Purification / Native Elution

PROTOCOL FOR:

Improved Native Isolation of Endogenous Protein A-Tagged Protein Complexes

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Legend

¡ATTENTION!
*HINT
REST

Background

This method utilizes cell powder produced by cryogenic grinding of cells (grindate) and rabbit IgG conjugated magnetic beads. A full description of procedures for preparing cell grindate and IgG conjugated Dynabeads can be found here: www.ncdir.org/public-resources/protocols/ (see sections Disruption of Yeast or Bacteria Cells and Isolation of Protein Complexes).

Reagents

- **0.5 M acetic acid at pH 3.4** (equilibrated with ammonium acetate)
- **500mM dithiothreitol (DTT)**
- **Desalting spin columns, 40 kDa MWCO** (see Peptide Removal section)
- **Extraction buffer**
- **Liquid nitrogen (N2)**
- **4x lithium dodecyl sulfate gel loading buffer** (Invitrogen, cat. #NP0007)
- **PEGylOx solution, saturated (> 2 mM) in e.g. 40 mM Tris pH 8.0, 100 mM**
- **NaCl, 0.01% Tween 20, 5% v/v EtOH** (see Native Elution section)
- **Rabbit IgG conjugated Dynabeads**

Procedure

Cell extract preparation

Optimized extraction conditions need to be determined on a case-by-case basis for each protein complex under investigation. When doing an affinity capture using SpA/IgG interaction for the first time we generally start with a 'standard' extraction buffer consisting of: 40 mM TRIS pH 8.0, 50 mM trisodium citrate, 300 mM NaCl, 0.1% v/v Tween 20. The SpA/IgG interaction is promoted by alkaline pH and the presence of e.g. sodium citrate or sulfate. After analyzing the results, further variations on conditions are often explored. All extraction buffers are also supplemented with protease inhibitor cocktails as a general rule.

¡ATTENTION! *Tubes containing the cell grindate stocks should always be held on N_2 or dry ice when not at -80°C – such as when dispensing powder for individual experiments.*

1. Weigh out up to 0.5 g of cell powder into a pre-cooled tube.

1.1. Tare analytical balance with the empty microfuge tube
1.2. Dispense grindate to an N_2 cooled tube using an N_2 cooled spoon or spatula
1.3. Check the mass of the grindate dispensed within tube on the analytical balance
1.4. Hold tubes containing grindate on N_2

*HINT: *To ease the weighing out of cell powders, small volumetric measuring spoons may be used. These have been found to give reproducible results (see equipment). In any case, weights of grindate dispensed to tubes are quickly checked on an analytical balance, as above. We have found excellent yields at readily Coomassie stainable levels (ca. 10s ~ 100s ng / band) of even low abundance proteins using a maximum of 0.5 g of cells; typically 0.25 g are sufficient for a Coomassie stain, and this amount of material is easily handled in standard volume microfuge tubes. For ease, larger scale purifications can be split across multiple tubes and pooled at the point of affinity isolation.*

¡ATTENTION! *We have found best results using screw-cap or "safe-lock" tubes. Residual pressure due to evaporating N_2, held within the cell grindate, can cause standard microfuge tubes to pop open during subsequent warming just prior to the addition of extraction buffer – potentially resulting in loss of the sample.*

2. Open the tube (or loosen screw-cap) with grindate and let stand at room temperature (RT) for 1 min. This will release pressure within the tube and prevent the immediate freezing of the extraction buffer when added to the grindate. No thawing is observed during this 1 min incubation.

3. Add 4 volumes of RT extraction buffer to cell grindate (i.e. 1:4 w:v, 1 ml to 0.25 g) and vortex briefly until powder is visibly dispersed homogenously (typically occurs very rapidly, within ~10 sec), and then hold on ice while proceeding to step 4.

*HINT: *The use of RT buffer encourages the rapid homogenous resuspension of the grindate. The fact that the grindate was at cryogenic temperature prior to buffer addition results in a cold mixture that is then handled cold for subsequent manipulations. The use of cold buffers encourages freezing of the mixture and can result in a heterogeneous frozen slurry, requiring extended mixing and thawing time. Hereafter e.g. washing buffer solutions are used cold or at RT only when complexes are known to be stable.*

4. Clarify the extract by centrifugation (e.g. 14k RPM, 7 min, 4°C, using Eppendorf 5417R centrifuge).

5. Remove the supernatant (clarified extract) and proceed to the affinity isolation by adding to pre-equilibrated Dynabeads (see below).

REST: *Clarified extract may be briefly held on ice prior to affinity isolation, as needed, but in this case it should be transferred away from the pellet to a fresh microfuge tube during the pause. Proceeding directly to affinity isolation is recommended.*

Affinity Isolation

Dynabeads (DBs) are manipulated using neodymium magnets in a specialized

microfuge tube holder (see equipment). When placed within the holder, beads are collected at the side of the tube under influence from the magnetic field. Solutions may then be removed without disturbing the beads.

6. Prior to adding the clarified extract to the tube containing DBs, they should be pre-washed. A 25 µl Dynabeads slurry, prepared as described in the background materials, is well calibrated for purifying protein complexes from 0.5 g grindate – scale-up according to need. Pre-wash the DBs slurry twice with 10 slurry volumes of 0.5 M acetic pH 3.4 (RT); then three times w/ 1 ml of extraction buffer (RT). Resuspend the beads by brief vortexing during each wash. Remove the entire wash buffer each time, and hold the beads on ice after removal of the last wash.

*HINT: *Dynabeads can be pre-washed while cell extracts are being centrifuged (step 4).*

7. Transfer the clarified cell extract to tube with beads and vortex briefly.

8. Incubate for up to 1 h at 4°C with constant mixing (rotator).

*HINT: *Incubation times of 30 min are typically sufficient to recover a large majority of your complex from the extract. We have observed increasing yield over periods of up to 2 hours, but the accumulation of nonspecific background tends to accelerate, relative to accumulation of the protein of interest, after roughly 30 min – 1 hr. 1 hr batch binding is a good starting point, but in the interest of time and purity this period can be reduced on a case by case basis.*

9. Aspirate the supernatant and wash the beads twice w/ 1 ml of extraction buffer – resuspend DBs by brief vortexing each time. On the second wash, transfer the beads to a fresh tube.

10. Aspirate the supernatant, and wash once more with 1 ml of extraction buffer, removing the final wash.

*HINT: *After mixing / vortexing, it is helpful to briefly pulse spin the tubes in a mini microcentrifuge to collect all contents at the bottom of the tube (beads will remains essentially suspended), prior to placement on the magnet and aspiration of the solution. Doing so ensures the minimum carryover of solutions between washes. We have found that transferring to a fresh tube prior to final washing and elution reduces non-specific background in purifications (although this is markedly more important when denaturing conditions are used for elution). At 1 g and higher purification scale the initial wash should be scaled up by 5-fold or more.*

Native Elution

The PEGylOx elution solution can be prepared in a buffer of your choosing. As long as the saturation concentration of peptide is above 2 mM, effective elution on a 15 min time-scale can be expected. Lot-to-lot variation in the concentration of a saturated solution has been observed (typically within the range of ~2.2 – 2.7 mM) and will also be buffer dependent. We recommend using a 40 mM Tris-buffered elution solution at pH 8.0 and ethanol at 5% v/v in any elution solution; these reagents maximize solubility of the peptide and stability of the resulting solution. Excellent results have also been achieved using HEPES at pH 7.4. The lyophilized peptide is acidic; ensure appropriate buffering strength. As a detergent we use Tween 20 at 0.01% v/v because at this concentration Tween 20 is readily depleted from the solution by spin column desalting to non-interfering levels for downstream in-solution proteolytic digestion and LC-MS/MS analyses. Higher levels of Tween 20 are otherwise perfectly acceptable and comparable performance has also been achieved with Triton X-100. In the conditions we have tested thus far, a detergent is required for stable high concentration solutions of PEGylOx. We have observed that the detergent CHAPS does not maximize peptide solubility, however.

11. Prior to applying the PEGylOx solution, wash the beads with 50 µl of the solution used to dissolve the peptide – this equilibrates the beads for the subsequent elution – remove the wash.

12. Native elution using PEGylOx is achieved by applying a saturated solution to the DBs and incubating 15 min at RT with gentle shaking (e.g. setting 3 on a vortex mixer).

*HINT: *26 µl of elution solution per 25 µl of DBs slurry used works very well, leaving negligible residual eluted protein on the beads (i.e. a subsequent wash with buffer only did not recover more protein; remaining protein was only significantly released by denaturation with LDS). Moreover, by adding 10 µl 4x LDS gel loading buffer and 4 µl 500 mM DTT, these samples can be applied to a 10-well, 1 mm thickness SDS-PAGE gel for direct analysis of the elution (the peptide does not interfere with electrophoresis at this abundance and migrates essentially at the front on standard protein gels). Smaller volumes, as little as 5 µl elution solution per 25 µl DBs used, elute proteins well enough for direct visualization by SDS-PAGE and provide for highly concentrated elution solutions at the sacrifice of total yield (at 5 µl elution volume the amount of solution retained by the beads is significant).*

13. Remove the supernatant – this is the natively eluted material – hold or otherwise store appropriately. You may subsequently wash the DBs with 1x LDS, incubating for 5-10 min at RT before recovering the supernatant, if you wish to determine what material remains on the beads after PEGylOx treatment.

¡ATTENTION! *Reducing agent should not be included during LDS elution from IgG conjugated DBs or excessive amounts of Ig chains will be co-eluted. This is particularly pronounced if the beads are also incubated at elevated temperature.*

REST: *Samples may be held at an appropriate temperature prior to subsequent manipulations depending on the time-scale – typically 4°C for hours, at -20°C for up to a few days or -80°C for extended storage until analysis is desired.*

*HINT: **Keep in mind, protein complexes do not always respond well to freeze-thaw cycles and may lose some of their native properties – the use of glycerol or other cryoprotectants may be advisable. We tend to initiate assays dependent on native properties within hours after elution, or to aliquot and snap freeze our samples in N_2, with subsequent long term storage at -80°C.**

Peptide Removal

It may be desirable to remove the peptide prior to downstream assays or storage. High concentrations of the peptide could potentially saturate reversed phase resins used for peptide sample cleanup or liquid chromatography (e.g. prior to LC-MS). Removal may also facilitate the re-use of the SpA-tag to affinity isolate the protein complex after e.g. reconstitution with other proteins.

14. Depending on the volume of the eluted fraction, samples may be applied to either Bio-Rad Micro Bio-Spin Columns with Bio-Gel P-30 (#732-6223) or Zeba Micro Spin Desalting Columns, 40K MWCO, 75µL (#87764) with equiv-

alent results. These columns deplete the peptide to better than 100-fold (outside the dynamic range of our assay) as determined by UV280 spectrophotometry and colloidal Coomassie staining. These columns should be pre-equilibrated to the desired buffer solution for exchange following the manufacturers instructions.

Equipment

- **1.5 – 2 ml microfuge tubes** (preferably screw-cap)
- **Bench-top refrigerated microcentrifuge**
- **Neodymium magnet** – e.g. Life Technologies DynaMag™-2 (#123-21D)
- **Sample rotator**
- **UV spectrophotometer**
- **Vortex mixer**
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Optional

- **Volumetric spoons –** our favorite: Norpro 3080 Mini Measuring Spoons, 5 Piece Set available though www.amazon.com

***HINT: We have found that one heaping scoop using 'smidgen' gives ~50 mg and one level scoop with 'dash' gives ~250 mg – an initial feel for the 'size' of the scoop must be made by the user.**