

High Efficiency Differential Ultracentrifugation of Small Sample Volumes Using the Thermo Scientific TFT 80.4 and 80.2 Rotors

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Key Words

- Differential Ultracentrifugation
- Small Volume Separation
- Centrifuge Theory
- Fixed-Angle Rotors

Introduction

As the technology and techniques for biochemical research advance, researchers are continually moving to smaller sample and reaction volumes. In fact, the specificity of enzymes and monoclonal antibodies has allowed biochemists to work on the μL scale for many protocols. To meet this demand, there are two fixed angle ultraspeed small volume rotors – the Thermo Scientific TFT– 80.4 and TFT– 80.2. Both of these rotors have low K factors and are ideally suited for differential ultraspeed centrifugation of small volumes. Since our lab has worked with the TFT-80.4 rotor, it will be featured in this discussion.

Our current ability to understand and control cadaveric organ graft rejection is still embryonic. As a result, our lab is developing improved methods for both the diagnosis and treatment of cadaveric organ graft rejection. Transplanting cadaver organs may be routine, but long-term survival is rare and cures are non-existent.

The TFT-80.4 rotor has been especially useful in evaluating monoclonal

antibodies (MAb) as a means to diagnose cardiac rejection *in situ*. We use a mouse IgG₁ MAb specific to mammalian cardiac myosin. In a rat heart transplant model, we have found that after I.V. injection of a radiolabeled form of the MAb, the MAb will circulate until it binds specifically to cardiac myosin that has been exposed in areas of the heart graft that are being rejected.¹

Materials and Methods

Immune ascities is prepared by loading ascities fluid directly onto a hydroxyapatite chromatographic column. Non-immunoglobulin proteins pass through the column and the proteins in the eluted peak can then be rapidly evaluated for purity on a ZORBAX GF-250 column (Agilent Technologies) or by the standard, but more laborious, technique of SDS-PAGE.²

Since the eluted IgG₁ is always more than 95% pure, we proceed directly to radiolabeling of the MAb with ¹²⁵I using chloramine-T. However, during storage of the pure MAb (frozen or at 4°C), multi-molecular

aggregates form which do not bind well to the cardiac myosin target molecules upon injection. Instead, the aggregates contribute unwanted background noise as they bind to receptors on sessile and circulating lymphoid and reticuloendothelial cells.

Since small amounts of the MAb are iodinated, the TFT-80.4 is used to clear the solution of MAb aggregates before iodination. 3 mL of the MAb (0.41 mg/mL) in 0.2 M sodium phosphate buffer (pH 7.4) are spun in Thermo Scientific Ultracrimp™ tubes (Cat. No. 03905) in a Thermo Scientific Sorvall® WX ultracentrifuge at 80,000 rpm (468,200 x g) for 1.25 hours.

This run time was determined as follows: The K factor for this rotor, tube and sample volume combination is 25.8. Assuming monomolecular MAbs have an S value of 7, and that we want to sediment all particles (aggregates) with S values of 20 or more, the minimum time needed is $K/S = 25.8/20 = 1.29$ hours (see Relationship Between Theory and Technique for a more complete discussion.)

The MAbs in the de-aggregated supernatant are radioiodinated using an optimized chloramine-T procedure. All the steps in this procedure must be performed in a vented enclosure with appropriate charcoal filtration to minimize personal uptake and environmental release.

10 μL of [¹²⁵I] sodium iodine (100 mCi/mL) are added to 0.5 mL of 0.2 M sodium phosphate buffer pH 7.4 in a 10 mL vial. 10 μL of a 50 mg/mL solution of chloramine-T in phosphate buffer are also added to the vial. After 5 minutes, 2 1.0 mL volumes of MAb solution are added to

TFT-80.4



the vial in succession while stirring. 5 to 10 μ L volumes of the chloramine-T solution are then added at 5 to 10 minute intervals during the next 30 to 40 minutes.

The amount of chloramine-T to be added and the total time for the reaction are determined by monitoring the extent of iodination before adding each new volume of chloramine-T. To determine how much of the MAb has been radioiodinated, 5 μ L amounts of the reaction mixture are removed every 5 to 10 minutes and spotted on a silica-impregnated glass fiber, thin-layer chromatography paper. The paper is developed with saline and the origin and solvent front assessed for radioactivity with a portable Geiger counter. As more of the ^{125}I is incorporated into the MAb, the proportion of radioactivity at the origin (iodinated protein) increases compared to the radioactivity at the solvent front (free ^{125}I). This chromatographic technique is more fully discussed in *Notes From the Radioiodination Laboratory* (Vol. 1, 1979) available from NEN.

The reaction is stopped by passing the contents of the vial over a Sephadex[®] G-25 column that has been preconditioned with phosphate buffer. 0.5 mL fractions from the column are collected and counted. The fractions comprising the radioactive peak containing the labelled MAb are pooled, counted and analyzed spectrophotometrically for optical density at 280 nm. This material is then ready for injection for *in vivo* detection of cardiac allograft rejection.

Using ultraspeed centrifugation to remove high molecular weight aggregates has other applications. For example, we use MAb *in vitro* to enumerate lymphoid cell subpopulations by ELISA.³ Ultracentrifugation of the MAbs in the TFT-80.4 rotor ensures that aggregates are removed and reduces non-specific Fc receptor binding, which causes high assay background.

We also use large proteins as immunosuppressive therapy; differential ultracentrifugation is used not only in the initial purification of the proteins, but also to remove aggregates that may cause adverse reactions just before I.V. treatment. In this situation, we usually purify a large batch of therapeutic protein with the high capacity T-8100 or T-890 rotor in a Sorvall WX ultracentrifuge. Then, after a small volume of the purified protein has thawed, it is de-aggregated by centrifuging the sample again in the WX ultracentrifuge, but this time using the TFT-80.4 rotor. We find it very convenient to use a single floor model ultracentrifuge for both our large and small volume centrifugation needs.

Theoretical Background

In contrast to isopycnic density gradient ultraspeed centrifugation, which separates particles (molecules, organelles, viruses) based on their densities, differential ultraspeed centrifugation relies on differences in sedimentation velocities (or sedimentation coefficients) among particles to concentrate certain particles in the pellet, while other particles remain in the supernatant fluid.

Under the same centrifugal force and in the same medium, the sedimentation velocities of two particles of equal density, but different size, will vary directly with the square of each particle's radius. As a result, after a given period of centrifugation, larger particles (comprising the pellet) will separate differentially from smaller particles (comprising the supernatant). This is the principle that makes differential ultraspeed centrifugation possible.

Since molecular weight and size are proportional for most large proteins, this technique can separate proteins by molecular weight. However, given sufficiently high centrifugal forces and enough time, even the low molecular weight proteins will sediment and join the high molecular weight pro-

teins in the pellet. Therefore, it is necessary to control the centrifugation time to maximize the separation between low and high molecular weight proteins. The time needed to pellet a protein or other particle is related to both its sedimentation velocity and to a clearing, or K factor. The K factor is independent of the physical characteristics of the protein, but does depend on the geometry of the rotor and its rotational speed. Specifically, the K factor varies directly and exponentially with the ratio of the maximum radii ($r_{\text{max}}/r_{\text{min}}$) of a given sample volume, as shown in Equation 1.

Equation 1:

$$\begin{aligned} r_{\text{max}} &= \text{maximum radius} \\ r_{\text{min}} &= \text{minimum radius} \\ K &= \frac{2.53 \times 10^{11} \ln (r_{\text{max}} / r_{\text{min}})}{(\text{RPM})^2} \end{aligned}$$

In addition to sample volume, r_{max} and r_{min} are defined by the dimension of the centrifuge tube, rotor geometry and presence of tube adaptors. The K factor also varies inversely with the square of the rotor's rpm. The lower the K factor, the more quickly a given particle will be cleared from the supernatant and sedimented in the pellet. Since low K factors are achieved by spinning small sample volumes at high speed, one can appreciate the value of rotors capable of meeting these criteria.

Relationship Between Theory and Technique

Now that the K factor has been defined, we can return to the important question of determining the optimum run time to achieve maximum separation of large particles away from small particles. The time for a given run to accomplish this purpose is determined by simply dividing the K factor by the sedimentation coefficient, S (in Svedberg units), of the large particles as shown in equation 2. The resulting time (in hours) is the minimum time needed to sediment the large particles.

Equation 2:

$$\text{Time (hrs)} = \frac{K}{S}$$

A more thorough treatment of the physical principles underlying centrifugation can be found in *Centrifugation in Biology and Medical Science*⁴ and www.thermo.com/rotors. RCF charts are also available, which list K factors for different Thermo Scientific ultracentrifuge rotors at discrete rpm's for specific tubes and sample volumes. Even though these charts do not give K factors for all possible speeds and sample volumes, K factor can be determined for any rpm, rotor and sample volume by using the Thermo Scientific Compass™ centrifugation software package available for the WX ultracentrifuge.

Conclusion

TFT-80.4 and TFT-80.2 fixed-angle rotors (Table 1) are ideal for small-volume differential ultracentrifugation. The high speed capability (80,000 rpm) and small sample volumes of these rotors results in low K factors, providing the researcher with an efficient and valuable tool for molecular separations. In addition, these two rotors expand the capabilities of floormodel ultracentrifuges making it possible to use a single instrument for small sample volume as well as scaled-up or large-volume procedures. From the brief discussion of centrifuge theory, and the applications discussed in this article, it is clear that TFT-80.2 and TFT-80.4 rotors are valuable additions to today's biochemical research laboratory.

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Table 1

Specifications for Thermo Scientific TFT-80.4 and TFT-80.2 Rotors

Rotors	Max. Speed (rpm)	RCF (x g)		Max. Volume (mL)	Radius (cm)	
		Max	Min		Max.	Min.
TFT-80.4	80,000	468,246	243,774	4.4	6.55	3.41
TFT-80.2	80,000	429,643	242,344	2.0	6.01	3.39

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