Chapter 11

Systematic Characterization of the Protein Interaction Network and Protein Complexes in *Saccharomyces cerevisiae* Using Tandem Affinity Purification and Mass Spectrometry

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Summary

Defining protein complexes is a vital aspect of cell biology because cellular processes are often carried out by stable protein complexes and their characterization often provides insights into their function. Accurate identification of the interacting proteins in macromolecular complexes is easiest after purification to near homogeneity. To this end, the tandem affinity purification (TAP) system with subsequent protein identification by high-throughput mass spectrometry was developed *(1, 2)* to systematically characterize native protein complexes and transient protein interactions under near-physiological conditions. The TAP tag containing two adjacent affinity purification tags (calmodulin-binding peptide and *Staphylococcus aureus* protein A) separated by a tobacco etch virus (TEV) protease cleavage site is fused with the open reading frame of interest. Using homologous recombination, a fusion library was constructed for the yeast *Saccharomyces cerevisiae (3)* in which the carboxy-terminal end of each predicted open reading frame is individually tagged in the chromosome so that the resulting fusion proteins are expressed under the control of their natural promoters *(3)*. In this chapter, an optimized protocol for systematic protein purification and subsequent mass spectrometry-based protein identification is described in detail for the protein complexes of *S. cerevisiae (4–6)*.

Key words: *Saccharomyces cerevisiae*, TAP tagging, Affinity purification, Mass spectrometry, LC-MS, MALDI-TOF, Protein complex

1. Introduction

Since protein interactions are important for most cellular processes, and multi-subunit protein machines actively participate in most or all such processes *(5–7)*, it is of immense significance to

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systematically characterize protein–protein interactions (PPIs) in various organisms. In the budding yeast *Saccharomyces cerevisiae*, large-scale PPI networks were first generated by systematically using the yeast two-hybrid technique *(8, 9)*. Subsequently, the tandem affinity purification (TAP) approach was developed for the purification of native yeast protein complexes *(2, 4)*. In this approach, TAP-tagged proteins are expressed under normal physiological conditions, purified ~10^6-fold via a two-step enrichment procedure, and then characterized by mass spectrometry. Because it is relatively simple to fuse affinity tags with target proteins, this approach has also been successfully applied to many other evolutionarily diverse organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Escherichia coli*, mammalian cells, and plants *(10–15)*. Exploring PPI networks on a large scale provides not only additional information about well-characterized proteins but also a rational framework for elucidating the biological functions of uncharacterized proteins based on the concept of "guilt by association" *(16)*.

The TAP tag consists of two affinity purification tags, a calmodulin-binding peptide (CBP) and *Staphylococcus aureus* protein A, separated by a tobacco etch virus (TEV) protease cleavage site *(2)*, to allow highly selective two-stage protein enrichment and to help reduce the number of nonspecifically bound proteins (**Fig. 1a)**. Each tagged protein is purified first by binding to beads containing immobilized IgG and subsequently by binding to beads containing immobilized calmodulin. To facilitate protein analyses on a global scale, Ghaemmaghami et al. *(3)* used homologous recombination to integrate a TAP-tag in-frame immediately after the stop codon of each predicted open reading frame in its natural chromosomal location in *S. cerevisiae*. The TAP-tagged fusion proteins are then expressed under the control of their natural promoters, and the abundance of each TAP-tagged fusion protein was assessed by quantitative Western blotting using an antibody that binds to the protein A component of the TAP tag *(3)*. The entire collection of TAP-tagged yeast strains has been made commercially available for academic use (http://www.openbiosystems.com/GeneExpression/Yeast/TAP/).

We purified a large number of the TAP-tagged bait proteins from 2 L yeast cultures under native conditions *(6)*. To increase the interactome coverage and confidence, the identities of the co-purifying proteins (preys) were determined using two complementary, highly sensitive mass spectrometry methods: gel-free liquid chromatography-tandem mass spectrometry (LC-MS/MS), and gel-based peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We attempted to purify 4,562 different soluble proteins, and an extensive PPI network containing high-confidence protein interactions generated from this

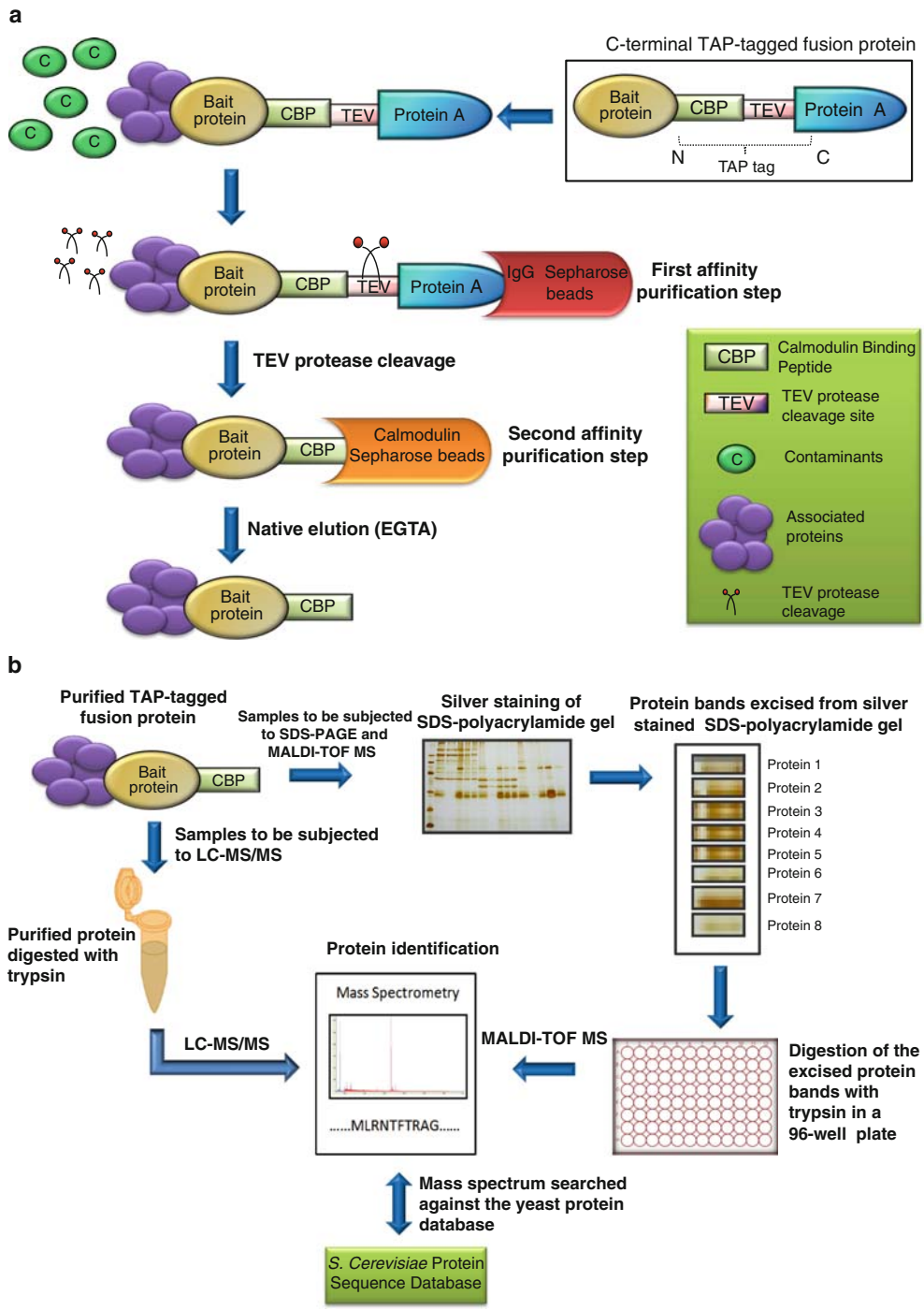

Fig. 1. Schematic representation of the procedures involved in the identification of protein complexes using the tandem affinity purification (TAP) approach followed by mass spectrometry analyses. The TAP-tag structure and an overview of the TAP strategy are shown in (**a**). Identification of the co-purifying proteins is performed using two complementary mass spectrometry methods (**b**). First, the purified TAP-tagged fusion protein and associated proteins are separated by SDS-PAGE followed by silver staining. The excised protein bands are digested with trypsin, and the resulting tryptic peptides are analyzed by peptide mass finger printing using MALDI-TOF MS. A yeast protein database is then searched using the Knexus software. Second, the purified TAP-tagged fusion proteins and associated proteins are digested with trypsin and analyzed by gel-free liquid chromatography-tandem mass spectrometry (LC-MS/MS). Searches are performed with the tandem mass spectra of the complete *S. cerevisiae* protein database using the SEQUEST computer algorithm to generate probable identifications of the proteins.

study was published in 2006 *(6)* at the same time as Gavin et al. *(5)*. These authors also used the TAP method and MALDI-TOF MS to elucidate a similarly extensive set of yeast PPI. More recently, the protein interaction data generated from these two large-scale studies were amalgamated into a single, more reliable collection of experimentally based PPIs using a novel purification enrichment (PE) scoring system *(17)*, and this larger, amalgamated set of PPI was used in conjunction with a Markov clustering algorithm to organize the *S. cerevisiae* proteome into a large number of more reliable protein complexes *(18)*.

Having attempted to purify all the yeast proteins predicted to be soluble, we are now purifying the many yeast proteins predicted to be membrane-associated (*see* **Note 1**). Purification of membrane proteins often poses unique challenges *(19–22)* because they are often not solubilized by the extraction buffer that we normally use for the TAP method. With some modifications of our standard procedures and with the addition of various nonionic detergents to our buffers, we are able to solubilize and purify the majority of the yeast membrane proteins. Currently, we are attempting to purify the ~1,600 *S. cerevisiae* proteins that are predicted to contain signal peptides or with at least one transmembrane domain. Strains for the vast majority of these purifications are already present in the collection of yeast TAP-tagged strains *(3)*. As of March 2009, more than 1,200 yeast membrane proteins had been purified using at least two different detergents.

In this chapter, detailed procedures are described for the purification of TAP-tagged bait proteins and subsequent identification of interacting protein partners by mass spectrometry. This method has been optimized for a well-characterized yeast laboratory strain and can be used to purify the subunits of high- or low-abundance yeast protein complexes. This basic approach can potentially be adapted for use in other organisms.

2. Materials

2.1. Purification of TAP-Tagged Proteins from S. Cerevisiae Strains

2.1.1. Culturing TAP-Tagged S. Cerevisiae Strains and Cell Lysis

1. *S. cerevisiae* strains used for culturing in which a bait protein is TAP-tagged have been described *(3)*.
2. Yeast extract–peptone–dextrose (YEPD) medium: Dissolve 10 g of yeast extract (Bioshop), 20 g of peptone (Bioshop), and 20 g of glucose in 800 ml of distilled water. Autoclave the media on a liquid cycle for 30 min.
3. 2 M Tris–HCl (pH 7.9) stock solution: Dissolve 242.2 g of Tris base in 800 ml of double distilled water, adjust to pH 7.9 with concentrated HCl, and make up the volume to 1 L.

4. 5 M NaCl stock solution: Dissolve 292.2 g of NaCl in 1 L of double-distilled water.
5. 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0) stock solution: Dissolve 186.1 g of EDTA in 800 ml of double-distilled water, adjust the pH to 8.0 using 1 M NaOH, and make up the volume to 1 L.
6. HEPES-KOH (pH 7.9) stock solution: Dissolve 238.3 g of HEPES in 800 ml of double-distilled water, adjust the pH to 7.9 using 5 M KOH, and make up the volume to 1 L.
7. 1 M $CaCl_2$: Dissolve 110.98 g of $CaCl_2$ in 800 ml of double-distilled water and make up the volume to 1 L.
8. 2 M KCl stock solution: Dissolve 149.1 g of KCl in 800 ml of double-distilled water and make up the volume to 1 L.
9. EDTA-free protease inhibitor tablets (Roche). Make sure to prepare YEB buffer with and without the EDTA-free protease inhibitor tablet.
10. YEB buffer with protease inhibitor: Dissolve one tablet of protease inhibitor in 50 ml YEB buffer.
11. Yeast extract buffer (YEB): Mix 36.6 ml of 2 M KCl, 3 ml of 0.5 M EDTA, 3 ml of 0.5 M ethyleneglycoltetraacetic acid (EGTA)-KOH (pH 7.9), and 30 ml of 1 M HEPES-KOH (pH 7.9) in 229 ml of double-distilled water. Store the solution at room temperature. Prior to use, add 750 µl of 1 M dithiothreitol (DTT; Bioshop) into the YEB buffer.
12. Dialysis buffer (DB): Mix 80 ml of 5 M NaCl, 20 ml of 2 M Tris–HCl (pH 7.9), 1.6 ml of 0.5 M EDTA, 800 ml of 100% glycerol, and 0.32 g of DTT with 3098.4 ml of double-distilled water. Store the solution at 4°C.

2.1.2. Purification of TAP-Tagged Proteins

1. Store 10% Triton X-100 solution (Sigma Aldrich) at 4°C.
2. IPP buffer: Add 50 µl of 2 M Tris–HCl (pH 7.9), 200 µl of 5 M NaCl, and 100 µl of 10% Triton X-100 to 9.65 ml of sterile distilled water.
3. TEV protease cleavage buffer: Add 250 µl of 2 M Tris–HCl (pH 7.9), 4 µl of 0.5 M EDTA, 200 µl of 5 M NaCl, 100 µl of 10% Triton X-100, and 10 µl of 1 M DTT to 9.7 ml of sterile distilled water.
4. Calmodulin binding buffer: Add 200 µl of 2 M Tris–HCl (pH 7.9), 800 µl of 5 M NaCl, 80 µl of 1 M $CaCl_2$, 400 µl of 10% Triton X-100, and 28 µl of β-mercaptoethanol solution to 38.9 ml of sterile distilled water.
5. Calmodulin wash buffer: Add 50 µl of 2 M Tris–HCl (pH 7.9), 200 µl of 5 M NaCl, 1 µl of 1 M $CaCl_2$, 100 µl of 10% Triton X-100, and 7 µl of β-mercaptoethanol solution to 9.6 ml of sterile distilled water.

6. Calmodulin elution buffer: Add 50 µl of 2 M Tris–HCl (pH 7.9), 200 µl of 5 M NaCl, 60 µl of 0.5 M EGTA, 7 µl of β-mercaptoethanol, and 100 µl of 10% Triton X-100 to 8.8 ml of sterile distilled water.

2.2. Protein Identification by MALDI-TOF Mass Spectrometry

2.2.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. SDS stock solution (10% (w/v)): 10 g SDS is dissolved in 100 ml double-distilled water. The solution is stored at room temperature.
2. Electrode running buffer (5×): (125 mM Tris–HCl, 960 mM glycine, 0.5% (w/v) SDS, pH 8.3). 30 g of Tris–HCl, 144 g of glycine, and 10 g of SDS are dissolved in 2 L of double-distilled water without pH adjustment and stored at 4°C.
3. Acrylamide: 30% acrylamide monomer, 0.8% *N,N′*-methylenebis-acrylamide (Bio-Rad). The solution is filtered through Whatman No.1 filter paper (Fischer Scientific) and stored at 4°C in the dark.
4. Separating buffer (4×): 1.5 M Tris–HCl (pH 8.7), 0.4% SDS. Store at room temperature.
5. Stacking buffer (5×): 0.5 M Tris–HCl (pH 6.8), 0.4% SDS. Store at room temperature.
6. Ammonium persulfate (10% APS): Mix 1 g of APS (Bioshop) in 10 ml of double-distilled water. Aliquot the solution into several microcentrifuge tubes in a volume of 200 µl. Store the aliquots at –20°C.
7. Water saturated *n*-butanol: Equal volumes of *n*-butanol (Sigma Aldrich) and double-distilled water are mixed in a glass bottle and left at room temperature to separate. Use the topmost layer containing *n*-butanol saturated with water. Store the solution at room temperature.
8. Resolving gel (12.5% polyacrylamide): Mix 7.2 ml of 4× separating buffer with 12 ml acrylamide solution, 9.6 ml double-distilled water, 120 µl 10% APS solution, and 20 µl tetramethylethylenediamine (TEMED, Bioshop).
9. Stacking gel (4.5% polyacrylamide): Mix 4 ml of 5× stacking buffer with 2.4 ml acrylamide solution, 9.6 ml double distilled water, 150 µl 10% APS solution, and 15 µl TEMED.
10. Sample stock buffer (3×): Mix 1.25 ml of 0.5 M Tris–HCl (pH 6.8), 1 ml of 100% glycerol, 1 ml of 10% SDS (w/v), and 20 mg of bromophenol blue (Bio-Rad).
11. 2× sample buffer: Add 6 ml of 3× sample stock buffer, 0.6 ml of β-mercaptomethanol, and 3.4 ml of distilled water.

2.2.2. Silver-Staining the SDS-Polyacrylamide Gel

1. Fixer: 50% methanol and 10% acetic acid (AA) are added to 400 ml sterile distilled water.
2. Sensitizer: 20 mg of fresh sodium thiosulfate (Sigma) is dissolved in 1,000 ml distilled water.

3. Silver nitrate solution: 2 g of silver nitrate (Fischer Scientific) is dissolved in 1,000 ml distilled water.
4. Developing solution: 1.4 ml of 37% formaldehyde and 30 g of sodium carbonate are added to 1,000 ml of distilled water.
5. Stop solution: 5 ml AA is diluted in 500 ml of distilled water.

2.2.3. Gel-Based MALDI-TOF Mass Spectrometry

1. An ULTRAFlex II MALDI-TOF instrument for acquiring spectral data from in-gel trypsin digested yeast samples (Bruker Daltonics, Billerica, MA).
2. MALDI target plate (Bruker).
3. Bulk C18 reverse phase resin (Sigma).
4. Knexus automation, a Windows-based program from Genomics Solutions Bioinformatics for database searches.
5. α-Cyano-4-hydroxycinnamic acid matrix solution (Fluka Buchs SG, Switzerland).
6. 1 mM HCl: Add 1 µl of 10 N HCl into 10 ml HPLC-grade water. Make a fresh solution each day and store on ice until use.
7. Trypsin stock solution: Dissolve 100 µg Boehringer Mannheim unmodified sequencing-grade trypsin (Roche) in 1 ml of 1 mM HCl. Store the trypsin stock solution at –80°C.
8. Digestion buffer: Mix 9.12 ml of 100 mM NH_4HCO_3, 9.12 ml of HPLC-grade water, 960 µl of 1% $CaCl_2$, and 1 ml of trypsin stock solution. Prepared a fresh solution each day and store on ice until use.
9. 100 mM NH_4HCO_3: Dissolve 0.79 g NH_4HCO_3 in 100 ml HPLC-grade water.
10. 100 mM NH_4HCO_3 containing 10 mM DTT: Add 100 µl of 1 M DTT to 9.9 ml of 100 mM NH_4HCO_3. Prepare a fresh solution each day and store in an amber-colored bottle at room temperature in the dark until use.
11. 100 mM NH_4HCO_3 containing 55 mM iodoacetamide (Sigma): Add 0.103 g of iodoacetamide to 10 ml of 100 mM NH_4HCO_3. Prepare a fresh solution every day and store in an amber-colored bottle at room temperature in the dark until use.
12. 66% Acetonitrile (ACN), 1% AA: Mix 66 ml HPLC-grade ACN (Sigma), 33 ml of HPLC-grade water, and 1 ml AA. Store the solution in a glass bottle for up to 1 month at room temperature.
13. 75% ACN, 1% AA: Mix 75 ml HPLC-grade ACN, 24 ml HPLC-grade water, and 1 ml AA. Store the solution in a glass bottle for up to 1 month at room temperature.

14. 2% ACN, 1% AA: Mix 2 ml of HPLC-grade ACN, 97 ml HPLC-grade water, and 1 ml AA. Store the solution in a glass bottle for up to 1 month at room temperature.
15. 1% $CaCl_2$: Dissolve 1 g of $CaCl_2$ in HPLC-grade water to a final volume of 100 ml. Store the solution in a glass bottle for up to 1 month at room temperature.
16. 0.1% Trifluoroacetic acid (TFA) stock solution: Dilute 0.25 µl of TFA in 250 µl of HPLC-grade water.
17. Peptide calibration standard (Bruker) solution: Lyophilized peptide standard is dissolved with 125 µl of 0.1% HPLC-grade TFA.

2.3. Protein Identification by Liquid Chromatography Mass Spectrometry (LC-MS/MS)

1. LTQ tandem mass spectrometer (Finnigan Corp, San Jose, CA, USA) to run the samples, and XCalibur software to acquire tandem mass spectra and to control the instrument.
2. Digestion buffr: Mix 599 µl of 50 mM NH_4HCO_3 and 1 µl of 1 M $CaCl_2$. Store the solution at 4°C prior to use.
3. Immobilized trypsin solution: Mix 18.7 µl of digestion buffer, 1.8 µl PIERCE immobilized trypsin beads (PIERCE), 0.9 µl immobilized trypsin beads (Applied Biosciences), and 0.06 µl of 1 M $CaCl_2$. Make sure that the pH of the immobilized trypsin solution is ~8.0.
4. 150 µm fused silica (Polymicro Technologies, Pheoenix, AZ, USA).
5. C18 reverse phase packing material (Zorbax eclipse XDB-C18 resin; Agilent Technologies, Mississauga, ON, Canada).
6. Solvent A: 5% ACN, 0.5% AA, and 0.02% heptafluorobutyric acid (HFBA)
7. Solvent B: 100% ACN
8. Proxeon nano HPLC pump (Proxeon Biosciences).

3. Methods

3.1. Purification of S. Cerevisiae TAP-Tagged Fusion Proteins

3.1.1. Culturing TAP-Tagged S. Cerevisiae Strains and Cell Lysis

1. Inoculate a loop of a glycerol stock of a TAP-tagged *S. cerevisiae* strain *(3)* into 10 ml YEPD liquid medium in a 20-ml sterile culture tube.
2. Grow the culture overnight and the next day at 30°C with shaking at 250 rpm until the OD_{600} reaches 1.0–1.5.
3. Inoculate 10 ml of the overnight culture into 2 L fresh YEPD liquid medium in a 4-L flask (*see* **Note 2**). The culture is grown overnight at 30°C with shaking at 250 rpm until the OD_{600} reaches ~1.0–1.5.

4. Transfer 2 L *S. cerevisiae* culture from the shaker to clean 1-L centrifugation bottles.
5. Centrifuge the bottles containing the *S. cerevisiae* culture in a Beckman J-20XP Avanti centrifuge at 3,993*g* for 5 min at 4°C.
6. Discard the supernatant and remove excess liquid by inverting the bottles on paper towels. Keep the centrifugation bottles on ice.
7. Add 10 ml cold distilled water to the centrifugation bottles and resuspend the *S. cerevisiae* cell pellets using a clean 25-ml pipette.
8. Transfer the cell lysates into 50-ml polypropylene Falcon tubes.
9. Centrifuge the Falcon tubes containing the cell lysates in a Beckman J-20XP Avanti centrifuge at 3,993*g* for 5 min at 4°C.
10. Decant the cold water from the Falcon tubes, and resuspend the cell pellets with an equal volume of cold YEB buffer without protease inhibitor using a clean 25-ml pipette.
11. Centrifuge the Falcon tubes containing the cell lysates in an Eppendorf centrifuge 5810R using an A-4–62 rotor at 751 × *g* (4,000 rpm) for 5 min at 4°C.
12. Add an equal volume of cold YEB buffer with protease inhibitor to the Falcon tubes containing the cell pellets and resuspend the pellets using a clean 25-ml pipette.
13. Repeat **step 11**.
14. Snap-freeze the Falcon tubes containing the cell pellets using liquid nitrogen. The frozen cell pellets are stored at –80°C for future use.
15. The Falcon tubes containing the frozen cell pellets are removed from the freezer and wrapped in several layers of paper towels.
16. Using a hammer, smash the Falcon tubes containing the frozen cell pellets into small pieces.
17. Transfer each yeast cell pellet (7–10 g) into a prechilled Krups coffee grinder (Krups, Model 203–70). Avoid transferring the broken pieces of plastic.
18. Add 25% dry ice into the coffee grinder containing 7–10 g yeast cell pellets.
19. Perform lysis by grinding the yeast cell pellets to a fine powder with dry ice using the Krups coffee grinder (*see* **Note 3**). This takes approximately 2–3 min.
20. Scrape the lysed powder into a 25-ml ultracentrifuge tube (25 × 89 mm, Beckman, Part No. 355642) placed on dry ice.

21. Resuspend the lysed powder by adding an equal volume of YEB buffer containing protease inhibitor into the 25-ml ultracentrifuge tubes (*see* **Note 4**).
22. Centrifuge the 25-ml ultracentrifuge tubes containing the cell lysates in a Beckman L8-M ultracentrifuge using a Type 70Ti rotor at 208,429 × *g* (45,000 rpm) for 1 h at 4°C.
23. The supernatants from the ultracentrifuge tubes are collected and transferred to dialysis tubes (Spectra/Por®, 29 mm in diameter). The supernatants are dialyzed against 4 L dialysis buffer containing 100 mM NaCl for 3 h at 4°C (*see* **Notes 5** and **6**).
24. After dialysis, the extracts are transferred from the dialysis tubes to sterile 25-ml ultracentrifuge tubes.
25. Centrifuge the 25-ml ultracentrifuge tubes containing the extracts in a Beckman L8-M ultracentrifuge using a Type 70Ti rotor at 208,429 × *g* (45,000 rpm) for 30 min at 4°C to remove any precipitated material.
26. Remove the extracts carefully from the Falcon tubes and transfer them to 15- or 50-ml (depending on the volume) sterile Falcon tubes.
27. Snap-freeze the Falcon tubes containing the samples in liquid nitrogen. The lysed frozen cell extracts are stored at –80°C for future use.
28. Thaw the frozen Falcon tubes containing the lysed cell extracts by placing the tubes in cold water. Centrifuge the tubes containing the lysed cell extracts in an Eppendorf centrifuge 5810R using a A-4–62 rotor at 751 × *g* (4,000 rpm) for 5 min at 4°C to remove any precipitated material.

3.1.2. Protein Purification

IgG-Sepharose Beads

1. All purification steps are performed at 4°C with precooled buffers and equipment.
2. The Falcon tubes containing the lysed frozen cell extracts are thawed by placing the tubes in cold water.
3. Prior to use, add 1 ml of IgG-Sepharose beads to a fresh 15-ml Falcon tube. The beads are washed with 3 × 5 ml of IPP buffer. Briefly mix the contents by tilting the Falcon tubes upside down.
4. Centrifuge the Falcon tubes containing the IgG-Sepharose beads with IPP buffer in an Eppendorf centrifuge 5810R using a A-4–62 rotor at 751 × *g* (4,000 rpm) for 5 min at 4°C. Discard the supernatants, taking care not to disturb the beads.
5. Add 100 µl of the washed IgG-Sepharose beads and 100 µl of IPP buffer to each Falcon tube containing cell extract. Briefly mix the contents by tilting the Falcon tubes upside down.

6. Rotate the Falcon tubes for 3 h at 4°C using a LabQuake shaker (Barnstead/Thermolyne).
7. Centrifuge the Falcon tubes containing the IgG beads and extracts in an Eppendorf centrifuge 5810R using a A-4–62 rotor at 751 × *g* (4,000 rpm) for 5 min at 4°C. Remove the supernatants as much and as carefully as possible, taking care not to disturb the loose bead pellets.
8. Resuspend the bead pellets in the leftover supernatants and transfer the beads into 0.8 × 4 cm Bio-Rad polypropylene prep columns (Bio-Rad). Make use of P1000 pipette tips clipped at the ends to transfer the beads into the prep columns. Remove the bottom outlet plugs of the columns and allow the eluates to drain by gravity flow.
9. Wash the columns five times with 200 µl of IPP buffer and twice with 200 µl of TEV cleavage buffer.
10. Close the bottom outlet of the column and add 200 µl of 1× TEV cleavage buffer and 5 µl of TEV protease (2 mg/ml). Close the top of the column with a cap and rotate the column overnight at 4°C.

Calmodulin-Sepharose Beads

1. Remove the top and bottom outlet plugs of the columns after incubation with the TEV protease and recover the eluates by gravity flow into Eppendorf tubes. Wash the columns with 200 µl TEV cleavage buffer and collect the eluates into the same Eppendorf tubes containing the eluates recovered after TEV cleavage. Mix the contents in the Eppendorf tubes by gently pipetting up and down.
2. To the eluates add 400 µl of calmodulin binding buffer and 3 µl of 1 M $CaCl_2$.
3. Transfer the mixtures into 0.8 × 4 cm Bio-Rad polypropylene prep columns containing 200 µl of calmodulin-Sepharose beads (Amersham Biosciences) washed twice with 5 ml of calmodulin binding buffer.
4. Close the tops of the columns with caps and rotate for 2 h at 4°C using a LabQuake shaker.
5. Remove the top and bottom plugs of the columns and drain the eluates by gravity flow.
6. The beads are washed five times with 200 µl of calmodulin binding buffer followed by three washes with 200 µl of calmodulin wash buffer.
7. The bound proteins are eluted in six fractions of 100 µl into fresh Eppendorf tubes using calmodulin elution buffer (*see* **Note 7**).
8. The eluted fractions (600 µl) are distributed into two separate Eppendorf tubes in equal volumes of 300 µl.

9. Dry down 300 µl of the eluted fractions using a Speedvac (Eppendorf Vacufuge). Add 60 µl of 2× SDS sample buffer to the dried eluate. The eluate and the sample buffer mixture are boiled for 5 min. The proteins are separated by SDS-PAGE and visualized by silver staining. Protein bands excised from the gel are analyzed by MALDI-TOF mass spectrometry.
10. In parallel with **step 9**, the other 300-µl eluted fraction is dried down using a Speedvac. This dried sample is then subjected to LC-MS/MS for protein identification.

3.2. Protein Identification by MALDI-TOF Mass Spectrometry

The tagged and purified *S. cerevisiae* proteins are separated by SDS-PAGE and stained with silver. The protein bands are excised, reduced, alkylated, subjected to in-gel tryptic digestion, and analyzed by MALDI-TOF mass spectrometry. The mass spectra are searched using Knexus automation against the complete yeast protein database to generate probable identifications of the proteins. The identified proteins are associated back to the purified protein bands (**Fig. 1b**). The various steps, such as silver staining, in-gel trypsin digestion, extraction and purification of tryptic peptide fragments, spotting samples on MALDI target plates, acquisition of spectra, and protein identification, are described below:

3.2.1. SDS-Polyacrylamide Gel Electrophoresis

1. The steps described below are carried out using the Whatman Model V16 (Gibco BRL) gel system. The glass plates, spacers, and combs should be clean and free of dried gel fragments, grease, and dust.
2. For each gel, lay out one small and one large glass plate, separated by spacers.
3. Carefully slide the glass plates into the holder by making sure that the glass plates are pushed all the way to the bottom.
4. Slide in the combs and mark lines at 2–3 cm from the bottoms of the combs.
5. Remove the combs gently and pour 12.5% polyacrylamide resolving gels up to the marked lines. Overlay a thin layer of water-saturated *n*-butanol on the top of each gel. Allow the gels to polymerize for about 30 min.
6. Invert the gels to remove the *n*-butanol. Touch with filter paper to wick off residual liquid.
7. Pour the 4.5% polyacrylamide stacking gels to fill the remaining space between the glass plates. Insert the combs and allow the gels to polymerize for another 30 min.
8. Once the stacking gels are polymerized, carefully remove the combs and use a 3-ml syringe fitted with a 22G needle to wash the wells with running buffer.
9. Place the gels in the unit and add running buffer to the upper and lower chambers of the unit.

10. Load each well with 60-μl samples suspended in 2× SDS sample buffer. Include high and low range Precision Plus prestained protein molecular weight standards (Bio-Rad) in two of the wells for each gel.
11. Complete the assembly of the gel unit by attaching the power cords first to the apparatus, and then to the power supply.
12. Turn on the power supply and run the gels at 150 V through the stacking gels and 200 V through the resolving gels. The gels are run until the blue dye front reaches the bottom.
13. After the electrophoresis, disassemble the gel plate from the apparatus. Use a thin spatula to carefully pry the upper glass plates away from the gels.
14. Transfer the gels immediately to clean the staining solution containing fixer and proceed immediately with the silver-staining protocol described below.

3.2.2. Silver-Staining the SDS-Polyacrylamide Gel

1. Agitate the gel in clean fixer for 20 min on a rocking shaker.
2. Rinse the gel with 20% ethanol for 10 min.
3. The gel is then washed twice with 500 ml double-distilled water for 10 min. Note that thorough rinsing gives a uniform, low background.
4. Remove the double-distilled water and agitate the gel in 500 ml sensitizer solution for 1 min.
5. Remove the sensitizer solution and rinse the gel twice again with double-distilled water for 20 s.
6. Pour off the water and incubate the gel in 200 ml of 0.1% silver nitrate for 30 min.
7. Discard the silver nitrate and rinse the gel once with distilled water for 20 s to remove excess silver nitrate.
8. Wash the gel with 50–75 ml of freshly prepared developing solution for half a minute. Replace with fresh developing solution and agitate the gel slowly by hand constantly (*see* **Note 8**).
9. When the desired staining intensity is achieved, discard the developing solution and add 80 ml of stop solution to the gel.
10. Incubate the gel with the stop solution for a minimum of 20 min before proceeding to excise the protein bands from the silver-stained gels.

3.2.3. Protein Identification by Gel-Based MALDI-TOF Mass Spectrometry

Reduction and Alkylation of Protein Bands

1. Protein bands are excised from silver-stained gels with clean razor blades. The gel slices are excised as closely as possible to the boundaries of the protein bands and stored in –80°C freezers in 96-well polypropylene plates (Nunc).
2. Gel slices in 96-well polypropylene plates are thawed for approximately 20 min. The liquid that accumulates during thawing is carefully removed.

3. Gel slices are shrunk with 200 µl of 100% ACN for ~10 min on an IKA Schuttler MTS 4 orbital shaker (VWR Scientific) at 700 rpm (*see* **Note 9**). Remove all liquid.
4. The gel slices are reduced with 75 µl of 100 mM NH_4HCO_3 containing 10 mM DTT for 30 min in a 50°C heating block. Note that the gel pieces should be covered with liquid. When hydrated, there should still be some fluid left.
5. Remove all liquid by centrifuging the plate at 58 × *g* (500 rpm) in a Beckman Allegra X-12 centrifuge using a SX4750 µ plate carrier for 3–5 min, and repeat **step 3**.
6. The gel slices are alkylated in the 96-well microtiter plate with 75 µl of 100 mM NH_4HCO_3 containing 55 mM iodoacetamide for 20 min in the dark at room temperature.
7. Centrifuge the plate at low speed 58 × *g* (500 rpm) for 3–5 min. Remove all liquid. Repeat **step 3**.

In-Gel Tryptic Digestion

1. The gel slices are hydrated with 60 µl of digestion buffer containing trypsin for 30–45 min on ice. Note that the gel pieces should be fully hydrated with trypsin solution on ice. After 15–20 min, if needed, add more digestion buffer containing trypsin to allow complete hydration.
2. Add 20 µl (if needed) of digestion buffer without trypsin and incubate samples overnight at 37°C (*see* **Note 10**).

Extraction of Tryptic Peptides

1. The extracted peptides are transferred into clean 96-well polypropylene plates.
2. Add 100 µl of 100 mM NH_4HCO_3 to the gel slices and extract peptides by shaking at 700 rpm on an orbital shaker for 60 min at room temperature.
3. Briefly centrifuge, transfer the extracted peptides into the clean 96-well polypropylene plates, and add 2.5 µl of 100% AA to the extracted peptides in each well.

Purification of Tryptic Peptides

1. Purification is performed using bulk C18 reverse phase resin (Sigma). Add 1.5 g of dry resin to a reservoir.
2. Wash the dry resin two times with HPLC-grade methanol and two times with HPLC-grade 66% ACN, 1% AA prior to use. Add 75% ACN, 1% AA to prepare 5:1 resin slurry.
3. Add 2.5 µl of C18 reverse phase resin slurry to the extracted peptides in the wells of the 96-well plate. The resin should float on top of the liquid. Shake the plate on an orbital shaker at 500–700 rpm for 45 min at room temperature. Discard the liquid underneath the beads by using a 200-µl multichannel pipette.
4. Add 200 µl of 2% ACN, 1% AA. Shake the plate for 5–15 min on an orbital shaker at 500–700 rpm at room temperature.

5. Prepare in advance a 384-well Melt Blown Polypropylene (MBPP) Whatman filter plate (Whatman). Place the MBPP filter plate on top of a 384-well collection plate (Whatman). Wash the MBPP filter plate wells with 15 µl of 66% ACN, 1% AA. Centrifuge the MBPP filter plate for 1–2 min at 1,000*g* and discard the filtrates collected in the collection plate.
6. Centrifuge the 96-well plates from **step 4** for 5–15 min at 1,000*g* at room temperature. Remove the supernatants with a 200-µl multichannel pipette and discard them.
7. Elute the peptides by adding 30 µl of 66% ACN, 1% AA. Shake the 96-well plate briefly at high speed on an orbital shaker. Ensure that all of the resin has entered into the slurry. Incubate the plates for approximately 5–10 min at room temperature. The resin should make a slurry and slowly pellet to the bottom.
8. Transfer the liquid supernatant with 50-µl multichannel pipette from the 96-well plates to the 384-well MBPP filter plate. Place a 384-well collection plate (Whatman) under the MBPP filter plate and centrifuge for 3–5 min at 2,000*g*. The filtrates collected in the 384-well collection plate are either spotted immediately onto the Bruker MALDI target plate or sealed with sealing foil and stored at –70°C until spotting onto MALDI targets.

Spotting Samples onto the MALDI Target Plates

1. Separate the Bruker MALDI target plate from its base.
2. Remove the previously spotted matrix spots from the MALDI target plate by washing the plate with 100% methanol followed by rinsing the plates gently with HPLC-grade water. Wipe the plate with 100% methanol using Kimwipes (*see* **Note 11**).
3. Spot 1 µl of α-cyano-4-hydroxycinnamic acid matrix (Fluka Buchs SG, Switzerland) solution and 1 µl of a purified trypsin-digested sample onto each target spot of the MALDI target plate. Allow the samples to dry at room temperature.
4. In the bottom-most row of the MALDI target plate, spot 1 µl of α-cyano-4-hydroxycinnamic acid matrix solution and 1 µl of peptide calibration standard (Bruker).
5. After the spotting is completed, reseal the 384-well trypsin-digested peptide plate (s) and store at –80°C. Make sure that all the spots on the MALDI target plate are dry before acquiring the spectra.

Acquiring Spectra from MALDI Target Plates

1. Double click on the flex control icon of an ULTRAFlex II MALDI-TOF instrument. When the log on information appears, Click OKAY. Open the FLEX control method: RP_ pepmix.par. Wait for the method to upload.

2. Insert the spotted MALDI target plate into the source chamber and click on the green load button on the instrument.
3. Wait until the system fully loads the MALDI plate. Once the plate is loaded, the system status light on the FLEX control should turn green and say "READY." The sample status light should turn green and say "IN."
4. The spectrum is acquired by clicking the start button and adjusting the laser intensity so that the peptide peaks can be visualized.
5. Click on "Add" to the sum buffer.
6. Click on the "calibration tab" and choose "peptidemix monoisotopic II" from the drop down menu, and a peptide reference list will appear.
7. Change the zooming factor to 0.5.
8. To manually calibrate the instrument, click on each peptide in the reference list. The red vertical line indicates the corresponding peptide. Click on the monoisotopic peak (left of the highest peak) to visualize each of the peptides.
9. Click on "accept fit result"; now the instrument is calibrated.
10. To automatically acquire the samples, click on the AutoXecute tab. Load the.txt file by clicking on the select button. Make sure that the method to be run is "autoXYMP." Check the box where it shows the autoX output.
11. Click on "START automatic run" and the system should automatically start acquiring the spectra.

MALDI-TOF Spectral Analysis and Protein Identification

1. MALDI spectral searches are performed using Knexus automation, a Windows-based program from Genomics Solutions Bioinformatics (Discovery Scientific, Inc., Vancouver, Canada) that selects the spectral peaks automatically and performs the searches.
2. After the installation of the Knexus program, the most recent Fasta sequences of the *S. cerevisiae* genome downloaded from the European Bioinformatics Institute database (http://www.ebi.ac.uk/) were uploaded into the machine using the Knexus Database Installation Wizard.
3. Protein identification is done using the ProFound search engine, which matches the observed peaks against the database of theoretical peaks.
4. The program runs ProFound on all the spectra using one set of conditions. In addition, a Java program was developed in-house, which automates the rerunning of Knexus. It uses 72 varying parameter sets and evaluates the aggregate results to produce a set of identified proteins in each case. Based on these results, it calculates an aggregate score for each protein.

5. Using graphical interface software developed in-house, the user is able to specify where the bands are located on the corresponding gel image. The entire area of the band is inputted so that its total intensity is measured (*see* **Note 12**). When all the data has been entered into the system for a given band, an annotated gel image and a plot are produced in JPEG format. Examples of affinity-purified protein complexes identified by in-gel trypsin digestion and MALDI mass spectral analysis are shown in **Fig. 2**.

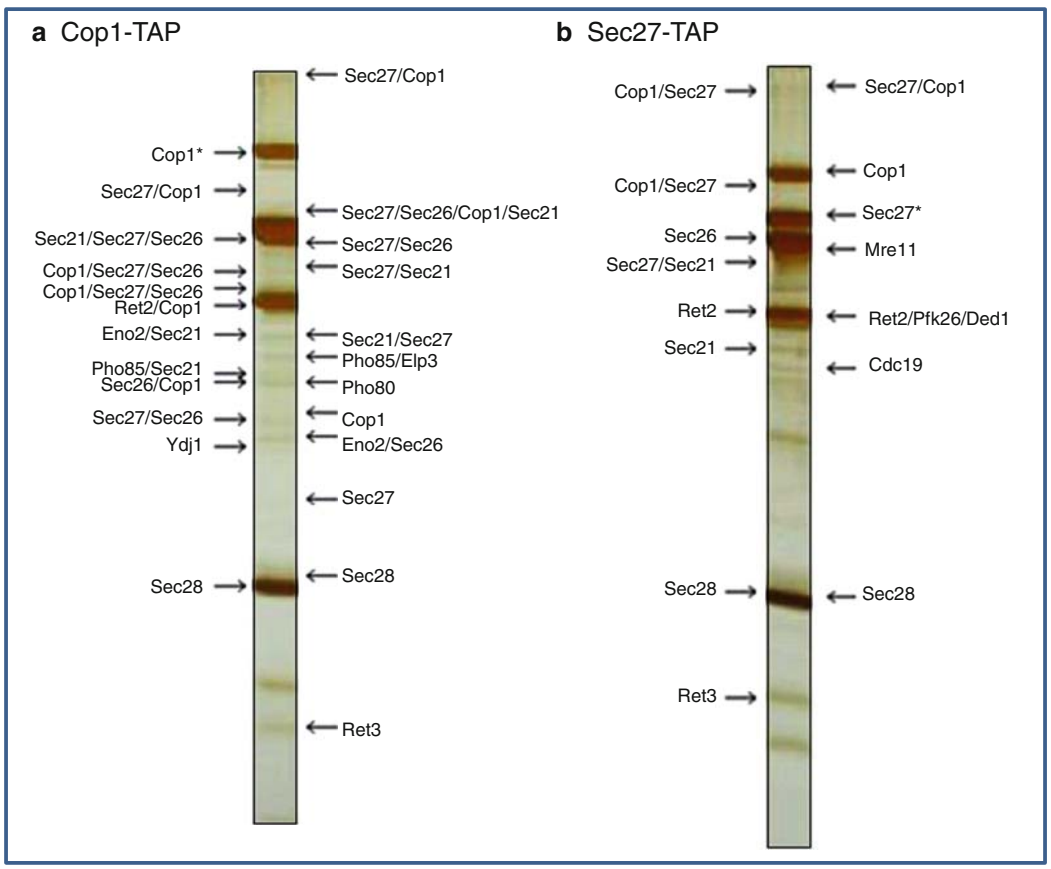

Fig. 2. Examples of silver-stained SDS polyacrylamide gel tracks containing COP1 coatomer protein complexes, which are involved in ER-to-Golgi and Golgi-to-ER transport, following affinity purification of *S. cerevisiae* TAP-tagged Cop1 (**a**) and Sec27 (**b**) proteins, respectively. Purifications of TAP-tagged Cop1 and Sec27 revealed other subunits of the COP1 coatomer protein complexes. The TAP-tagged bait proteins are specified at the top of each lane. Individual subunits of the purified complex and various co-purifying contaminants, all indicated by *arrowheads*, were identified by in-gel trypsin digestion followed by MALDI-TOF mass spectrometry. *Asterisks* designate the intact tagged bait protein recovered from each purification; proteolytic fragments of various coatomer subunits are also often identified. The known coatomer subunits include Cop1, Sec21, Sec26, Sec27, Sec28, Ret2, and Ret3. These purified preparations of coatomer complex also contained small amounts of the contaminating proteins Eno2, Pho80, Pho85, Elp3, Ded1, Mre11, Ydj1, Pfk26, and Cdc19.

3.3. Protein Identification by Gel-Free Liquid Chromatography-Tandem Mass Spectrometry

3.3.1. Proteolysis and Sample Preparation for LC-MS/MS

1. Three hundred µl eluted fractions (described in **step 10** in "Calmodulin-Sepharose Beads") in microcentrifuge tubes were dried down using a Speedvac.
2. The dried down samples are dissolved in 20 µl of the immobilized trypsin solution. Make sure to dissolve all the dried down sample in the immobilized trypsin solution. Using a P1000 pipette tip, pipette the samples up and down.
3. The samples are incubated overnight at 30°C with rotation or agitation.
4. Add 20 µl of the LC-MS solvent A to stop digestion.
5. The peptide mixtures are centrifuged for 5 min at maximum speed using an Eppendorf centrifuge.
6. Carefully transfer 10–20 µl of the supernatants containing the peptide mixtures into fresh microcentrifuge tubes and immediately analyze by LC-MS, or store the samples at –20°C prior to use.

3.3.2. LC-MS Spectral Analysis and Protein Identification

1. Protein samples are analyzed using single-dimension reverse phase chromatography coupled online to ion trap tandem mass spectrometry using standard conditions.
2. The microcolumns are packed with ~10 cm of 5-µm Zorbax eclipse XDB-C_{18} resin and are interfaced to a custom electrospray ion source.
3. The packed microcolumn is placed in line with the LC-MS instrument.
4. A Proxeon nano HPLC pump is used to deliver a stable tip flow rate of ~300 nl/min during the peptide separations.
5. Peptide elution is achieved using the following gradient which may be increased or decreased according to the complexity of the sample: 15% of solvent B from 0 to 30 min, 40% of solvent B from 31 to 49 min, 80% of solvent B from 50 to 55 min, 80% of solvent B from 56 to 60 min, 100% of solvent A from 61 to 65 min, 45% solvent B from 66 to 70 min, 80% solvent B from 71 to 75 min, 80% solvent B from 76 to 80 min, 100% solvent A from 81 to 85 min, 100% solvent A from 86 to 105 min. The flow rate at the tip of the needle is set to 300 nl/min for 105 min.
6. The mass spectrometer cycles run through successive series of 11 scans as the gradient progresses. The first scan in a series is a full mass scan and is followed by successive tandem mass scans of the most intense ions.
7. Proteins from the mixture are identified using the SEQUEST computer algorithm *(23)* and validated using the STATQUEST *(24)* probabilistic scoring program (*see* **Note 13**).

4. Notes

1. A similar TAP strategy is being used to purify the membrane-associated proteins except that all the purification steps are performed in the presence of a nonionic detergent.
2. Although low-abundance proteins can be detected using highly sensitive mass spectrometers, it is important to start with enough cells for the purification. This can be achieved by noting the expression level of the tagged protein *(3)* or by examining the literature to identify conditions that are suitable for the preparation of cell extracts specifically for the target protein. It is possible in some cases to successfully purify and identify by mass spectrometry a tagged protein that is not detectable by Western blotting.
3. If using the same coffee grinder to grind multiple protein samples, make sure not to clean the grinder with water because it will freeze. Use wipes and paper towels to clean the grinder.
4. Make sure to mix the YEB buffer with the lysed powder until it thaws. The tube is then inverted several times at room temperature to ensure proper mixing.
5. In some cases, however, it is necessary to use a higher salt concentration to reduce nonspecific binding of proteins.
6. It is not mandatory to perform the dialysis step in the preparation of extracts.
7. Make sure to elute the samples with calmodulin elution buffer, as it is a very critical step in releasing the complexes from the resin. Yields of the bound proteins can sometimes be improved either by increasing the salt concentration or by increasing the EGTA concentration in the calmodulin elution buffer.
8. When the developing solution becomes cloudy, replace it with new solution. Make sure to develop all the gels for about the same time.
9. The gel slices will become white and should feel gritty like grains of sand. Make sure to remove all the liquid when the gel slices are completely shrunk.
10. Make sure that in each well there is enough liquid for the gel slices to be completely submerged.
11. If the spots still remain on the MALDI target plate, sonicate the plate for 10 min with 100% methanol followed by 5 min sonication with HPLC water and 100% methanol, respectively.

12. The band location algorithm tries to identify the complete area of each band. Make sure to confirm that the location of the band that the computer identifies matches the band in the gel image.
13. The acquired spectra are used to search the *S. cerevisiae* protein database downloaded from the European Bioinformatics Institute (http://www.ebi.ac.uk/) using the SEQUEST search algorithm ***(23)*** to identify the proteins from which they originated. Confidence scores for all putative matches were evaluated and assigned using a probabilistic STATQUEST ***(24)*** scoring program. Proteins detected with two or more high-confidence peptide matches and with a minimum likelihood threshold cut-off of 90% or greater probability were considered as a positive identification.

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