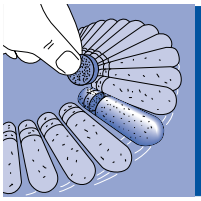

Application Information

Ultracentrifugation

Rapid Plasmid Isolations Using Efficient Sedimentation Program Overspeed Control in the Optima™ XL Ultracentrifuge from Beckman

Anasuya S. Patel, Allen Furst, and Paul Voelker
Beckman Instruments, Inc.

Introduction

The ESP™ Efficient Sedimentation Program from Beckman is a computer algorithm that simulates centrifugal separations. It can be used to model rapid separation protocols, or to provide automated instrument control. ESP models the redistribution of a gradient-forming salt, such as CsCl, in a centrifugal field, and simultaneously models macromolecules undergoing separation in the changing gradient.

In conventional separations using self-forming gradients, ESP demonstrates that most macromolecules, when initially dispersed homogeneously with the gradient salt, form discrete bands well before the gradient reaches equilibrium. Thus, most such separations can be terminated without a loss of resolution before isopycnic equilibrium is attained. Modeling of the separation allows the researcher to estimate purity and to stop the separation at earlier times.

In addition, ESP allows a further reduction in run times by initially running CsCl gradients at speeds that, if continued for prolonged intervals, would lead eventually to precipitation of the gradient salt (Overspeed Control). The presence of precipitates in a centrifuge tube spinning at high speeds may result in rotor failure due to localized overstressing. ESP allows rotor speed to be reduced automatically before precipitation can occur. Since the time required for gradient formation decreases with faster rotor speeds, this approach results in more rapid separations. In general, for fixed angle rotors, rotor speed is limited by the need to avoid precipitation of the gradient salt at the bottom of the tube, where the concentration is greatest as the gradient develops. By simulating the time-dependent changes in salt concentration throughout the gradient, ESP allows one to design protocols in which the rotor speed is maximized, without gradient salt precipitation.

Simulated Plasmid Isolation Runs

ESP was used to model optimized run conditions for CsCl-ethidium bromide gradients typically used for isolations of supercoiled plasmid DNAs.[1] Initial CsCl concentration was uniform throughout the tube and corresponded to a density of 1.55 g/mL, with ethidium bromide present at a saturating concentration of 0.6 mg/mL. Under these conditions, supercoiled plasmid DNAs band at a density of 1.575 g/mL, whereas the nicked and linear forms, which can bind greater quantities of the dye, band at 1.535 g/mL.

To model the time-dependent changes in the distribution of macromolecules in the centrifuge, the ESP algorithm establishes a finite number of radial boundaries within the confines of the centrifuge tube, and calculates the flux of molecules across each boundary during a series of time intervals that comprise the run. Each molecular species is characterized by its sedimentation and diffusion coefficients, as well as its partial specific volume, from which the buoyant density is determined. Tube geometry is accurately modeled, and portions of the algorithm permit conversion of radial distances to equivalent volumes within the tube after the run has stopped and the gradient has reoriented.

The ESP-generated protocols described in this publication include a progressive, automatic decrease in rotor speed to avoid precipitation of the gradient salt.

Results and Discussion

Isolation of Plasmid DNA Modeled by ESP

An ESP simulation of the isolation of supercoiled plasmid DNA allows one to define a number of distinct stages during the separation (Figure 1). During the earliest stages of centrifugation, the CsCl gradient develops most markedly at the innermost (r_{min}) and outermost (r_{max}) radial positions in the tube. Only later does a gradient of appreciable slope develop near the center of the tube.

As centrifugation progresses, the macromolecules first migrate away from the most extreme inner and outer radial positions, where the gradient is steepest, and begin to accumulate at their isopycnic positions. Eventually, the simulation demonstrates a situation where the two species have completely separated. We define this time point as the initial separation time.

At initial separation, the bands remain relatively broad. This is especially true of the upper band which is composed of species with lower sedimentation coefficients than the more compact supercoiled species in the lower band. The degree of separation at this time point will depend upon the quantity of material loaded. While the ESP algorithm assumes ideally dilute solutions of macrosolutes, laboratory verifications of these predictions show that these simulations are representative of the degree of separation for loads as high as 200 µg per tube.

Following initial separation, the bands continue to separate and become more compact. Eventually, a distribution is achieved that will not change appre-

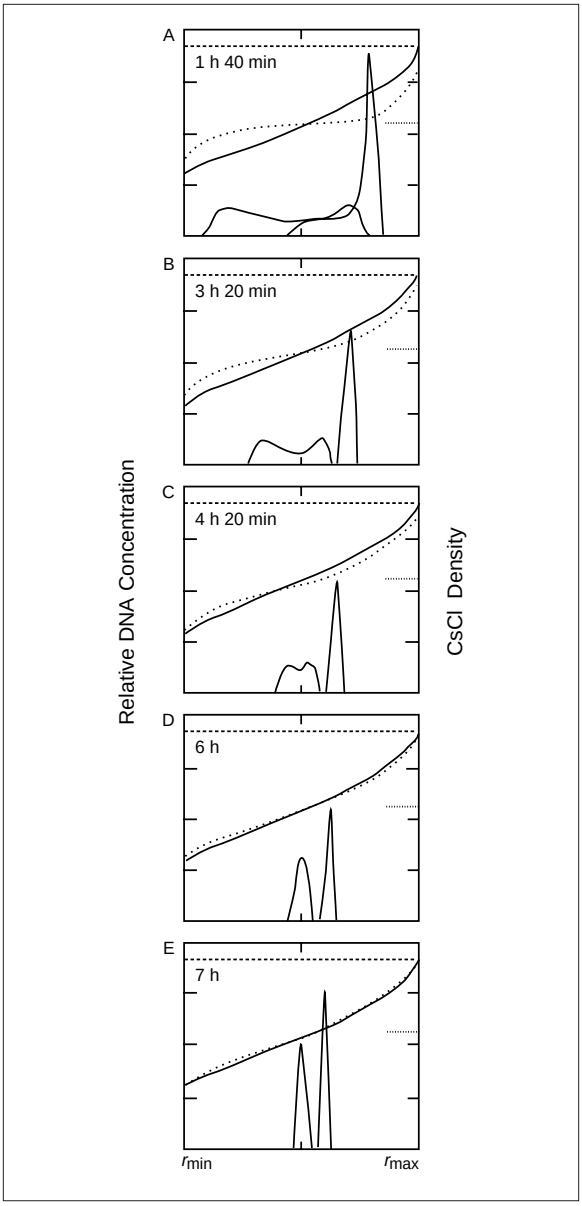

Figure 1. *ESP simulation of plasmid DNA isolation in the Type 100 Ti rotor using the 6.0-mL tube. The ESP algorithm was used to automatically develop a protocol permitting maximum rotor speed with periodic decelerations to avoid precipitation of CsCl. Each panel in the figure shows the status of the simulated separation at the indicated time. In each case, the upper curve represents the CsCl density gradient, and the lower curves the distribution of DNA. The partial dashed line at the right of each panel shows the starting concentration of CsCl (corresponding to a density of 1.55 g/mL), and the dashed line across the top of each panel shows the point at which CsCl precipitates at 25°C. The algorithm predicts complete separation of the supercoiled DNA from the nicked and linear contaminants in 7 h using this protocol (Panel E). Experiments described in the text confirm that this is the case, although nearly pure supercoiled DNA can be recovered as early as 6 h (Panel D).*

ciably as centrifugation proceeds toward gradient equilibrium. We define this as the stable separation time. Band compactness at the stable separation time provides an optimal situation for recovery of an individual molecular species.

Plasmid Isolation Times Determined by ESP

Table 1 offers a comparison of minimal separation times in several rotors, as determined by the ESP algorithm and confirmed in the laboratory.[2-4] Several points are evident from the data in this table. First, the shortest run times will be obtained by using vertical and near vertical tube rotors. This is because the radial distance subtended by the gradient in these rotors is minimized, allowing the fastest possible gradient formation. Near vertical tube rotors, in particular, are ideal for this application, since they combine a short radial span with a slight inclination of the tube, which prevents pelleted RNA from contacting the DNA bands when the rotor comes to a stop. Second, faster rotors permit the fastest isolations, since the greater force that they generate causes the gradient to form most rapidly. Thus ESP's Overspeed Control feature, which allows application of the greatest possible force, permits a further, significant reduction in run times.

Verification of ESP Predictions

Predictions made by the ESP algorithm have been confirmed in the laboratory. The details of this work have been published elsewhere[5] and are summarized here. In brief, defined mixtures of supercoiled and nicked + linear plasmid molecules were combined in equal ratios in CsCl/ethidium bromide solutions and centrifuged following the appropriate protocol. The centrifugation was terminated at the times predicted for initial separation, stable separation, or after 24-48 h to approximate equilibrium. Separa-

Table 1. Plasmid Separation Times

Rotor	*Tubes*		*Standard Protocol*		*Separation by ESP Overspeed Protocol*	
	Volume (mL)	*Number of Tubes*	*Speed (rpm)*	*Time (h:min)*	*Initial Time (h:min)*	*Stable Time (h:min)*
Fixed Angle Rotors						
Type 100 Ti	6.0	8	62,000	7:00	6:00	7:00
	5.1*		67,000	6:30	4:40	5:50
	3.5*		78,000	3:30	2:00	3:30
	2.0*		80,000	2:30	1:30	1:50
Type 90 Ti	13.5	8	52,000	20:00	11:00	14:30
	10.0*		56,000	17:00	8:00	11:00
	6.3*		62,000	7:00	4:00	5:50
	4.2*		67,000	5:00	2:30	3:50
Type 80 Ti	13.5	8	48,000	24:00	11:30	13:30
Type 75 Ti	13.5	8	49,000	24:00	12:00	16:30
Type 70.1 Ti	13.5	12	49,000	24:00	11:30	15:30
Vertical Tube Rotors						
VTi 90	5.1	8	90,000	2:30	1:30	2:10
VTi 80	5.1	8	80,000	3:00	2:00	2:30
VTi 65	5.1	8	65,000	4:00	2:30	3:30
VTi 65.1	6.3*	8	65,000	4:30	3:00	4:30
VTi 65.2	5.1	16	65,000	4:00	2:30	3:10
Near Vertical Rotors						
NVT 90	5.1	8	90,000	3:00	2:00	2:40
NVT 65	6.3*	8	65,000	4:30	3:00	4:30
NVT 65. 2	5.1	16	65,000	4:30	3:00	4:30

**g*-Max™ tube.

tions were characterized by fluorometry by piercing tubes at the bottom and passing the gradient through a flow fluorometer. Electrophoresis on agarose gels was used to demonstrate purity of isolated plasmid conformational isomers (supercoiled, nicked, and linear).

A quantitative evaluation of the simulation is shown in Figure 2, where the actual distribution of DNA in each tube is compared to the computer-generated predictions corresponding to initial separation and stable separation. The accuracy of predictions concerning the time-dependence of band formation is borne out by this comparison. At the initial separation, the bands are distinct, but broad and incompletely resolved. The extent of baseline overlap increases with increasing load. At stable separation, the bands have compacted as predicted by the simulation.

Purity of the Isolated Supercoiled Plasmid DNA

Supercoiled plasmid DNA isolates recovered at both initial and stable separation have been examined by agarose gel electrophoresis in order to assess the degree of purity achieved in these rapid preparations. The results are shown in Figure 3. The three plasmid conformational isomers are identified unambiguously by running previous isolates of supercoiled, nicked, or linearized plasmid molecules as standards. Starting materials for the centrifugations are shown and contrasted to material recovered from the lower band of tubes centrifuged to the initial separation times and stable separation times, respectively. No linear material is detected in these isolates. The amount of nicked material is estimated as less than 5% of the total. Some nicked material may arise through postcentrifugal handling of the samples. These results demonstrate that high-quality, accelerated separation protocols can be attained by modeling with the ESP algorithm.

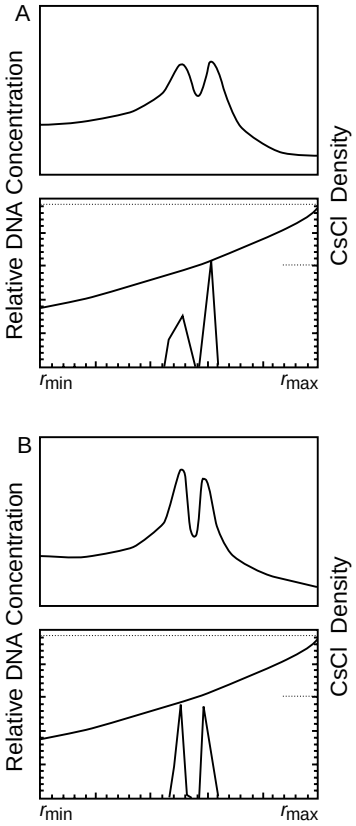

Figure 2. *Fluorescence profiles of ESP-controlled isolations of supercoiled plasmid DNA. Separations were performed in 13.5-mL tubes in the Type 90 Ti rotor, for the times indicated in Table 1. The ESP simulation predicts a usable purification of supercoiled DNA in 13 h (A, lower panel), and complete isolation in 16 h (B, lower panel). Confirmation of the prediction is obtained by fluorometric detection of the DNA/ethidium bromide complex (A and B, upper panels). The sloping baseline in the fluorographs is due to an inverse gradient of ethidium bromide which develops as centrifugation progresses.*

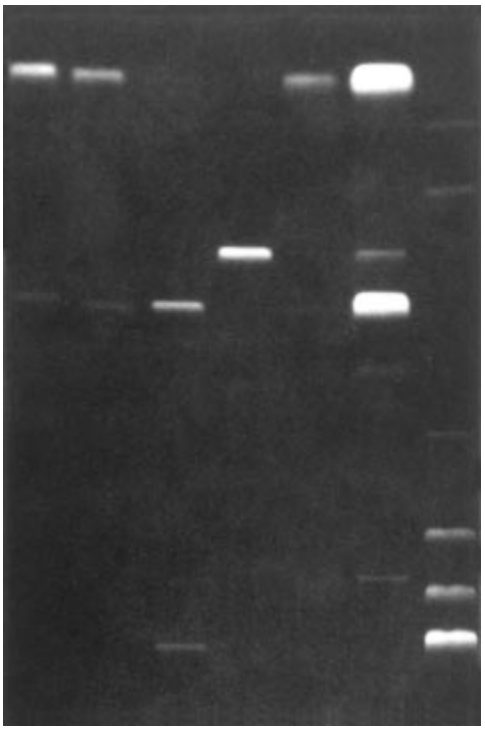

Figure 3. *Agarose gel electrophoresis demonstrates the purity of ESP-isolated supercoiled DNA. Lane 1,* Hin*dIII digest of lambda DNA; Lane 2, crude lysate; Lane 3, supercoiled material isolated as lower band in a previous isopycnic centrifugation; Lane 4, plasmid DNA linearized by restriction endonuclease digestion; Lane 5, nicked material isolated as the upper band in a previous isopycnic centrifugation; Lane 6, lower band isolated from 16 h (stable separation time) long-tube ESP centrifugation described in the text; Lane 7, lower band isolated from 5-h (initial separation time) short-tube ESP centrifugation described in the text.*

References

1. Bauer, W., Vinograd, J. The interaction of closed circular DNA with intercal-ative dyes. I. The superhelix density of SV40 DNA in the presence and absence of dye. *J. Mol. Biol. 33*, 141-171 (1968)
2. Little, S. E. Plasmid separations in NVT™ near vertical tube rotors. *Applications Data Sheet DS-770*, Beckman Instruments, Inc. (1990)
3. Little, S. E., McRorie, D. K. Rapid separation of plasmid DNA in preparative ultracentrifuge rotors. *Applications Data Sheet DS-734A*, Beckman Instruments, Inc. (1989)
4. Little, S. E., McRorie, D. K. High-efficiency plasmid separations using the VTi 90 vertical tube rotor. *Applications Data Sheet DS-751A*, Beckman Instruments, Inc. (1989)
5. Voelker, P., Furst, A. ESP allows rapid isolation of plasmid DNA in the Type 90 Ti rotor by avoidance of CsCl precipitation. *Applications Data Sheet DS-756*, Beckman Instruments, Inc. (1989)

BECKMAN

Beckman Instruments, Inc. • 2500 Harbor Boulevard, Box 3100 • Fullerton, California 92634-3100
Sales: 1-800-742-2345 • Service: 1-800-551-1150 • Internet: http://WWW.BECKMAN.COM • Telex: 678413 • Fax: 1-800-643-4366

Worldwide Offices: Africa, Middle East, Eastern Europe (Switzerland) (22) 994 07 07. **Australia** (61) 02 816-5288. **Austria** (2243) 7292164. **Canada** (800) 387-6799. **China** (861) 5051241-2. **France** (33) 1 43 01 70 00. **Germany** (49) 89-38871. **Hong Kong** (852) 814 7431. **Italy** (39) 2-953921. **Japan** 3-5352-2825. **Mexico** 525 575 5200, 525 575 3511. **Netherlands** 02979-85651. **Poland** 408822, 408833. **Singapore** (65) 339 3633. **South Africa** (27) 11-805-2014/5. **Spain** (1) 358-0051. **Sweden** (8) 98-5320. **Switzerland** (22) 994 07 07. **Taiwan** (886) 02 378-3456. **U.K.** (01494) 441181. **U.S.A.** 1-800-742-2345.

SBU4/95079-CB-10

Printed in U.S.A. on recycled paper