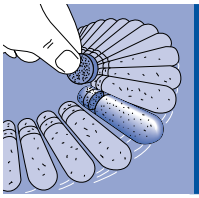

Application Information

Ultracentrifugation

A Rapid Method for Ribosome Subunit Isolation: Part 2—Using the High-Capacity Swinging Bucket MLS-50 Rotor in an Optima™ MAX Tabletop Ultracentrifuge

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Introduction

The separation performance of the Optima™ MAX ultracentrifuge is demonstrated by isolating a bacterial ribosome using two high-capacity rotors. This work is described in two separate application information bulletins:

- Part 1 (document number A-1850A) describes conditions for pelleting an intact 70 *S* ribosome using differential centrifugation with a fixed angle MLA-80 rotor and an 8-mL tube.
- Part 2 (this document) describes conditions for preparing and separating 30 *S* and 50 *S* ribosomal subunits using rate zonal density gradient centrifugation with a swinging bucket MLS-50 rotor and a 5-mL tube.

Results for part 2 show approximately 3 mg of dissociated ribosomes are separated as 30 *S* and 50 *S* subunits with negligible cross-contamination using this rotor/tube combination. A 5-mL tube replaces the 2.2-mL tube as the largest available size and allows for greater throughput than was possible previously in a tabletop ultracentrifuge. The subunits were analyzed by analytical ultracentrifugation.

Method

The 70 *S* ribosome was dissociated and separated into the 30 *S* and 50 *S* subunits following standard procedures.[1,2,3] (Note: preparation of the intact 70 *S* species is described in Application Bulletin A-1850A.) An example of a step-wise approach used to prepare and isolate ribosomal subunits is presented below. All procedures were performed at 5°C unless otherwise indicated.

Buffers

D: 20 mM $MgCl_2$ 50 mM NH_4Cl, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl (pH 7.4)

E: 20 mM $MgCl_2$ 100 mM NH_4Cl, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl (pH 7.4)

F: 1 mM $MgCl_2$ 500 mM NH_4Cl, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl (pH 7.4)

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1. A bacterial pellet was resuspended in an equal volume of buffer D, then ruptured in a cell disruption bomb using nitrogen at 2500 psi. To maximize the percentage of ruptured cells, the cells were passed through the bomb twice.
2. A solution of 1 µg/mL of DNAse (in water) was added to the cell homogenate.
3. The cell homogenate was divided into two aliquots and spun in two 50 mL bottles at 105,000 × *g* for 4 hours in an Avanti® J-30I centrifuge from Beckman Coulter with a JA-30.50 rotor. The supernatant was discarded.
4. The pellet was resuspended in the same buffer, clarified for 30 minutes at 20,000 × *g*, and precipitated with ammonium sulfate (49 g of solid ammonium sulfate per 100 mL of ribosome suspension with a concentration of approximately 1 mg/mL) and stored overnight at 4°C.
5. The precipitate was collected by spinning for 30 minutes at 30,000 × *g*.
6. The resulting ribosomal pellet was resuspended in a small volume of buffer E, dialyzed against the same buffer, and then against buffer F for dissociation into subunits.
7. The suspension was again clarified by spinning for 30 minutes at 30,000 × *g*. An additional pelleting step was required to concentrate the solution of subunits into a smaller volume—the solution was centrifuged in an MLS-50 rotor at 268,000 × *g* (50,000 rpm) for 5 hr 15 min at 5°C. The pellet was resuspended in 1 mL of buffer F and stored at 5°C.
8. The individual subunits were isolated by layering the ribosomal suspension (in a 250-µL volume) onto a 5-mL tube containing a linear 25-40% sucrose gradient in buffer F and spinning in an MLS-50 rotor at 268,000 × *g* (50,000 rpm) for 5 hr at 5°C.
9. Following centrifugation, the tube bottom was pierced and fractions of approximately 20 drops were collected. Fractions were dialyzed against buffer F and stored at 5°C.

The physical properties of 1) dissociation and 2) separation were determined with an Optima™ XL-I analytical ultracentrifuge from Beckman Coulter using a sedimentation velocity protocol. Runs were made at 30,000 rpm at 20°C in buffer F. Data were measured using interference optics and the size distributions were determined using the time derivative analysis of the sedimentation velocity profiles.[4] Data are presented graphically as peaks. The areas under the peaks are used to estimate the relative yield of each subunit.

Results/Discussion

The dissociation of bacterial ribosomes (70 *S*) produced the two subunits (30 *S* and 50 *S*) in near quantitative yield (Figure 1). A 2:1 ratio of 50 *S* to 30 *S* subunits (measured as weight % by the XL-I) with a A_{260}/A_{280} ratio of 1.95 provided an estimate of sample purity (a ratio for $A_{260}/A_{280} > 1.8$ is an acceptable criterion for a pure ribosome preparation).[5]

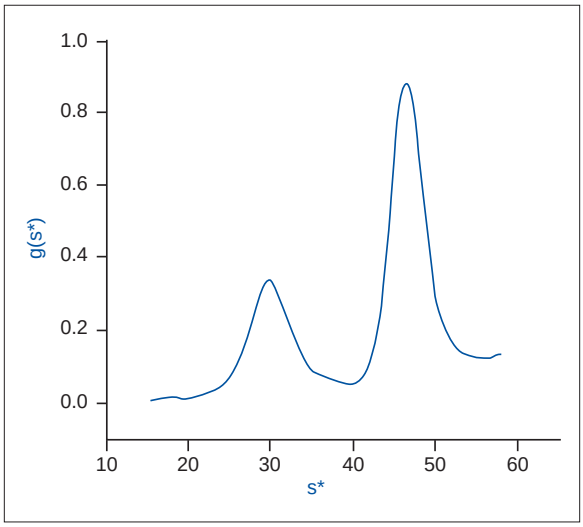

Figure 1. *Dissociation of bacterial ribosomes (70* S*) into the 50* S *and 30* S *subunits. A 2:1 ratio of 50* S *to 30* S *subunits (measured as weight % by the Optima™ XL-I) was estimated by measuring the areas under the peaks. Sedimentation coefficients (s*) were measured in aqueous buffer at 20°C.*

The two ribosomal subunits were separated in 5 hours using a 25-40% sucrose density gradient in a 5-mL tube with a swinging bucket MLS-50 rotor at 5°C. (A 10-40% gradient produced band separation near the tube bottom rather than the middle under the same conditions.) A swinging bucket rotor is considered the rotor of choice for rate zonal density gradient centrifugation. At speed, the horizontal orientation of the bucket ensures that a sample moves through the gradient without encountering any disruptive wall effects.

Sample capacity of the 5-mL tube was determined by loading various amounts of a ribosomal mixture to the gradient and measuring the resolution between the two subunits. The sample mixture was quantified by measuring the absorbance at 260 nm (one A_{260} unit equals approximately 0.26 mg of bacterial pellet—using reported values of 69 pmol and 34.5 pmol for 30 *S* and 50 *S* subunits, respectively).[5] Optimal sample capacity was found to be approximately 3 mg with a loading volume of

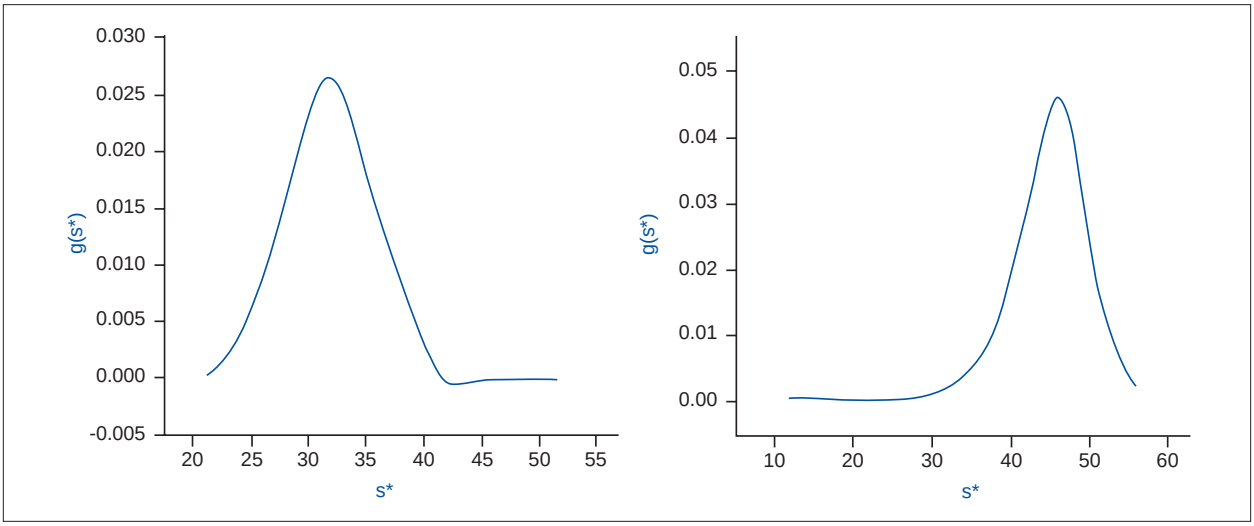

Figure 2. *Isolation of a ~3 mg mixture of ribosomal subunits by rate zonal density gradient centrifugation. The 30* S *(left-hand plot) and 50* S *(right-hand plot) subunits were separated with negligible cross-contamination. Sedimentation coefficients (s*) were measured in aqueous buffer at 20°C.*

250 µL. Analysis of the separation showed the ribosomal mixture was isolated as two distinct subunits with negligible cross-contamination (Figure 2). The two subunits were collected in three successive fractions corresponding to a separation occurring near the middle of the tube. Table 1 shows that the 50 *S* and 30 *S* subunits were recovered in quantities of 1.85 mg and 0.85 mg, respectively, with an overall yield of 2.7 mg (86%). A ratio of 2:1 at A_{260} for 50 *S* to 30 *S* and a ratio of about 2 for A_{260}/A_{280} for each of the subunits were consistent with expectations.

Table 1. Rate Zonal Separation of Bacterial Ribosomal Subunits Using an Optima™ MAX Tabletop Ultracentrifuge[1]

	Recovery (mg)	***A_{260}***	***A_{280}***
70 *S*[2] (starting material)	3.13	0.98	0.5
30 *S*[3]	1.85	0.33	0.15
50 *S*[3]	0.85	0.72	0.33
	(total) 2.7		

1. The two subunits were separated in 5 hours using a 25-40% sucrose density gradient in a 5-mL tube with an MLS-50 rotor.
2. A_{260} and A_{280} measurements correspond to a dilution of 1:100.
3. A_{260} and A_{280} measurements correspond to a dilution of 1:10.

The separation was repeated with 6 mg of a ribosomal mixture (also in a 250 µL volume) loaded on the gradient. Results (not shown) produced a separation with significant cross-contamination of the two subunits. In addition, the two subunits were distributed over a larger number of tube fractions (four instead of three). A separation with 1.5 mg of ribosomal mixture was comparable in quality to a 3 mg sample load but made use of only half the loading capabilities of the tube.

Conclusions

Before introduction of the MLS-50 rotor, separation of subcellular organelles (and other biological material) using rate zonal density gradient centrifugation in a swinging bucket rotor was generally performed in a floor model ultracentrifuge because of the availability of larger-capacity rotors. With the introduction of the MLS-50 swinging bucket rotor for the Optima MAX tabletop ultracentrifuge, this type of separation is possible using a smaller instrument. The availability of a 5-mL tube in a tabletop instrument provides additional capacity over a 2.2-mL tube and allows for separations on the same scale as those obtained in rotors in a floor model ultracentrifuge.

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