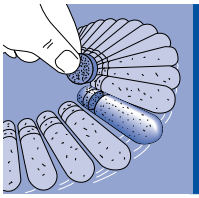

Technical Information

High Speed Centrifugation

Developing Elutriation Protocols

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A heterogeneous cell suspension may be separated into its constituent cell populations by a process known as counter-current centrifugal elutriation. In the elutriation process, the cell mixture is placed into a specially designed centrifuge rotor/chamber and subjected to a centrifugal field. Cells are then sequentially washed out of the rotor based on their size (smaller cells first), using a buffer stream that flows in the direction opposite the centrifugal field. By balancing centrifugal force against the opposing buffer flow, cells of any predetermined size may be selectively removed from the mixture (see Figure 1).

Because elutriation is a gentle process that uses physiological media, normal cell viability and function are maintained. Consequently, the cells are not usually activated or artificially stimulated. Thus, cells purified by elutriation are suitable for studies in genetics, immunology and other disciplines where cell purity and unaltered cell function are critical.

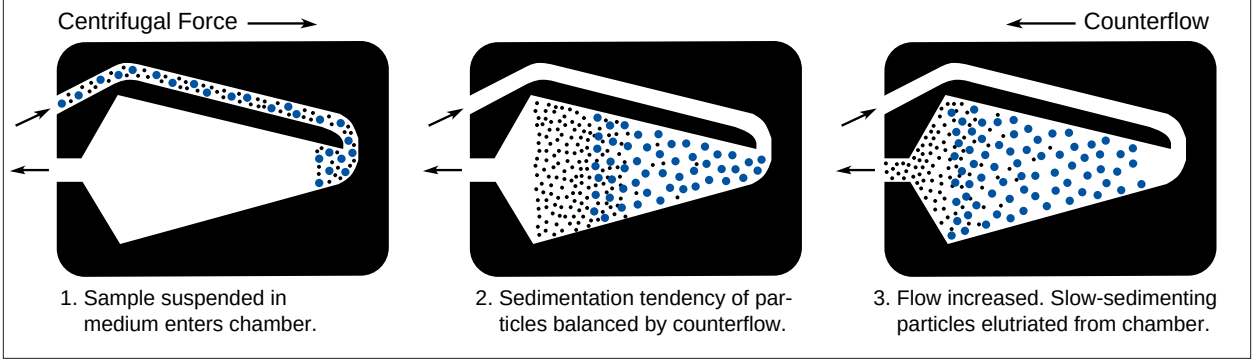

Figure 1. *Diagrammatic representation of the elutriation process.*

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In the development of an elutriation protocol, several variables of the elutriation process must be considered:

A. The strength of the centrifugal force field in the elutriation chamber (*g*-force);
B. The effect of the counterflow buffer stream in the elutriation chamber (counterflow velocity);
C. The size distribution (in microns) of the cells in the mixture;
D. The geometry of the elutriation chamber (especially the cross-sectional area of the chamber at its widest point); and
E. The density of the elutriation buffer.

The relationship of some of these variables is expressed in the formula (Stokes' Law):

$$SV = \left(\frac{d^2(\rho_p - \rho_m)}{18\eta} \right) \omega^2 r \qquad (1)$$

where SV = the sedimentation velocity
d = the diameter of the particle
ρ_p = the density of the particle
ρ_m = the density of the buffer
η = the viscosity of the buffer
r = the radial position of the particle
ω = the angular velocity in radians/second

Although Stokes' Law accurately describes the behavior of rigid spherical particles, it is somewhat less accurate in describing the sedimentation velocity of cells or particles that are not rigid and occasionally not spherical. Nevertheless, it is useful because it deals with those aspects of the system that influence the behavior of a sedimenting particle. Note that two properties of a spherical cell affect its sedimentation velocity: its size and its density. Size plays a more important role, however, since the diameter value is raised to the second power. Because cell populations often do not differ much with respect to density, cell separation by sedimentation velocity is based mostly on size differences.

In counter-current centrifugal elutriation, the forces that result in cell sedimentation in a radial direction are balanced by the velocity of fluid flowing in the opposite direction. The flow velocity, *V*, at any point is equal to the flow rate, *F*, divided by the cross-sectional area at that point, *A*.

$$V = \frac{F}{A} = \left(\frac{d^2(\rho_p - \rho_m)}{18\eta} \right) \omega^2 r \qquad (2)$$

The flow rate in the chamber is the same at every point; *i.e.*, $V_1A_1 = V_2A_2$. Therefore, changes in the cross-sectional area produce changes in the flow velocity. Where the cross-sectional area is small (for example, near r_{max}) the flow velocity is highest. At the elutriation boundary, where the cross-sectional area is greatest, the fluid velocity is lowest. Thus, there is a velocity gradient in the elutriation chamber.

Similarly, there is a gradient of centrifugal force, increasing from the elutriation boundary r_{eb}, to r_{max}. Where the centrifugal force field is greatest, the fluid velocity is also greatest; as r decreases, the cross-sectional area of the chamber increases and the fluid velocity decreases. Under the influence of the equal but opposing forces of the gravitational field and the fluid flow, small (lower sedimentation velocity) cells are in equilibrium nearest r_{eb} where the centrifugal force field and fluid velocity are low. Thus, separations are the result of cells of different sedimentation velocities being in equilibrium at different radial positions in the chamber. When the flow rate is increased (or the speed is decreased), cells that were in equilibrium near the elutriation boundary are washed out of the chamber, and the distribution of cells at equilibrium shifts toward the center of rotation. Subsequent increases in flow rate and/or decreases in speed elute populations of cells in order of increasing size.

The nomogram in Figure 2 allows you to determine flow rate and speed combinations with which cells of a given size will either be retained or swept out of the chamber. It is based on equation (2) where F/A is substituted for V and the relationship solved for F:

$$F = Ad^2 \left(\frac{\rho_p - \rho_m}{18\eta} \right) \omega^2 r \qquad (3)$$

Assuming that $\rho_p - \rho_m = 0.05$ g/mL, $\eta = 1.002$ mPa/s, and combining these with A (the cross-sectional area of the chamber at the elutriation boundary), r = the radius at the elutriation boundary, and constants that convert ω to rpm (Table 1), yield a chamber constant, X. Equation (3) then becomes:

$$F = Xd^2 \left(\frac{RPM}{1000} \right)^2 \qquad (4)$$

an expression relating flow rate, cell diameter and rotor speed.

Table 1. Chamber Constants for Various Chambers

40-mL large chamber	1.73×10^{-1}
5-mL standard chamber	5.11×10^{-2}
5-mL Sanderson chamber	3.78×10^{-2}

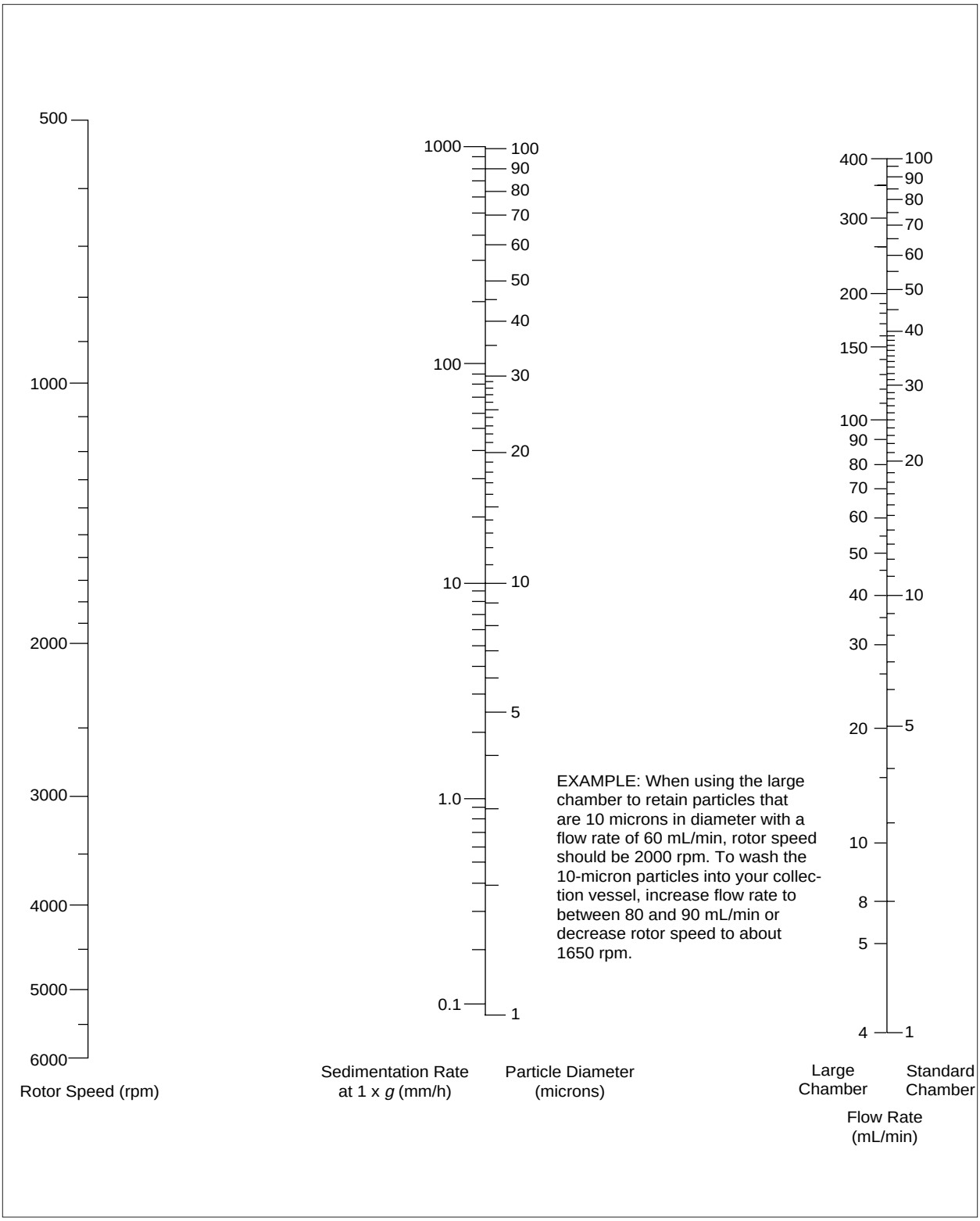

Figure 2. *Rotor speed and flow rate nomogram. Use a straightedge to connect a flow rate and rotor speed so that this line inersects the particle axis at a point corresponding to the smallest, lightest particles to be retained for the chamber you are using. For example, to retain all particles 10 microns and larger in the large chamber at a rotor speed of 2000 rpm, use a flow rate of 70 mL/min. To collect these particles, increase flow rate or decrease speed.*

The sedimentation velocity scale of the nomogram assumes that the sedimentation velocity of the cell was measured in a fluid having the same density and viscosity as the fluid being used for elutriation. If this is not so, the sedimentation velocity should be adjusted for elutriation conditions using the following formula:

$$SV_{adj} = SV_{determined}\left(\frac{\Delta\rho_{elutriation\ fluid}}{\Delta\rho SV_{determined}}\right)\left(\frac{\eta SV_{determined}}{\eta_{elutriation\ fluid}}\right) \quad (5)$$

When the particle diameter scale is used instead of the sedimentation velocity scale, flow rates from the graph may require adjustment if the viscosity of the elutriation medium and the difference in density between the particle and the medium differ significantly from the assumptions made to construct the nomogram (1.002 mPa/s and 0.05 g/mL, respectively). The following formula should be used for that adjustment:

$$SV_{adj} = SV_{determined}\left(\frac{\Delta\rho \text{ in g/mL}}{0.05 \text{ g/mL}}\right)\left(\frac{1.002 \text{ mPa/s}}{\text{viscosity in mPa/s}}\right) \quad (6)$$

For the design of any individual protocol, consideration must be given to ease of use, survival rates of cells subjected to constant handling, effects of time and elutriation buffer on cell functions, etc. In general, the less the cells are handled and the less time they spend out of culture conditions, the better the survival rates and the less the cell function is disrupted.

It is important to control all variables as closely as possible when confirming flow rate and rotor speed. For example, buffer temperature must be constant from reservoir to elutriation chamber if an accurate chamber temperature is to be derived for elution parameters. The centrifuge must maintain accurate temperature and speed for the rotor, and this should be calibrated at least once a year. All air should be purged from the rotor before loading cells into the chamber (verified by a "0" reading on the in-line pressure gauge). Elutriation chambers and rotors must be kept clean and free of endotoxins and contamination. Pumps need calibration routinely to ensure accurate flow rates, and electronic particle counters and sizers need routine calibration to deliver proper readout.

Example 1

We wish to derive a set of elutriation parameters for the separation of two particular cell species in a heterogeneous mixture (*e.g.*, lymphocytes and monocytes) obtained by a Ficoll density gradient separation of peripheral blood cells. The gradient separation yields a mixture having the following components: 32% monocytes, mean cell diameter 9.0 microns; and, 68% lymphocytes, mean cell diameter 2.8 microns.

We choose to use PBS as the elutriation buffer and perform elutriation at a constant 2500 rpm, at 20°C, in a 5-mL Sanderson chamber. (In this example, we arbitrarily selected the rotor speed and chamber for the purpose of illustration. Actual selection of these parameters should be made after consulting available literature pertaining to the specific cells to be isolated.) We calculate the buffer flow rates using equation (4):

$$F = 0.0378 \times (5.0)^2 \left(\frac{2500}{1000}\right)^2$$

$$= 6 \text{ mL/min for the 5.0-micron lymphocytes}$$

$$F = 0.0378 \times (9.0)^2 \left(\frac{2500}{1000}\right)^2$$

$$= 19 \text{ mL/min for the 9.0-micron monocytes}$$

We can now set the initial buffer flow to 6 mL/min, which will establish elutriation boundary conditions for the lymphocytes when they are loaded into the elutriation chamber. Increasing the flow rate by 1 or 2 mL/min after the elutriation boundary is established will then wash the lymphocytes out of the chamber but retain cells with a diameter that exceeds 5.0 microns. After we collect a 150-mL fraction of the 5.0-micron cells, we then increase the buffer flow rate to 19 mL/min to collect any cells from the mixture that fall in the range of greater than 5.0 microns but less than 9.0 microns in diameter. We collect 150 mL of these cells (which are discarded) and then increase the buffer flow to 20–21 mL/min and collect a 150-mL fraction of the 9.0-micron monocytes. [Fraction size is determined by measuring the volume of buffer used to elute at least 90% of the cell population. This volume can be inferred from periodic sampling of the effluent stream. When the cell count falls to less than 10% of the initial elution count (cells/mL of effluent), the fraction is deemed eluted from the chamber. In a 5-mL chamber, this elution volume is usually no greater than 150 mL.]

Example 2

We wish to isolate 6.0–7.5-micron cells from a mixture with a range of cell sizes from 2.5–10.3 microns. There are two ways to accomplish this:

1. We can use a one-step elutriation method that removes the cell range directly by varying the flow rate, or
2. We can use a two-step protocol that requires us to collect and reprocess the cells, when greater purity is desired.

One-Step Method

By using the formulas for flow rate as in Example 1, we calculate that at 2500 rpm the flow rate for eluting 5.9-micron cells is 8.0 mL/min, and for 7.5-micron cells, it is 13 mL/min. Therefore, we load at 8.0 mL/min and collect a load fraction of 150 mL. This fraction is discarded because it contains cells less than 6.0 microns. We then increase the buffer flow to 13–14 mL/min and collect a second fraction of 150 mL. This fraction contains the 6.0–7.5-micron cells. What remains in the chamber is washed out by stopping the rotor and allowing the buffer flow to continue. This fraction is also discarded because it contains cells larger than 7.5 microns.

The effect of increasing rotor speed is to increase the range of flow rates required to elute cells of differing size. For example, to elute the same 6.0–7.5-micron cell population at 3400 rpm would require flow rates of 16–25 mL/min (*vs.* 8–13 mL/min at 2500 rpm). This fact can be used to advantage when separating cells with small size differences; *i.e.*, the higher the centrifugal force, the easier it is to resolve the two cell lines due to the greater difference in flow rate to wash the cells out of the chamber.

Two-Step Method

We know that the flow rate used at 2500 rpm for eluting cells up to 7.5 microns is 13 mL/min. The cells are therefore loaded at 13 mL/min, and 150 mL of the load fraction are collected and saved. What remains in the chamber is washed out by stopping the rotor and allowing the buffer flow to continue. These are cells above the 7.5-micron range, and this fraction is thus discarded. The first fraction (load fraction) is spun to concentrate the cells to 10 mL and reinjected into the rotor at 13 mL/min, but at an increased rotor speed of 3200 rpm. This higher speed determines that the previous flow rate

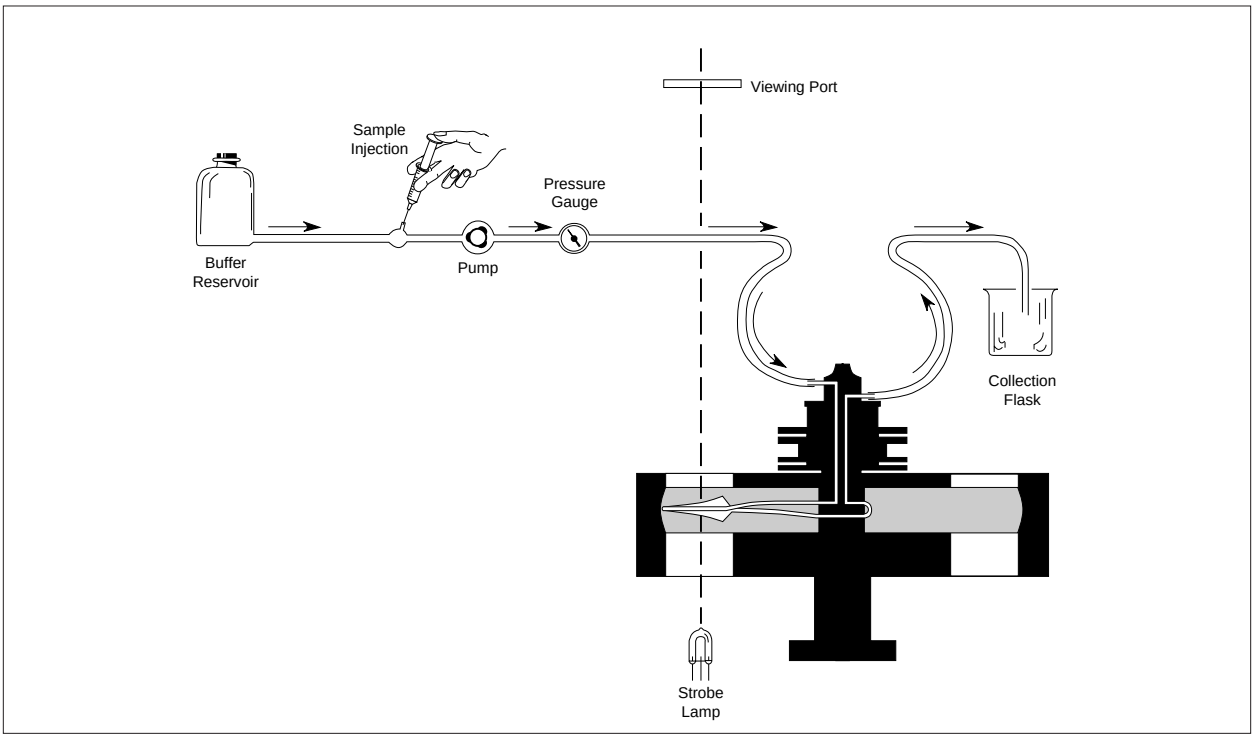

Figure 3. *The JE-6B Elutriator System. Essential elements of the system are shown. The buffer reservoir, pump, sample injection syringe, and collection flask are provided by the operator. Beckman provides all other components, including a harness which is a combined sample injection inlet/sample reservoir. When the injection harness is used, injection may be done downstream of the pressure gauge, but the pressure gauge must always be located downstream from the pump. If desired, a flow meter may also be included by the operator.*

of 13 mL/min elutes cells up to 5.9 microns, but not larger. A load fraction of 150 mL is collected and discarded. The chamber contents are then washed out by stopping the rotor and allowing the buffer flow to continue. These elutriated cells are in the range of 6.0–7.5 microns.

First Time Operation

These steps should be followed when first attempting a new protocol:

1. Connect the elutriation apparatus as shown for the JE-6B rotor in Figure 3 (the setup for the JE-5.0 rotor is similar).
2. Fill the rotor with elutriation buffer. Bring the rotor to operating speed and begin the flow of elutriation buffer through the rotor while stopping and starting the rotor at least twice to purge air from the system. When all air is purged, the pressure gauge will read "0" at operating speed. This is its normal operating value.
3. Determine the operating speed of the centrifuge and the pump speed to be used for the protocol. Bring the rotor to operating speed.
4. Set the buffer flow to the velocity necessary to establish the elutriation boundary conditions for the smallest diameter cells to be elutriated from the cell mixture. Introduce the mixture into the small (5-mL) elutriation chambers in an injection volume of 10 mL. For the 40-mL chamber, an injection volume of 30 mL should be used to avoid crowding the cells. In any case, it is desirable to use as low a volume as practicable to inject cells into the chamber. Observe the cells entering the elutriation chamber and check to see that the elutriation boundary has been formed and is stable. If the elutriation boundary fails to form, then either there are too few cells in the chamber to produce a visible boundary, or the cells are not behaving according to the mathematical model used to construct the nomogram. Generally, decreasing the loading rate (mL/min) or increasing rotor speed will cause the elutriation boundary to form. A check on the size of the cells exiting the rotor will confirm that one of these parameters needs adjustment. It is good practice to determine cell sizes for each eluted fraction to verify that the nomogram model is working.
5. Increase the buffer flow 1 mL/min and collect a 50-mL sample of the effluent at this buffer flow. Perform a cell differentiation and count on a Coulter cell sizer (or equivalent) and record the results.
6. For the 5-mL chambers, increase the buffer flow in 1-mL/min increments, collecting 50-mL samples for each incremental increase in buffer flow. For the 40-mL standard chamber, incremental flow rates should be higher (*i.e.*, 5 mL/min) and fraction sizes should be 100–200 mL. Determine the mean cell size and number for each sample when it is collected. When the proportion of cells of desired size in the mixture reaches 50%, this becomes the penultimate fraction and a 0.5–1.0-mL increase in buffer flow should be used to elute this size population of cells from the chamber. A collection volume of 150 mL should be sufficient to wash the entire cell population from the chamber (250–350 mL in the large 40-mL chamber).
7. Proceed to increase the buffer flow to the rate that establishes elutriation boundary conditions for the next size cell population to be eluted from the mixture, and perform steps 5 and 6 to clear this population of cells from the chamber.
8. Repeat steps 5–7 until all of the desired cells are cleared from the chamber.
9. Stop the centrifuge and wash the remaining cells from the chamber by continuing the buffer flow and collecting at least 100 mL of effluent wash volume.
10. Note the flow rates for each population of cells. This is the elutriation protocol.

Suggested Reading

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