Ultracentrifugation

Plasmid Separations in NVT™ Near Vertical Tube Rotors

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There are three major types of preparative ultracentrifuge rotors: swinging bucket, fixed angle, and vertical tube. Each of these rotor classes has different strengths and weaknesses for any given centrifugal separation. Occasionally, a particular separation would be accomplished best by a rotor with a combination of characteristics from these classes. Consider, for example, isopycnic separations in which a few bands form, but some sample components with densities outside the gradient range are deposited on the centripetal or centrifugal wall of the tube. The selection of the rotor type then depends on which characteristics are deemed more important. Vertical tube rotors (0° relative to the axis of rotation) allow shorter run times, but fixed angle rotors (typically about 25°) keep the pelleted material farther away from the banded material and do not allow the pelleted material to fall through the banded material.

Plasmid DNA separations in CsCl-ethidium bromide gradients provide a model system for testing the effect of tube angle on the conflicting goals of decreasing run time and decreasing contact between pelleted (RNA) and banded (DNA) components. During the separation two DNA bands form, the lower of which is the plasmid DNA. The upper band is linear or nicked DNA. Often there are classes of particles that are more dense or less dense than the extremes of the gradient range, and consequently either pellet or float.

We have studied the effect of various tube angles on the banded and pelleted components in plasmid DNA separations, and report here on the development of a new class of rotors: the NVT rotors. NVT rotor designs are optimal for those separations that require the speed and efficiency of vertical tube rotors; at the same time some of the advantages of a fixed angle rotor are retained. The use of low concentrations of Triton X-100 to prevent the contamination of bands by materials that pellet during isopycnic separations is also described.

Methods

To study the effect of tube angle on plasmid DNA separations, rotors were fabricated with 5°, 7.5°, 10° and 12.5° tube angles. These prototype rotors were based on a modified VTi 65 design. During analysis of the performance of these prototype designs, a Triton lysis method was used to extract the plasmid DNA from *E. coli* (Ausubel *et al.*, 1987). Based on the success of the tests using the prototype rotors, several NVT rotor have been released as products: the NVT 65, the NVT 90, and the TLN-100. All evaluations of these production rotors were performed using the following SDS-alkaline lysis method.

E. coli containing either of two plasmids, pUC9 (BRL) or Bluescript (Stratagene) with a 160-basepair (bp) insert (pBS9), were cultured overnight. The NaOH-SDS method of Birnboim and Doly, 1979; Ausubel *et al.*, 1987; Little and McRorie, 1989a, b was used to obtain a crude nucleic acid preparation. This crude nucleic acid pellet was dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.6 (TE). One gram of CsCl was added for each 1 mL of solution. Then 0.8 mL of a 10-mg/mL ethidium bromide solution was added for each 10 mL of this solution. The resulting solution density of 1.55 g/mL was checked using a refractometer (RI - 1.3860) and pipetted into polyallomer Quick-Seal® tubes. As described later, Triton X-100 was added to some of the centrifuge tubes prior to centrifugation. Centrifugal run conditions arc given in the figure legends.

To study the position of the contaminants in these plasmid preparations, a low-speed spin was not employed to remove contaminating material. No RNase, phenol, or other purification procedure was used. These preparations were purposely “dirty,” with roughly 40% of the tube containing flocculent material before ultracentrifugation. Other conditions, including additions or changes to the above methods, are given in the figure legends.

Results and Discussion

Shallower tube angles decrease the sedimentation pathlength and also allow higher rotor speeds due to the decreased risk of CsCl precipitation. The net result is that shallow angles yield shorter run times. When the Triton X-100 lysis method was used with the prototype rotors, the high-density material pelleted towards the bottom of the tube. With tube angles of ≥7.5°, the flocculent material, hereafter referred to as RNA, and the material of lighter density, commonly referred to as protein, did not contact the DNA bands. Thus, we chose the 7.5° tube angle for the NVT 65 rotor, since this was the shallowest angle that did not allow the pelleted RNA and plasmid DNA to come in contact. Slightly larger tube angles were selected for the other two NVT tube rotors, which carry small volume tubes, in order to accommodate the RNA pellet in a proportionally larger amount of the tube volume. The NVT 90 and TLN-100 rotors have tube angles of 8° and 9°, respectively.

If plasmid separations are performed with samples prepared by the alkaline SD method without the addition of Triton X-100, the RNA is very tenacious and adheres to virtually the entire length of the centrifugal tube wall (Figure 1). Tests were conducted over a range of Triton X-100 concentrations to determine the optimum conditions for avoiding an adherent pellet.

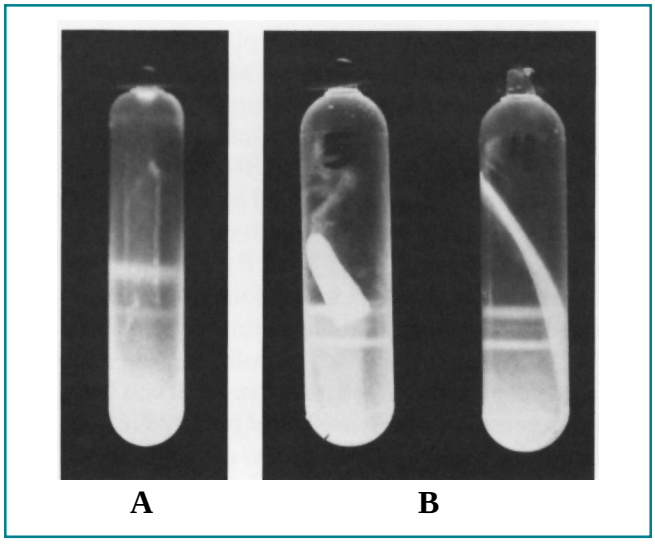

Figure 1. *RNA pellets in near vertical and vertical tube rotors (NaOH-SDS protocol, without Triton X-100, used for sample preparation). (A) RNA pellet on side of NVT 65 rotor tube. (B) RNA pellets falling through the DNA bands in VTi 80 rotor tubes.*

The results in Figure 2 show that Triton X-100 in concentrations higher than 0.001% causes the RNA to pellet below the lower DNA band in the TLN-100 rotor. At Triton X-100 concentrations of 0.1% or higher, a significant amount of Triton came out of solution. At higher concentrations (most notably at 1% Triton X- 100), the floating Triton X-100 layer

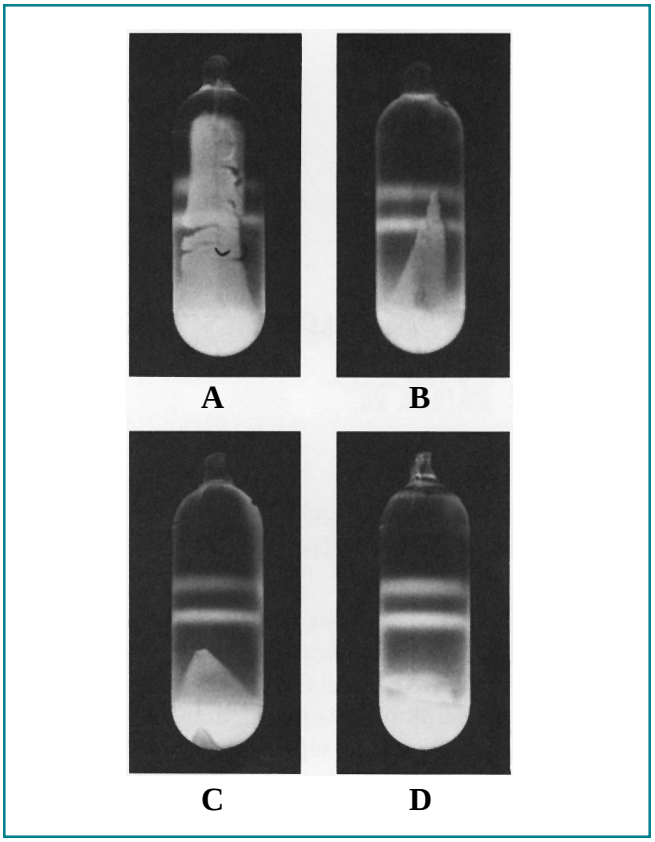

Figure 2. *Effect of Triton X-100 on RNA pellet in the TLN-100 rotor. The final concentration of Triton X-100 in these tubes is: (A) 0%, (B) 0.0001%, (C) 0.001%, (D) I%. As the Triton X-100 concentration increases, the pellet adhesion decreases. High Triton X- I 00 levels (0.I % to I %) produce a visible reddish band at the top of the tube.*

was colored red with ethidium bromide. The addition of Triton X-100 also helped to solve two other common problems. In the presence of Triton X-100 concentrations higher than 0.001%, if the RNA pellet detached from the wall as the gradient reoriented during deceleration, it settled towards the bottom of the tube and did not fall through the DNA bands. The pellet reoriented at the same time as the gradient instead of afterwards. On occasion, a portion of the flocculent material does not form a pellicle or pellet, but floats in the gradient. The use of ≥0.01% Triton X-100 concentrations dispersed this flocculent material or caused it to band below the plasmid band in each of the three Triton X-100 concentration tests where the flocculent material was observed in the control tubes (0% Triton X-100). Triton X-100 in the concentration range of 0.001 to 0.1% will achieve the desired results. The best separations were observed at a concentration of about 0.01%.

The effect of Triton X-100 on subsequent manipulations of the sample was not examined. The final concentration of Triton X-100 in the sample is much less than the concentration used during the Triton lysis method.

Methods used to remove the CsCl should also remove the Triton X-100. Thus, we do not foresee any problems with the addition of this amount of Triton X-100 to the sample.

The maximum speed at which a homogeneous solution of CsCl with a density of 1.55 g/mL can be run without salt precipitation in the NVT 90 rotor is 78,000 rpm. Using a simulation algorithm to model the separation, the five-step program given in Table 1 was developed to provide a rapid plasmid separation at 20°C that avoids the precipitation of CsCl during the run.

Table 1. Five-Step Program for Plasmid Separation in the NVT 90 Rotor

Speed (rpm)	Step Time (h)	Total Elapsed Time (h)
90,000	1.50	1.50
87,000	0.25	1.75
83,000	0.25	2.00
81,000	0.50	2.50
80,000	0.50	3.00

This separation is shown in Figure 3. The protocol may be modified slightly if desired. However, to avoid precipitation of CsCl, do not run the last step at 80,000 rpm for longer than 1 h. Speeds over 78,000 rpm will cause CsCl precipitation in the NVT 90 rotor if a starting density of 1.55 g/mL CsCl is used. A run performed at a constant speed of 78,000 rpm takes 4 h to achieve a similar separation of plasmid and linear DNA (Figure 4).

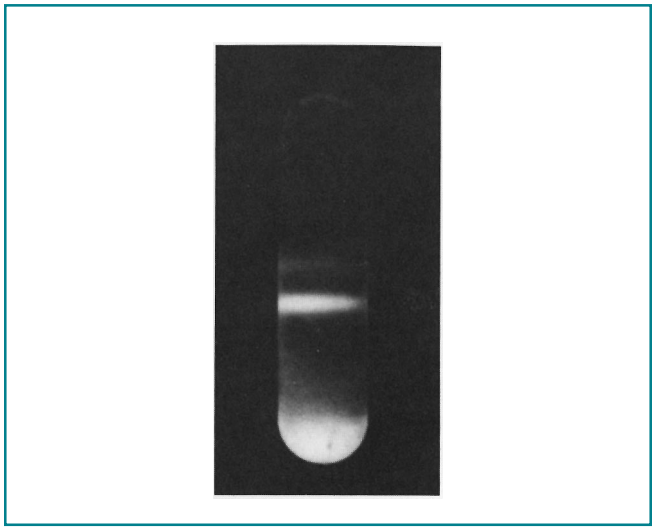

Figure 3. *Three-hour plasmid preparation designed by using a simulation algorithm. The NVT 90 rotor was centrifuged in an Optima™ XL-90 Ultracentrifuge using the time steps given in Table 1. The last step at 80, 000 rpm can be run for 1 h; if times longer than 1 h are used, the last time-step should be set at 78, 000 rpm.*

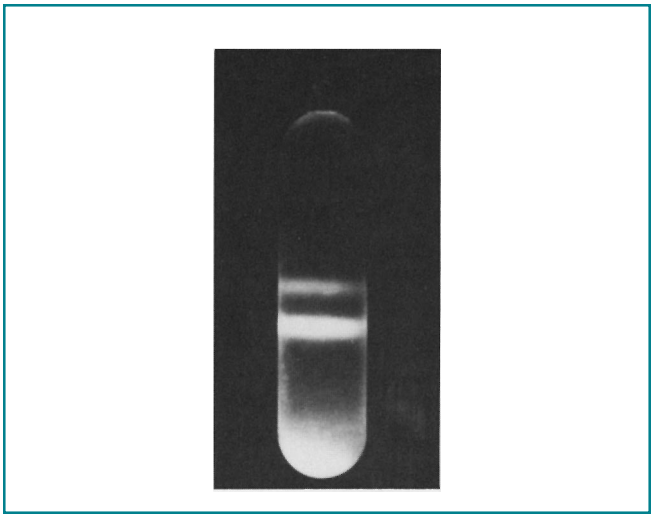

Figure 4. *Plasmid DNA separation in the NVT 90 rotor run at a constant speed of 78, 000 rpm for 4 h.*

Summary

In order to optimize plasmid DNA separations, a new type of rotor has been developed: the near vertical tube rotor. Whereas NVT rotors do not have an optimal design for general pelleting-type separations, their small tube angles are ideally suited for rapid isopycnic separations where it is desirable to prevent contact between banded particles of interest with either pelleted or floating contaminants.

The addition of small amounts of Triton X-100 to samples permits the rapid separation of plasmid DNA with minimal preultracentrifugation cleanup and with no contact, or minimal contact, between the plasmid DNA band and contaminating materials. The conditions given in Table 2 have been found to give good results starting with homogeneous mixtures of CsCl, ethidium bromide and nucleic acids.

Triton X-100 (0.01%) can be added easily to the centrifuge tube with the following protocol: 10 µL of a 1:100 dilution of Triton X-100:H_2O (or 1 mM Tris, 10 mM EDTA, pH 7.4 instead of H_2O) are added per mL of tube volume. For the 3.9-mL tubes used in the TLN-100, 39 µL of 1:100 Triton X-100 were pipetted into the 3.9-mL Quick-Seal tube, the tube sealed and inverted several times to mix. Specific volumes of Triton X- 100 for full-sized tubes are given for the NVT 65, NVT 90, and TLN-100 rotors in Table 2.

Table 2. Plasmid Separations in NVT Rotors at 20°C

Rotor	Volume of 1: 100 Triton X- 100 (µL)	Speed (rpm)	Time (h)	Five-Step Program Time[1] (h)
NVT 65	135	65,000	4	-
NVT 90	51	78,000	4	3
TLN-100	39	100,000	4	

[1]See Table 1.

References

Ausubel, F. M. and others, editors. *Current Protocols in Molecular Biology,* pp. 1.7.1-1.7.5. New York, Wiley, 1987.

Birnboim, H. C., Doly, J. A. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res. 7*,1513-1523 (1979)

Little, S. E., McRorie, D. K. Rapid separation of plasmid DNA in preparative ultracentrifuge rotors. *Applications Data DS-734*, Palo Alto, Calif., Beckman Instruments, Inc., Spinco Division, 1989a.

Little, S. E., McRorie, D. K. High-efficiency plasmid separations using the VTi 90 vertical tube rotor. *Applications Data DS-751.* Palo Alto, Calif., Beckman Instruments, Inc., Spinco Division, 1989b.

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