Protocol

Improved Tandem Affinity Purification Tag and Methods for Isolation of Proteins and Protein Complexes from *Schizosaccharomyces pombe*

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The tandem affinity purification (TAP) method uses an epitope that contains two different affinity purification tags separated by a site-specific protease site to isolate a protein rapidly and easily. Proteins purified via the TAP tag are eluted under mild conditions, allowing them to be used for structural and biochemical analyses. The original TAP tag contains a calmodulin-binding peptide and the IgG-binding domain from protein A separated by a tobacco etch virus (TEV) protease cleavage site. After capturing the Protein A epitope on an IgG resin, bound proteins are released by incubation with the TEV protease and then isolated on a calmodulin matrix in the presence of calcium; elution from this resin is achieved by chelating calcium with EGTA. However, because the robustness of the calmodulin-binding step in this procedure is highly variable, we replaced the calmodulin-binding peptide with three copies of the FLAG epitope, (3× FLAG)–TEV–Protein A, which can be isolated using an anti-FLAG resin. Elution from this matrix is achieved in the presence of an excess of a 3× FLAG peptide. In addition to allowing proteins to be released under mild conditions, elution by the 3× FLAG peptide adds an extra layer of specificity to the TAP procedure, because it liberates only FLAG-tagged proteins.

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.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

3× FLAG peptide (A6001, APExBIO)

This is a synthetic peptide with the amino acid composition MDYKDHDGDYKDHDIDYKDDDDK.

Anti-FLAG M2 affinity gel (A2220, Sigma-Aldrich)

Dithiothreitol (DTT)

IgG sepharose 6 Fast Flow (17-0969-01, GE Healthcare Life Sciences)

IgG wash buffer <R>

Modified TAP (tandem affinity purification) lysis buffer <R>

Omit protease inhibitors when preequilibrating the IgG Sepharose resin (see Step 15).

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From the Fission Yeast collection, edited by Iain M. Hagan, Antony M. Carr, Agnes Grallert, and Paul Nurse.

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Modified tobacco etch virus (TEV) cleavage buffer <R>
Protein assay reagent (e.g., Bradford, BCA, Lowry)
TEV protease (TurboTEV; 1500020012, Eton Bioscience)
Yeast extract with supplements (YES) (4×)

Prepare the standard YES recipe <R> using a fourfold increase of all components.

Yeast strains, untagged (control) and tagged (experimental)

(3× FLAG)–TEV–Protein A cassettes for carboxy-terminal tagging of genes in the pFA6a vectors containing the hphMX (hygromycin resistance) or the natMX (clonNAT resistance) markers have been constructed and are available from Addgene (http://www.addgene.org/browse/pi/1385/). These cassettes can be used to generate strains of interest, as described by Bähler et al. (1998).

Yeast suspension buffer <R>

Equipment

Automatic pipettor and pipettes
Beakers (250-mL)
Centrifuge (floor)
Centrifuge bottles (0.5-L)
Centrifuge rotors (50–100 mL tube capacity)
Centrifuge tubes (disposable, 50-mL)
Culture flasks (2-L)
Light microscope
Liquid nitrogen
Microcentrifuge
Microcentrifuge tubes (disposable, 1.5-mL)
Mortar Grinder RM 200 (Retsch)

Grinding can also be performed with a manual mortar and pestle.

Polypropylene column (5-mL) (29922, Pierce)
Retort stand and clamps
Rotating wheel
Strainer

METHOD

Detergents interfere with mass spectrometry. If this method is to be used to produce material for analysis by mass spectrometry, detergents should be omitted.

Growth, Harvesting, and Storage of Cells

1. Grow 2 L each of the relevant yeast strains (e.g., untagged control and tagged experimental) in 4× YES to an OD_{600} of ~8.

   Although 2 L of culture grown to an OD600 of ~8 is probably sufficient in most cases, the exact amount of cells to use will mostly depend on the abundance of the TAP-tagged protein of interest.

2. Transfer cultures to centrifuge bottles. Chill on ice for 15 min.
3. Harvest cells by centrifugation at 500*g* for 5 min at 4°C.
4. Wash cells once with 0.5 L of ice-cold water.
5. Resuspend cells with one-quarter cell-pellet volumes of ice-cold yeast suspension buffer.
6. Using a 5-mL pipette, slowly drip cell suspension in liquid nitrogen to make "*pombe* popcorn."
7. Scoop "popcorn" from the liquid nitrogen using a strainer. Transfer to vented 50-mL tubes. Store at −80°C.

Cell Lysis

8. Chill the grinder by filling the mortar to the brim with liquid nitrogen. Allow the nitrogen to evaporate completely. Repeat twice more.
9. Add "popcorn" to mortar. Lower pestle just enough to pulverize it (~10 min).
   At this stage, mortar can be kept full of liquid nitrogen.
10. Gradually increase pressure to maximum. Grind until >75% of cells have been broken (~1 h), as estimated by light microscopy.
    At this stage, add just enough liquid nitrogen to keep the cell powder in a state resembling cookie dough.

Whole Cell Lysate Preparation

One hundred microliters of lysate at 10–15 mg of protein/mL can be prepared routinely using the procedure described here.

11. Place the ground "*pombe* popcorn" in a beaker. Add 80–90 mL of ice-cold modified TAP lysis buffer. Stir gently at room temperature until the powder has thawed.
12. Transfer lysates (~100 mL) to suitable tubes. Centrifuge at 20,000*g* for 1 h at 4°C.
    Alternatively, if using an ultracentrifuge, centrifuge at 100,000g for 1 h.
13. Determine the protein concentration of the control and experimental lysates using a protein assay of choice (e.g., Bradford, BCA, Lowry).
14. Equalize protein concentration of the lysates with modified TAP lysis buffer.

Protein A Capture

15. For each sample, preequilibrate 0.5 mL of the IgG Sepharose resin by washing three times with 10 mL of cold modified TAP lysis buffer without added protease inhibitors.
16. Incubate lysates with IgG Sepharose resin overnight at 4°C with gentle rotation.
17. Transfer IgG resin to a 5-mL polypropylene column.
18. Wash by gravity flow at 4°C.
    i. Wash three times with 2 mL of IgG wash buffer.
    ii. Wash once with 2 mL of 50% IgG wash buffer/50% modified TEV cleavage buffer.
    iii. Wash once with 2 mL of modified TEV cleavage buffer.

Cleavage by TEV Protease

Cleavage by TEV protease is probably the most variable step in the TAP method. The exact amount of protease to use and the digestion time will need to be determined empirically.

19. Resuspend the IgG Sepharose resin with 1.5 mL of TEV cleavage buffer.
20. Add DTT to a final concentration of 0.75 mM. Add 500 U of TEV protease.
21. Incubate for 4 h at room temperature with gentle rotation.
22. Collect the column eluate by gravity flow.
23. Wash the IgG resin by gravity flow at room temperature three times with 0.5 mL of modified TEV cleavage buffer. Pool these washes with the column eluate from Step 22.

FLAG Tag Capture

24. For each sample, preequilibrate 0.25 mL of anti-FLAG resin by washing three times with 10 mL of cold modified TEV cleavage buffer.
25. Combine the IgG resin eluate and washes (from Step 23) with the preequilibrated anti-FLAG resin in a 5-mL polypropylene column. Incubate overnight at 4°C with gentle rotation.

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26. Wash the anti-FLAG resin by gravity flow at room temperature three times with 1 mL of modified TEV cleavage buffer.
27. Incubate the anti-FLAG resin with 0.25 mL of modified TEV cleavage buffer supplemented with 0.5 µg/mL 3× FLAG peptide for 20 min with gentle rotation.
28. Collect the column eluate by gravity flow.
29. Repeat Steps 27 and 28 four more times. Pool all five eluates.

Eluted proteins can be used as a native preparation or they can be precipitated by incubating on ice for 1 h with TCA (25% final concentration).

RECIPES

IgG Wash Buffer

1 mM EDTA
10% (v/v) glycerol
20 mM HEPES (pH 7.5)
150 mM NaCl
0.1% (v/v) NP-40

Prepare fresh before use (recommended).

Modified TAP Lysis Buffer

1 mM EDTA
10% (v/v) glycerol
20 mM HEPES, pH 7.5
150 mM NaCl
0.8% (v/v) NP-40
1 mM phenylmethylsulfonyl fluoride
1× Complete Protease Inhibitor Cocktail, EDTA-free (11873580001, Roche)

Prepare fresh before use (recommended).

Modified TEV Cleavage Buffer

1 mM EDTA
20 mM HEPES, pH 7.5
150 mM NaCl
0.1% (v/v) NP-40

Prepare fresh before use (recommended).

Yeast Extract with Supplements (YES)

Reagent	Amount to add (1 L)
Glucose	30 g
Yeast extract	5 g
Adenine	0.2 g
Uracil	0.2 g
Histidine	0.2 g
Leucine	0.2 g
Lysine	0.2 g
Agar (for solid medium only)	20 g
H_2O	to 1 L

Prepare 1 L of YES by combining the reagents listed above. Sterilize by autoclaving at 10 psi for 15 min. Store at 4°C. (Note that the yeast contains sufficient thiamine to repress *nmt1* promoters.)

Yeast Suspension Buffer

20 mM HEPES, pH 7.6
50 mM NaCl
Prepare fresh before use (recommended).

REFERENCES

Bähler J, Wu J, Longtine MS, Shah NG, McKenzie A III, Steever AB, Wach A, Philippsen P, Pringle JR. 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14:** 943–951.

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