Protocol

Native Elution of Yeast Protein Complexes Obtained by Affinity Capture

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This protocol describes two options for the native (nondenaturing) elution of protein complexes obtained by affinity capture. The first approach involves the elution of complexes purified through a tag that includes a human rhinovirus 3C protease (PreScission protease) cleavage site sequence between the protein of interest and the tag. Incubation with the protease cleaves immobilized complexes from the affinity medium. The second approach involves the release of protein A–tagged protein complexes using a competitive elution reagent called PEGylOx. The degree of purity of the native assemblies eluted is sample dependent and strongly influenced by the affinity capture. It should be noted that the efficiency of native elution is commonly lower than that of elution by a denaturing agent (e.g., SDS) and the release of the complex will be limited by the activity of the protease or the inhibition constant (K_i) of the competitive release agent. However, an advantage of native release is that some nonspecifically bound materials tend to stay adsorbed to the affinity medium, providing an eluted fraction of higher purity. Finally, keep in mind that the presence of the protease or elution peptide could potentially affect downstream applications; thus, their removal should be considered.

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

Desalting spin columns (40 kDa molecular mass cutoff) (optional; see Step 14)

Depending on the volume of the eluted fraction, use either Micro Bio-Spin Columns with Bio-Gel P-30 (Bio-Rad 732-6223) or Zeba Micro Spin Desalting Columns, 75 µL, with 40 kDa molecular mass cutoff (Thermo Scientific 87764). These columns give equivalent results, depleting the peptide >100-fold.

Digestion buffer (for Steps 1–10 only)

Determine the optimal composition of this buffer for each protein complex of interest. A suggested formulation to work from is 20 mM K-HEPES (pH 7.4), 150 mM sodium chloride, 110 mM potassium acetate, 2 mM magnesium chloride, 0.1% Tween 20, and 1 mM DTT.

PEGylOx native elution solution (saturated solution; >2 mM) (for Steps 11–15 only)

PEGylOx is an amino-terminally PEGylated peptide of primary sequence DCAWHLGELVWCT, cyclized by oxidation of the cysteines to cystine. It can be synthesized by standard Fmoc solid-phase synthesis methods. The PEGylOx solution can be prepared using a solvent of your choosing. As long as the saturation concentration of

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peptide is ~2 mM (which will be solvent dependent), effective elution can be expected. We recommend using a 40 mM Tris–Cl buffered elution solution at pH 8.0 and ethanol at 5% (v/v) in any elution solution. Na-HEPES at pH 7.4 can be substituted for Tris-Cl. As a detergent, we use Tween 20 at 0.01% (v/v); a detergent is required for stable high concentration solutions of PEGylOx. The concentration of the peptide solution is monitored by UV spectrophotometry at 280 nm using an estimated molar extinction coefficient (ε280) of 11,125 M^{-1} cm^{-1}.

PreScission protease (human rhinovirus 3C protease) (GE Healthcare Life Sciences) (for Steps 1–10 only)

PreScission protease inhibitors (optional; see Step 9)

Yeast protein complexes attached to antibody-conjugated Dynabeads as prepared in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015)

Equipment

Low protein-binding tubes (e.g., Sorenson Biociences 39640T) (optional; see Step 14)

Microcentrifuge (benchtop; refrigerated)

Microcentrifuge tubes (1.5–2 mL)

Neodymium magnet (e.g., DynaMag-2 from Life Technologies)

Sample mixing block

Vortex mixer

METHOD

Select either Steps 1–10 or Steps 11–15.

Native Elution of Protein Complexes Using a Cleavable Tag

This protocol is suitable for eluting complexes purified through a tag that includes a human rhinovirus 3C protease (PreScission protease) cleavage site sequence (GLEVLFQGPS) between the protein of interest and the tag (Cordingley et al. 1989, 1990; Walker et al. 1994). This approach has also been successful using a cleavage site for tobacco etch virus protease (Dougherty et al. 1989; Polayes et al. 1994).

1. Prepare 1.25 µL of digestion mixture (PreScission protease in digestion buffer at a concentration of 0.08 units/µL) per 5 µL of Dynabeads slurry used in the affinity capture. This solution will be added directly to the beads containing immobilized complexes obtained at the end of Step 11 in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015). Keep the digestion mixture on ice.

   We suggest preparing a small excess of digestion mixture to compensate for pipetting errors. If the protease activity (units) is not known, try 0.08 µg/µL.

2. If the digestion buffer is different from the wash buffer used in Step 11 in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015), wash the slurry of protein complexes attached to beads once with 10 slurry volumes of cold digestion buffer.

3. Add digestion mixture from Step 1 to the beads. Use 1 µL per 5 µL of Dynabeads slurry used in the affinity capture. Tap the tube until the beads are fully resuspended. Do not vortex.

4. Incubate with gentle agitation for 30–45 min at 4°C.

5. Centrifuge the sample at 3500*g* for 1 min at 4°C to pellet the beads. Place the tube in the magnet, and transfer the supernatant to a clean tube on ice.

6. Add digestion buffer (no enzyme) to the beads. Use 1 µL per 5 µL of Dynabeads slurry used in the affinity capture. Tap the tube until the beads are fully resuspended. Do not vortex.

7. Centrifuge the sample at 3500*g* for 1 min at 4°C to pellet the beads. Place the tube in the magnet, and transfer the supernatant to the same tube that contains supernatant from Step 5.

8. Centrifuge the combined eluted fraction at 20,000*g* for 10 min at 4°C to pellet residual magnetic beads and particulates.
9. Transfer the supernatant to a clean tube—this is the natively eluted material.

   The sample should be used as soon as possible to avoid disintegration of the purified complex, but depending on the stability of the complex the sample could be held on ice or at 4°C for up to several hours if further processing by, for example, density gradient centrifugation will be carried out. The sample may be stored at −20°C or lower for longer term, but freeeze–thaw cycles may adversely affect native structures. Consider the addition of a cryoprotectant such as glycerol or ethylene glycol to the sample before storage at −20°C if, for example, enzymatic assays will be conducted on the sample. If the material is not going to be used immediately in subsequent procedures, consider adding PreScission protease inhibitors to the sample. The manufacturer suggests 4 mM Pefabloc or 100 μM chymostatin.

   To prepare a more homogeneous solution of protein complexes (i.e., one without fragments caused by degradation during the purification procedure), subject the eluate to rate-zonal density gradient ultracentrifugation as described in Protocol: ***Density Gradient Ultracentrifugation to Isolate Endogenous Protein Complexes after Affinity Capture*** *(Fernandez-Martinez et al. 2015).*

10. Analyze a fraction of this sample by SDS–PAGE as in Step 12 in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015).

    To determine what material remains on the beads after protease cleavage, wash the Dynabeads with SDS–PAGE sample loading buffer.

    See Troubleshooting.

Native Elution of Protein A–Tagged Complexes Using the Competitive Elution Reagent PEGylOx

This procedure uses a synthetic peptide, PEGylOx, whose production, handling and usage is described in LaCava et al. (2013). The peptide competitively releases protein A–tagged complexes from interaction with the IgG constant (Fc) region, and it can be removed from the eluted fraction by spin column gel filtration.

11. Wash protein complexes immobilized on Dynabeads (from the last wash in Step 11 in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** [LaCava et al. 2015]) with 50 μL of the solution used to dissolve PEGylOx, and then remove the wash solution. Fifty microliters of solution is sufficient for up to 25 μL of Dynabeads slurry used in the affinity capture. Scale up the wash if necessary.

    This step is to equilibrate the beads with the elution solution, while keeping the beads and complexes highly concentrated. It can be omitted if the elution solution and the extraction solvent are the same.

12. Add 26 μL of PEGylOx native elution solution to up to 25 μL of the Dynabeads slurry; scale up as needed. Incubate with gentle shaking (e.g., setting three on a vortex mixer) for 15 min at room temperature.
13. Apply a brief pulse of low-speed centrifugation to ensure all of the slurry is at the bottom of the tube. Place the tube in the magnet, and transfer the supernatant to a clean tube. To ensure no beads have carried over, place the sample on the magnet again, and transfer the supernatant to a fresh tube—this is the natively eluted material.

    The samples should be used as soon as possible to avoid disintegration of the purified complex, but depending on the stability of the complex the sample could be held on ice or 4°C for up to several hours if further processing by, for example, PEGylOx removal and/or density gradient centrifugation will be carried out. The sample may be stored at −20°C or lower for longer term, but freeze–thaw cycles may adversely affect native structures. Consider the addition of a cryoprotectant such as glycerol or ethylene glycol to the sample prior to storage at −20°C if, for example, enzymatic assays will be conducted on the sample. Bear in mind that PEGylox solubility at low temperatures may vary with the composition of the elution solution used—the PEGylOx solution compositions suggested in Materials are stable at the above storage temperatures.

14. (Optional) To deplete the peptide >100-fold, apply the natively eluted samples to gel filtration spin columns with a nominal molecular mass cutoff of 40 kDa. Pre-equilibrate these columns with the desired buffer solution for exchange following the manufacturer's instructions. If necessary, use this step as an opportunity to exchange the solution the sample is in for downstream assays. Consider using low protein-binding tubes to collect the flowthrough.

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15. Analyze a fraction of the eluate by SDS–PAGE as in Step 12 in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015). To determine what material remains on the beads after PEGylOx treatment, wash the Dynabeads in the pellet with SDS–PAGE sample loading buffer.

TROUBLESHOOTING

Problem (Step 10): Protein complexes are only weakly detected or absent.

Solution: The composition of the digestion buffer may not be optimal. The composition of the digestion buffer is complex dependent and will have to be formulated for each protein complex of interest. Follow these guidelines:

- Do not include protease inhibitors in the buffer.
- To ensure optimal efficiency of the PreScission protease, add DTT at a final concentration of 1 mM.
- As a general rule, prepare the digestion buffer so that its composition is similar to the buffer used for affinity capture (i.e., the extraction solvent used in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** [LaCava et al. 2015]). Ensure that the ionic strength is enough to elute the complex from the magnetic matrix without causing dissociation of the protein components. Add a low concentration of detergent (e.g., Tween 20) to help release the complex and avoid nonspecific adsorption to the affinity medium.

RELATED INFORMATION

A procedure for eluting affinity-captured protein complexes in a denaturing solvent (SDS–PAGE sample buffer) is included in Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015).

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