

Chapter 22

Sequential Peptide Affinity Purification System for the Systematic Isolation and Identification of Protein Complexes from *Escherichia coli*

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Summary

Biochemical purification of affinity-tagged proteins in combination with mass spectrometry methods is increasingly seen as a cornerstone of systems biology, as it allows for the systematic genome-scale characterization of macromolecular protein complexes, representing demarcated sets of stably interacting protein partners. Accurate and sensitive identification of both the specific and shared polypeptide components of distinct complexes requires purification to near homogeneity. To this end, a sequential peptide affinity (SPA) purification system was developed to enable the rapid and efficient isolation of native *Escherichia coli* protein complexes (J Proteome Res 3:463–468, 2004). SPA purification makes use of a dual-affinity tag, consisting of three modified FLAG sequences (3X FLAG) and a calmodulin binding peptide (CBP), spaced by a cleavage site for tobacco etch virus (TEV) protease (J Proteome Res 3:463–468, 2004). Using the λ -phage Red homologous recombination system (PNAS 97:5978–5983, 2000), a DNA cassette, encoding the SPA-tag and a selectable marker flanked by gene-specific targeting sequences, is introduced into a selected locus in the *E. coli* chromosome so as to create a C-terminal fusion with the protein of interest. This procedure aims for near-endogenous levels of tagged protein production in the recombinant bacteria to avoid spurious, non-specific protein associations (J Proteome Res 3:463–468, 2004). In this chapter, we describe a detailed, optimized protocol for the tagging, purification, and subsequent mass spectrometry-based identification of the subunits of even low-abundance bacterial protein complexes isolated as part of an ongoing large-scale proteomic study in *E. coli* (Nature 433:531–537, 2005).

Key words: Affinity purification, Protein complex, *E. coli*, SPA-tagging, λ -Red recombination, Mass spectrometry, LC-MS, MALDI-TOF

1. Introduction

Since virtually all cellular processes involve physical associations between proteins, it is of immense importance to systematically map protein-protein interaction (PPI) networks as a means of investigating the mechanistic basis of biological systems. Large-scale PPI networks were initially described for *Saccharomyces cerevisiae* using yeast two-hybrid analysis (1, 2), and, more recently and far more comprehensively, by purifying soluble stable protein complexes on a global scale using the tandem affinity purification (TAP) approach (3, 4), wherein the tagged proteins are expressed under normal physiological conditions and then purified $\sim 10^6$ -fold via a two-step enrichment procedure. The flexible and robust TAP method was subsequently adapted for use in flies, worms, and human cells (5–8). Our group has developed the sequential peptide affinity (SPA) system, a variant of TAP in which a smaller 3X FLAG tag is substituted for the original protein A moiety and the system is optimized for creating C-terminal fusions via recombining in suitably engineered bacterial strains (9). Since interacting proteins usually function in the same biological process, computational assessment of protein complexes and PPI networks provides a rational framework for systematically inferring the biological functions of uncharacterized proteins (guilt by association).

We have used SPA tags to purify hundreds of tagged proteins and their associated protein partners from *Escherichia coli*, a model microbe well suited to genomic investigations of the fundamental physiology and conserved molecular pathways of prokaryotes. As of April 2009, more than 2,000 *E. coli* open reading frames (ORFs) have been SPA-tagged using this system, with over 1,800 of these proteins purified successfully, together with their endogenous interacting partners, as judged by successful detection by mass spectrometry. An extensive PPI network, consisting of highly conserved and essential protein complexes, was reported previously (10). The entire collection of SPA-tagged strains has now been made commercially available for unfettered academic use (<https://www.openbiosystems.com/GeneExpression/NonMammalian/Bacteria/EcoliTaggedORFs/>). Recently we performed an extensive proteomic survey using ~ 2000 affinity-tagged *E. coli* strains, including proteins of unknown function, and generated comprehensive genomic context inferences to derive a high-confidence putative physical interactions, most of which are novel (11). Membrane-associated protein complexes are far more difficult to purify, however, and specialized methods for these proteins are currently under development.

SPA purification utilizes dual affinity tags, 3X FLAG, and a calmodulin binding peptide (CBP), separated by a tobacco etch virus (TEV) protease cleavage site ([9], Fig. 1a), to allow for highly selective two-stage protein enrichment. We chose to use the 3X FLAG affinity tag for three reasons: first, the FLAG epitope has been widely used for tagging, monitoring, and purifying proteins from various organisms; second, there are far fewer amino acid residues (22 residues) in 3X FLAG than the 137 residues in the protein A moiety of the original TAP tag, which lessens the chance of functional perturbation and misfolding/instability of certain tagged proteins; finally, 3X FLAG is sensitively detected with a high-affinity monoclonal antibody, M2, which is commercially available and known to work well in Western blots and immunoprecipitation experiments (12).

Homologous recombination is used to target the SPA-tag construct into chromosomal loci of interest so as to produce C-terminal fusions without perturbing the nearby transcriptional promoter or terminator sequences of the targeted bacterial operon. Sequence-specific linear PCR products encoding the affinity purification tag and a selectable marker are transformed into the cell and undergo recombination at the stop codon of the target gene in the chromosome of the lysogenic *E. coli* strain DY330, which harbors the highly efficient λ -phage-encoded homologous recombination enzymes *exo*, *bet*, and *gam* of the “Red” system under the control of the temperature-sensitive *CI857* repressor ([13], Fig. 1b). Strains in which the PCR product has integrated are subjected to antibiotic selection, and tagged protein expression is confirmed by Western blotting. The tagged bait proteins are then isolated by affinity purification (Fig. 1c) from crude whole cell lysates prepared from cells harvested from large-scale cultures. Two complementary and highly sensitive mass spectrometry methods are used to ensure identification of co-purifying interacting proteins: gel-free liquid chromatography–tandem mass spectrometry (LC-MS), and gel-based peptide mass fingerprinting using matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry (MALDI-TOF).

In this chapter, we describe detailed step-by-step procedures for effective tagging of individual *E. coli* ORFs, purification of the resulting bait proteins, and subsequent identification of interacting protein partners by mass spectrometry methods. While this method has been expressly optimized for a well-characterized *E. coli* laboratory strain, we note that the basic approach can potentially be adapted for use in any other closely related strain or other bacteria that can be cultured under standard laboratory conditions.

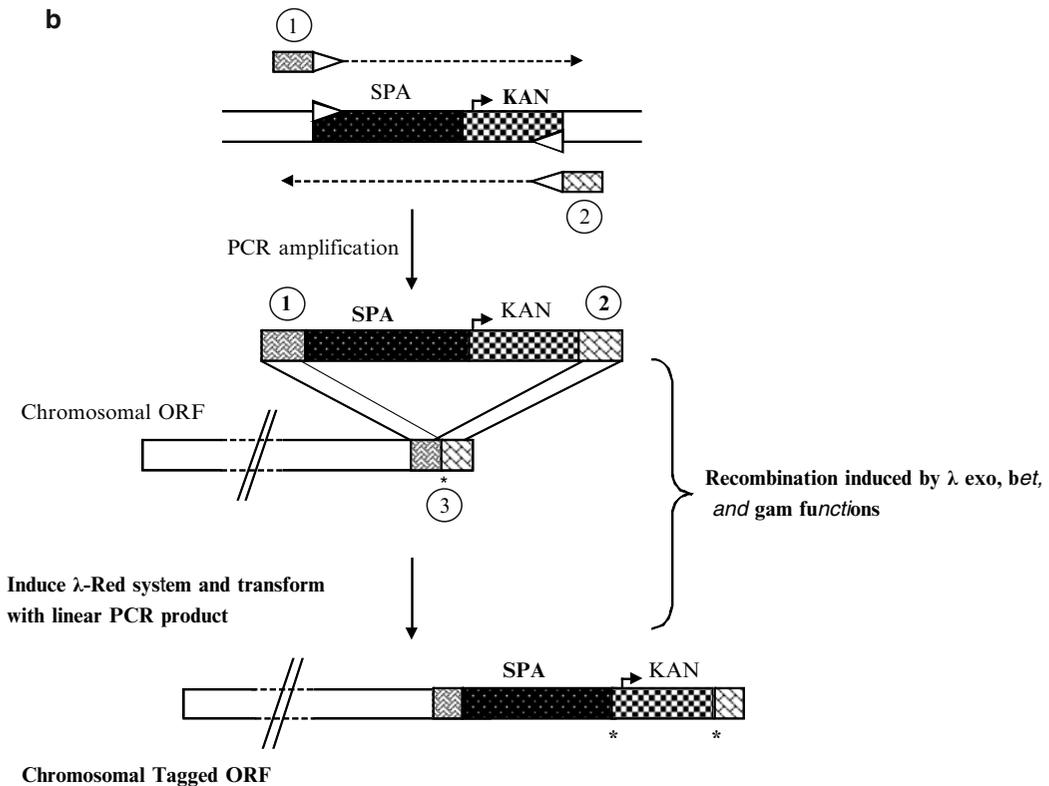
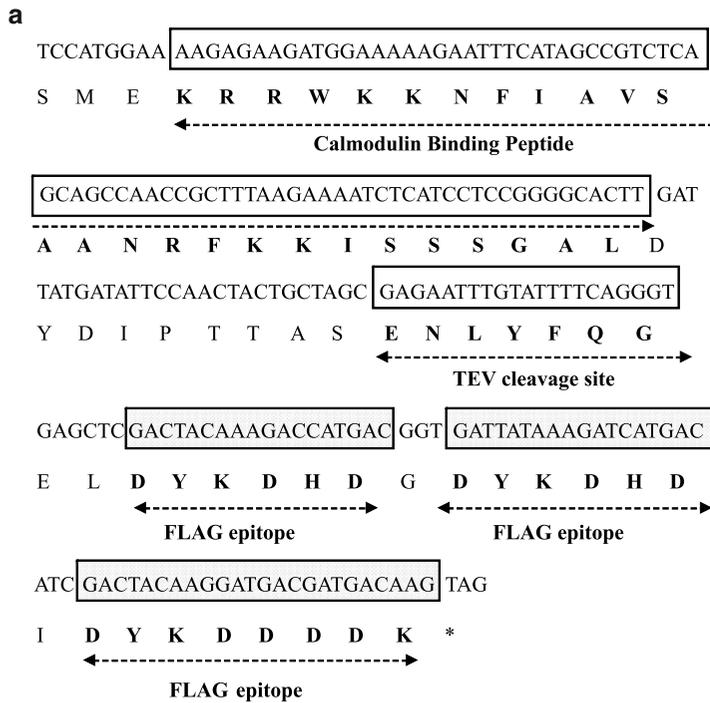


Fig. 1. (a) Schematic representation of the sequence of the SPA-tag. The asterisk represents a stop codon. (b) Gene-specific affinity-tagging cassettes produced by the polymerase chain reaction (PCR) using primers (9,13) homologous to the regions on either side of the target gene translational termination codon (10) were integrated into the *E. coli* chromosome using the λ -Red recombination system. SPA, Sequential Peptide Affinity system; Kan, Kanamycin resistance cassette; *, stop codon.

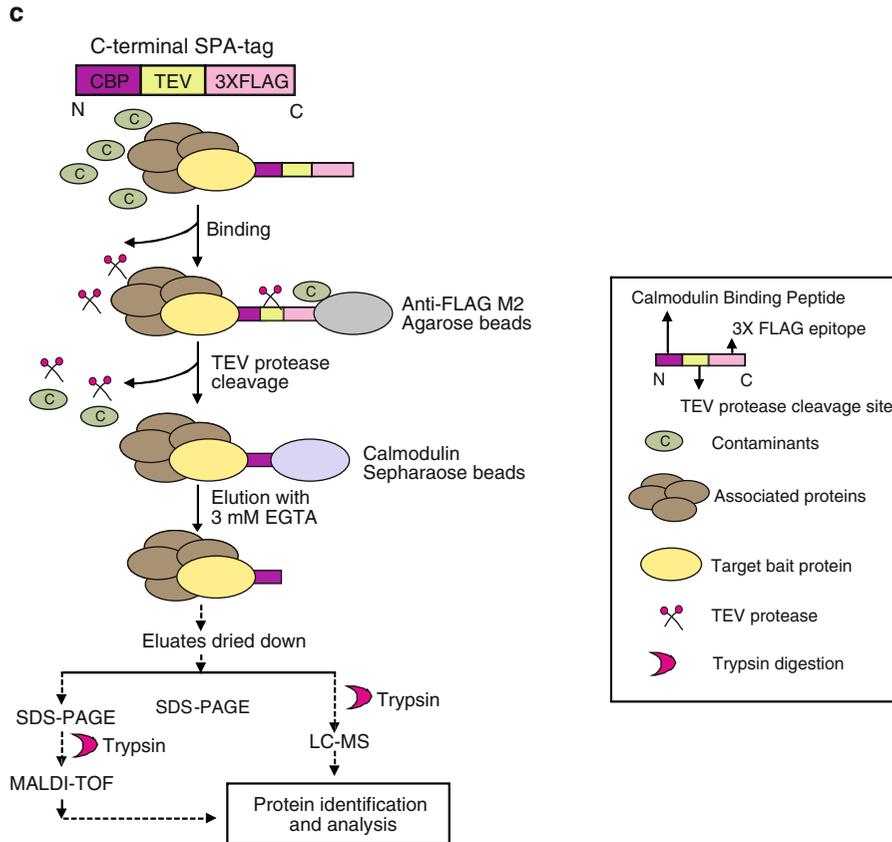


Fig. 1. (continued) (c) Overview of the SPA-tag affinity purification procedure.

2. Materials

2.1. SPA Vector Construction and Gene-Specific SPA-Tagging in *E. coli*

1. The plasmid pJL148 was created by replacing the TAP tag from the plasmid pJL72 (14) with the SPA-tag from the vector pMZ13F (14).
2. All the oligonucleotides used in this study were synthesized by Invitrogen Canada Inc. (Burlington, Ontario).
3. Restriction enzymes were from New England BioLabs Ltd. (Pickering, Ontario).
4. Isolation of plasmid DNA or purification of PCR products was performed using Qiagen products (Qiagen Inc., Mississauga, Ontario).
5. One molar Tris-HCl (pH 7.5) stock solution: Dissolve 121.1 g of Tris base in 800 mL of double distilled water, adjust to pH 7.5 with concentrated HCl and make up the volume to 1 L.

2.2. Induction of λ -Red Recombination Functions, and Preparation and Transformation of Competent *E. coli* Cells

6. EDTA (0.2 M, pH 8.0) stock solution: Dissolve 74.44 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.
1. Luria-Bertani (LB) Medium: Solid medium is prepared by dissolving 25 g of LB powder (Bioshop cat#LBL405) and 20 g of agar (Bioshop cat#AGR003) in 1,000 mL of distilled water. Liquid LB medium is prepared without agar.
 2. The Red-expressing *E. coli* strain DY330 was a kind gift from Donald L.Court (National Cancer Institute, Frederick, MD 21702).
 3. SOC medium was obtained from Invitrogen Inc. (Invitrogen cat#15544-034).
 4. Sample stock buffer (3X): Prepare 10 mL of 3X sample stock buffer containing 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 cat#161-0404).
 5. To prepare 30 mL of 1X sample buffer, add 9 mL of 3X sample stock buffer, 1 mL of β -mercaptomethanol, and 20 mL of distilled water.
 6. Kanamycin (Sigma cat# 60615) antibiotic stock: Dissolve 100 mg/mL of kanamycin in double distilled water and filter-sterilize using a 0.22 μ m millipore filter. The filter-sterilized kanamycin stock solution is stored in single use aliquots at -20°C .

2.3. SDS-Polyacrylamide Gel Electrophoresis

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 (5X): [125 mM Tris-HCl, 960 mM glycine, 0.5% (w/v) SDS, pH 8.3]. Thirty gram 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 cat#161-0125). The solution is filtered through Whatman No.1 filter paper (Fischer Scientific cat#09-806A) and stored at 4°C in the dark.
4. Separating buffer (4X): 1.5 M Tris-HCl (pH 8.7), 0.4% SDS. Store at room temperature.
5. Stacking buffer (5X): 0.5 M Tris-HCl (pH 6.8), 0.4% SDS. Store at room temperature.
6. Ammonium persulfate (APS): Prepare 20% APS (Bioshop cat#AMP001) solution by mixing 2 g of APS in 10 mL of double distilled water. Aliquot the solution into several microcentrifuge tubes in a volume of 200 μ L. Store the aliquots at -20°C .

7. Water saturated *n*-Butanol: Mix equal volumes of *n*-butanol (Sigma Aldrich cat#B7906) and water in a glass bottle and leave it for a while to separate. Use topmost layer containing *n*-butanol saturated with water. Store the solution at room temperature.
8. Resolving gel (12.5%): Prepare 1.5 mm thick, 12.5% polyacrylamide resolving gel by mixing 7.2 mL of 4X separating buffer with 12 mL acrylamide solution, 9.6 mL double distilled water, 100 μ L 20% APS solution, and 20 μ L TEMED (Bioshop cat#TEM001).
9. Stacking gel (7.5% polyacrylamide): Prepare the stacking gel by mixing 4 mL of 5X stacking buffer with 2.4 mL acrylamide solution, 9.6 mL double distilled water, 100 μ L 20% APS solution, and 10 μ L TEMED.

2.4. Confirmation of SPA-Tagging in Transformants Using Western Blotting with Chemiluminescent Method

1. Trans-Blot[®] Cell system (Bio-Rad cat#170-3853) was used to transfer the protein samples from the SDS-polyacrylamide gels to nitrocellulose membranes (Bio-Rad cat#162-0115).
2. Transfer stock buffer: Prepare 10X transfer stock buffer with 120 g Tris-HCl and 576 g glycine in distilled water.
3. To prepare 1X transfer buffer, dilute 400 mL of 10X transfer stock buffer with 800 mL methanol and 2800 mL of sterile distilled water. Store the buffer at room temperature.
4. TBS buffer: Prepare 5X TBS stock buffer with 48.44 g Tris and 584.4 g NaCl.
5. Wash buffer: TBS buffer containing 0.05% Tween-20.
6. Blocking buffer: 5% (w/v) non-fat dry milk in TBS buffer containing 0.1% Tween-20.
7. Primary antibody: M2 antibody (Sigma cat#F3165) against the FLAG epitopes of the SPA-tag.
8. Primary antibody buffer: TBS buffer supplemented with 1% gelatin, 0.05% Tween-20, and 0.02% NaN₃.
9. Secondary antibody: Antimouse IgG conjugated to Horseradish Peroxidase (HRP; Amersham Biosciences cat#NA931V).
10. Secondary antibody buffer: 5% (w/v) non-fat dry milk in TBS buffer containing 0.1% Tween-20.
11. Chemiluminescence reagent (PIERCE cat#1856136) is prepared (0.125 mL of chemiluminescence reagent per cm² of membrane) by mixing equal volumes of the enhanced luminol reagent and the oxidizing reagent.
12. Kodak X-OMAT autoradiography film (Clonex Corp., cat#CLEC810).

13. Stripping buffer: 62.5 mM Tris-HCl (pH 6.7), 2% (w/v) SDS, and 100 mM β -mercaptoethanol. Store the buffer at room temperature.

2.5. SPA-Tagged Protein Purification

2.5.1. Culturing SPA-Tagged E. coli Strains and Sonication

1. *E. coli* strains are used for culturing in which a bait protein is SPA-tagged.
2. Terrific Broth (TB) medium is prepared by dissolving 11.07 g of tryptone (Bioshop cat#TRP402), 22.13 g of yeast extract (Bioshop cat#YEX401), and 30 mL of 50% glycerol in 830 mL of double distilled water.
3. Potassium salt stock solution: Dissolve 18.48 g of monobasic KH_2PO_4 and 100.24 g of dibasic K_2HPO_4 in 800 mL of double distilled water.
4. Sonication stock buffer is prepared by dissolving 9.6 mL of 2 M Tris-HCl (pH 7.9), 19.2 mL of 5 M NaCl, 384 μL of 0.5 M EDTA, and 192 mL of 50% glycerol in 738.2 mL of double distilled water. Store the stock solution at 4°C.
5. Sonication working buffer is prepared by mixing 300 mL of sonication stock buffer with seven protease inhibitor tablets (Roche cat#800-363-5887) and 150 μL of 0.5 M dithiothreitol (DTT; Bioshop cat# DTT001). Aliquot the sonication working buffer into polypropylene Falcon tubes (25 mL in each) and keep them refrigerated at 4°C.

2.5.2. Purification of SPA-Tagged Proteins

1. Protease inhibitor stock solution (25X): Dissolve 1 tablet of protease inhibitor (Roche cat#11 873 580 001) in 2 mL of sterile distilled water. This stock solution is stable for at least 12 weeks when stored at -15°C to -25°C .
2. Two molar 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.
3. Five molar NaCl stock solution: Dissolve 292.2 g of NaCl in 1 L of double distilled water.
4. Ten percent Triton X-100 solution from Sigma Aldrich (cat#93443) is stored at 4°C.
5. EDTA (0.5 M, 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. AFC buffer 10X stock solution: Prepare 40 mL of 10X AFC buffer stock solution containing 2 mL of 2 M Tris-HCl (pH 7.9), 8 mL of 5 M NaCl, 4 mL of 10% Triton X-100, and 26 mL of sterile distilled water.
7. To prepare 40 mL of 1X AFC working buffer, add 4 mL of 10X AFC buffer stock solution, 200 μL of 0.2 M DTT, 1.6 mL of protease inhibitor stock solution, and 34.2 mL of sterile distilled water.

8. TEV cleavage 10X stock buffer: Prepare 40 mL of 10X TEV cleavage buffer stock solution containing 10 mL of 2 M Tris-HCl (pH 7.9), 8 mL of 5 M NaCl, 160 μ L of 0.5 M EDTA, 4 mL of 10% Triton X-100, and 17.84 mL of sterile distilled water.
9. To prepare 25 mL of 1X TEV cleavage buffer, add 2.5 mL of 10X TEV cleavage stock buffer, 125 μ L of 0.2 M DTT, 1 mL of protease inhibitor stock solution, and 21.4 mL of sterile distilled water.
10. Calmodulin binding 10X stock buffer: Prepare 40 mL of 10X calmodulin binding stock buffer solution containing 2 mL of 2 M Tris-HCl (pH 7.9), 8 mL of 5 M NaCl, 800 μ L of 1 M CaCl_2 , 4 mL of 10% Triton X-100, and 25.2 mL of sterile distilled water.
11. To prepare 12 mL of 1X calmodulin binding buffer, add 1.2 mL of 10X calmodulin binding stock buffer, 8 μ L of β -mercaptoethanol, 480 μ L of protease inhibitor stock solution, and 10.32 mL of sterile distilled water.
12. Calmodulin wash 10X stock buffer: Prepare 10 mL of 10X calmodulin wash stock buffer containing 500 μ L of 2 M Tris-HCl (pH 7.9), 2 mL of 5 M NaCl, 10 μ L of 1 M CaCl_2 , and 7.5 mL of sterile distilled water.
13. To prepare 10 mL of 1X calmodulin wash buffer, add 1 mL of 10X calmodulin wash stock buffer, 7 μ L of β -mercaptoethanol, and 8.3 mL of sterile distilled water.
14. Calmodulin elution 1X stock buffer: Prepare 10 mL of 1X calmodulin elution stock buffer containing 50 μ L of 2 M Tris-HCl (pH 7.9), 1 mL of 1 M ammonium hydrogen carbonate (NH_4HCO_3), 150 μ L of 0.2 M EGTA, 7 μ L of 14 M β -mercaptoethanol, and 8.8 mL of sterile distilled water.
15. NH_4HCO_3 (1 M): Dissolve 0.395 g of NH_4HCO_3 in 5 mL sterile distilled water. Make sure to use freshly prepared NH_4HCO_3 solution each time.

2.6. Protein Identification by Gel-Based Peptide Mass Fingerprinting Using Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry

2.6.1. Silver Staining the SDS-Polyacrylamide Gel

1. Fixer: 50% methanol and 10% acetic acid (AA) in 500 mL of sterile distilled water.
2. Sensitizer: Prepare 20 mg of fresh sodium-thiosulfate (Sigma cat#S-7143) in 1,000 mL of distilled water.
3. Silver nitrate solution: Dissolve 2 g of silver nitrate (Fischer Scientific cat#S181-100) in 1,000 mL of distilled water.
4. Developing solution: Add 1.4 mL of 37% formaldehyde and 30 g of sodium carbonate to 1,000 mL of distilled water. Avoid using glutaraldehyde as it might crosslink the proteins to the gel.
5. Stop solution: Dilute 5 mL of AA in 500 mL of distilled water.

2.6.2. Gel-Based MALDI-TOF Mass Spectrometry

1. ULTRAFlex II MALDI-TOF instrument (Bruker Daltonics, Billerica, MA) to run the samples and acquire spectral data.
2. MALDI target plate from Bruker (Bruker, cat#209519).
3. Bulk C18 reverse phase resin from Sigma (cat#H8261).
4. Knexus automation, a Windows-based program from Genomics Solutions Bioinformatics to perform automatic protein searches and to evaluate MALDI spectral results.
5. α -Cyano-4-hydroxycinnamic acid matrix solution (Fluka Buchs SG, Switzerland cat# 28480).
6. One millimolar HCl: Dilute 1 μ L of 10 N HCl into 10 mL HPLC grade water. Solution is made fresh each day and stored on ice until use.
7. Trypsin stock solution: Dissolve 100 μ g Boehringer Mannheim unmodified sequencing grade trypsin (Roche cat#1047 841) in 1 mL of 1 mM HCl. Store the trypsin stock solution at -80°C .
8. Digestion buffer: Add 9.12 mL of 100 mM NH_4HCO_3 , 9.12 mL of HPLC grade water, 960 μ L of 1% CaCl_2 , 1 mL of trypsin stock solution. Prepare the solution fresh each day and store on ice until use.
9. One hundred millimolar NH_4HCO_3 : Dissolve 0.79 g of NH_4HCO_3 in 100 mL of HPLC grade water.
10. Ten milliliter of 100 mM NH_4HCO_3 containing 10 mM DTT is prepared by adding 100 μ L of 1 M DTT to 9.9 mL of 100 mM NH_4HCO_3 . Solution is prepared fresh each day and stored in amber colored bottle at room temperature in dark until use.
11. Ten milliliter of 100 mM NH_4HCO_3 containing 55 mM iodoacetamide (Sigma cat#16125) is prepared by adding 0.103 g of iodoacetamide to 10 mL of 100 mM NH_4HCO_3 . Solution is prepared fresh every day and stored in amber colored bottle at room temperature in dark until use.
12. Sixty-six percent acetonitrile (ACN), 1% AA: 66 mL HPLC grade ACN (Sigma cat#A998-4), 33 mL of HPLC grade water, 1 mL AA. Store the solution in a glass bottle for up to 1 month at room temperature.
13. Seventy-five percent ACN, 1% AA: 75 mL HPLC grade ACN, 24 mL HPLC grade water, 1 mL AA. Store the solution in a glass bottle for up to 1 month at room temperature.
14. Two percent ACN, 1% AA: 2 mL of HPLC grade ACN, 97 mL HPLC grade water, 1 mL AA. Store the solution in a glass bottle for up to 1 month at room temperature.
15. One percent 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. Trifluoroacetic acid (TFA) (0.1%) stock solution: Dilute 0.25 μL of TFA in 24.75 μL of HPLC grade water.
17. Peptide calibration standard (Bruker cat#206195) solution: Dissolve the lyophilized peptide standards with 125 μL of 0.1% HPLC grade TFA.

2.6.3. Gel-Free Liquid Chromatography–Tandem Mass Spectrometry

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 buffer: Prepare the digestion buffer by mixing 50 mM NH_4HCO_3 and 1 mM CaCl_2 . Store the solution at -4°C prior to use.
3. Immobilized trypsin solution: Add 18.7 μL of digestion buffer, 1.8 μL PIERCE immobilized trypsin beads (PIERCE cat#20230), 0.9 μL immobilized trypsin beads (Applied Biosciences cat# 2-3127-00) and 0.06 μL of 1 M CaCl_2 . Make sure that pH of the immobilized trypsin solution is ~ 8.0 .
4. One hundred and fifty micrometer fused silica (Polymicro Technologies, Phoenix, AZ, USA).
5. C18 reverse phase packing material (Zorbax eclipse XDB-C18 resin) from Agilent Technologies (Mississauga, ON, Canada).
6. Solvent A: 5% ACN, 0.5% AA, and 0.02% Heptafluorobutyric acid (HFBA; Sigma Aldrich cat#77247).
7. Solvent B: 100% acn.
8. Proxeon nano HPLC pump (Proxeon Biosciences).

3. Methods

3.1. SPA Vector Construction and Gene-Specific SPA-Tagging in *E. coli*

3.1.1. E. coli SPA Vector Construction

1. All amplifications were performed using Bio-Rad PCR system (Bio-Rad, cat#170-8703). Following are the PCR cycling conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 68°C for 2 min 10 s, followed by 68°C for 10 min.
2. The kanamycin-resistance (Kan^{R}) cassette was PCR-amplified from the plasmid pKD4 (12) with primers ECTAP-Forward (5'-CTAGATATCGTGTAGGCTGGAGCTGCTTC-3') and ECTAP-Reverse (5'-CGCGGGCCCATATGAATATCCTCCTTAGTTC-3').
3. The amplified PCR product was cut with restriction enzymes EcoRV and ApaI and ligated into the plasmid pBS1479 (15) containing the TAP-tag cassette. The resulting plasmid was

named as pJL72 and contains the TAP-tag and the “Kan^R” marker for selection in *E. coli* (see **Note 1**).

4. The SPA-tag was amplified (using the above PCR conditions) from the plasmid pMZ13F (**I4**) by PCR using the ECSPA-Forward (5'-CGCGGATCCATGGAAAAGAGAA-GATGGAA-3') and ECSPA-Reverse (5'-CTAGATATCTC-CCCTCAGGTCACACTTGT-3') primers.
5. The amplified SPA-tag product was then cut with restriction enzymes BamHI and EcoRV and ligated into the plasmid pJL72 by replacing the TAP-tag with the SPA-tag between the sites 2,205 and 2,433. The resulting ~4.5 kb construct was designated as pJL148. In all cases, PCR-amplified products were purified using the Qiaquick PCR purification kit (Qiagen).

3.1.2. Gene-Specific SPA-Tagging in *E. coli*

1. The plasmid pJL148 construct enabled the cloning of the SPA-tag downstream of the target gene and production of the target protein as a C-terminal SPA fusion.
2. To increase the efficiency of recombination, we used the SPA template instead of the plasmid pJL148 as DNA template in PCR reactions. To generate the SPA template, the 27 bp SPA-Forward (5' AGCTGGAGGATCCATGGAAAAGAGAAG 3') and 27 bp SPA-Reverse (5' GGCCCCATATGAATATCCTCCTTAGTT 3') primers homologous to the 5' and 3' end of the SPA cassette with ~10 bp of the vector sequences, respectively, were designed to amplify the SPA-tag. The tag was amplified by PCR using the cycling conditions shown in **Subheading 3.1.1, step 1**.
3. The amplified PCR products were purified using the Qiaquick PCR purification kit (Qiagen).
4. The purified PCR products were run on a 1% DNA agarose gel at 100 V for 1 h. The 1.7 kb amplified SPA cassette was gel-extracted and purified using the Qiagen purification kit. The purified SPA-tag amplicon was suspended in 200 µL of sterile water or TE buffer (10 mM Tris-HCl, pH 7.5/1 mM EDTA) and quantified by spectrophotometry (Fisher Scientific, cat#S42669ND). The purified SPA-tag amplicon was stored at -20°C and served as a template for the gene-specific SPA-tagging.
5. In each case, we used a gene-specific forward primer containing 45 bp of nucleotide sequence immediately upstream of the target gene stop codon (variable region forward, VF) in frame with the tag-specific TF (5'-TCCATGGAAAAGAGAAG-3') constant priming region, and a gene-specific reverse primer containing 45 bp of nucleotide sequence immediately downstream of the target gene stop codon

(variable region reverse, VR) in frame with the tag-specific TR (5'-CATATGAATATCCTCCTTAG-3') constant priming site. The primer pairs VF-TF and VR-TR were used to amplify the SPA cassette and Kan^R resistance gene flanked by gene-specific 45 bp sequences. These sequences are identical to regions flanking the target gene stop codon and are substrates for the λ -Red homologous recombination machinery.

6. The PCR product was purified using a Qiaquick PCR purification kit (Qiagen) to remove salts that might interfere with electroporation.
7. The purified PCR product was then introduced by electroporation into *E. coli* strain DY330, in which the λ -Red system was induced (*see Subheading 3.2.1* for details).
8. After transforming the linear PCR product, cells which had recombined the tag into the chromosome were selected by their resistance to kanamycin, and were screened for the expression of a tagged fusion protein by Western blot.
9. Strains expressing fusion proteins with C-terminal tags were then cultured on a larger scale to allow isolation of potential protein complexes by SPA purification.

3.2. Induction of λ -Red Recombination Functions, Preparation and Transformation of the Competent *E. coli* Cells

*3.2.1. Induction of λ -Red Recombination Functions and Preparation of competent *E. coli* cells*

1. The Red expressing strain DY330 was grown overnight in 2 mL of LB medium at 32°C with shaking at 180 rpm.
2. Inoculate 1.4 mL of the overnight culture into 70 mL fresh LB in a 500 mL conical flask. The inoculum was grown at 32°C with shaking at 180 rpm until OD₆₀₀ reached 0.5 to 0.8.
3. Induction was performed by transferring the culture into a 250 mL conical flask. The flask was incubated in a water bath at 42°C by gently shaking at 200 rpm for 15 min.
4. Immediately after the 15 min induction, the flask was incubated in an ice water slurry bath for at least 30 min with gentle shaking.
5. The cooled 70 mL culture was poured into pre-chilled 50 mL polypropylene tubes and the culture was centrifuged at $3,993 \times g$ for 6 min at 4°C.
6. The cell pellets were resuspended in 50 mL ice-cold sterile water and centrifuged once again at $3,993 \times g$ for 6 min at 4°C. **Subheading 3.2.1, step 6** was repeated one more time.
7. The cell pellets were resuspended in 1 mL of cold water, transferred to a 1.5 mL Eppendorf tube, and centrifuged at maximum speed for 20 s at 4°C.
8. After this series of washing steps, the cell pellets were finally resuspended in 700 μ L of ice-cold sterile water (*see Note 2*).

3.2.2. Cell Transformation and Selection Procedure

1. Mix 1 μL (~100 ng) of the purified linear donor DNA with 40 μL of the electro-competent cells in Eppendorf tubes. Incubate the cells on ice for 5 min. Transfer the mixture into a 0.2 cm precooled electroporation cuvette.
2. Place the cuvette in the cuvette holder and electroporate the cells with the DNA in a Bio-Rad GenePulser® II electroporator set at 2.5 kV, 25 μF with pulse controller of 200 Ω .
3. Immediately, add 800 μL of SOC medium to the cells in the cuvette and mix the contents by gently pipetting up and down. Transfer the cell contents from the cuvette into a 12 mL culture tube.
4. Incubate the transformed cells at 32°C with shaking at 190 rpm for 1–2 h prior to the selection of recombinants.
5. After 1–2 h of shaking, centrifuge at $3,000 \times g$ for 3 min. Remove the excess SOC and mix the cells gently by pipetting up and down 3–4 times. One hundred microliters of the transformed cells are spread onto the LB agar plates containing 50 $\mu\text{g}/\text{mL}$ kanamycin and are incubated overnight at 32°C to obtain Kan^R colonies.

3.2.3. Confirmation of Recombinants

1. Two colonies were randomly picked from each transformed plate.
2. A single transformed colony was inoculated into 2 mL LB medium containing 50 $\mu\text{g}/\text{mL}$ kanamycin and grown overnight at 32°C with shaking at 190 rpm.
3. One milliliter of the overnight culture was centrifuged for 30 s at $15,900 \times g$. The cell pellet was resuspended in 200 μL of 1X SDS sample buffer and boiled for 5 min.
4. Load 20 μL of the cell pellet resuspended in 1X SDS sample buffer onto a 12.5% SDS gel. After electrophoresis, immediately transfer the gel onto a nitrocellulose membrane and perform Western blotting.
5. If the Western blotting result indicates successful tagging of the target bait protein, add 1 mL of 50% glycerol to the leftover 1 mL of overnight culture, and store it in a cryogenic tube in –80°C.

3.3. SDS-Polyacrylamide Gel Electrophoresis

1. The following steps were performed using the Mini Protean 3 Cell (Bio-Rad cat# 165-3301) gel system. Make sure the glass plates, spacers, and combs are clean and free of dried gel fragments, grease, and dust.
2. For each gel, lay one small and one large glass plate, separated by spacers and an alignment card. Make sure to place the small glass plate on top of the spacers so that both sides and the bottom of the plates and the spacers are even.

3. Slide glass plates into the holder without tightening the screws. Make sure the glass plates are pushed all the way to the bottom before tightening the screws. If the assembly is correct, then the whole setup should snap into place when placed above the gray gasket.
4. Remove the alignment card. Now slide in the comb and mark a line at 2-3 cm from the bottom of the comb. Make sure that the comb thickness is the same as that of the spacers.
5. Carefully remove the comb. Pour the 12.5% polyacrylamide resolving gel up to the marked line. Overlay a thin layer of water-saturated *n*-butanol on the top of the gel. Allow the gel to polymerize for about 30 min.
6. Invert the gel to remove the *n*-butanol. Touch with filter paper to wick off residual liquid.
7. Pour the 7.5% polyacrylamide stacking gel to fill the remaining space between the glass plates. Insert the comb and allow the gel to polymerize for another 30 min.
8. Once the stacking gel has set, carefully remove the comb and use a 3-mL syringe fitted with a 22-gauge needle to wash the wells with running buffer.
9. Place the gel in the unit and add running buffer to the upper and lower chambers of the unit.
10. Load each well with 20 μ L samples suspended in 1X SDS sample buffer. Make sure to include in two of the wells high and low range precision plus pre-stained protein molecular weight standards (Bio-Rad cat#161-0363). Complete the assembly of the gel unit and attach the power cords first to the apparatus, then to the power supply.
11. Turn on the power supply and run the gel at 150 V through the stacking gel and 200 V through the resolving gel. The gel is run until the blue dye front reaches the bottom.
12. After the electrophoresis, unclamp the gel plate assembly from the apparatus. Place the gel plate assembly on a paper towel. Use a thin spatula to carefully pry the upper glass plate away from the gel.
13. Immediately transfer the gel to the nitrocellulose membrane following the Western transfer protocol indicated below.

3.4. Western Blotting with the Chemilumi- nescent Method

3.4.1. Western Blot Transfer

1. Fill the Bio-ice cooling unit with water and store it at -20°C until ready to use. These instructions assume the use of a Trans-Blot[®] Cell system.
2. Cut the nitrocellulose membrane slightly larger than the dimension of the gel.

3. Equilibrate the gel and soak the membrane, four pieces of Quick Draw™ blotting paper (Sigma Aldrich cat#P7796), and two scotch-brite® fiber pads in transfer buffer for 15 min. Make sure to wear gloves at all times when handling the membrane. Mark one side of the membrane for future reference.
4. Preparation of the gel sandwich is as follows:
 - (a) Place the cassette with the gray side down on a clean surface.
 - (b) Place one pre-wetted scotch-brite® fiber pad on the gray side of the cassette.
 - (c) Put two pieces of wet blotting paper on the scotch-brite® fiber pad.
 - (d) Place the equilibrated gel on top of the filter paper.
 - (e) Place the pre-wetted nylon membrane on top of the gel.
 - (f) Place the other two pieces of blotting paper over the membrane.
 - (g) Be sure to remove any air bubbles trapped between the gel, membrane, and blotting paper layers. This is easily done by rolling a clean pipet over the sandwich. Complete the sandwich with the second Scotch-Brite® fiber pad.
5. Close the cassette firmly being careful not to move the gel and the filter paper sandwich. Lock the cassette with the white latch.
6. Insert the sandwich into the transfer apparatus with the membrane positioned between the gel and the appropriate electrode. Most polypeptides are eluted from SDS-polyacrylamide gels as anions and therefore the membrane should usually be placed between the gel and the anode.
7. Fill the transfer apparatus with transfer buffer. Pour the transfer buffer slowly to prevent bubble formation. Put the lid on the tank and activate the power supply. Transfers are accomplished at either 30 V overnight or 100 V for 1 h. Make sure to add a standard stir bar to help maintain even buffer temperature and ion distribution in the tank.
8. After the transfer is complete, unclamp the blot sandwich, remove the membrane, and allow it to air dry at room temperature. Make sure to mark the side of the membrane that is facing the gel. Mark the positions of the pre-stained markers, since they may fade away during detection.

3.4.2. Confirmation of the Tagging in Transformants with M2 Antibody Against the FLAG Epitopes of the SPA-Tag

1. After transferring the proteins to the nitrocellulose membrane, block non-specific binding sites on the membrane by incubating the membrane in blocking buffer for 1 h at room temperature.
2. Rinse the membrane briefly with wash buffer prior to adding the primary antibody. The primary anti-FLAG M2 antibody is usu-

ally diluted 1:5,000 in primary antibody buffer and incubated with the membrane for 1 h by shaking gently using a C2 platform rocking shaker (New Brunswick Scientific, Edison, NJ, USA).

3. Rinse the membrane with wash buffer once for 15 min, and then twice for 5 min.
4. Dilute 2 μL of HRP (horseradish peroxidase) - labeled secondary antibody in 40 mL of secondary antibody buffer and incubate with the membrane for 45 min at room temperature by shaking gently using a rocking shaker.
5. Rinse the membrane with wash buffer once for 15 min and then twice for 5 min.

3.4.3. Image Processing

1. Transfer the membrane to a shallow tray and incubate the membrane in the chemiluminescence reagent for 5 min by gently shaking (in the dark, if possible).
2. Remove excess chemiluminescence reagent by draining or blotting.
3. Cover the membrane with Saran wrap.
4. Expose the membrane to Kodak X-OMAT Blue Autoradiography film for 30 s. If necessary, expose the membrane for up to another 30 min.

3.4.4. Stripping and Re-probing the Blots

1. After the exposure of the film, wash the membrane for 20 min in TBS buffer.
2. Incubate the membrane for 30 min at 50°C in the stripping buffer.
3. Wash the membrane for 20 min in TBS buffer.
4. Incubate the membrane for 1 min in the Western lightningTM chemiluminescence reagent. Expose the film for at least 30 min to 1 h to make sure that the original signal is removed.
5. Wash the membrane again for 20 min in TBS buffer. The membrane is now ready for reuse.

3.5. Purification of SPA-Tagged Proteins

3.5.1. Culturing SPA-Tagged *E. coli* Strains and Sonication

1. Inoculate 100 μL of a SPA-tagged *E. coli* glycerol stock into 45 mL TB liquid medium supplemented with 5 mL of potassium salt solution and 25 μL of 100 mg/mL kanamycin solution in a 250 mL conical flask.
2. Grow the culture overnight at 32°C until late log phase. Note that the OD₆₀₀ of the overnight culture should be ~5 to 6.
3. Inoculate 50 mL overnight culture into 900 mL fresh TB supplemented with 100 mL of potassium salt and 25 $\mu\text{g}/\text{mL}$ kanamycin in a 4 L flask. The culture is grown at 32°C with shaking at 250 rpm until the OD₆₀₀ reaches to ~4 to 5.
4. Transfer 1,000 mL *E. coli* cultures from the shaker to clean centrifugation bottles.

5. Centrifuge the *E. coli* cultures in a Beckman J6-HC centrifuge at $3,993 \times g$ for 15 min.
6. Discard the supernatants and remove excess liquid by inverting the bottles on paper towels. Keep the centrifugation bottles on ice.
7. Add 25 mL of sonication working buffer to the centrifugation bottles and resuspend the *E. coli* cell pellets using a clean 25 mL pipet.
8. Transfer the resuspended cultures into 50 mL polypropylene Falcon tubes and snap freeze the Falcon tubes using liquid nitrogen. Frozen, resuspended cell pellets are stored at -80°C for future use.
9. Remove the Falcon tubes containing the frozen cell pellets from the freezer and place the Falcon tubes in cold water to minimize thawing time. Keep the samples at all times on ice when they are completely thawed.
10. Ensure that the notch of the flat-tip in the sonicator is fastened securely. Set the sonicator (Branson Ultrasonic Sonifier 450 analog, cat#23395) controls to Duty cycle: "50"; Timer: "Hold" and Output control: "7."
11. Transfer the cell samples to the stainless steel cup for sonication.
12. Place the stainless steel cup on ice in an appropriate sized box. Make sure to place the box on top of the sand at an appropriate height so that the flat tip is submerged into the liquid but not too close to the bottom of the stainless steel container. Try to avoid contact between the flat tip and the stainless steel container.
13. When ready, turn on the sonicator. Set timer to 5 min. Sonication is done for 3 min followed by 2 min of cooling to prevent overheating of the samples (*see Note 3*).
14. Pour the sonicated cell lysates into pre-chilled centrifugation tubes and place them on ice.
15. Centrifuge the lysates at $35,267 \times g$ (16,000 rpm) for 30 min using a JA-17rotor (Beckman).
16. Remove the supernatants carefully from the centrifugation tubes, transfer them to 50 mL Falcon tubes, and snap freeze the samples with liquid nitrogen. The sonicated frozen cell extracts are stored at -80°C for future use.

3.5.2. SPA-Tag Purification

Anti-Flag M2 Agarose Beads

1. Prior to use, 100 μL of anti-Flag M2 agarose beads are transferred into a column and the beads are washed twice with 1 mL of AFC buffer without DTT.
2. The Falcon tube containing the sonicated frozen cell extract is thawed by placing the tube in cold water.

3. The cell extracts are incubated with 3 μL of benzonase nuclease (Novagen cat#70746; 25 U) for 30 min at 4°C.
4. Add 500 μL of 10% non-ionic detergent Triton X-100 (final concentration of Triton X-100 should be 0.1%) and 150 μL of anti-flag M2 agarose beads (Sigma cat#A2220) to the Falcon tube containing the cell extracts. Briefly mix the tube contents by tilting the Falcon tube upside down, then rotate the Falcon tube for 3 h at 4°C using a LabQuake shaker (Barnstead/Thermolyne, cat#59558).
5. Centrifuge the tubes at $1,700 \times g$ for 6 min. Carefully remove as much supernatant as possible, taking care not to disturb the loose bead pellet.
6. Resuspend the pellets in the remaining supernatant and transfer the beads into 0.8 \times 4 cm Bio-Rad polypropylene prep columns (Bio-Rad cat#732-6008). Remove the bottom outlet plugs of the columns and allow the eluates to drain by gravity flow.
7. Wash the columns five times with 200 μL of 1X AFC working buffer and twice with 200 μL of TEV cleavage buffer.
8. Close the bottom outlet of the column. Cleavage is done in the same column by adding 200 μL of 1X TEV cleavage buffer and 5 μL (50 units) of TEV protease. Close the top of the column with a cap.
9. The column containing the beads is rotated overnight at 4°C.

Calmodulin-Sepharose
Beads

1. Remove the top and bottom outlet plugs of the column after incubation with TEV protease and drain the eluates into fresh columns.
2. One hundred microliter of Calmodulin-Sepharose beads (Amersham Biosciences cat#17-0529-0), corresponding to 200 μL of bead suspension, is transferred into a column and washed twice with 10 mL of 1X calmodulin binding buffer.
3. Four hundred microliters of 1X TEV cleavage buffer and 1.2 μL of 1 M CaCl_2 are added to the eluate recovered after TEV cleavage.
4. The mixture is then transferred to the column containing the washed Calmodulin-Sepharose beads. After closing the column, rotate for 3 h at 4°C.
5. Remove the top and bottom plugs of the column and drain the eluate by gravity flow.
6. The beads are washed four times with 200 μL of 1X calmodulin binding buffer followed by two washes with 200 μL of calmodulin wash buffer.

7. The bound proteins are eluted in six fractions of 50 μL in a fresh eppendorf tube using 1X calmodulin elution buffer.
8. Distribute the eluted fractions into two separate microcentrifuge tubes in volumes of 125 μL and 175 μL , respectively.
9. Dry the 175 μL eluted fractions to 50 μL using a Speedvac (Eppendorf Vacufuge cat#5301). Add half the volume of 3X SDS sample buffer to 50 μL of the dried eluate. The eluate and the sample buffer mixture are boiled for 5 min and load onto an SDS polyacrylamide gel, silver stained and the sliced peptide bands are analyzed by MALDI-TOF mass spectrometry.
10. In parallel with **step 9**, dry down the other 125 μL eluted fraction using a Speedvac. This dried sample is subjected to LC-MS for protein identification.

3.6. Protein Identification by Gel-Based Peptide Mass Fingerprinting Using MALDI-TOF Mass Spectrometry

The tagged and purified *E. coli* bait protein is separated by silver staining on an SDS-polyacrylamide gel, and peptide bands are cut out and analyzed by MALDI-TOF mass spectrometry. The mass spectrometry data is further analyzed to produce probable identifications of the proteins. Protein identification is finally linked back to the purified protein band. The various procedures, 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 summarized below.

3.6.1. Silver Staining the SDS-Polyacrylamide Gel

1. Place the gel in a clean staining solution containing fixer and agitate the gel on a shaker for 20 min.
2. Rinse the gel in 20% ethanol for 10 min.
3. Wash the gel twice with 500 mL double distilled water for 10 min. Note that thorough rinsing gives a low uniform background.
4. Discard the distilled water and agitate the gel in 500 mL sensitizer solution for 1 min.
5. Discard the sensitizer solution and rinse the gel twice in 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 fresh 50 to 75 mL developing solution for half a minute. Replace with fresh developing solution and agitate the gel slowly by hand constantly (*see Note 4*).
9. When desired intensity is achieved, discard the developing solution, and add 80 mL of stop solution to the gel. Incubate the gel with stop solution for a minimum of 20 min.

3.6.2. Gel-Based MALDI-TOF Mass Spectrometry

Preparation of In-Gel Digests

1. Protein bands were excised from silver stained gels with a clean, sharp straight edge razor blade. The gel slices were excised as close as possible to the boundaries of the protein band and stored in -80°C freezers in 96-well polypropylene plates (Nunc cat#249946).
2. Gel slices stored in the -80°C freezer in a 96-well polypropylene plate are allowed to thaw for ~ 20 min. Remove the liquid that accumulates during thawing.
3. Shrink the gel slices with $200\ \mu\text{L}$ of 100% ACN for ~ 10 min on an IKA Schuttler MTS 4 orbital shaker (VWR cat #82006-096) at 700 rpm (*see* Note 5). Remove all liquid.
4. Reduce the gel slices with $75\ \mu\text{L}$ of 100 mM NH_4HCO_3 containing 10 mM DTT for 30 min in a 50°C heating block. 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 ~ 500 rpm for 3–5 min and repeat **step 3**.
6. Alkylate the gel slices in the 96-well microtiter plate with $75\ \mu\text{L}$ of 100 mM NH_4HCO_3 containing 55 mM iodoacetamide for 20 min in the dark.
7. Centrifuge the plate at low speed (~ 500 rpm) for 3–5 min. Remove all liquid. Repeat **step 3**.

In-Gel trypsin Digests

1. Hydrate the gel slices with $60\ \mu\text{L}$ of digestion buffer containing trypsin for 30–45 min on ice. Gel pieces should be fully hydrated with trypsin solution on ice. Check after 15–20 min and add more digestion buffer containing trypsin, if necessary, to allow complete hydration.
2. Add $20\ \mu\text{L}$ (if needed) of digestion buffer without trypsin and incubate samples overnight at 37°C (*see* Note 6).

Extraction of Tryptic Peptide Fragments

1. Transfer the extracted peptides into a clean 96-well polypropylene plate.
2. Add $100\ \mu\text{L}$ 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 96-well polypropylene plate, and add $2.5\ \mu\text{L}$ of 100% AA to the extracted peptides.
4. Repeat **steps 2 and 3** one more time.

Purification of Tryptic Fragments

1. The following purification uses bulk C18 reverse phase resin from Sigma. Add 1.5 g of dry resin to a reservoir.
2. Wash 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 $2.5\ \mu\text{L}$ of C18 slurry to the extracted peptides into the 96-well plate, the resin should float on top of the liquid. Shake

at moderate speed on an orbital shaker at 500 to 700 rpm for 45 min at room temperature.

4. Discard the supernatant by placing the pipet tips below the surface of the liquid to avoid aspirating any resin. Add 200 μL of 2% ACN, 1% AA. Shake briefly for 5 to 15 min at moderate speed of 500 to 700 rpm on an orbital shaker at room temperature.
5. Prepare in advance a 384-well Melt Blown Polypropylene (MBPP) Whatman filter plate (Whatman cat#7700-2117). Place the MBPP filter plate on top of a 384-well collection plate (Whatman cat#7701-3100). Wash the MBPP filter plate wells with 15 μL of 66% ACN, 1% AA. Centrifuge the MBPP filter plate for 1-2 min at $1,000 \times g$, and discard the filtrate collected in the collection plate.
6. Remove the supernatant from **step 4** by centrifuging the 96-well plate briefly for 5-15 min at $1,000 \times g$ at room temperature.
7. Elute the peptides by adding 30 μL of 66% ACN, 1% AA. The resin should make a slurry and slowly pellet to the bottom. Shake the 96-well plate at high speed on an orbital shaker and incubate approximately for 5 to 10 min at room temperature. Ensure that all of the resin has entered into the slurry.
8. Transfer the liquid from the 96-well plate to the MBPP filter plate. Place 384-well collection plates (Whatman cat#7701-3100) under the MBPP filter plate and centrifuge for 3-5 min at $2,000 \times g$. The filtrate collected in 384-well collection plates is 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. Then, gently rinse the plates with HPLC grade water. Wipe the plate with 100% methanol using Kimwipes (*see Note 7*).
3. Spot 1 μL of α -cyano-4-hydroxycinnamic acid matrix (Fluka Buchs SG, Switzerland cat#28480) solution and 1 μL of the purified trypsin sample onto the MALDI target plate. Make sure that there are no air bubbles and the tip does not touch the MALDI target plate. Allow samples to dry at room temperature.
4. For the bottom-most row, 1 μL of α -cyano-4-hydroxycinnamic acid matrix solution and 1 μL of peptide calibration standard (Bruker cat#206195) are spotted onto the MALDI target plate.

Acquisition of Spectra from
MALDI Target Plates

5. On completion of spotting, seal the protein plate (s) and store at -80°C . Wait until the spots on the MALDI target plate dry before acquiring the spectra.
1. Double click on the flex control icon on an ULTRAFlex II MALDI-TOF instrument. When the log on information appears, Click OKAY. Open the FLEX control method: RP_pepmix.par.\
2. Wait for the method to upload.
3. Insert the spotted MALDI target plate into the source chamber and click on the green load button on the instrument.
4. Wait for the system to fully load 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."
5. Acquire the spectra by clicking the start button and adjust the laser intensity such that the peptide peaks can be visualized.
6. Click on "Add" to the sum buffer.
7. Click on the "calibration tab" and choose peptide mix monoisotopic II from the drop down menu and a peptide reference list will appear.
8. Change the zooming factor to 0.5.
9. 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.
10. Click on accept fit result; now the instrument is calibrated.
11. 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 "autoXtest." Check the box where it shows the autoX output.
12. Click on START automatic run and the system should start acquiring the spectra automatically.

MALDI-TOF Spectral
Analysis and Protein
Identification

1. MALDI spectral searches were performed using Knexus automation, a Windows-based program from Genomics Solutions Bioinformatics (Discovery Scientific, Inc., Vancouver, Canada) that automatically selects the spectral peaks and performs the searches.
2. After the installation of the Knexus program, the Fasta sequences of the *E. coli* genome were downloaded from the European Bioinformatics Institute database (<http://www.ebi.ac.uk/>) and 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 automates the running of ProFound on more than one spectrum using one set of conditions. In addition, a Java program was developed in-house, which automates the re-running 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 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 8*). When all the data have 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 analyzed by SDS-PAGE and silver staining followed by protein identification by trypsin digestion and MALDI spectral analysis are shown in **Fig. 2**.

3.6.3. Gel-Free Liquid Chromatography–Tandem Mass Spectrometry

Proteolysis and Sample Preparation for LC-MS

1. One hundred and twenty-five microliters of eluted fractions (described in **step 10** under the section Calmodulin-Sepharose Beads) were dried down using a Speedvac.
2. Dissolve the pellets in 20 μL of the immobilized trypsin solution.
3. Incubate the samples overnight at 30°C with rotation or agitation.
4. Stop digestion by adding 20 μL of the LC-MS solvent A and centrifuge the peptide mixtures for 5 min at maximum speed using an Eppendorf centrifuge.
5. Carefully recover 10–20 μL of the peptide mixtures into microcentrifuge tubes and immediately analyzed by LC-MS or store the samples at –20°C prior to use.

LC-MS Analysis and Protein Identification

1. Proteins are analyzed using single-dimension reverse phase chromatography coupled online to ion trap tandem mass spectrometry using standard conditions.
2. The micro-columns are packed with ~10 cm of 5 μm Zorbax eclipse XDB-C₁₈ resin and are interfaced to a custom electro-spray ion source.
3. Place the packed column 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. Elution of the peptide is achieved using the following gradient that may be increased or decreased according to the sample

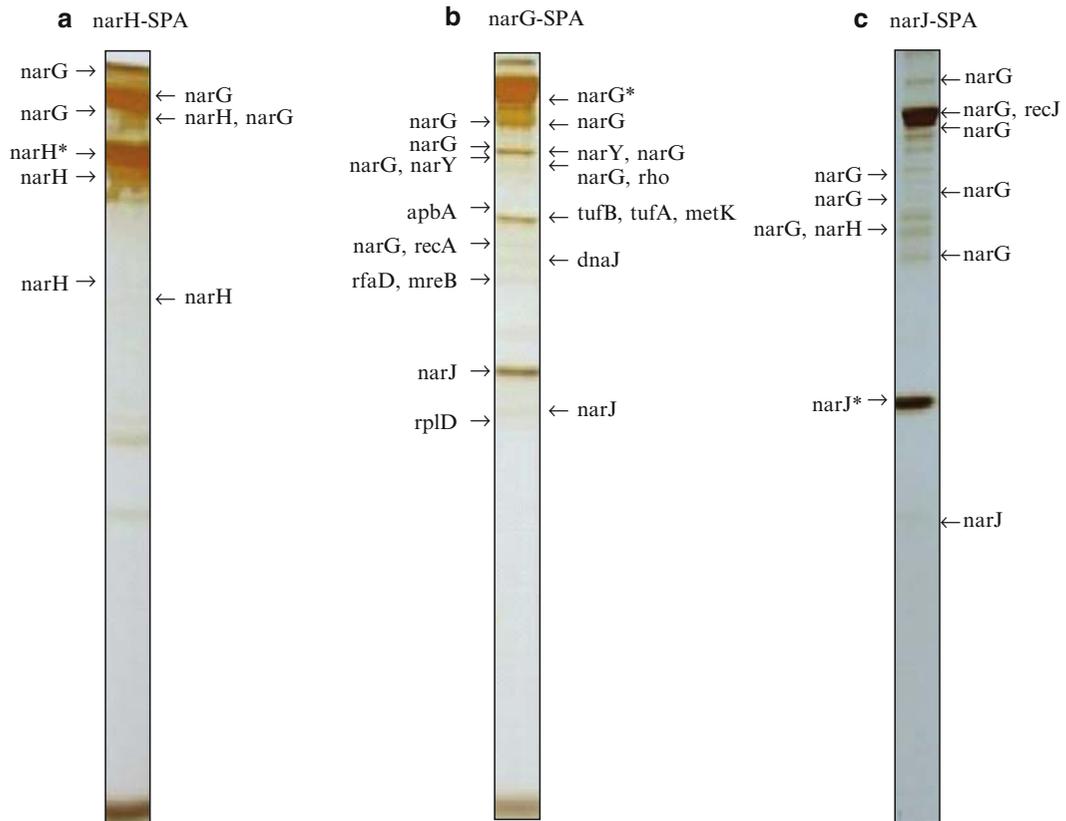


Fig. 2. Examples of silver-stained SDS polyacrylamide gels portraying NarHGJ (nitrate reductase) protein complexes recovered following affinity purification of *E. coli* SPA-tagged NarH, NarG, and NarJ, respectively. The SPA-tagged bait proteins are indicated at the top of each lane. Distinct gel bands (indicated by arrowheads) containing individual subunits of the purified complexes were identified by in-gel trypsin digestion followed by MALDI-TOF mass spectrometry. Note that the observed interactions in any given lane are validated by reciprocal SPA-tagging and purification of proteins shown in the other lanes. Asterisks indicate the recovery of the intact tagged bait protein from each purification. Protein degradation products are also routinely identified.

complexity: 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 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 (16) and validated using the STATQUEST (17) probabilistic scoring program (see Note 9).

4. Notes

1. The plasmid pJL72 contains a version of the TAP cassette (14) that was placed adjacent to a selectable Kan^R marker for use in *E. coli* and related bacteria.
2. Forty microliters of competent cells are sufficient for each standard electroporation reaction. For a greater number of reactions, larger numbers of cultures are prepared. It is very important to make sure that the electrocompetent cells are prepared fresh each time prior to transformation.
3. Avoid sonicating the samples continuously for 3 min as overheating of the instrument might lead to proteolysis of the proteins in the samples.
4. The developing solution must be made fresh each time. When the solution becomes cloudy, replace it with new developing solution. Make sure that all the gels are developed for about the same time.
5. 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.
6. In each well ensure that there is enough liquid for the gel slices to be completely submerged.
7. If spots still remain on the MALDI targets, sonicate the plate for 10 min with 100% methanol followed by 5-min sonication with HPLC water and 100% methanol, respectively.
8. The band location algorithm tries to identify the complete area of each band. It is very important to look at the location that the computer has chosen for each band and make sure that it matches the gel image and accurately includes the full area of the band.
9. The SEQUEST database search algorithm was used to match the acquired spectra to peptide sequences encoded in the *E. coli* protein database downloaded from the European Bioinformatics Institute (<http://www.ebi.ac.uk/>) database. The probabilistic STATQUEST scoring program was programmed to evaluate and assign confidence scores to all putative matches. Proteins were considered as positive if detected with two or more high confidence peptide candidates, each passing with a minimum likelihood threshold cut-off of 90% or greater probability.

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