Protocol

Density Gradient Ultracentrifugation to Isolate Endogenous Protein Complexes after Affinity Capture

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This protocol describes the isolation of native protein complexes by density gradient ultracentrifugation. The outcome of an affinity capture and native elution experiment is generally a mixture of (1) the complex(es) associated with the protein of interest under the specific conditions of capture, (2) fragments of the complex generated by degradation or disassembly during the purification procedure, and (3) the protease or reagent used to natively elute the sample. To separate these components and isolate a homogeneous complex, an additional step of purification is required. Rate-zonal density gradient ultracentrifugation is a reliable and powerful technique for separating particles based on their hydrodynamic volume. The density gradient is generated by mixing low- and high-density solutions of a suitable low-molecular-weight inert solute (e.g., sucrose or glycerol). The gradient is formed in a solvent that could be any of the solvents used for the affinity capture and native elution and should help to preserve the structure and activity of the assembly.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Buffer for preparing gradients

As a general rule, the buffer used to prepare the gradients should be the same composition as the one used to purify and stabilize the protein complex (i.e., the one used during the native elution during Protocol: ***Native Elution of Yeast Protein Complexes Obtained by Affinity Capture*** *[LaCava et al. 2015]). However, the buffer composition can be modified depending on the downstream sample processing requirements, bearing in mind that the stability and activity of the complex in the new composition should be tested beforehand.*

Natively eluted yeast protein complexes prepared by either method described in Protocol: **Native Elution of Yeast Protein Complexes Obtained by Affinity Capture** (LaCava et al. 2015)

Protease inhibitor cocktail (e.g., Sigma-Aldrich P8340)

Sucrose (66%, w/w) or glycerol

Prepare a solution of 66% (w/w) sucrose by dissolving 1710 g of sucrose (molecular biology grade) in 900 mL of high-performance liquid chromatography (HPLC)-grade water. Double-check the concentration using a refractometer and correct if necessary by adding sucrose or water. This solution can be stored at 4°C indefinitely because the low available water in the solution prevents bacterial growth.

As an alternative to sucrose, glycerol is a solute very commonly used to generate density gradients.

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Equipment

Filter (Millex-GP Syringe Filter Unit [0.22-µm] from EMD Millipore)
Gradient Master (BioComp Instruments)
Piston Gradient Fractionator (BioComp Instruments) (optional; see Step 10)
Polyclear centrifuge tubes (13 × 51-mm) (Seton Scientific)

When purchased from BioComp Instruments, Inc. (cat. no. 151-513), the tubes are tested for tolerance and compatibility with piston fractionation.

Refractometer (Bausch & Lomb)
SW 55 Ti Rotor (Beckman Coulter)
Ultracentrifuge (Beckman Coulter)

METHOD

Gradient Preparation

Prepare two gradients by following the instructions in the BioComp Gradient Master operator's manual, which are briefly summarized here. The small gradient volumes (~5 mL each) help to maintain the concentration of the purified complex within a reasonable range, as usually the amount of purified native protein assemblies is very limited.

1. Prepare 10 mL each of 5% and 20% (w/w) sucrose solutions in a buffer with the same composition as the one used during the native elution. See Griffith (1986, p. 49) for a chart indicating the appropriate dilutions of a 66% (w/w) sucrose solution to prepare a 5% solution (1/20 dilution) and a 20% solution (1/4.1 dilution). Filter the solutions through a 0.22-µm filter. Verify the final concentration using a refractometer.

   The concentrations of the gradient should be adjusted to the size of the protein complex that needs to be isolated. 5%–20% (w/w) sucrose works well for complexes of 200–800 kDa. For larger complexes, a 10%–40% (w/w) sucrose gradient would be more suitable. If the gradient is prepared using glycerol, the most common gradient used for separation of protein complexes is 10%–30% (v/v).

2. Add protease inhibitor cocktail to each sucrose solution to give a final concentration of 0.001×, and mix carefully by rocking to avoid the formation of air bubbles. Add other kinds of chemical compounds depending on the enzymatic activities that need to be inhibited or preserved.
3. Place two polyclear centrifuge tubes into the SW 50/SW 55 marker block and, using a marker, draw a fine line following the top of the block. Fill each tube with 5% (w/w) sucrose solution until the liquid reaches ~2 mm above the half-full mark. Fill a 10-mL syringe with the 20% (w/w) sucrose solution and attach a blunt-end steel cannula to its tip. Being careful to avoid air bubbles and not to disturb the interface, insert the cannula quickly to the bottom of the tube and slowly layer the 20% solution until the interface formed with the 5% reaches the half-full mark. Quickly withdraw the cannula and cap the tubes using the short rubber caps.
4. Place the tubes in the tube holder and use the Gradient Master to rotate and mix the solutions as outlined in the operator's manual to generate a linear gradient.
5. Take the formed gradients and let them cool down for 45 min to 1 h at 4°C. At this time, ensure that the rotor is also precooled at 4°C.

   The gradients are stable for up to 3 h at 4°C.

Centrifugation

During this series of steps, avoid heating the gradients by working fast or in a cold room.

6. Carefully remove the caps from the gradient tubes, and slowly load between 50 and 200 µL containing 10–100 pmol of natively eluted yeast protein complexes (from Protocol: **Native Elution of Yeast Protein Complexes Obtained by Affinity Capture** [LaCava et al. 2015]) on top of one of the gradients, trying not to disturb it.

   Although these ranges are desirable, lower amounts can be successfully analyzed. We recommend using gel-loading tips for this task.

7. Use the other gradient to balance the rotor; add a volume of water or buffer equal to the volume of sample so as to keep them balanced.

   Alternatively, to analyze several samples, distribute the samples across multiple gradients.

8. Balance the tubes before loading them into the swing-out rotor. Check that the gradients are of comparable weight using a balance, keeping them within ±100 mg of one another by adding solution as needed.

9. Centrifuge at 59,000–237,000*g* in an SW 55 Ti rotor and Beckman L-80 ultracentrifuge for 6–20 h at 4°C.

   These centrifugation conditions are commonly used, but the time and speed of centrifugation must be determined empirically for each protein assembly because their sedimentation rate depends not only on their size, but also on their shape. Ideally, the complex should move about two-thirds of the way down the gradient to obtain good separation.

10. After centrifugation, fractionate the gradients at 4°C by one of the following methods.
    - Use a BioComp Piston Gradient Fractionator to collect fractions in 2-mm increments (i.e., ~20 fractions of ~225 µL each).
    - Manually fractionate by unloading from the top using a pipette or from the bottom of the tube using a capillary and a peristaltic pump. Collect 12 fractions of 410 µL each or 24 fractions of 205 µL each, depending on how precisely the peak of the complex needs to be determined.

      For assays requiring native complexes (e.g., assays of enzymatic activity or protein complex structure), analyze the fractions immediately. If this is not possible, store for short periods (no more than a few hours) at 4°C, or flash-freeze in liquid nitrogen and store for days to months at –80°C. For assays that do not require native complexes (e.g., sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] analysis), store the fractions at –20°C until analysis.

Analysis of Fractions

11. Analyze an aliquot of each fraction by SDS-PAGE protein staining (e.g., Coomassie blue, silver, or SYPRO Ruby) to visualize the components and localize the complex of interest.

    In the event that the final sample concentration in the fractions is insufficient for visualization upon direct loading of an aliquot, the fractions can be effectively precipitated with methanol/chloroform (Wessel and Flügge 1984).

    Data from an experiment in which a native protein complex was purified by affinity capture, released by PreScission protease cleavage, and enriched by density gradient ultracentrifugation is presented in Figure 1.

12. Characterize the purified native complexes as desired.

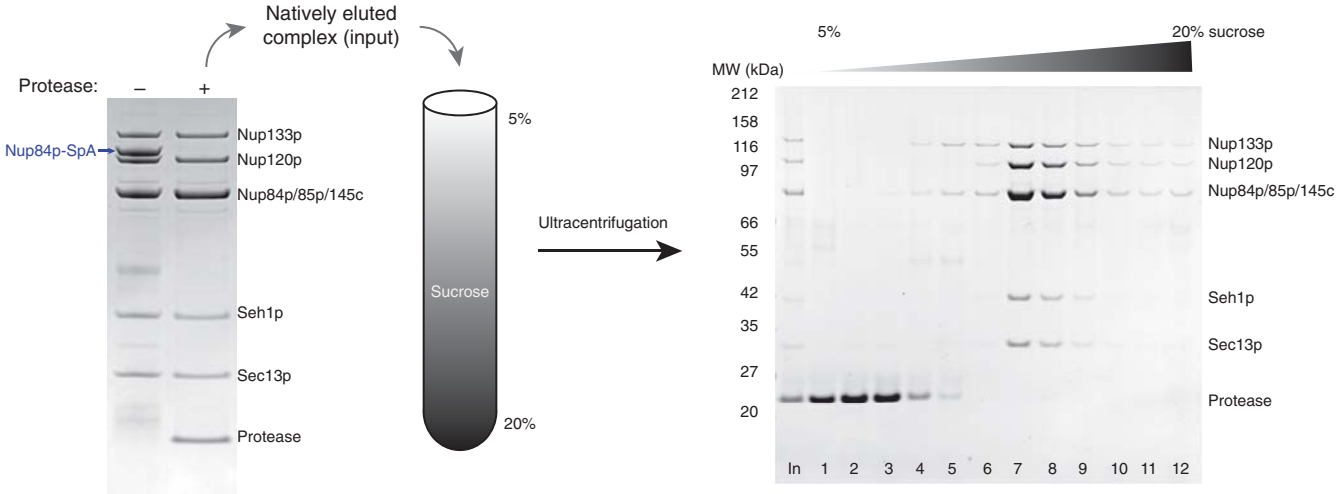

FIGURE 1. The endogenous heptameric Nup84-complex purified by affinity capture, released by PreScission protease cleavage, and enriched by fractionation across a 5%–20% (w/w) sucrose density gradient.

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Mass spectrometry is the ideal method to identify the proteins present in each fraction. Depending on the downstream application, the sucrose or glycerol used in the gradient may need to be removed by dialysis, gel filtration, or ultrafiltration. For examples of downstream structural and functional characterization using protein complexes purified with this procedure, see Erickson (2009) and Luhrmann and Stark (2009).

RELATED INFORMATION

For further information regarding the density gradient ultracentrifugation procedure, consult Griffith (1986) and Erickson (2009).

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