Chapter 8

Tandem Affinity Purification (TAP) of Low-Abundance Protein Complexes in Filamentous Fungi Demonstrated Using *Magnaporthe oryzae*

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Abstract

Protein–protein interactions underlie cellular structure and function. In recent years, a number of methods have been developed for the identification of protein complexes and component proteins involved in the control of various biological pathways. Tandem affinity purification (TAP) coupled with mass spectrometry (MS) is a powerful method enabling the isolation of high-purity native protein complexes under mild conditions by performing two sequential purification steps using two different epitope tags. In this protocol, we describe a TAP-MS methodology for identifying protein-protein interactions present at very low levels in the fungal cell. Using the 6xHis-3xFLAG double tag, we start the affinity purification process for our protein of interest using high-capacity Ni^{2+} columns. This allows for greatly increased sample input compared to antibody-based first-step purification in conventional TAP protocols and provides a large amount of highly concentrated and preliminarily purified protein complexes to be used in a second purification step involving FLAG immunoprecipitation. The second step greatly facilitates the capture of low-level interacting partners under in vivo conditions. Our TAP-MS method has been proven to secure the characterization of low-abundance protein complexes under physiological conditions with high efficiency, specificity, and economy in the filamentous fungus *Magnaporthe oryzae* and might benefit gene function and proteomics studies in plants and other research fields.

Key words Tandem affinity purification, Mass spectrometry, Low-abundance protein complexes, Filamentous fungi, *Magnaporthe oryzae*

1 Introduction

Genes encode proteins that often function in the context of protein–protein interactions (PPIs) [1]. PPIs underlie a broad range of biological processes, including physiological and pathological control [2]. Identification of the constituents of protein complexes is crucial to gain insight into the function of the target gene. One of the most widely used methods for uncovering protein–protein interactions is co-immunoprecipitation (co-IP), in which an antibody, directed against an endogenous protein or an epitope-tagged

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protein, is used to capture the protein complexes associated with the protein of interest [3]. However, although performing co-IP is straightforward, nonspecific binding of the antibodies to unrelated proteins frequently occurs, especially when co-IP is carried out in batch leading to potentially large numbers of false-positive PPIs [4]. To reduce the amount of nonspecific binding, tandem affinity purification (TAP), performed with two successive purification steps based on two different affinity tags fused to the bait protein (*see* **Note 1**), was first developed in yeast [5] and then used in mammalian cells [6–8], bacteria [9–11], and plants [12–15]. The additional purification step in the TAP method increases the selectivity of the desired protein complexes and greatly reduces the contamination from sticky and highly abundant cellular proteins [16].

Conventional TAP method typically purifies more protein complexes of interest than co-IP and other methods. Sometimes, however, it is still unsatisfactory in isolating very low-abundant protein complexes, even following enrichment processes [5, 7]. For example, the target signal is not visible on Coomassie blue dye or silver-stained SDS-PAGE gel. This is especially a problem for the study of very low-abundant PPIs in filamentous fungi, where protein yields under native cell lysis conditions, in our experience, are much lower than that in plant cells. This is likely due to the thick fungal cell wall that is essential for maintaining structural integrity and is of vital importance for protection against environmental stressors, such as osmotic stress or antifungal substances that disrupt fungal cell wall integrity [17, 18]. One way of tackling very low-abundant PPIs is to increase the initial amounts of tissues used in the cell lysis procedure, but this is limited by the high cost of antibody-coupled beads used in the first-step affinity purification of TAP. To obviate this problem, during a recent study on the regulation of the low-abundant fungal sirtuin protein Sir2 in the filamentous fungus *Magnaporthe oryzae* [19], we experimentally optimized the two affinity purification steps and established an effective TAP methodology for identifying Sir2 interacting partners that control protein stability. This was achieved by fusing a double affinity tag 6xHis-3xFLAG to Sir2. Through TAP purification coupled with mass spectrometry (MS) analysis, we successfully identified a set of E3 ubiquitin protein ligases that are weakly expressed in *M. oryzae* but play essential roles in modulating Sir2 protein abundance under normal and oxidative stress conditions. This was demonstrated to be vital for establishing the biotrophic stage of *M. oryzae* during rice infection. Here, we describe in detail the whole procedure for our optimized TAP purification system, with tips for effective cell lysis, protein complex isolation and enrichment, as well as subsequent data analysis using the filamentous fungus *M. oryzae*. This methodology will facilitate researchers in identifying low-abundant PPI complexes in other fungi and might also be relevant to plant and mammal cells.

2 Materials

High-grade chemicals are the preferred choice. General lab equipment such as pipettes, scales, stir sticks, and fume hoods are not mentioned.

2.1 Fungal Hyphae Preparation

1. 500 mL graduated cylinder.
2. Narrow-neck 500 mL glass flask bottles.
3. Liquid complete medium (CM). 10 g/L glucose, 2 g/L peptone, 1 g/L yeast extract, 1 g/L casamino acids, 0.1% (v/v) trace elements, 0.1% (v/v) vitamin supplement, 6 g/L $NaNO_3$, 0.5 g/L KCl, 0.5 g/L $MgSO_4$, 1.5 g/L KH_2PO_4; adjust to PH 6.5 with 10 M NaOH, and then sterilize by autoclaving at 121 °C for 30 min [20].
4. Trace elements (working concentration for each component in CM: g/mL).
   $ZnSO_4 \times 7H_2O$ (0.0022%), H_3BO_3 (0.0011%), $MnCl_2 \times 4H_2O$ (0.0005%), $FeSO_4 \times 7H_2O$ (0.0005%), $CoCl_2 \times 6HOH$ (0.00017%), $CuSO_4 \times 5H_2O$ (0.00016%), $Na_2MoO_4 \times 2H_2O$ (0.00015%), Na_4EDTA (0.005%).
5. Vitamin supplement (working concentration for each component in CM: g/mL).
   Biotin (0.0001%), pyridoxine (0.0001%), thiamine (0.0001%), riboflavin (0.0001%), PABA (*p*-aminobenzoic acid) (0.0001%), nicotinic acid (0.0001%).
6. Orbital incubator shaker.
7. Miracloth.
8. Sterile distilled water.
9. Tissue paper.
10. Liquid nitrogen.

2.2 Total Protein Preparation for TAP

1. Cold room (4 °C).
2. Mortar and pestle.
3. 50 mL conical centrifuge tubes.
4. 15 mL conical centrifuge tubes.
5. PE buffer. 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 10 mM imidazole, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail, 50 μM MG132, and 1 mM phenylmethylsulfonyl fluoride (PMSF) in water (*see* **Note 2**).
6. Vortex mixer.
7. High-speed centrifuge tubes.
8. Ultracentrifuge (e.g., Beckman Coulter).

9. 1.5 mL low-retention microcentrifuge tubes (e.g., Protein LoBind conical tubes).
10. Protein quantification kit (e.g., Bradford Protein Assay Reagent).

2.3 Ni^{2+} Affinity Chromatography

1. Ni^{2+} affinity column (e.g., HisTrap™ High Performance, 1 mL, GE Healthcare).
2. HW buffer. 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 10 mM imidazole in water (*see* **Note 3**).
3. HE buffer. 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 500 mM imidazole, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail, 50 μM MG132, in water (*see* **Notes 2** and **4**).
4. 1.5 mL low-retention microcentrifuge tubes (e.g., Protein LoBind conical tubes).

2.4 FLAG Immunoprecipitation

1. 1.5 mL low-retention microcentrifuge tubes (e.g., Protein LoBind conical tubes).
2. ANTI-FLAG M2 Affinity Agarose Gel.
3. Benchtop centrifuge with temperature control.
4. Tube rotator.
5. 1× TBS (pH 7.5). 50 mM Tris-HCl (pH 7.5), 150 mM NaCl in water.
6. 0.1 M glycine–HCl (pH 3.5). 0.1 M glycine–HCl in water; adjust to pH 3.5 with HCl.
7. FW buffer. 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail in water.
8. 3× FLAG peptide solution. 300 ng/μL 3× FLAG peptide, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl in water.
9. Neutralization buffer. 0.5 M Tris–HCl (pH 7.4), 1.5 M NaCl in water.
10. 2× LDS sample buffer. 20% glycerol, 2% lithium dodecyl sulfate (LDS), 0.4 M triethanolamine–HCl (pH 7.6), 2% Ficoll 400, 0.0125% phenol red, 0.0125% Coomassie G250, 1 mM EDTA in water.
11. Heated water bath at 95 °C.

2.5 Data Analysis

1. Search engine (e.g., Mascot).
2. Protein database for *M. oryzae* (e.g., downloaded from the Broad Institute (https://www.broadinstitute.org/genomics) or the UniProt (www.uniprot.org)).
3. Protein database for common contaminants (e.g., the cRAP).

4. A custom protein database containing the user-supplied fusion protein sequence.
5. Proteome software (e.g., Scaffold).
6. Statistical analysis tools (e.g., Microsoft Excel, R, or Python).

3 Methods

PPI is a function-driven molecular event. To perform specific biological functions, proteins interact with their partners dynamically and transiently [21], so the temporal nature of protein interactions, the decisive factor of the components of protein complexes, should be considered prior to PPI investigation. For example, in our recent paper [19], a time course expression study under oxidative stress conditions was first carried out, and then, based on those results, the time point at 40 min after H_2O_2 treatment was chosen as the optimal condition for *SIR2* complex isolation. In addition, the spatial expression patterns of the gene of interest (GOI) should be determined before TAP, which is necessary for tissue-specific expressed genes. In this respect, we used vegetative hyphae for preparing total proteins for TAP because we found the *SIR2* gene was expressed in the vegetative hyphae under both normal and oxidative stress conditions [19]. In addition to gene transcriptional analysis, the solubility of the protein needs to be considered for optimizing TAP, especially for membrane-associated proteins, for which the concentration of non-denaturing detergents should be increased to break protein–lipid interactions. Furthermore, it is better to incorporate a control protein, e.g., green fluorescent protein (GFP), in the whole process of TAP, in order to differentiate background proteins during data analysis.

3.1 Fungal Hyphae Preparation

1. Inoculate the fungal strains expressing the GOI (gene of interest) fused to *6xHis-3xFLAG*, and a control stain expressing GFP^{6H3F}, in 350 mL liquid CM, and grow at 25 °C for 48 h with continuous shaking at 140 rpm (*see* **Note 5**).
2. Harvest the mycelium with Miracloth, wash excessively with sterile distilled water, pat dry with tissue paper, flash freeze in liquid nitrogen, and store at −80 °C until use.

3.2 Total Protein Preparation for TAP

Perform all steps from now on in a cold room with the temperature set to 4 °C unless stated otherwise.

1. Grind the frozen fungal mycelium to fine powder in liquid nitrogen using a prechilled mortar and pestle.
2. For each strain, carefully transfer 12 g of finely ground mycelium to a prechilled 50 mL conical centrifuge tube followed by

adding precold 20 mL of PE buffer. Mix gently with a sterile stirring rod followed by a short vortexing which can help to homogenize the mycelium in the PE buffer (*see* **Note 6**).

3. Transfer the mixture to a high-speed centrifuge tube, and ultracentrifuge at >20,000 × *g* at 4 °C for 30 min.
4. After removal of insoluble cell debris by centrifugation, transfer the supernatant (crude extract) to a precold 50 mL conical centrifuge tube. Save 100 μL as the loading control (L) for Ni^{2+} affinity chromatography.
5. Measure protein concentration using Bradford Protein Assay. For the test strain and the control strain, adjust the protein concentration to a comparable level with PE buffer (*see* **Note 7**).

3.3 Ni^{2+} Affinity Chromatography

1. Wash the 1 mL HisTrap HP column first with 5 mL of sterile distilled water and then equilibrate with 10 mL of HW buffer (*see* **Note 8**).
2. Load the crude extract through the equilibrated column into another 50 mL conical tube on ice. Save the flow-through for quality controls (His-FT).
3. Wash the column with 20 mL of PE buffer. Save the wash for quality controls (His-W).
4. Sequentially elute bound proteins off the column with 5 mL of HE buffer into ten 1.5 mL low-retention tubes (E1–E10) with 500 μL per fraction. Save 10 μL of each fraction for quality controls. Place the samples E1–E10 on ice until use.
5. Determine in an immunoblot analysis of E1–E10 using anti-FLAG antibody the fractions in which the His-FLAG-tagged proteins are enriched (*see* **Note 9**).
6. Pool two fractions containing most of the enriched His-FLAG-tagged proteins as FLAG-IP loading and proceed to FLAG immunoprecipitation (*see* **Note 10**). Save 10 μL of FLAG-IP loading for quality controls.

3.4 FLAG Immunoprecipitation

1. Remove 50 μL ANTI-FLAG M2 Affinity Agarose Gel (bead slurry) to a 1.5 mL low-retention tube (*see* **Note 11**).
2. Wash the resin twice with 1 mL of 1× TBS (pH 7.5) followed by 0.5 mL of 0.1 M glycine–HCl (pH 3.5) once to remove any trace of amounts of unbound antibody from the resin suspension (*see* **Note 12**). Next, wash the resin three times with 1 mL of 1× TBS (pH 7.5) (*see* **Note 13**). Between the washing steps, spin down the resin at 3000 × *g* for 30 s at 4 °C. Carefully remove the supernatant with a pipette after each centrifugation.

3. Add 1 mL of HE buffer to the resin for equilibration and then pellet the beads by spinning at 3000 × *g* for 30 s at 4 °C. Carefully remove the supernatant with a pipette.
4. Add the combined two fractions from Subheading 3.3, **step 6** to the washed resin. Agitate all samples and controls gently at 4 °C for 2 h by end-over-end rotation on a tube rotator.
5. At the end of incubation, pellet the resin at 3000 × *g* for 30 s at 4 °C. Carefully remove the flow-through to another 1.5 mL low-retention tube without touching the resin. Save 4 μL of flow-through for quality controls (FLAG-FT).
6. Wash the resin four times in 500 μL of FW buffer and then wash four times in 500 μL of 1× TBS buffer (pH 7.5). Save 4 μL of the first (W1) and the second (W2) wash for quality control. For each wash, gently resuspend the resin with wash buffer followed by gentle rotation for 5 min at 4 °C on a roller shaker.
7. Centrifuge the resin at 3000 × *g* for 30 s at 4 °C. Carefully remove the supernatant using a pipette without taking any resin.
8. Elute immunoprecipitated proteins of FLAG resin with either 3× FLAG peptide solution, with 0.1 M glycine–HCl (pH 3.5), or with 2× LDS sample buffer (*see* **Note 14**). Save 1 μL of each eluate for quality controls (FLAG-E). Store the eluate at 4 °C for immediate use or −20 °C for long-term storage.
   (a) *Elution with 3× FLAG peptide solution*: add 100 μL of 3× FLAG peptide solution (300 ng/μL) to the resin followed by gentle shaking for 30 min at 4 °C, and then centrifuge the resin at 8200 × *g* for 30 s at 4 °C. Carefully transfer the supernatant to a fresh 0.5 mL low-retention tube labeled with FLAG-E_{pep}. Save 4 μL of the sample for quality controls.
   (b) *Elution with 0.1 M glycine (pH 3.5)*: add 100 μL of 0.1 M glycine (pH 3.5) to the resin followed by gentle shaking for 5 min at room temperature, and then centrifuge the resin at 8200 × *g* for 30 s. Carefully transfer the supernatant to a fresh 0.5 mL low-retention tube containing 10 μL of neutralization buffer. Label the tube with FLAG-E_{acid} and save 4 μL for quality controls.
   (c) *Elution with 2× LDS sample buffer*: add 50 μL of 2× LDS sample buffer (*see* **Note 15**) to the resin followed by incubating in the water bath at 95 °C for 3 min, and then centrifuge the resin at 8200 × *g* for 30 s. Carefully transfer the supernatant to a fresh 0.5 mL low-retention tube labeled with FLAG-E_{LDS}. Save 4 μL of the sample for quality controls.

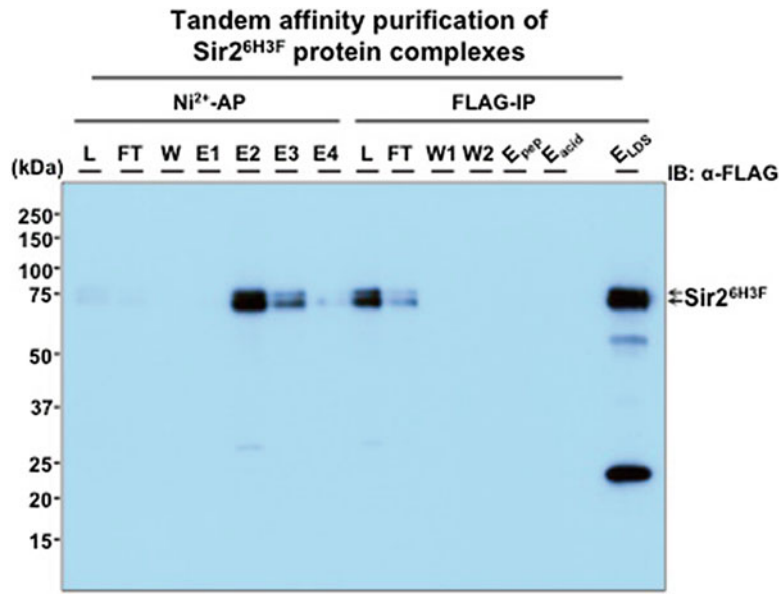

Fig. 1 Quality evaluation of tandem affinity purification procedures for isolating Sir2-associated protein complexes. Vegetative hyphae of the *Δsir2* complementation strain expressing *SIR2*^{6H3F} were cultured in liquid CM at 25 °C for 48 h and then subjected to oxidative stress treatment with 12 mM H_2O_2 for 40 min. Following harvesting of the hyphae, total proteins were isolated with native lysis buffer containing the protease inhibitor cocktail, phosphatase inhibitor cocktail, and the proteasome inhibitor MG-132. After protein preparation, tandem affinity purification was carried out using Ni^{2+} affinity purification followed by FLAG-immunoprecipitation. Note that, based on our experience of the fraction distribution patterns of Sir2^{6H3F} in the eluates of Ni^{2+}-AP characterized by the pilot experiment, elution fractions E2 and E3 were directly chosen, combined, and used in the subsequent FLAG-IP directly after the Ni^{2+}-AP. The image shown here is the result of immunoblotting analysis of the purification quality of the Sir2^{6H3F} protein complexes throughout our TAP workflow. L, FT, W, and E indicate loading, flow-through, wash, and eluate, respectively; Epep, Eacid, and E_{LDS} denote elution with 3× FLAG peptide solution (300 ng/μL in 1×TBS), acidic condition (0.1 M glycine, pH 3.5), and 2× LDS sample buffer, respectively. Please note that Sir2^{6H3F} protein complexes can only be eluted off FLAG resin by 2× LDS sample buffer but not the 3× FLAG peptide solution or the 0.1 M glycine, pH 3.5. *IB* immunoblotting. (This image is an overexposed version of the one used in Fig. S5 of our recent paper [19])

9. After the FLAG immunoprecipitation, evaluate the quality of the TAP and the efficiency of the final elution of the FLAG-IP by analyzing the saved small aliquots of each fraction using immunoblotting analysis (an example is given in Fig. 1). If the quality and amount is satisfactory, analyze the majority of the FLAG-E fraction by Coomassie blue staining, silver staining, or mass spectrometry (*see* **Note 16**). In this case, use the control protein (e.g., GFP^{6H3F}) for differentiating

nonspecifically bound background proteins and thus facilitate the identification of the interaction partners of the tagged GOI[6H3F].

3.5 Data Analysis

1. After the MS data are collected, analyze all MS/MS samples using Mascot or other search engines. These softwares use mass spectrometry data to perform database search for identification of proteins from peptide sequence databases. For identification of *M. oryzae* proteins, search against the protein database MGx in the Broad Institute (https://www.broadinstitute.org/genomics) or UniProt (www.uniprot.org), along with a database for common contaminants, e.g., the cRAP, and a custom database containing the user-supplied fusion protein sequence . For any search engine used, related search parameters, e.g., the digestion enzyme(s) used to generate the peptides prior to MS analysis, the fragment ion mass tolerance, and the parent ion tolerance, should be set up so as to filter out the low-scoring matches and remove the redundancy, all of which can greatly affect the final output.
2. Following database search, use proteome software, e.g., Scaffold, to validate MS/MS-based peptide and protein identifications. In the case of the Scaffold, peptide identifications are accepted if they could be established at greater than 95% probability by the PeptideProphet algorithm [22] with Scaffold delta-mass correction. Protein identifications are accepted if they could be established at greater than 95% probability and contain at least 5–10 identified peptides (*see* **Note 17**). Protein probabilities are assigned by the ProteinProphet algorithm [23]. Proteins that contain similar peptides and cannot be differentiated based on MS/MS analysis alone are grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence are grouped into clusters.

   Proteins identified through TAP may be involved in direct or indirect protein-protein interactions; if the direct interaction needs to be determined, other experimental strategies, e.g., yeast two-hybrid analysis, should be further employed. Moreover, as MS analysis is based on peptide identification, confirm authentic interactions using the in vivo pull-down assay between the bait and the prey proteins and antibody-based immunoblotting analysis. In addition, it is worth noting that the extra purification step in TAP increases the manipulation time period which may result in the loss of transient interaction partners; thus, interactive review of the TAP and co-IP data is suggested for a comprehensive understanding of the PPI networks of interest [19].

4 Notes

1. Two different tags used for TAP are usually fused at one side (N- or C-terminus) of the bait protein. The fusion should be tested first by complementation assays in a respective mutant background, and only the strain with the functional fusion can be used for TAP.
2. Protease inhibitor cocktail, phosphatase inhibitor cocktail, and MG132 should be EDTA- or EGTA-free as chelating agents will disturb the Ni^{2+} affinity chromatography. PMSF should be freshly prepared and added just before use.
3. The NaCl concentration in the HW buffer can be set to 150–300 mM depending on the stability of the target protein complexes.
4. The NaCl concentration in the HE buffer can be set to 150–300 mM depending on the stability of the target protein complexes.
5. In our case, we fused the tandem affinity tags 6xHis-3xFLAG at the C-terminus of our bait protein Sir2 and used it for TAP as we verified $Sir2^{6H3F}$ is functional in complementing the defects of the pathogenicity of Δ*sir2* knockout mutant strains.
6. Avoid sonication during homogenization of the mycelium because the heat gradient from the probe may damage proteins and disrupt PPIs under non-crosslinking conditions.
7. Adjusting the protein concentration of the test strain and control strain to comparable levels will help differentiate background proteins identified in TAP.
8. Use a syringe or a pump for protein purification using a 1 mL HisTrap HP column. Suggested flow rates for using a 1 mL HisTrap HP column throughout the whole process of Ni^{2+} affinity chromatography are 0.5–1 mL/min.
9. To save time for this step, perform a pilot experiment to determine the tagged protein distribution in elution fractions because the fraction distribution of a specific protein is repeatable if the elution volume is close to 500 μL. Target fractions expected to contain the enriched tagged proteins can be chosen directly for FLAG-IP. Confirm the protein distribution later by immunoblotting with the saved 10 μL aliquot of each fraction.
10. According to our experience, the 500 mM imidazole in the HE buffer does not interfere with the FLAG-IP of interacting proteins significantly, so imidazole does not have to be removed here.
11. When handling agarose beads, it is better to cut the end of the pipette tips before taking the beads to ensure the uptake of the correct volume of the beads.

12. Do not leave the beads in glycine–HCl for more than 20 min. Remove the glycine–HCl as soon as possible after washing.
13. To carefully remove the supernatant after each centrifugation without losing any resin, narrow-end pipette tips are suggested, which can be made using forceps to pinch the opening of the pipette tip until it is partially closed.
14. Considering the difference in protein characteristics, it is better to try eluting the tagged proteins with various elution conditions as sometimes 3× FLAG peptide or 0.1 M glycine may fail in elution of target protein off the agarose resin (see an example given in Fig. 1).
15. To minimize the denaturation and elution of the immobilized M2 antibody, do not include reducing agents, including 2-mercaptoethanol or DTT, in the LDS sample buffer.
16. If the purified proteins are eluted using anionic detergents, e.g., SDS or LDS, remove the detergents prior to LC-MS/MS analysis. This can be achieved by running an SDS-PAGE followed by in-gel digestion, or by TCA precipitation, etc. If the in-solution digestion is preferred before LC-MS/MS, use the acid labile surfactants, e.g., RapiGest SF, PPS Silent Surfactant, or ProteaseMAX for protein elution from FLAG resin. These surfactants are compatible with the most commonly used proteolytic enzyme trypsin and can be easily removed through hydrolysis in acidic conditions before LC-MS/MS.
17. For regular one-step affinity purification, e.g., co-IP, the protein identifications can be accepted if they contain at least two identified peptides. However, for TAP, it is better to drive up the standards, i.e., the identified proteins should contain at least 5–10 identified peptides, as the constituents of the protein complex are enriched by an additional purification step in TAP, though the expression level of the proteins should also be considered.

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