for separation. The top of the gradient must be dense enough to support the sample and the column must have enough of a density gradient to remain stable during the centrifugation and fractionation of the tube. Thus, a 15-20% sucrose gradient will be much less stable than a 5-45% gradient.

You can appreciate the need for stability by thinking of the contents of the tube as centrifugation begins and ends. There is a period at both ends of a run when the tube is rotating in a vertical position with centrifugal force applied to its side, and two other periods when this force is gradually transferred to or from the vertical axis of the tube as the bucket begins to swing out at the beginning of the run or down at the end of the run. These are difficult times for the gradient and the only thing that prevents vertical mixing is the steepness of the gradient. Moral: the greater the change in % between the top and bottom of a gradient, the more stable it is. As a general rule, the delta % should be  $\geq 20\%$  and as high as 40%.

### 1.1 The choice of solute; density, viscosity, osmolarity

The choice of solute is dictated by the type of separation desired. Density gradients have been constructed from many solutes in the past 40 years so it might be useful to list some of the more common ones and describe their uses and limitations:

**Sucrose:** By far and away the most common solute. It is cheap, very soluble, chemically inert and does not absorb in the UV. It has very high osmolarity, so it tends to be unkind to living cells, and at its maximum density is incapable of supporting biomolecules other than lipids, so its use is limited to rate zonal gradients for the most part.

**Glycerol:** A substitute for sucrose, sharing most of its properties. Used in cases where sucrose is harmful or insoluble in the downstream treatment of fractions.

**CsCl** (NaCl, KCl, KBr, LiCl): These salt gradients are useful when nucleic acids are banded, since the high solubility and density of some of these solutes can float these dense biomolecules. These gradients lack the viscosity of sucrose gradients and are less stable as a result (handle with care). The denser of these solutes (CsCl and LiCl) will form gradients on their own under centrifugal force, so it is possible to simply mix the salt directly in the sample and let the gradient form during an overnight run. The steepness of the gradient depends on the speed of the rotor (the x Gs applied to the tube).

**K Acetate:** Used in velocity banding of DNA/RNA fragments which are recovered from the fractions by adding isopropanol and precipitating the nucleic acid.

**Percoll™:** Pharmacia introduced Percoll many years ago as a low osmolarity solute suitable for whole cell purification. It is widely used in cell biology. It has one unusual property that has limited its use: It is actually a suspension of tiny silica particles coated with 3 polyvinylpyrrolidone (PVP) that can not be removed by dialysis. It self-forms gradients under centrifugation.

**Nycodenz™:** Originally developed as a radio-opaque dye for blood work, this Nycomed (now Axis-Shield) product has seen many years of use in cell purification. It has low osmolarity, is dialysable and its gradients are self-forming.

**Optiprep™** (iodixanol): A recent upgrade from Nycomed developed as a radio-opaque dye but currently enjoying robust gradient use, it shares many of Nycodenz's properties and is less toxic in some applications.

**Renografin™** (tri-iodinated benzoic acid, also called Angiografin, Hypaque, Renografin, Uroografin, Urovison): An early radio-opaque dye used for density gradients, it is no longer seeing much use, since it had some serious health issues and is difficult to source.

**Metrizamide** (Amipaque): The earliest radio-opaque dye. Same as Renografin, but with a slightly different chemical structure.

**Ficoll™:** A high molecular weight sucrose polymer used in some cell separations.

**Dextran:** A high molecular weight polymer of glucose. See Ficoll.

With so many choices, it is difficult to choose the right medium for the job. Your best bet is to use PubMed and search with the words “gradient” AND “your particle of interest” and see what others have done, i.e. “gradient AND polysomes”.

If you are intent on striking out on your own, choose the solute that best matches your needs. If purifying a proteinaceous particle, sucrose or glycerol are usually the solutes of choice. If you want to separate membrane fractions, you can sometimes still use these two solutes, but if the membranes contain more proteins than these solutes can support, then a denser medium is needed and the iodinated compounds are used. Whole cell preparations require the higher density, low osmolarity solutes.

## 1.2 Rate zonal (velocity) vs. Isopycnic (equilibrium) centrifugation

The kind of gradient you should use depends on the particle of interest and the other particles it is being separated from.

**Velocity** or **rate zonal** runs separate things on the basis of their mass and to a lesser extent on their density and shape. With these gradients, there is no point in the gradient capable of supporting the density of the particle of interest. Consequently, the particle will continue to sediment through the gradient as long as centrifugal force is applied. Separation is based on a particle's S value (Svedberg units). This type of gradient is used when the particle has a unique S value. Ribosomes (30S, 50S, 70S), phage and virus particles and sub assemblies (15S - 1200S) are typical examples. The separation is exquisitely sensitive to subtle changes in mass, density and shape and can reveal much in the way of a particle's composition, conformation and assembly.

The % sucrose or glycerol used can have a large impact on the separation. For separations of particles with similar S values, a relatively shallow gradient is used (i.e. 5-30%), whereas if the particles have very different S values (120S and 1200S), a steeper gradient is used (i.e. 5-45%). The viscosity of sucrose and glycerol solutions increase exponentially at high concentrations (they become syrupy), and this greatly increases the drag on the faster particles as they enter the bottom half of the gradient, allowing the lower S value particles to continue to separate from the cellular debris in the top half.

Often overlooked is the need for the top of the gradient to support the sample as it is layered on before the run. 5% solute is usually dense enough, but in cases of very concentrated extracts, 10% solute might be needed.

Given that steeper gradients are usually better than shallow ones, the lowest % top solute needed will give a higher slope. More on this in the layering section below.

The name “**rate zonal**” derives from the fact that the particles are applied to the top of the gradient as a thin “zone” and migrate through the gradient as distinct zones. Sample size obviously matters here if the zone is to be thin in the first place, hence we have designed the Short cap (-R) which leaves 4 mm or room for the sample when it is removed (see below). We have shown that samples less than 3 mm in height as they sit on top of the gradient give essentially the same bands and that steep gradients can actually compress bands as they migrate through the tube (see the data below).

A little known fact about rate zonal gradients is the observation that sample losses become serious as the particles sediment to the bottom of the tube. The source of these losses is unknown, but may involve the collision of the particles with the wall and their subsequent loss from the band. The rule of thumb is to use the top 2/3 of the tube whenever possible for your best separation.

**Equilibrium** or **isopycnic** runs are a completely different from rate zonal ones. As their name implies, the particles of interest come to rest at their “isopycnic point”, the point in the density gradient that matches their density. No matter how long they are centrifuged after that, they remain in the same position.

Consequently, sample size is irrelevant so long as the gradient contains the isodense (isopycnic) zone capable of floating the particle. Since sample volumes are generally much greater than in rate zonal runs, we have designed a **Long cap (-L, 10 mm)** for our gradient forming system (see below).

Samples can be applied to the top of the gradient or the bottom (in a suitable dense solution) from whence they “float” to their position. They can also be mixed with the solute in the case of self-forming gradients like CsCl and placed in the tube without a gradient being preformed. The speed of the rotor is very important here for it influences the rate at which the particles are driven to their isopycnic point and also because it can also impact on the shape of the gradient.

As noted above, most of the equilibrium density media like CsCl or Optiprep are selfforming, so that if you centrifuge 12-16 hr, a gradient will form at equilibrium with a shape that is dependent on the speed of the rotor: higher speeds yield steeper gradients. Pre-forming density gradients for isopycnic runs drastically reduces the run time, since particles begin to move immediately rather than waiting for the gradient to be established.

## Section 2. Forming density gradients

### 2.0 Step gradients

Step gradients are of very special but limited utility. The goal is to create a series of steps in density that will separate the particle of interest from its undesirable neighbors in a quick and dirty way. For example, if the particle density is 1.12 g/cc, the steps of 1.09 and 1.15 g/cc would trap these particles at the interface between the two steps in the very thin zone where this density exists. The downside is that these gradients are often used to separate membrane fractions when no real classes exist: they take a smear and make it look deceptively good by concentrating into a series of steps.

When layering these gradients, start with the lightest step and underlay the next heavier step to avoid disturbing the step above. Withdraw the needle as you layer, keeping just below the interface of the two steps.

### 2.1 Non-linear gradients; exponential/isokinetic

In the 1960's and 70's, there was some interest in these unusual gradients because they matched the increasing rate of sedimentation at higher radii with an exponential increase in solute and viscosity, giving a dead heat: particles sedimented at the same rate over the entire length of the tube. The gradient forming part was very tricky, with the mixing chamber of the two chamber device (see below) sealed off to give a constant volume that the other solution diluted exponentially rather than linearly. The fad seems to have passed.

### 2.2 Linear gradients (two chambers, freeze thaw, lay it flat)

Linear gradients are the most widely used type, both for rate zonal and equilibrium runs. They can be formed a number of different ways with varying degrees of difficulty and reproducibility. The simplest way to make them is with the standard two-chamber device in use since the dawn of centrifugation. Equal volumes of the heavy and light solution are placed in adjacent chambers and allowed to mix in one chamber on their way into the tube. Done carefully, this is the best way to make a linear gradient. However, there are many problems with this method: the chambers don't always empty at the same rate, one can only make a single gradient at a time.

A while back, someone discovered that you could freeze and thaw a homogeneous solution of sucrose in a tube a couple of times and the exclusion of the solute as the water froze into ice would lead to pure water and concentrated solute, eventually forming a gradient. Before you head off to your local freezer, however, beware of the technique that seems to be too good to be true. It is! The gradients are non-linear, they take days

to cycle through the multiple freeze-thaws and most importantly, they are a gradient of everything, not just sucrose. All solutes are excluded from the ice and all end up forming a gradient, so you end up with a buffer gradient, a salt gradient and perhaps a bit of a pH gradient as well. The top of these gradients is essentially pure water, which is incapable of supporting a sample layered on top. Do not use these gradients!

Finally, the other way that has been proposed to make gradients is to layer the two solutions in the tube and cap it and lay it on its side and let the two solutions diffuse into a gradient. Needless to say, the timing of the diffusion would need to be carefully controlled and the result is decidedly non-linear.

### 2.3 Tilted tube rotation

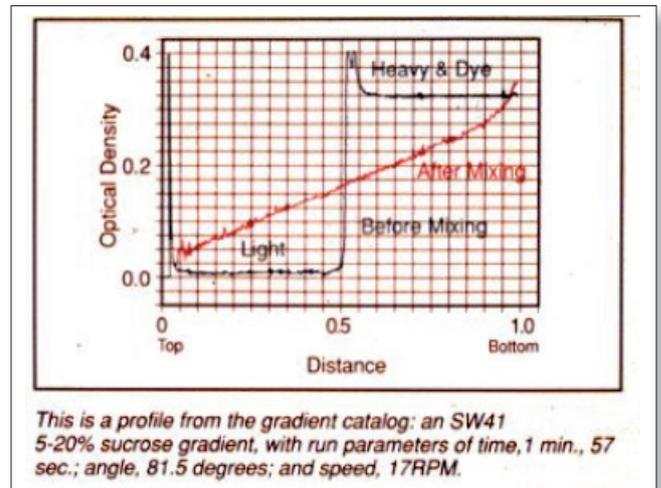
This brings us to a completely different way to form linear gradients that is as non-intuitive as it is fast and reproducible. We call it tilted tube rotation. The two end point solutions are layered into the tube, the tube is capped to lock in the solutions and out any bubbles, placed in a holder and rotated at a high tilt angle for a fixed time and speed. Lacking any sophisticated training in fluid dynamics, the process is essentially empirical and unpredictable; there is no way of knowing what time/angle/speed combination will work for a particular tube, cap size and solute concentration, but once it is determined it is known forever.



**The Gradient Master**

The marker block is used to mark the half-full point on the tube for layering. The caps (short or long) retain the gradient during tilted rotation. **Short caps** are used for rate zonal runs where a small sample is needed, and **long caps** are used in isopycnic or equilibrium runs where sample size is less important.

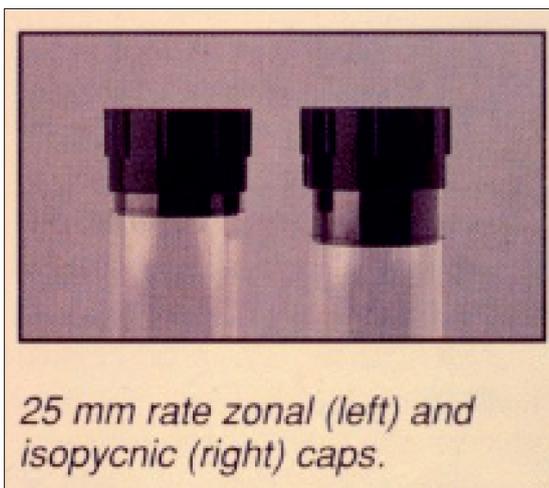
A before and after scan of a gradient showing how tilted tube rotation can quickly, accurately and reproducibly produce a linear gradient from the two endpoint solutions layered in the tube.



**Marker block, MagnaBase Tube Holder, Caps**

**2.4 Cushions and shelves**

Cushions are often applied to the bottom of gradients to trap particles that might otherwise pellet to the bottom. The best way of doing this is to remove the cushion's volume from the bottom of a finished gradient and layer the cushion solution under the gradient. Its volume and density are determined by the type of particles to be trapped.



**Short (-R) & Long (-I) Caps**

**2.5 Temperature**

Temperature is an important part of gradient use, since some particles are very thermolabile. There are two ways to make gradients that will be used at non-ambient temperatures: Make them in the cold room with cold solutions and keep them cold until use or make them warm and then chill them before use. While the "all cold" method is theoretically the best, it requires unreasonable temperature rigor to reproduce, since temperature has such a drastic effect on the density and viscosity of density media. The chilling of room temperature gradients will lead to some convective mixing, but experience has shown that it is minimal. Since virtually all the run parameters for gradient forming were developed at room temperature, chilling the room temperature gradients is the way to go.

## Section 3. Layering and handling gradients

### 3.0 Sample preparation

Whatever type of fractionation you plan to use, it is a good idea to apply a sample that is devoid of cell debris to the gradient. Give the sample a good 30 sec spin in a microfuge to clean it and be careful not to disturb the pellet as you harvest the sample before layering. Note that the % solute on the top of the gradient must be able to float your sample. 5% solute is usually enough, but beware of extracts derived from large numbers of cells; they can exceed the 5% density and fall through the tube until they find their own density, destroying your resolution in the process. To test this, start with a 5% sucrose solution in a small beaker and hold it up to the light as you gently layer some sample into the center of the liquid. Watch for the sample to rise (OK) or sink (not OK).

### 3.1 Sample layering for velocity runs

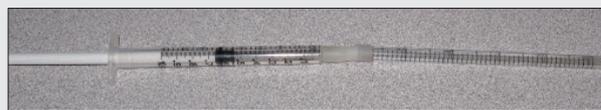
If you want the highest possible resolution in your velocity (rate zonal) runs, you need to start with the thinnest possible sample on the top of the gradient. If your sample is smeared or overloaded, the bands overlap and the resolution drops precipitously. Illustrated below is a simple layering device that outperforms automatic pipettors and pasteur pipettes, is inexpensive and reusable if needed.

**3.1.a. Gradient preparation:** First, make your gradients with the short caps and remove 0.1 ml less than the sample you plan to apply. If you are going to layer 0.3 ml samples, remove ~0.2 ml from one of the gradients you have just formed. Drop this tube into a beaker with a Kimwipe pushed into it with your finger to support the tube upright and TARE them on a balance. Bring all the other tubes to within 0.05 g of the tared weight. This will allow you to load a constant volume of sample on the gradients and avoid having to rebalance the tubes before the run.

When removing the top of the gradient, use the same technique illustrated below to avoid disturbing or creating a discontinuity in the gradient: pull the interface up the wall with the device shown and then suck off the required amount. If your gradients will be run at 4°C, now is the time to put them well spaced and exposed in a refrigerator for 30-45 min to equilibrate at this temperature.

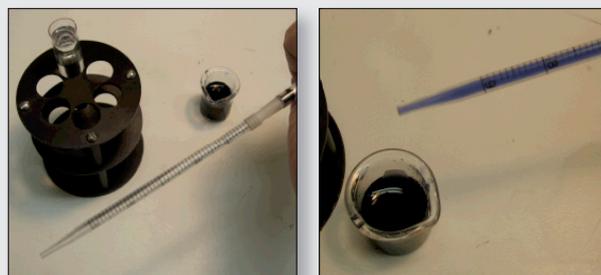
**3.1.b. The layering device:** Take a sterile plastic 1 cc pipette and roll a razor blade over the 0.4 ml line to etch it, then snap the pipette in half at this line. Connect it to a 1 cc syringe with a short piece

of flexible tubing (silicone is best) as shown below. Make as many of these as you have different samples to layer.



**3.1.c. Sample preparation:** Always resuspend your sample in slightly more buffer than you plan to layer so you'll be sure to have enough of every sample. If you want to layer 0.3 ml, resuspend your sample pellet in 0.4 or 0.45 ml. When the cells are thoroughly resuspended and lysed, give them a 30-60 sec spin in microfuge tubes at 14,000 rpm to pellet the large debris.

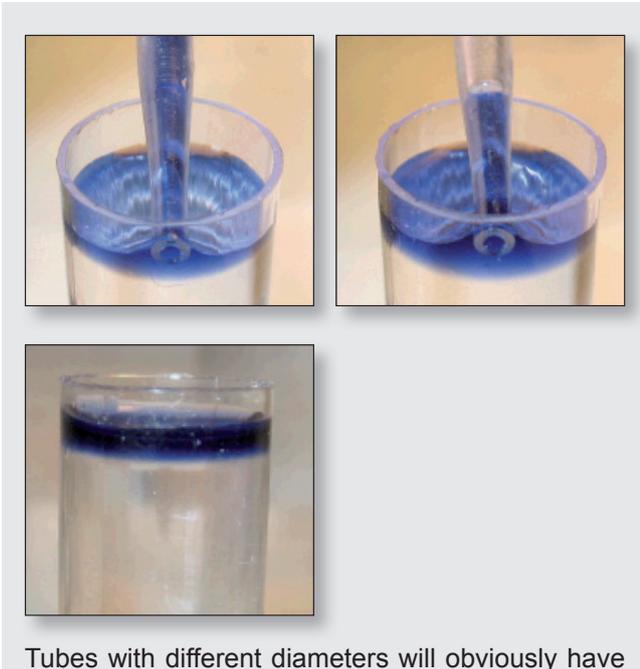
**3.1.d. Sample layering:** Start by pulling 0.05 ml of air into the syringe to allow you to expel the last bit of sample when layering. Now, with the pellet on the high side of the microfuge tube, suck off the supernatant to the desired volume on the pipette (0.3 ml in the photos below). Leave no air in the tip of the pipette as shown: sample is flush with the end.



**This is the critical part: with the pipette nearly horizontal, insert the tip into the top of the gradient on the far wall and tilt the pipette to pull the meniscus up the wall.**



Start layering. Keep raising the tip up the wall to keep it above the level of the sample in the tube. When you are done and the last bit of sample has been expelled from the pipette, you are finished.



Tubes with different diameters will obviously have different sample capacities. As a general rule, your sample should be no more than 2.5 mm high to give the highest resolution. Samples that are higher than this will degrade resolution to some extent, depending on the endpoints of the gradient: Note that higher bottom endpoints (45%) preserve resolution.

### 3.2 Gradient buffers

Be creative here (or not). The buffer in the gradient can selectively preserve or disrupt the particle of interest. Be aware of the effects of divalent cations, ionic strength, pH and detergents.

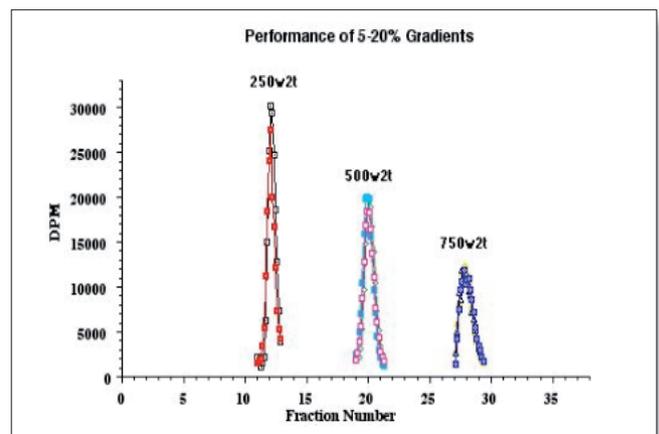
### 3.3 Sample size, layering and band compression

The volume of the sample that can be loaded on the top of a gradient depends greatly on the type of gradient you are running. For rate zonal runs, the sample should lie on the top of the gradient in a layer that is 1-3 mm thick. Oddly, smaller samples volumes than this do not yield sharper bands. However, thicker samples will degrade band sharpness. Steep gradients will compress the leading edge of bands and allow larger sample heights, as well as preventing sample loss to the wall during the run. See the detailed discussion below. Isopycnic runs have no real restrictions on sample volume and can be loaded on the top, bottom or mixed with the solute if the gradient is self forming. The only thing that really matters is that the area of the gradient where the particle will band has a steady, and fair-

ly steep density gradient to it. Remember that, given 12-16 hr, self forming solutes will come to their own gradient profile depending on the speed and the rotor .

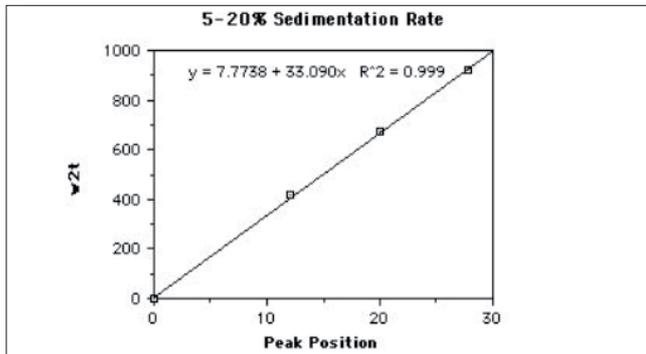
#### 3.3.a The value of a steep gradient

One of the most widely used density gradients is the 5-20% (w/v) sucrose gradient. While it may perform adequately for some applications, as we show below, it has many faults compared to its 5-45% cousin.



**Figure 1.** Performance of 5-20% sucrose gradients. 35S-labeled T4 phage were centrifuged on the L5-75 ultracentrifuge using the integrator preset at the indicated  $\Omega^2t$  values. Two 250  $\Omega^2t$  gradients were run and 3 each of the 500 and 750  $\Omega^2t$  samples were run together. The sample volume was 0.1 ml and the tube was run in the SW41 rotor. The horizontal axis is calibrated in old "crank" units, where one turn of the crank handle or fraction = 1.90mm.

The figure demonstrates three important points. First is the loss of peak height the further down the tube the band sediments. The reduction arise from two sources; sample loss and band spreading. Of the 100% of counts loaded on the top of the gradient, 63% are present in the 250  $\Omega^2t$  sample, 53% in the 500  $\Omega^2t$  sample and only 38% in the 750  $\Omega^2t$  sample. The most likely source of the loss of counts is sample adsorption to the wall of the tube. Any particle that diffuses laterally into the wall will either adsorb to it or sediment to the bottom of the tube and be lost from the band. The band spreading evident in the lower bands arises from top to bottom band diffusion. It has been known for some time that 5-20% sucrose gradients in a variety of rotors are isokinetic; that is, the sedimentation distance of a particle is directly proportional to sedimentation time at any given speed. This is born out in a plot of the peak positions in Figure 1 vs the total  $\Omega^2t$  they experienced, including acceleration and braking.

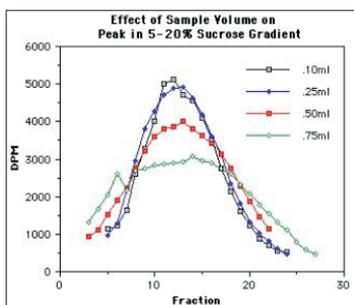


**Figure 2.** Peak position vs. total sedimentation. This type of plot can be useful in two ways. If you know how far a particle sediments at a given speed or time of centrifugation, you can easily calculate how far it will travel if you change a run parameter. Distance will be directly proportional to  $\Omega^2t$  or approximately to the run time at a given speed, and inversely proportional to the square of the rotor speed. If you increase rpms from 33,000 to 41,000, run time will be  $(332)/(412) = 0.648$  x the original run time at 33K rpm.

Another consequence of the isokinetic property of a 5-20% gradient is that particle S values are directly proportional to distance travelled, so the S value of an unknown can be calculated by determining the relative sedimentation rate of a known particle in the same gradient. Thus if a 70S particle sediments 47 mm into a gradient, a particle banding at 22 mm has an S value of  $(22/47)*70=33S$ .

Figure 1 also illustrates the high level of reproducibility of the Total System. Using our "First Drop" method of aligning the top of each gradient, we have achieved  $\pm 0.5$  mm reproducibility between tubes in the same run. Thus, only when ultra-fine fractions (0.2-0.4 mm) are being taken is it necessary to include a marker in each tube to align the gradient profiles.

The loading capacity of the 5-20% gradient is illustrated in Figure 3. An identical amount of labelled phage was diluted to the indicated volumes (0.1, 0.25, 0.5, 0.75 ml) and loaded on 5-20% gradients. All 4 gradients were run together and the peaks aligned on the spread sheet to illustrate the band spreading evident in larger samples.



**Figure 3.** Loading volume influences band width; 5-20% gradient. While the 0.1 and 0.25 ml samples gave identical peak shapes, the 0.5 and 0.75 ml sample volumes clearly lead to a broadening of the band and the result-

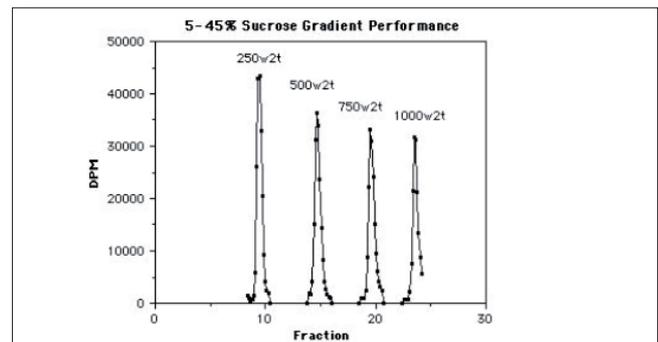
tant loss of resolution. Thus, for the SW41 rotor tubes, sample volumes in 5-20% gradients must be limited to 0.25 ml and fairly short runs if high resolution is required. These gradients were fractionated using the hand crank (pre-motorized).

A final limitation of these gradients will become evident when examining the 5-45% profiles below. The range of S values that can be separated on a given gradient is limited here because the low viscosity and density of the 20% end point does not retard the sedimentation of large particles. In Figure 1, for example, particles with an S value of 250-750S would have been separated adequately but 1000S particles would have pelleted in this run.

### Steep Gradients Offer Many Advantages

We have used 5-45% (w/v) sucrose gradients for many years and find their resolving capabilities far superior to 5-20% gradients. The advantages stem from two main properties. The increased steepness of the gradients makes them inherently more stable during sample layering, handling and centrifugation. The increased viscosity and density of the 45% endpoint reduces diffusion, causes band compression and increases the range of sizes that can be separated on a single gradient.

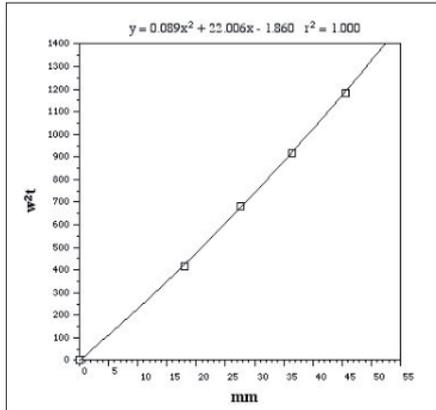
Figure 4 demonstrates several of these points. T4 phage samples (0.1 ml) were layered on identical 5-45% sucrose gradients and run to the indicated preset integrator values as before.



**Figure 4.** Performance of 5-45% sucrose gradients. Of 100% of the counts loaded on the top of each gradient, the amount remaining in the band is 77% in the 250  $\Omega$ t sample; 66%, 59% and 48% in the 500, 750 and 1000  $\Omega$ t bands, respectively. Thus sample recovery is significantly better here, perhaps because the increased viscosity in the bottom half of the tube decreases the diffusion and loss of sample from particles colliding with the wall.

Also notice that rather than increasing in volume as the bands sediment further into the gradient, there is actually a small band compression evident here.

The range of sizes separated is also greater, so, for example, particles from 250S to 1000S would all be well resolved on the same gradient, and increase of 30% in range over 5-20% gradients. Of course, this increase range comes with a slight degradation of the isokinetic property as illustrated below.

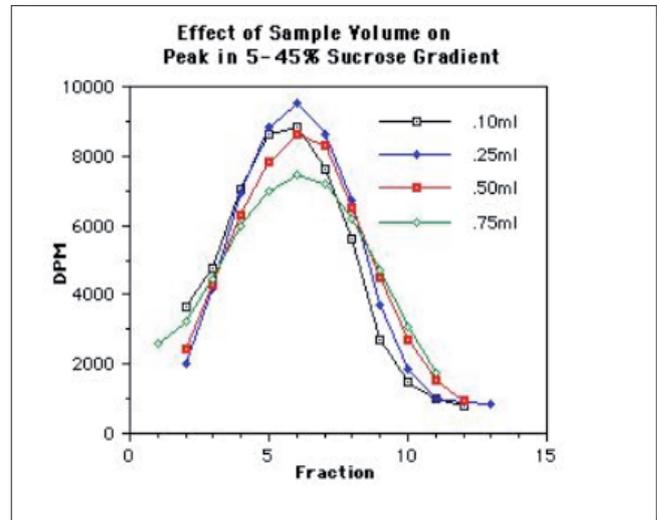


**Figure 5.** Peak position vs. total sedimentation; 5-45% gradient. Here we have plotted the position of the T4 phage peaks at various  $\Omega 2t$ . The function above the figure is a perfect fit for the data points

and reveals the slightly non-isokinetic behavior of this steeper gradient. However, it is still possible to determine the S value of a particle relative to a known S marker. Suppose that our marker, a 950S particle (T4 phage), sediments to position 39.2 mm at 1000  $\Omega 2t$ . A particle sedimenting to position 15.7 in the same gradient has reached the position of our phage marker sedimented for 365  $\Omega 2t$  (solve the equation for  $x = 15.7$ ). The unknown therefore has an S value  $365/1000 \times$  our marker (950S) = 347S. Using this logic, the graph can be converted into an S value graph by fixing the  $\Omega 2t$  value (say at 1000) and converting the Y axis to S value. Thus the 1000  $\Omega 2t$  position becomes 950S, the 365  $\Omega 2t$  position becomes 347S and so on. The present technique has the inherent advantage of being empirically determined under your actual run conditions. Of course, the availability of an S value marker is required.

### Effect of Sample Volume in 5-45% Gradients

The sample capacity of 5-45% is quite remarkable. As illustrated in Figure 6 below, when volumes ranging from 0.1 to 0.5 ml are layered on top, the bands are indistinguishable. Even at 0.75 ml, while the peak height is slightly reduced, the band volume is about the same; 0.3 ml. Thus the compression is a real advantage since large samples can be loaded with little or no loss of resolution.



**Figure 6.** Effect of sample volume on peak width in 5-45% gradients. The band spreading evident in the 5-20% gradients is almost completely absent here. In fact, bands loaded in  $>0.5$  ml samples volumes are recovered in less volume than the sample. This is the result of the compression of the leading edge of the band as it continually enters zones of significantly increased viscosity and density in the steep gradient.

Thus, resolution, sample recovery, band compression and S value range of a 5-45% gradient are all improved over the 5-20% gradient. The price one pays for the isokinetic property of a 5-20% gradient is clearly not worth the modest gain in mathematical ease it offers.

The graphs and tips shown here illustrate the value of reproducible gradient forming and fractionation. The full potential of gradients to separate and analyze macromolecular structures cannot be realized until these processes are completely standardized.

### 3.4 Loading the tubes in the rotor, running, unloading

Once the sample has been loaded, the gradients must be handled with great care to avoid mixing the sample with the upper layers, especially in rate zonal runs. The same is true with gradients that have been run and are awaiting fractionation. It is a good idea to keep the fractionator close to the centrifuge to avoid long and dangerous trips down crowded hallways. The sensitivity of the sample to acceleration and braking will depend on the centrifuge and the gradient. A steep, viscous gradient will be fairly resistant to disruption, while a shallow one may not be. A good idea is to establish the sharpness of your bands with gentle acceleration and braking and then seeing if faster starts and stops hurt the resolution.

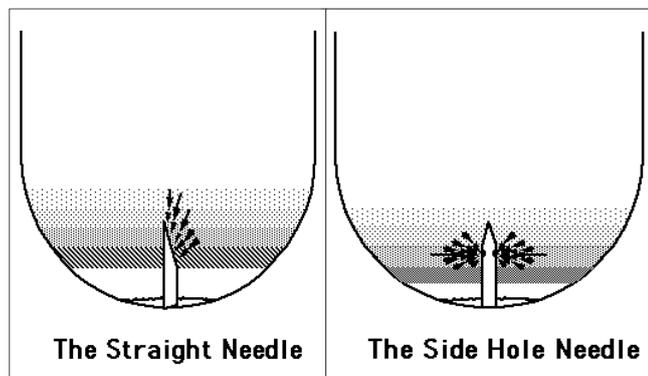
## Section 4. Fractionating gradients

### 4.0 The essential problem: Laminar capillary flow; getting a flat band into a thin column

There is a round hole/square peg problem that has plagued gradient work from its inception: the bands are thin horizontal discs and the devices used to retrieve them are invariably thin tubes at some point. Thus the problem is one of conversion between the two geometries. In addition to this, the tubing exhibits laminar capillary flow, where the central lamina has the highest velocity and the zero boundary adhered to the wall is stationary. This makes the conversion even more difficult than it already is and is a little-appreciated part of gradient fractionation because the high velocity central lamina tends to pull gradient solution from directly in front of itself, reaching far beyond the zone that is supposed to be entering the tubing system. This degrades resolution because different layers are being sampled at the same time.

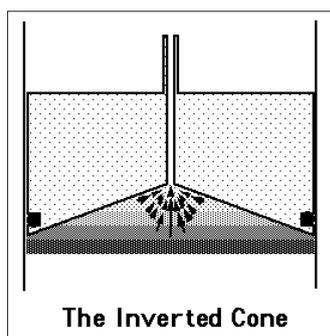
#### 4.1 Needles; straight and side hole:

As shown by the figures, these are the poorest ways to fractionate a gradient. The central lamina is offered unfettered access to layers far beyond the one next to the tip of the needle and the resultant loss of resolution is severe.



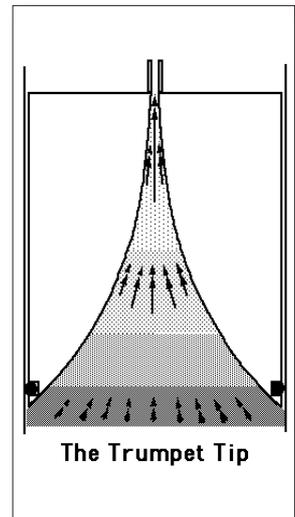
#### 4.2 Cones:

Cones used in many fractionation devices are a first attempt to deal with the central lamina problem. Unfortunately, at anything but the slowest speeds, they smear out your fractions, albeit to a lesser extent than the needles.

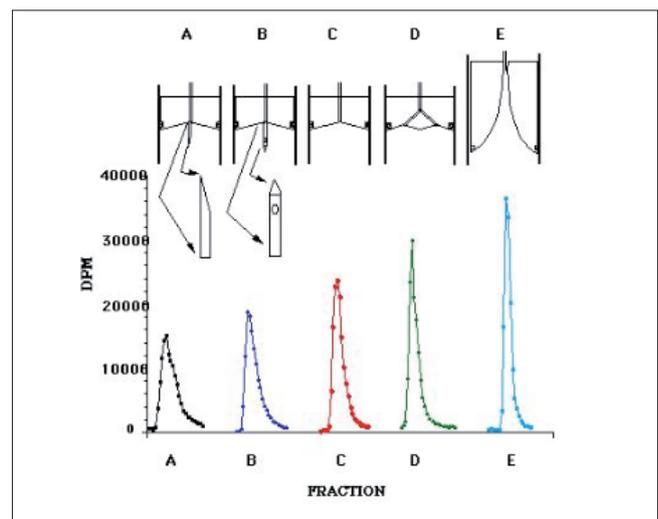


### 4.3 The Trumpet Tip™

The trumpet shape of the tip restricts the flow into the tubing to the central lamina. The gradual taper is designed to take advantage of the gradual spread of the fastest flowing lamina to ever wider zones as the distance from the narrowest diameter increases. The cost here is that the trumpet has its own zero boundary layer over this larger wetted area, but the resulting increase in resolution is far superior to the cone.



#### 4.4 A Comparison of different tip designs



**Figure 2. The Evolution of the Fractionator**

These are five identical gradients containing a T4 phage peak fractionated with different tip configurations at the same speed with a motorized fractionator. Peak fractions are displayed at the same scale in this composite view. Above each peak is a diagram of the fractionation tip used to harvest the gradient. As the tip is pressed down into the tube, the gradient is displaced into collection tubing connected to the outlet needle at the top of the tip. **Tip A** is the standard beveled needle frequently used to puncture the bottom of the tube. In this case it has been inserted into the center of the single hole cone tip to provide a direct comparison to the other tips. **Tip B** is the side hole needle often used in simple puncture fraction collectors. It also has been inserted into the cone tip. **Tip C** is the basic cone design with a single collection hole in the center. **Tip D** is the multi-hole tip first developed and patented in 1976. **Tip E** is the patent-pending Trumpet Tip™ developed by BioComp.

### 4.5 The Piston Fractionator

The basic design of the Piston Fractionator involves forcing a trumpet tipped piston down into the tube to displace the gradient from top to bottom. The piston is driven by a high resolution stepper motor coupled to a screw and offers 10 micron resolution with 99.9% accuracy.



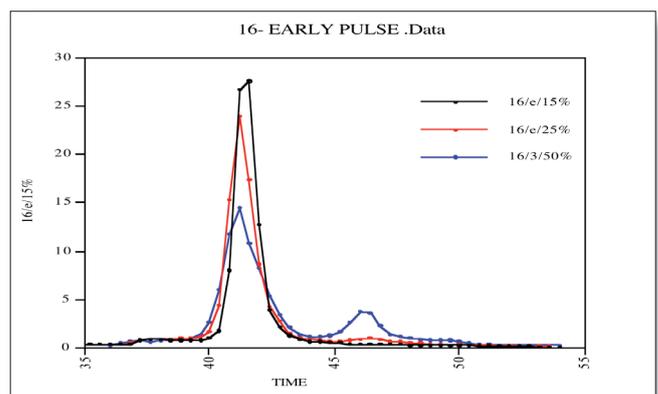
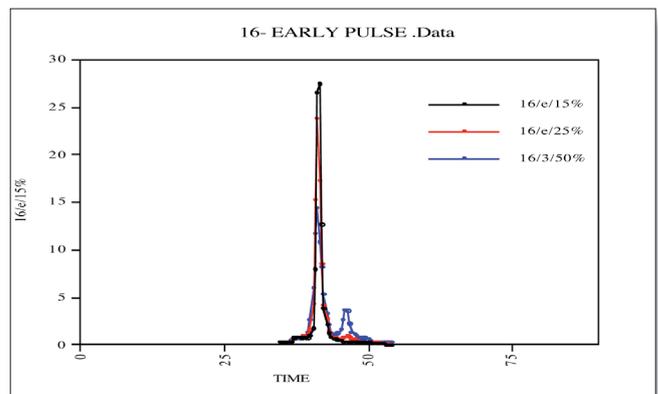
#### The Gradient Station Forms and Fractionates Gradients

### 4.6 The highest resolution fractionation ever achieved.

Never before has gradient fractionation offered resolution comparable to HPLC. Using the Trumpet Tip™ described above to nearly eliminate smearing of peaks, and the unique rinse/air cleaning system described below, you can now routinely achieve ultrahigh resolution profiles of your gradients.

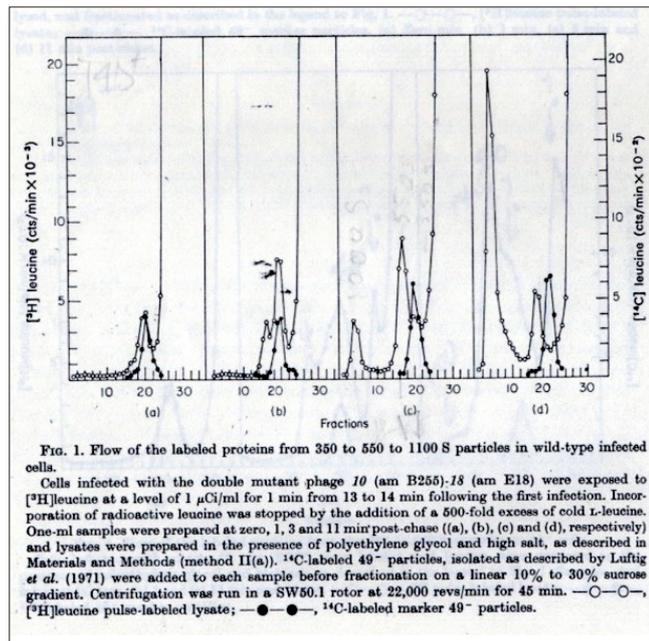
#### 4.6.a. High resolution and reproducibility:

Examine the three gradients superimposed below. The top version shows how the gradients look at 1X, where the X-axis represents the length of the tube. The bottom version shows the same data plotted to include just the area that was sampled. This illustrates another important advantage of this system: you can concentrate your analysis on one or more areas of the gradient with high resolution while ignoring unimportant areas.

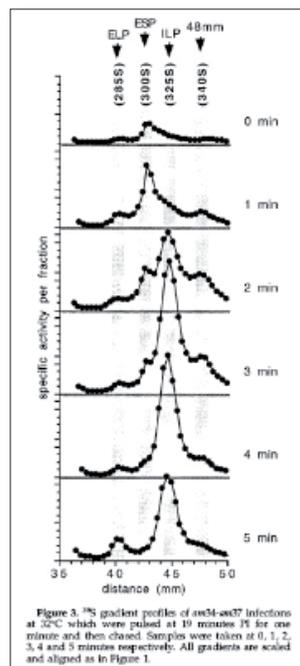


4.6.b. Teasing out new species:

Laemmli & Favre (1973: JMB 80:575)



Here is another example (Jardine & Coombs, 1998) of a series of gradients used to identify the elusive, but critically important phage T4 head packaging intermediate, the ISP (initiated small particle) following a pulse-chase of infected cells. The transient shoulder on the right side of the graph labelled 48 mm is the peak in question. This experiment follows the same protocol used by Laemmli & Favre (1973), but their analysis obtained by dripping from the bottom and showed only two large peaks. Just imagine what this kind of resolution could do for your experiments.



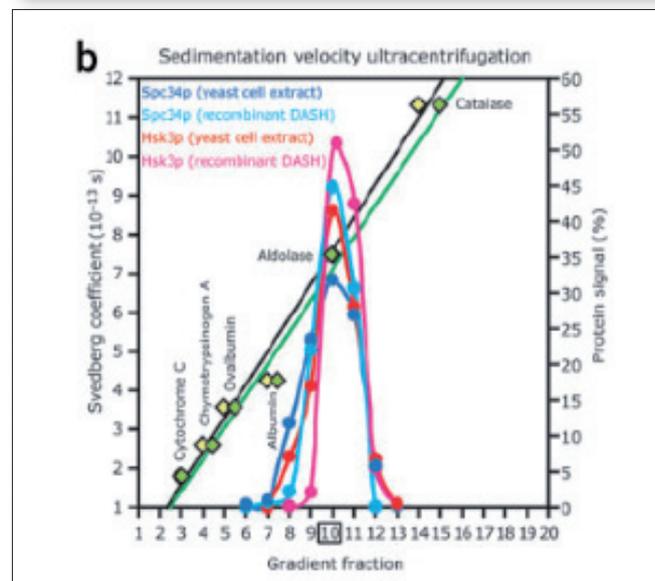
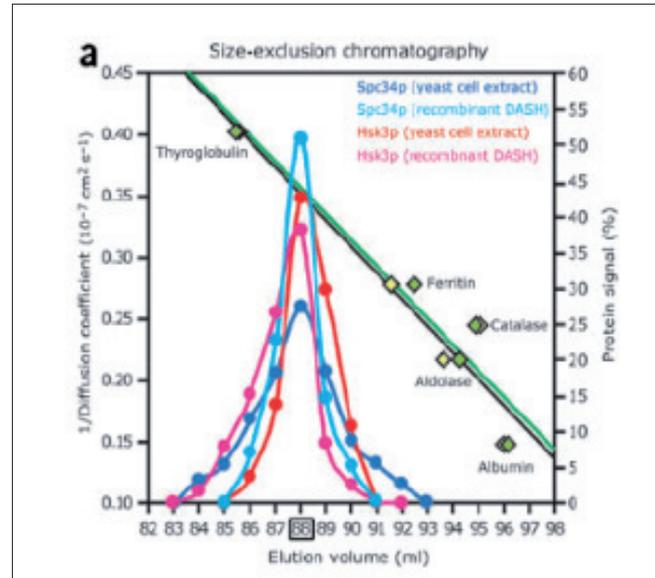
Jardine & Coombs (JMB: 1998, 284:661)

4.6.c. Resolving small protein complexes:

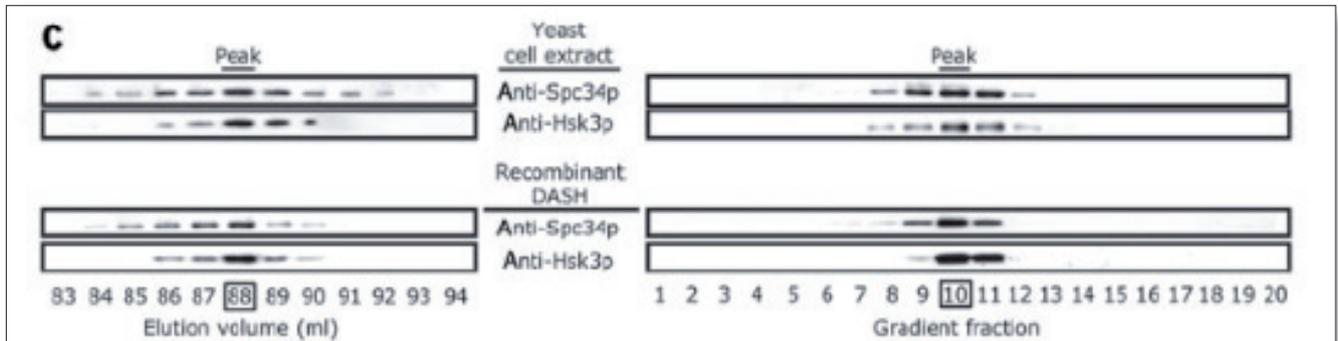
The figure below from Peter Sorger's lab, then at MIT, shows the purification of a small protein complex (210 kD heterodecamer; 7.4S) on glycerol gradients using the Gradient Master and Piston Fractionator. This rate zonal gradient clearly outperformed columns in the purification, both in peak width and r2 values for the marker proteins on two separate runs. (Miranda J.J., De

Wulf, P., Sorger, P.K., Harrison, S.C. 2005. The yeast DASH complex forms closed rings on microtubules Nat Struct Mol Biol. Feb;12(2):138-43.)

Figure1 Hydrodynamic analysis of recombinant and native DASH.



(a) Elution profiles from size exclusion chromatography. Yellow and green diamonds, elution positions of five standard proteins as obtained from two independent experiments. Black and green regression lines ( $r^2 = 0.91$ , and  $0.92$ , respectively) yield the diffusion coefficient of a protein from its elution volume. (b) Sedimentation profiles from sedimentation velocity ultracentrifugation. Yellow and green diamonds, sedimentation positions of six standards proteins as obtained from two independent experiments. Black and green regression lines ( $r^2 = 0.98$ , and  $0.99$ , respectively) yield the Svedberg coefficient of a protein from its position in the glycerol gradient.

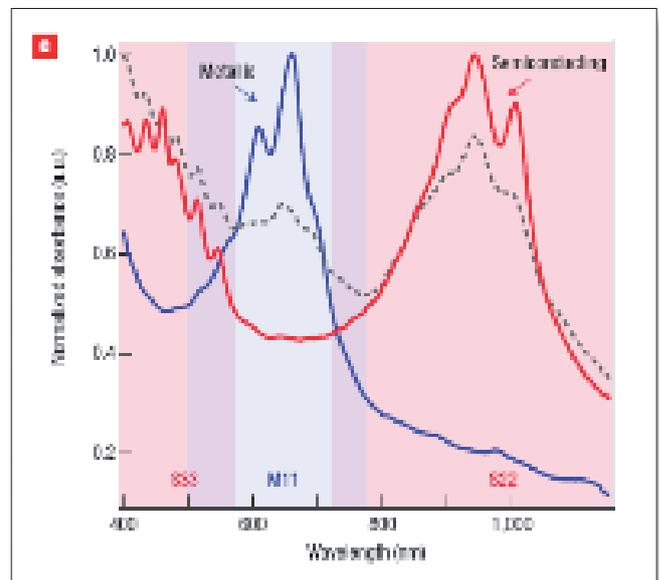
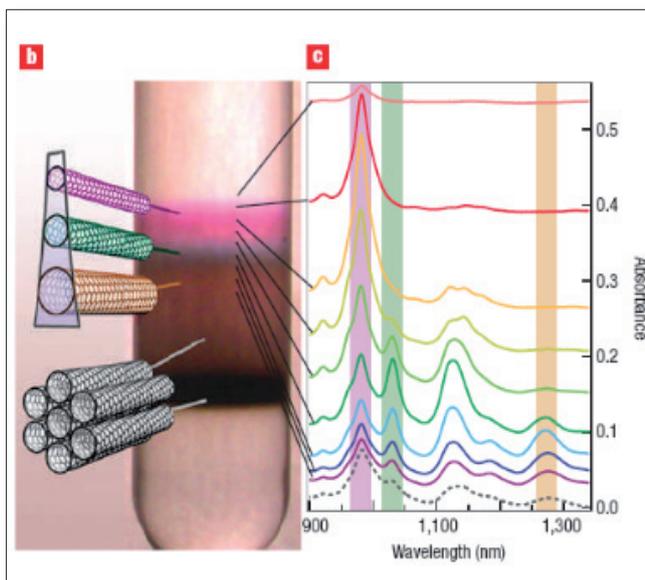
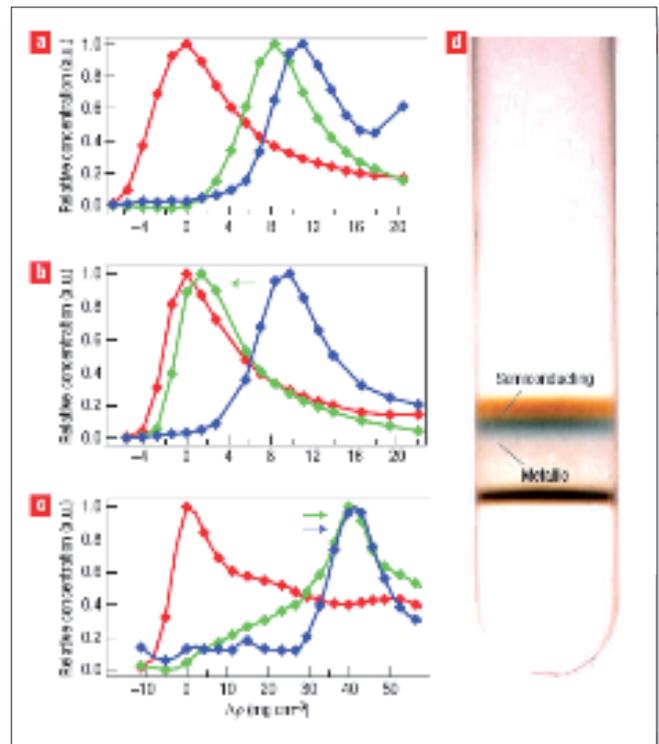


(c) Western blots of column and gradient fractions obtained with polyclonal antibodies raised against Spc34p and Hsk3p. Quantitation of the band intensity is plotted in a and b.

**4.6.d . Resolving carbon nanotubes**

A recent development in the field of physics is the use of gradients to purify the various morphs of carbon nanotubes. The work was pioneered by Dr. Mike Arnold during his PhD work in Mark Hersam’s lab at Northwestern (Arnold et al, 2006, Nature Nanotechnology. 1:60). Mike used a clever coating technique and isopycnic Optiprep (iodixanol) gradients to sort the various diameter nanotubes, and was able to test their very different properties. This spawned a whole new field as many labs adopted this technique to purify nanotubes. Mike used the Piston Fractionator to achieve the resolution he needed:

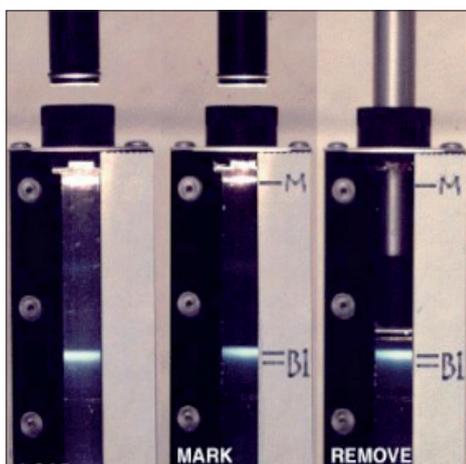
**4.7 The band visualization system and manual fractionation**



Particles larger than ribosomes in sufficient concentrations scatter visible light to such an extent that they can be viewed with the unaided eye. The piston fractionator makes three significant improvements in this area. First, the tube holder surrounds the tube in black to offer the best background for viewing. Second, the holder is designed to permit the tube to be immersed in water to eliminate light scattering on the surface of the tube. This feature greatly increases the sensitivity of the light scattering bands. The third improvement is the elimination of the lens effect that the bottom of the tube has on the illumination from below. The entire length of the tube is illuminated.

The fractionator kit includes a small T-square to allow the user to mark the position of the bands on a piece of tape placed alongside the gradient. The marks at the top and bottom of each band guide the manual recovery of any number of bands from a tube. The piston tip is lowered to the top line, the tubing is rinsed and dried with air, the piston is moved to the bottom line and the sample still in the tubing is recovered with a short burst of air. Sample recovery is complete since the piston displaces the entire band.

Piston



T4 phage band

#### 4.8 First drop/Reset; synchronizing gradient profiles.

A close inspection of balanced tubes reveals some variation in the height of the meniscus. If all gradients were fractionated from a fixed vertical spot, this variation would offset the resulting profiles. To counter this effect, we eliminate any offset by lowering the piston into the tube manually, slowing down as the piston approaches the meniscus and then proceeding on down until the first drop appears at the end of the sample tubing. At this point, the display is reset to 0.00 mm and fractionation proceeds, with all gradients reasonably well syn-

chronized. There is still some variation between profiles ( $\pm 0.5$  mm), and if needed, this can be eliminated by the introduction of a sedimentation or density marker.

#### 4.9 Fraction size

The question often arises as to what the most advantageous sample size is, given that the 10 micron resolution is obviously overkill. In our experience the smallest sample worth taking is 0.3 mm/fraction. Since the amount of protein, nucleic acid or dpm declines with the sample volume, there is a point where these become limiting. A good rule of thumb from the HPLC folks is to ensure that each peak has about 10 data points to describe it. Practice will reveal the sample size that gives that resolution.

Another factor to consider is the reason for doing the gradient. If the purpose is to survey the entire gradient in X number of samples, measure the distance from the bottom of the piston tip at reset (see above) to the bottom of the cylindrical part of the tube and divide this distance by X. On the other hand, most gradients have large areas of little or no interest. In this case, set up a multistep run where a few large fractions are taken (and discarded) and fine fractionation begins where the important particles are.

#### 4.10 Sampling the bottom of the tube

A word on the inaccessible round bottom of the tube. Some users want to fractionate right to the last drop in the tube but the piston is not able to penetrate into the round end. It may take some convincing, but particles that have entered this round zone are not worth fractionating since they have encountered the wall pinching in from the sides and are no longer representative of the same forces that the rest of the gradient has experienced. There is also the danger of damaging the piston tip seal as it is compressed in the round end.

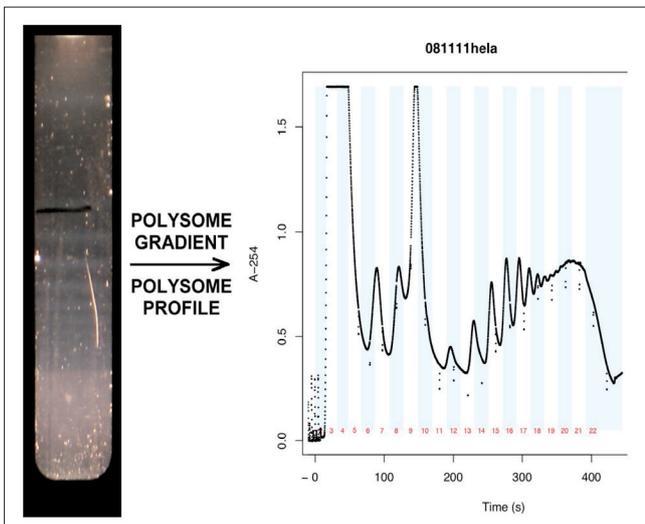
#### 4.11 U.V. Fractionation

Large numbers of users are interested in viewing the gradient profile by UV. The fractionator accommodates this role effortlessly, since it is programmable to take the entire contents in one large fraction at a single fixed speed. We have been recommending the BioRad EM-1 Econo UV Monitor, with its flow cell attached to the moving piston head so that the tubing connection, with its unavoidable smearing, is kept to an absolute minimum. The output from the EM-1 is an analog 1 V signal that we convert to digital with 1:10<sup>TM</sup> (should be exp 6, superscript) resolution for importing into an Excel spreadsheet. This way, no runs are lost because the strip chart was set wrong and the profile can be

formatted for publication. Here is a photo of the EM-1 attached to the fractionator's moving head.

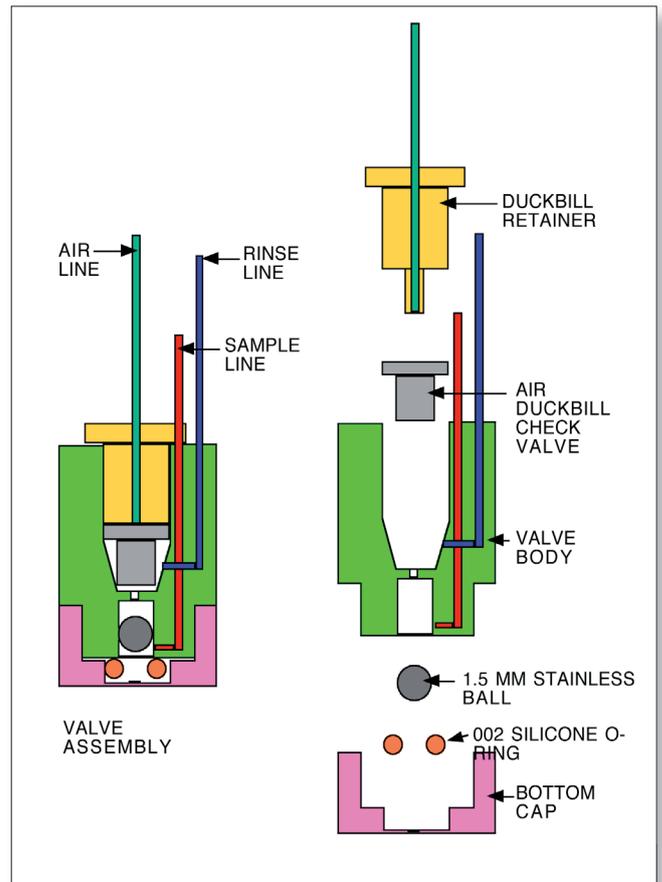


Incredibly, even polysomes are visible to the naked eye with the visible light scattering system. The same gradient gave the profile shown to the right.



#### 4.12 The rinse/air valve system.

The piston fractionator offers something no other fractionator can: the ability to rinse and dry the tubing between fractions from the point of gradient capture to then end of the tubing. The result is even sharper resolution with little or no smearing of bands. The key here is to tailor the automatic rinse protocol to match your needs.



How the valve works: The valve is located at the tip of the piston, and the different size tip/seals are screwed onto it. As the piston descends into the tube, liquid is forced up through the bottom O-ring, lifting the ball off the O-ring as the piston moves down into the gradient. The liquid then passes out through the **red SAMPLE line** to the collector. Between fractions, **AIR** is pumped down through the rubber duckbill and into the ball chamber, forcing the ball onto the Oringand sealing off the gradient below. **Air** then passes out through the **red sample line**. **RINSE** is pumped into the air's duckbill chamber, down into the ball chamber and out the **red sample line**. Rinsing with water and then blowing the sample tubing dry eliminates cross contamination between fractions.

## Section 5. Post-gradient sample manipulations

### 5.1 TCA/SDS-PAGE

Add 1/10 vol of 100% w/v TCA, ice the sample for 30 min, spin for 5 min, rinse with 10% TCA, Acetone-Tris, dry and you have samples ready for SDS PAGE.

### 5.2 Native gels

native gels are a little trickier because the samples may need to be concentrated without resorting to TCA. The best solution we have found is the Centricon-type centrifugal filter.

### 5.3 Scintillation counting

The most perfect profiles obtainable come from counting the whole sample with a small volume rinse included with each fraction. Most scintillation cocktails can tolerate the rinse with no quenching. Alternatively, you can fractionate and then sample the fractions with a pipette so that you can analyze the contents of peak fractions by PAGE.

### 5.4 UV detection

Take undiluted fractions to the spectrophotometer for UV profiles when the need arises in the absence of a flow cell.



**Gradient Master**



**Fractionator**



**Gradient Station**



**Navigator**

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