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Improved tandem affinity purification tag and methods for isolation of proteins and protein complexes from *S. pombe*

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Abstract

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 TEV protease cleavage site. After capturing the Protein A epitope on an IgG resin, bound proteins are released by incubation with 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 (Rigaut *et al.*, 1999){Rigaut, 1999 #1}{Rigaut, 1999 #1}{Rigaut, 1999 #1}{Rigaut, 1999 #1}. 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. We constructed (3×FLAG)-TEV-Protein A cassettes for C-terminal tagging of genes in the pFA6a vectors containing the *hphMX* (hygromycin resistance) or the *natMX* (clonNAT resistance) markers, which are available from Addgene (<http://www.addgene.org/browse/pi/1385/>). These cassettes are used to generate strains of interest as described (Bahler *et al.*, 1998) (Chapter 4).

Reagents

4× YES – four fold increase of all components of YES (Chapter 4)

Protein assay reagent (Bradford, BCA, Lowry, *etc.*)

IgG Sepharose 6 Fast Flow (17-0969-01, GE Healthcare)

Dithiothreitol (DTT)

TEV protease (1500020012, Eton Bioscience)

Anti-FLAG[®] resin (A2220, Sigma-Aldrich)

3×FLAG[®] peptide (MDYKDHDGDYKDHDIDYKDDDDK – A6001, APExBIO)

Resuspension buffer – 20 mM HEPES pH 7.6, 50 mM NaCl

Lysis buffer – 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.8% *v/v* NP-40, 10% *v/v* glycerol, 1 mM PMSF, 1× Protease inhibitor cocktail (11873580001, Roche)

IgG wash buffer – 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% *v/v* NP-40, 10% *v/v* glycerol

TEV cleavage buffer – 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% *v/v* NP-40, 1 mM EDTA

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

Materials

Culture flasks (2 L)

0.5 mL centrifuge bottles, 50-100 mL centrifuge tubes and compatible rotors

Floor and tabletop centrifuges

Automatic pipettor and pipettes

Liquid nitrogen

Strainer to collect cell popcorn

1.5 mL and 50 mL disposable tubes

Retsch automated pestle and mortar grinder (can be done with manual pestle and mortar).

Conventional light microscope to check cell disruption

250 mL beakers

Rotating wheel

5-mL polypropylene column (29922, Pierce)

Retort stand and clamps

Protocol

1. Growing, harvesting and storing cells
 1. Grow 2 L of relevant strains (e.g. untagged control and tagged experimental) in 4× YES to an OD₆₀₀ of ~8
 2. Transfer cultures to suitable centrifuge bottles and 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 ¼ cell pellet volumes of ice-cold resuspension buffer

6. Using a 5 mL pipette, slowly drip cell suspension in liquid nitrogen to make “cell popcorn”

7. Transfer popcorn to vented 50 mL tubes and store at -80 °C

Note: 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. Cell lysis in a mortar grinder

1. Chill grinder by filling mortar to the brim with liquid nitrogen and letting it completely evaporate, three times

2. Add popcorn to mortar and lower pestle just enough to pulverize it (~10 min). At this stage, mortar can be kept full of liquid nitrogen

3. Gradually increase pressure to maximum and 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 cell powder in a state resembling cookie dough

3. Whole cell lysate preparation

1. Place ground cell popcorn in a beaker, add 80-90 mL of ice-cold lysis buffer and gently stir at room temperature until the powder has thawed

2. Transfer lysates (~ 100 mL) to suitable tubes and centrifuge at 4 °C for 1 h at 20,000 *g*, or if available, in an ultracentrifuge at 100,000 *g*

3. Measure protein concentration of the control and experimental lysates using a protein assay of choice (*e.g.* Bradford, BCA, Lowry, *etc.*)

4. Equalize protein concentration of the lysates with lysis buffer

Note: 100 mL of lysate at 10-15 mg of protein mL⁻¹ can be routinely made using the protocol described here

4. Protein A capture

1. Incubate lysates overnight at 4 °C with 0.5 mL of IgG resin, pre-equilibrated in lysis buffer without protease inhibitors, with gentle rotation

2. Transfer IgG resin to a 5-mL polypropylene column and wash it by gravity flow at 4 °C: three times with 2 mL of IgG wash buffer, once with 2 mL of 50% IgG wash buffer/50% TEV cleavage buffer, and once with 2 mL of TEV cleavage buffer

5. Cleavage by TEV protease

1. Resuspend IgG resin with 1.5 mL of TEV cleavage buffer

2. Add DTT to a final concentration of 0.75 mM and 500 U of TEV protease. Incubate at room temperature for 4 h with gentle rotation
3. Collect column eluate by gravity flow
4. Wash IgG resin by gravity flow at room temperature three times with 0.5 mL of TEV cleavage buffer, and pool these washes with the column eluate from 5.3

Note: 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.

6. FLAG[®] tag capture

1. Combine IgG resin eluate from 5.4 with 0.25 mL of anti-FLAG[®] resin pre-equilibrated in TEV cleavage buffer in a 5-mL polypropylene column. Incubate overnight at 4 °C with gentle rotation
2. Wash anti-FLAG[®] resin by gravity flow at room temperature three times with 1 mL of TEV cleavage buffer
3. Elute anti-FLAG[®] resin by incubating it five times with 0.25 mL of TEV cleavage buffer supplemented with 0.5 µg mL⁻¹ 3×FLAG[®] peptide for 20 min with gentle rotation. Pool these eluates together and use as a native preparation or precipitate protein with TCA (25% final concentration, on ice 1 h)

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References

- Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. A generic protein purification method for protein complex characterization and proteome exploration. *Nature biotechnology*. 1999; 17:1030–1032.
- Bahler J, Wu J, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philleppsen P, Pringle JR. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast*. 1998; 14:943–951. [PubMed: 9717240]