A method to resolve the composition of heterogeneous affinity-purified protein complexes assembled around a common protein by chemical cross-linking, gel electrophoresis and mass spectrometry

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Protein complexes form, dissociate and re-form in order to perform specific cellular functions. In this two-pronged protocol, noncovalent protein complexes are initially isolated by affinity purification for subsequent identification of the components by liquid chromatography high-resolution mass spectrometry (LC-MS) on a hybrid LTQ Orbitrap Velos. In the second prong of the approach, the affinity-purification strategy includes a chemical cross-linking step to 'freeze' a series of concurrently formed, heterogeneous protein subcomplex species that are visualized by gel electrophoresis. This branch of the methodology amalgamates standard and well-practiced laboratory methods to reveal compositional changes that occur in protein complex architecture. By using mouse N-terminally tagged streptavidin-binding peptide–hemagglutinin–TANK-binding kinase 1 (SH-TBK1), we chemically cross-linked the affinity-purified complex of SH-TBK1 with the homobifunctional lysine-specific reagent bis(sulfosuccinimidyl) suberate (BS^3), and we separated the resultant protein complexes by denaturation and by silver-stained one- and two-dimensional SDS-PAGE. We observed a range of cross-linked TBK1 complexes of variable pI and M_r and confirmed them by immunoblotting. LC-MS analysis of *in situ*–digested cross-linked proteins shows differences in the composition of the TBK1 subcomplexes. The protocol is inherently simple and can be readily extended to the investigation of a range of protein complexes. From cell lysis to data generation by LC-MS, the protocol takes approximately 2.5 to 5.5 d to perform.

INTRODUCTION

Tandem affinity purification (TAP) is a two-step purification protocol originally developed by Seraphin *et al.*[1] to enrich and isolate functional noncovalent protein complexes under near-physiological conditions[2–5]. Combining TAP with LC-MS has proven to be a powerful approach for generating large-scale protein-protein interaction networks in yeast[6–10] and humans[11–13] in order to understand disease-associated proteins[14–16] and delineate signaling pathways[17–21]. On a more focused level, TAP-MS has also resulted in the successful charting of selected molecular machines[15,16,22–32].

Historical development

Seraphin *et al.*[1] assessed a number of commonly used high-affinity tags and showed that the C-terminal-TAP tag (CBP-TEV-ProtA) had the highest recovery efficiency and enabled the rapid purification of functional complexes from a relatively small number of cells. When combined with protein fractionation via denaturing one-dimensional SDS gel electrophoresis (1D SDS-PAGE), *in situ* tryptic digestion and MS, the TAP strategy enabled the identification of the constituent proteins in the noncovalent, biochemically active complex.

Although TAP-MS was relatively straightforward in yeast, difficulties were encountered with other cell types. Successful studies using retroviral-mediated gene transfer of TAP-tagged proteins in HEK293 cells required a large number of cells (typically 5×10^8–1×10^9 cells per TAP)[18,19]. To address this limitation, Bürckstümmer *et al.*[24] compared the original yeast TAP-tag (y-TAP) with four newly designed TAP constructs. The improved construct consisted of two IgG-binding motifs of Protein G (ProtG) from *Streptococcus* sp. and a single streptavidin-binding peptide separated by a tobacco etch virus (TEV) protease cleavage site. By using the optimized GS-TAP tag, complex yields were obtained that were an order of magnitude greater than the original y-TAP tag. The subsequent increase in efficiency of the affinity purification enabled the enrichment and identification of protein complexes from 5×10^7 HEK293 cells. As for previous TAP-MS strategies, the constituents of the noncovalently interacting protein complexes were identified via separation by 1D SDS-PAGE, *in situ* tryptic digestion and analysis by LC-MS.

Other shortcomings of the TAP-tag technology included the following: (i) the labor-intensive generation of large collections of cell lines expressing epitope-tagged bait proteins; (ii) the low yield of the protein complexes isolated from the cell lines; (iii) the loss of protein via gel-based separation; and (iv) the sensitivity of available MS technology. Glatter *et al.*[11] overcame some of these major limitations by the combination of (i) the tetracycline-inducible Flp-In recombination system that enabled rapid generation of human cell

lines expressing TAP-tagged proteins, (ii) a SH-TAP tag to increase sample recovery and reproducibility, and finally (iii) the elimination of gel-based analysis in favor of gel-free LC-MS that reduces protein loss and improves the overall sensitivity of the protein identification. The recommended quantity of cells for purifying protein complexes via SH-TAP is 3×10^7 cells. These investigators, however, demonstrated successful identification of the constituents of a protein complex from 4×10^6 HEK293 cells. In addition to the TAP-tags discussed above, a number of other tags exist and are described in a recent review on TAP-tag technology[5].

To obtain information on protein complex architecture, stoichiometry and dynamics, our approach combines these improved affinity purification techniques with a chemical cross-linking strategy and latest-generation high-sensitivity mass spectrometry. This protocol (**Fig. 1**) provides researchers with tools to identify the constituents of noncovalently interacting functional protein complexes to build interactome networks and/or to trap multiple, concurrently formed, heterogeneous subcomplex species via the introduction of a cross-linking agent to further dissect the multiple layers of the interactome. The cross-linker aids in stabilizing the alternate configurations to identify the compositional makeup of the subcomplex species. A step-by-step description of how to determine the composition of protein complexes should enable a researcher to disentangle and distinguish subtle variations in protein subcomplexes associated with the same protein that are physically separated by gel electrophoresis and identified by mass spectrometry. Ultimately, our protocol will assist in furthering our understanding of the action of dynamically interacting protein complexes that are in a constant state of flux. We have applied this methodology, and parts thereof, to published[13,15,16,31] and ongoing large-scale interactome investigations in our laboratory.

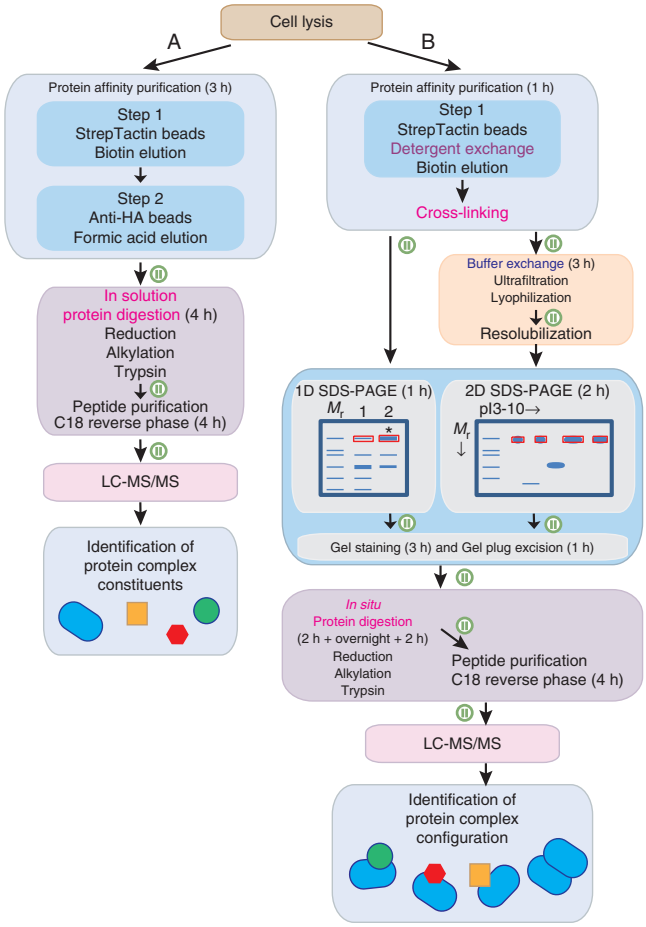

Figure 1 | Schematic overview of the entire protocol. After cell lysis, there are two possible directions to follow: option A, two-step protein affinity purification to purify noncovalently interacting functional complexes to identify the protein constituents alone, and/or option B, one-step protein affinity purification followed by chemical cross-linking to trap and stabilize protein complex configurations and subsequently identify the compositional makeup of the subcomplex species. Timing for the individual sections of the protocol and possible pause points (symbols shown in green) are indicated.

Applications of the method

The formation of multiple, heterogeneous, simultaneous protein subcomplexes around a single central protein implies alternate cellular functions for each subcomplex. Protein subcomplex information may aid in furthering our understanding of phenotypic outcomes (disease) following mutation(s). A single mutation may either only affect the formation, and thus the function, of one particular complex, or it may affect a number of subcomplexes that could explain interference with several cellular processes and the cause of pleiotropic effects. Other applications relate to the remodulation of heterogenous protein complexes emanating from a central, unchanging protein under the influence of external stimuli, e.g., drug perturbation, viral infection or agonist stimulation. TBK1, for example, is involved in the innate immune response, cell proliferation and oncogenesis, which are clearly different cellular functions that must involve different interacting partners. Separating protein subcomplexes will provide information on the role a specific protein plays in a cell, at different time points or under different influences, which is not attainable using other methods for probing protein interactomes and complex composition. With follow-up biological validation of identified protein subcomplexes, it is feasible that particular cellular functions can be assigned to specific subcomplexes.

Cross-linking of SH-TAP–tagged complexes formed *in vivo* can also be combined with the SH-TAP protocol to enrich stabilized complexes, but there is a risk of introducing a cross-link within the SH-TAP epitope. Thus, the binding efficiency during the enrichment procedure is decreased or even abolished. Possible solutions are to use a cross-linker that does not modify specific amino acid residues located in the TAP-tag epitope, or to forego any purification of the *in vivo* cross-linked protein complex and analyze the entire cell lysate by 2D SDS-PAGE. Regions of the gel corresponding to immunoblotting against the SH-TAP tag can be excised, digested and analyzed by LC-MS. The cross-linking of protein complexes and separation via 2D SDS-PAGE could also be applied to antibody-based immunoprecipitation of native protein complexes without an SH-TAP tag. The disadvantages of both latter applications are that the contamination levels of nonspecifically interacting proteins are high.

Comparison with other methods

There are numerous mass spectrometry–based proteomic methods to probe protein interactomes and complex compositions[3,33–36]. These are becoming robust and more widely used in the research community. However, without the level of coverage and density of

interaction information (such as that obtained from yeast), such classical approaches cannot yet reveal the partitioning of proteins in different, concurrent complexes.

The only other methods currently available that can reveal protein complex information are the separation of affinity-purified complexes by native PAGE or native capillary isoelectric focusing (CIEF)[37] and size exclusion chromatography (or similar techniques), or SILAC (stable isotope labeling by amino acids in cell culture)-based protein profiling of partly purified subproteomes[38].

An alternative to TAP is traditional antibody immunoprecipitation. The greatest advantages of an immunoprecipitation versus a TAP is that no tag is introduced into the target proteins and overexpression can be avoided. Thus, the complex formed *in vivo* is truly in a native state, and no questions can be raised concerning the effect the tag has on protein complex fidelity. A major disadvantage with antibody enrichment of protein complexes is that the resultant eluate is usually contaminated by numerous nonspecifically interacting proteins. Highly sensitive LC-MS exacerbates this drawback. Although a single-step affinity purification using only one of the tag epitopes also produces an extensive protein list by LC-MS, this is still considerably less complex than that obtained via antibody enrichment.

The addition of a cross-linking component to the TAP strategy has the advantage that transient interactors that are normally lost in the two-step process are irreversibly trapped and remain as part of the complex for subsequent identification by LC-MS. In addition, the separation of the cross-linked protein complex(es) by 2D SDS-PAGE has the benefit of physically separating and delineating protein complexes of similar molecular weight. An added advantage of our approach is that it is not necessary to establish a series of stable cell lines expressing reciprocal SH-TAP–tagged proteins to confirm protein complex identification and composition.

Limitations

Current limitations of the protocol are primarily related to: (i) the expression level of the SH-tagged protein of interest; (ii) the quantity of the enriched, purified protein complex(es); (iii) the optimization of the purification conditions for different protein classes; (iv) the nature of the cross-linker; (v) the sensitivity of the mass spectrometer; (vi) the size of the SH-TAP–tagged protein; and (vii) the size of the cross-linked protein complex(es) for separation by 1D and/or 2D SDS-PAGE.

The expression level of an SH-TAP–tagged protein of interest can vary considerably. This effect is exacerbated if it is not feasible to use the Flp-In system, and the SH-TAP–tagged protein has to be introduced into the cell using retroviral-mediated gene transfer. Generating Flp-In cells is not trivial; however, there are some cell lines available on the market from Invitrogen and Life Technologies. Overall, low expression levels of the SH-TAP–tagged proteins result in a decreased quantity of the final purified product, and this must be taken into consideration when following our protocol. The choice of an appropriate cross-linking reagent is also important and should be based on any information that has already been accumulated concerning the protein complex and expected or predicted interaction partners. The availability of cross-linker–reactive amino acid residues in the protein sequence should also be considered. Ideally, but only if practical, a range of cross-linking reagents can be assessed in parallel for new SH-TAP–tagged proteins and the subsequently formed complex(es). This will aid in determining the most favorable cross-linking reagent to use for further experiments. There are numerous cross-linking reagents available on the market with different reactivities (e.g., BS^3, bis(sulfosuccinimidyl) suberate, 1,4-bismaleimidobutane (BMB)), linker arm length (e.g., disuccinimidyl suberate (DSS), disuccinimidyl glutarate (DSG)), solubility (e.g., DSS, BS^3), thiol-cleavable (e.g., dithiobis[succinimidyl propionate] (DSP), (3,3′-dithiobis [sulfosuccinimidylpropionate])(DTSSP)[39–41]) and so on.

Although TAP is very efficient for a wide range of proteins, some classes may require additional effort to determine the optimal purification conditions. For example, tightly bound membrane proteins are often difficult to isolate, and thus more stringent conditions (e.g., additional or alternative detergents) can lead to the loss of associated complex components. Nevertheless, detergents such as *n*-dodecyl-β-maltoside (DDM) are effective in solubilizing membrane proteins but are still sufficiently gentle to maintain the noncovalent protein-protein interactions. TAP is a powerful system for identifying strongly associated protein complexes, but a single-step purification is preferable when the aim is to identify very weak and transiently interacting proteins.

The success of the protocol is dependent on the sensitivity of the LC-MS system available for analysis of the protein components from the SH-TAP. For the protocol given here, 3% (vol/vol) of the final eluate from the two-step TAP analyzed on a hybrid LTQ-Orbitrap Velos provides results in a deep analysis of the total composition of the proteins in the complexes that are purified by the tandem affinity procedure. For the one-step purification followed by cross-linking and SDS-PAGE, a minimum of 40 mg of total cell extract is used. Here the limitation is the sensitivity of the staining procedure that is necessary to visualize the separated proteins and complex(es) that are subsequently isolated from the gel and analyzed by LC-MS.

Another limitation is the size of the protein of interest. Retroviral-mediated gene transfer is more restrictive with respect to the size of the protein that can be encoded. The Flp-In system enables incorporation of much larger proteins. Finally, the size of the cross-linked protein complex must be taken into account. If a complex is too large, then either the proteins simply do not enter the gel or, if so, it is simply not feasible to show a clear separation of the subspecies via SDS-PAGE. Standard limitations of SDS-PAGE technology also apply, e.g., the difficulties of highly hydrophobic proteins.

A final, potential shortcoming is that during the denaturing acidic elution of the two step–purified protein complex, the proteins may precipitate *in situ*, preventing elution from the HA-agarose. If this occurs, then an alternative elution strategy (compatible with gel-free LC-MS analysis) must be considered. Examples of denaturing regents that can disrupt the noncovalent interaction between the HA-tag on the protein of interest and the anti-HA antibodies on the agarose beads are urea, guanidinium HCl and reducing agents such as dithiothreitol and tris(2-carboxyethyl)phosphine.

Experimental design

To obtain the expression vector for tetracycline-controlled expression of an N- and/or C-terminally SH-tagged version of the protein of interest, the corresponding cDNA must be cloned into the destination vector pcDNA5/FRT/TO/SH/GW (ref. 11) or pcDNA5/FRT/TO/C-SH/GW. The expression vectors are compatible with the Gateway technology (Invitrogen). The cDNA of interest is initially cloned using BP recombination into a Gateway-compatible donor vector (e.g., pDONR221, Invitrogen). An LR recombination is then performed between the donor vector and

TABLE 1 | SH-TAP vectors that are currently available.

Vector	Expression system	Protein expression	Source
pcDNA5/FRT/TO/SH/GW	Flp-In T-Rex	Inducible (N-terminal tag)	ETH, Zurich[11]
pcDNA5/FRT/TO/C-SH/GW	Flp-In T-Rex	Inducible (C-terminal tag)	ETH, Zurich[a]
pfMSCV-SH IRES GFP	Retroviral	Constitutive (N-terminal tag)	CeMM, Vienna[b]
pfMSCV-C-SH IRES GFP	Retroviral	Constitutive (C-terminal tag)	CeMM, Vienna[15,16]

SH-TAP, streptavidin-binding peptide-hemagglutinin tandem-affinity purification.
[a]Available upon request from M. Gstaiger, ETH, Zurich, Switzerland. [b]Available upon request from G. Superti-Furga, CeMM, Vienna, Austria.

the destination vector. We optimized the protocol described here using recombinant N-terminally SH-tagged mouse TANK-binding kinase 1 (SH-TBK1_M) inducibly expressed in HEK293 Flp-In cells. We generated a stable cell line using the Flp-In T-Rex-293 cell line, Flp recombinase expression vector pOG44 (Invitrogen) and the pcDNA5/FRT/TO/SH/GW expression vector. Depending on the cell type, stable cell lines can also be generated by standard procedures such as transfection or retroviral infection. **Table 1** shows the SH-TAP vectors that are currently available. Approximately five confluent 15-cm plates of cells are recommended (equivalent to 5×10^7 cells or 40–50 mg whole-cell lysate). The final number of cells required for a single SH-TAP purification is largely dependent on the type of target cells, the level of recombinant protein expression and the sensitivity of the LC-MS system. Data obtained from our laboratory have shown that it is feasible to perform a successful SH-TAP from as little as 5–12.5 mg of total protein input (equivalent to 0.5–1 × 15 cm culture plate or $4–8 \times 10^6$ cells)[16].

Our protocol can be extended to quantification using well-known approaches and established methodology. These include label-free approaches via peak intensity[42] or normalized sequence abundance factor[43], as well as stable-isotope labeling techniques such as isobaric tags for relative and absolute quantification (iTRAQ)[44] (**Box 1**), TMT[45] and SILAC[46], and absolute quantification[47].

Box 1 | Relative quantification of affinity-purified protein complexes with iTRAQ ● TIMING 1–3 d

The protocol can be extended to add quantitative information using 4-plex or 8-plex iTRAQ reagents (and other stable-isotope, chemically derivatizing agents such as TMT). Quantification of the proteins in the complexes provides information on the relative changes that can occur at the protein level when a complex is, e.g., perturbed or stimulated.

1. Select the samples for which quantitative information is required. For example, for a 4-plex iTRAQ experiment, choose biological replicates of an untreated SH-tagged protein complex compared with biological replicates of the same SH-tagged protein complex following treatment with a drug.
2. Take the remaining tryptically digested samples from PROCEDURE Step 32.
3. Acidify the samples with TFA to a final concentration of 1% (vol/vol) and perform a solid-phase extraction (SPE) of the peptide digests according to the manufacturer's instructions.
4. Chemically derivatize the digested samples with the 4-plex iTRAQ reagents according to the manufacturer's instructions (a maximum of 100 µg of protein per reagent vial).
   - Only use buffers that do not contain primary amines, e.g., TEAB. Buffers with primary amines, e.g., ammonium bicarbonate (NH_4HCO_3), compete as an acceptor of the iTRAQ label with the tryptic peptides.
   - The pH must be between 8 and 9.
   - The final buffer concentration after the addition of the reagent must be at least 20 mM.
   - For 4-plex iTRAQ labeling, the ethanol concentration must be >65%.
5. Quench the reaction with 5% (vol/vol) formic acid.
6. Pool the four samples, evaporate the organic solvent and perform SPE on the mixed, labeled peptides.
7. Resolubilize iTRAQ-labeled peptides in 20 mM amine-free buffer, e.g., TEAB.
8. Rebuffer the sample with 20 mM ammonium hydroxide (NH_4OH), pH 10, before separation by C18 reversed-phase chromatography, pH 10, and collect 20 time-based fractions.
9. Lyophilize the collected fractions in a vacuum concentrator and add 5% (vol/vol) formic acid.
10. Analyze the samples by online LC-MS with higher-energy collision-induced fragmentation on a hybrid LTQ Orbitrap Velos mass spectrometer to identify the proteins, and determine the relative quantities of the proteins in the complexes.

MATERIALS

REAGENTS

Affinity purification

- Anti-hemagglutinin (HA) agarose (Sigma-Aldrich, cat. no. A2095) ▲ CRITICAL This is only required for a two-step affinity purification without cross-linking.
- Avidin (IBA, cat. no. 2-0204-015)
- D-(+)-Biotin (Alfa Aesar, cat. no. A14207/L05109) ▲ CRITICAL Use dextrorotatory biotin, as it has a higher solubility than levorotatory biotin.
- Bovine serum albumin (BSA) standards, prediluted set (Thermo Scientific, cat. no. 23208)
- Formic acid, 98–100%, Suprapur (Merck, cat. no. 1.11670.1000) ! CAUTION Formic acid is corrosive. Handle it in a fume hood; wear protective gloves and clothing and safety glasses. ▲ CRITICAL Formic acid is only required for a two-step affinity purification without cross-linking.
- Protein assay dye reagent concentrate (Bio-Rad, cat. no. 500-0006), based on the Bradford method
- DDM, >99.5% (Affymetrix, cat. no. D310A) ▲ CRITICAL DDM is only required for a one-step affinity purification with cross-linking.
- EDTA (Merck, cat. no. 1.08454.0250)
- HEPES (Merck, cat. no. 1.10110.0250)
- Monoclonal antibody anti-HA peroxidase conjugate clone HA-7 (Sigma-Aldrich, cat. no. H6533)
- NP-40 alternative (Calbiochem, cat. no. 492016)
- NuPAGE LDS sample buffer (4×; Invitrogen, cat. no. NP0007)
- PMSF (Sigma-Aldrich, cat. no. 78830) ! CAUTION PMSF is toxic and corrosive; wear gloves and safety glasses when handling it, and use a respirator when handling the powder.
- Protease inhibitor cocktail (Sigma-Aldrich, cat. no. P8340)
- Sodium chloride (NaCl), ≥99.8% (Sigma-Aldrich, cat. no. 31434-5KG-R)
- Sodium fluoride (NaF) ≥99% (Sigma-Aldrich, cat. no. S7920-500G) ! CAUTION NaF is toxic and an irritant. Wear protective gloves, clothing, and eye and face shields.
- Sodium orthovanadate (Na_3VO_4; Sigma-Aldrich, cat. no. S6508) ! CAUTION Na_3VO4 is toxic. Wear protective gloves, clothing, and eye and face shields.
- StrepTactin Sepharose (IBA, cat. no. 2-1201-010)
- Triethylammonium bicarbonate (TEAB), 1 M (Sigma, cat. no. 17902-100ML) ▲ CRITICAL TEAB is only required for a two-step affinity purification without cross-linking. ▲ CRITICAL Check the expiry date and order a new solution if it is older than 3 months.
- Cellular material to be analyzed (see Experimental design)
- Liquid nitrogen

Cross-linking

- BS^3 (no-weigh format; Thermo Scientific, cat. no. 21585) ! CAUTION The compound is toxic. Avoid breathing dust.
- HiMark prestained protein standard, 250 µl (Invitrogen, cat. no. LC5699)
- NuPAGE LDS sample buffer (4×; Invitrogen, cat. no. NP0007/1071-8414)
- NuPAGE Novex 3–8% (wt/vol) Tris-acetate gel 1.0 mm, 10 well (Invitrogen, cat. no. EA0375BOX/1076-9993)
- NuPAGE Tris-acetate SDS running buffer (20×; Invitrogen, cat. no. LA0041)
- Tris (Trizma base) (Sigma-Aldrich, cat. no. T1503) ! CAUTION Tris is toxic. Avoid breathing dust, and wear eye protection, gloves and a dust mask.
- Buffer III, Buffer IV, Tris-HCl, BS^3, CH_3CN in Tris-HCl (see Reagent Setup)

Gel plug excision and *in situ* tryptic digest

- Ammonium hydrogen carbonate (NH_4HCO_3; AppliChem, cat. no. A3689, 0500) ! CAUTION NH_4HCO_3 is toxic. Wear eye protection, gloves and a dust mask; wash skin thoroughly after handling.
- DL-dithiothreitol (DTT), for molecular biology, minimum 99% titration (Sigma-Aldrich, cat. no. D9779-5G) ! CAUTION DTT causes acute toxicity. Wear gloves and safety glasses when handling it.
- Ethanol (Merck, cat. no. 1.00983.1000; see Reagent Setup) ! CAUTION Ethanol is toxic and flammable; keep it away from open flames. Wear gloves and safety glasses when handling ethanol.
- Formic acid, 98–100%, Suprapur (Merck, cat. no. 1.11670.1000) ! CAUTION Formic acid is corrosive and flammable. Handle it in a fume hood, and wear gloves and safety glasses.
- Iodoacetamide (IAA; Sigma-Aldrich, cat. no. I1149-5G) ! CAUTION IAA causes acute toxicity and damage to the organs. Wear gloves and safety glasses when handling it, and use a respirator when handling the powder.
- Trifluoroacetic acid (TFA; Uvasol, for spectroscopy; Merck, cat. no. 1.08262.0025) ! CAUTION TFA is corrosive. Handle the compound in a fume hood, and wear gloves and safety glasses.
- Trypsin, sequencing grade modified, frozen (Promega, cat. no. V5113) ▲ CRITICAL Use freshly thawed aliquots of trypsin. Discard the aliquots after use.
- Water, LiChrosolv (Merck, cat. no. 1.15333.2500)
- Alkylation solution, digestion buffer, digestion solution, extraction solution, reduction stock, reduction solution and wash solution (see Reagent Setup)

High-performance LC-MS

- Formic acid, 98–100%, Suprapur (Merck, cat. no. 1.11670.1000) ! CAUTION Formic acid is corrosive. Handle the compound in a fume hood, and wear gloves and safety glasses.
- Isopropyl alcohol ($(CH_3)_2CHOH$) LC-MS grade (Sigma-Aldrich, cat. no. 34965, 2.5 l) ! CAUTION $(CH_3)_2CHOH$ is toxic and flammable. Keep it away from open flames. Wear gloves and safety glasses when handling.
- Methanol (CH_3OH) LC-MS grade (Fisher Scientific, cat. no. M/4062/17, 2.5 l) ! CAUTION CH_3OH causes acute toxicity and damage to organs. The compound is flammable, and hence keep it away from open flames. Wear gloves and safety glasses when handling.
- TFA (Uvasol, for spectroscopy; Merck, cat. no. 1.08262.0025) ! CAUTION TFA is corrosive. Handle the compound in a fume hood, and wear gloves and safety glasses.
- Water, LiChrosolv (Merck, cat. no. 1.15333.2500)

Solution tryptic digest

- DTT, for molecular biology, minimum 99% titration (Sigma-Aldrich, cat. no. D9779-5G) ! CAUTION DTT causes acute toxicity. Wear gloves and safety glasses when handling.
- IAA (Sigma-Aldrich, cat. no. I1149-5G) ! CAUTION IAA causes acute toxicity and damage to organs. Wear gloves and safety glasses when handling, and a respirator when handling the powder.
- Trypsin, sequencing grade modified, frozen (Promega, cat. no. V5113) ▲ CRITICAL Use freshly thawed aliquots of trypsin. Discard the aliquots after use.
- Water, LiChrosolv (Merck, cat. no. 1.15333.2500)
- Alkylation solution, reduction solution (see Reagent Setup)

Stage tip purification

- Acetonitrile (CH_3CN; Fisher Scientific, cat. no. A/0638/17, 2.5 l) ! CAUTION CH_3CN causes acute toxicity and is flammable. Keep it away from open flames. Wear gloves and safety glasses when handling the compound.
- Formic acid 98–100%, Suprapur (Merck, cat. no. 1.11670.1000; see Reagent Setup) ! CAUTION Formic acid is corrosive. Handle the compound in a fume hood, and wear gloves and safety glasses.
- CH_3OH, LC-MS grade (Fisher Scientific, cat. no. M/4062/17, 2.5 l) ! CAUTION CH_3OH causes acute toxicity and damage to organs. The compound is flammable, and hence keep it away from open flames. Wear gloves and safety glasses when handling CH_3OH.
- TFA (Uvasol, for spectroscopy; Merck, cat. no. 1.08262.0025; see Reagent Setup) ! CAUTION TFA is corrosive. Handle the compound in a fume hood, and wear gloves and safety glasses.
- Water, LiChrosolv (Merck, cat. no. 1.15333.2500)
- Stage tip solution, stage tip elution (see Reagent Setup)

2D SDS-PAGE

- CHAPS (Sigma-Aldrich, cat. no. C3023) ! CAUTION CHAPS causes acute toxicity, and is hazardous to health on aspiration. Wear gloves and safety glasses when handling it, and use a respirator when handling the powder.
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126-25G)
- Complete mini protease inhibitor tablets (Roche, cat. no. 04693124001)
- Criterion Tris-HCl gel, 4–15% (wt/vol), IPG+1 well (Bio-Rad, cat. no. 345-0103)
- DeStreak reagent (GE Healthcare, cat. no. 17-6003-18)
- DTT, for molecular biology, minimum 99% titration (Sigma-Aldrich, cat. no. D9779-5G) ! CAUTION DTT is toxic. Wear gloves and safety glasses when handling it.
- Electrode wicks (Bio-Rad, cat. no. 164-6031)
- EDTA (Merck, cat. no. 1.08454.0250)
- Glycerol (Merck, cat. no. 1.04094.1000)
- HCl (Merck, cat. no. 1.09063.1000) ! CAUTION HCl is toxic and corrosive. Handle it in a fume hood, and wear gloves and safety glasses.

- IAA (Sigma-Aldrich, cat. no. I1149-5G) ! CAUTION IAA is toxic and harmful. Wear gloves and safety glasses when handling it.
- Laemmli sample buffer (Bio-Rad, cat. no. 161-0737) ! CAUTION Laemmli sample buffer causes acute toxicity; it is hazardous and corrosive. Contains 2-mercaptoethanol. Wear gloves and safety glasses when handling it.
- Mineral oil (Bio-Rad, cat. no. 163-2129)
- HiMark prestained protein standard, 250 µl (Invitrogen, cat. no. LC5699)
- Pharmalyte 3-10 (GE Healthcare, cat. no. 17-0456-01)
- Plastic cover sheets (high-quality, dust-free; available at any office supplies retailer)
- ReadyStrip IPG strips, pH 3–10, 11 cm (Bio-Rad, cat. no. 163-2014)
- SDS (Sigma-Aldrich, cat. no. L3771) ! CAUTION SDS causes acute toxicity and is flammable. It is a skin, eye and airway irritant. Wear gloves and safety glasses when handling it, and use a respirator when handling the powder.
- Thiourea (Sigma-Aldrich, cat. no. 88810) ! CAUTION Thiourea causes acute toxicity and is a health and environmental hazard. Wear gloves and safety glasses when handling it.
- Tris (Sigma-Aldrich, cat. no. 93352) ! CAUTION Tris causes acute toxicity. Wear gloves and safety glasses when handling it.
- Urea (Sigma-Aldrich, cat. no. 51456)
- Water, LiChrosolv (Merck, cat. no. 1.15333.2500)
- 2D sample buffer, rehydration buffer, equilibration buffers (see Reagent Setup)

EQUIPMENT

▲ CRITICAL Do not use autoclaved plasticware, glassware, buffers or solutions, as this can introduce unwanted chemical contamination into the samples, which compromises the analysis by LC-MS. If absolutely necessary, sterilize stock solutions by filtration. Minimize contamination by keratin by keeping consumables in a dust-free environment and always wearing nonpowdered gloves.

Affinity purification and cross-linking

- Amicon Ultra-0.5, Ultracel-10 membrane, 10-kDa cutoff (Millipore, cat. no. UFC501008)
- ART low retention pipette tips, 20 µl (Fisher Scientific, cat. no. 10251772)
- ART low retention pipette tips, 100 µl (Fisher Scientific, cat. no. 10314042)
- ART low retention pipette tips, 200 µl (Fisher Scientific, cat. no. 10333322)
- BioSpin columns, 5 ml (Bio-Rad, cat. no. 732-6008) ▲ CRITICAL An alternative column can be used, but it must be stable at acidic pH. If this is not the case, plasticizers will be extracted from the column during the second-step elution with acid and will severely compromise the analysis by LC-MS.
- Centrifuge (Sigma-Aldrich, 3-18K, cat. no. 10290, rotor: 19776-H)
- Centrifuge (Eppendorf centrifuge, cat. no. 5417R, rotor: F45-30-11)
- Conical tubes, screw cap, polypropylene, 50 ml (BD Biosciences, cat. no. 352070)
- Conical tubes, screw cap, polypropylene, 15 ml (BD Biosciences, cat. no. 352097)
- HPLC screw-cap vial, amber, 100/pk (Agilent Technologies, cat. no. 5182-0716)
- CHRIST vacuum concentrator, Alpha 1-2 LD (Martin Christ Gefriertrocknungsanlagen, cat. no. Alpha 1-2 LD), or similar
- Syringe filters, sterile, nylon membrane, membrane diameter 25 mm, pore size 0.2 µm (Corning, cat. no. 43122)
- Parafilm (Sigma-Aldrich, cat. no. P7668)
- Pipette tips, Safe-Seal, 10 µl (Biozym Scientific, cat. no. 770010)
- Pipette tips, Safe-Seal, 1 ml (Biozym Scientific, cat. no. 770600)
- Protein LoBind tubes, 1.5 ml (Eppendorf, cat. no. 0030 108.116)
- Roto-Shake Genie (Scientific Industries, cat. no. SI-1100), or similar
- Safe-Lock tubes, 1.5 ml (Eppendorf, cat. no. 0030 120.086)
- Screw caps, blue, 100 per package (Agilent Technologies, cat. no. 5182-0717)
- Ultrafiltration tube, 100-kDa cutoff (Millipore, cat. no. UFC510008)
- Vortex mixer (Stuart Equipment, cat. no. SA8), or similar

Gel plug excision and *in situ* tryptic digest

- Disposable grid gel cutter (1 mm × 5 mm, 50 rows, 1 column) and mount (Gel Company, cat. no. MEE1-5-50 and MEF50-5), or similar
- Hood with laminar flow to minimize keratin contamination (Erlab), or similar
- Mini-incubator (Labnet International, cat. no. I5110), or similar
- Prolite Basic 2 light box (Kaiser Fototechnik, cat. no. 2403), or similar
- Protein LoBind tubes, 1.5 ml (Eppendorf, cat. no. 0030 108.116)

Stage tip purification

- C18 high-performance extraction discs, 47 mm, Empore (3M, cat. no. 2215)
- KF needle gauge 17 (Hamilton, cat. no. 90517/00)
- HPLC screw-cap vial, fixed insert, clear, 100 per package (Agilent Technologies, cat. no. 5188-6591)
- Pipette tips (2–200 µl, yellow; Plastibrand, VWR, cat. no. 612-5725)
- Screw caps, blue, 100 per package (Agilent Technologies, cat. no. 5182-0717)
- Centrifuge (Eppendorf centrifuge, cat. no. 5810, rotor:A-2-DWP), or similar
- Vacuum concentrator 5301 (Eppendorf, cat. no. 5301), or similar

2D SDS-PAGE

- Conical tubes with screw caps, 15 ml (BD Falcon, cat. no. 352196)
- Isoelectric focusing (IEF) device (Bio-Rad, cat. no. 164-6001), or similar
- Focusing tray, 11 cm (Bio-Rad, cat. no. 164-6111), or tray matching the IEF device
- Forceps for handling IPG strips (Bio-Rad, cat. no. 165-4070)
- Cell culture dishes, diameter 15 cm (PAA Laboratories, cat. no. PAA20151X) or Criterion stain/blotting trays (Bio-Rad Laboratories, cat. no. 345-9920)
- Criterion cell (Bio-Rad, cat. no. 164-6001), or similar
- Power supply, universal (Bio-Rad, cat. no. 164-5070), or similar
- Roller mixer, RM5-40 (Finemech, cat. no. 60205-00), or similar
- Shaker VXR VIBRAX (IKA, cat. no. 2819000), or similar

High-performance LC-MS

- HPLC trap column, Zorbax 300SB-C18 5 µm, 5 mm × 0.3 mm (Agilent Technologies, cat. no. 5065-9913) ▲ CRITICAL The trap column can be replaced by any other column designed for proteomic analyses.
- Customized nanoflow HPLC separation/analytical column, ReproSil-Pur 120 C18-AQ 3 µm, 160 mm × 0.05 mm (Dr. Maisch, cat. no. r13.aq) ▲ CRITICAL The separation column can be replaced by any other reversed-phase column designed for low flow rates (50–300 nl min^{-1}).
- Uncoated nanoelectrospray borosilicate emitters (New Objective, cat. no. FS360-20-10-N-20) ▲ CRITICAL Alternative emitters can be used depending on the configuration of the LC-MS system.
- Nanoflow high-performance liquid chromatography (nano-HPLC 1200 series) system (Agilent Technologies) ▲ CRITICAL The nano-HPLC can be replaced by any other system designed for low flow rates (50–300 nl min^{-1}).
- Hybrid linear ion trap-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray (nanoESI) ion source with a liquid junction (Proxeon Biosystems) ▲ CRITICAL Protein samples can be analyzed on all modern mass spectrometers. ▲ CRITICAL The sensitivity of the analysis is dependent on the type of mass spectrometer used.
- Xcalibur software (ThermoFisher Scientific)

REAGENT SETUP

▲ CRITICAL Prepare all solutions with extreme care in order to minimize contamination with keratin. Wear nonpowdered gloves at all times.

HEPES-NaOH (pH 8.0), 750 mM To sterilize and prolong the shelf life of HEPES-NaOH, use a 0.2-µm nylon filter. The solution can be stored at 4 °C for 3 months. ▲ CRITICAL Do not autoclave the solution, as degradation can occur.

NaCl, 5 M Store NaCl at room temperature (RT, 23–26 °C) for up to 6 months.

EDTA (pH 8.0), 0.5 M EDTA aliquots can be stored in plastic tubes at −20 °C for 1 year.

NaF, 0.5 M NaF aliquots can be stored in polypropylene tubes at −20 °C for 1 year. ▲ CRITICAL Do not store NaF in glass, as the solution will etch the surface; protect the solution from light during storage.

NP-40, 10% (vol/vol), in water NP-40 must be freshly prepared on the day before the experiment.

Na_3VO_4, 10 mM Na_3VO_4 aliquots can be stored at −20 °C for 1 year. ▲ CRITICAL Vanadate should be activated for maximal phosphotyrosil-phosphatase inhibition as described by Gordon[48]; see **Box 2**. Discard the solution when it becomes yellow.

PMSF, 100 mM Dissolve the PMSF in ethanol or isopropanol. Aliquots can be stored at −20 °C for 1 year.

Protease inhibitor cocktail Aliquots of the commercial solution can be stored at −20 °C for 4 years. ▲ CRITICAL For all frozen aliquots, once they are thawed, discard them after use.

TEAB Aliquot 125 µl of 1 M TEAB into amber HPLC vials. Prepare sufficient TEAB for the required number of affinity purifications to be performed ▲ CRITICAL TEAB is only required for a two-step affinity purification without cross-linking.

Stock D-biotin solution Dissolve 0.0061 g of D-biotin (25 mM biotin) in 1 ml of 100 mM HEPES-NaOH (pH 8.0). ▲ CRITICAL Prepare the solution freshly immediately before use and discard the unused portion.

Lysis buffer Add avidin to an appropriate quantity of buffer I to obtain a final concentration of 1 µg ml^{-1}. Freshly prepare the buffer immediately before use.

Box 2 | Procedure for vanadate activation ● TIMING ~4 h

1. Prepare a 10 mM Na_3VO_4 solution by dissolving 0.1839 g of sodium orthovanadate in 100 ml of water.
2. Measure the pH. The pH should be >11.
3. Adjust the pH to 10.0 using 1 N HCl. The solution will turn yellow.
4. Boil the solution in a microwave (remove as soon as bubbles begin to form). Alternatively, the solution can be placed in a water bath to boil. Heat the solution until it turns colorless.
5. Cool the solution to ambient RT on ice.
6. Adjust the pH to 10.0 with 1 N NaOH. The solution may become yellow again.
7. Repeat the boiling and adjustment of pH until the solution remains colorless and the pH stabilizes at 10.0 at RT.
8. Aliquot the solution into conical tubes and store them at −20 °C for up to 1 year.

Store it at 4 °C during use and discard the unused portion.
▲ CRITICAL Approximately 1 ml of lysis buffer is required per 15-cm cell culture dish.

Buffer I Mix 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% (vol/vol) NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitors (1:1,000) and water. The lysis buffer may need to be modified on the basis of the nature of the protein of interest, e.g., the use of high-salt concentration for purification of nuclear proteins[49] or the use of DDM for the extraction of membrane proteins[50]. Salt, DDM or other reagents should be added to buffer I. The table shown below is an example for preparing 100 ml of the standard buffer I.

100 ml Buffer I

		ml	Stock
50 mM, pH 8	HEPES-NaOH	6.66	750 mM
150 mM	NaCl	3	5 M
5 mM	EDTA	1	0.5 M
0.5% (vol/vol)	NP-40	5	10% (vol/vol)
50 mM	NaF	10	0.5 M
1 mM	Na_3VO_4	10	10 mM
1 mM	PMSF	1	100 mM
1:1,000	Protease inhibitors	100 µl	
	Water	63.34	

▲ CRITICAL Freshly prepare the buffer from stock solutions; store it at 4 °C during use and discard the unused portion after completion of the purification. ▲ CRITICAL Keep protease inhibitors and PMFS cold at all times and add them immediately before using the buffer. The half-life of PMSF is short in aqueous solutions (110 min at pH 7, 55 min at pH 7.5, 35 min at pH 8, complete at 25 °C).

Buffer II Mix 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 5 mM EDTA and water. As for buffer I, modifications of the lysis buffer on the basis of the nature of the protein of interest may be required.

100 ml Buffer II

		ml	Stock
50 mM, pH 8	HEPES-NaOH	6.66	750 mM
150 mM	NaCl	3	5 M
5 mM	EDTA	1	0.5 M
	Water	89.34	

▲ CRITICAL Freshly prepare buffer II from stock solutions; store the buffer at 4 °C during use and discard the unused portion after completion of the purification. ▲ CRITICAL Buffer II is only required for a two-step affinity purification without cross-linking.

D-Biotin elution Mix 2.5 mM D-biotin, 1 ml of D-biotin stock solution and 9 ml of buffer I (pH 8.0). ▲ CRITICAL Freshly prepare the solution immediately before use and discard the unused portion.

Formic acid, 100 mM Mix 39.85 g of water and 0.185 g of formic acid. Prepare formic acid on the day before purification; store the solution at 4 °C in glass and discard the unused portion. This reagent is only required for a two-step affinity purification without cross-linking.
▲ CRITICAL Do not use plastic tips to pipette 100% acid; always use glass in order to prevent acid extraction of plasticizers.

Buffer III Prepare buffer I, but without NP-40. Freshly prepare the buffer from stock solutions; store the solution at 4 °C during use and discard the unused portion.

Buffer IV Make buffer I with 1% (wt/vol) DDM at a final concentration of 0.02% (wt/vol) instead of NP-40. Freshly prepare buffer IV from stock solutions; store it at 4 °C during use and discard the unused portion.
▲ CRITICAL Freshly prepare DDM stock, keep it cold and add it to buffer I immediately before use.

BS^3, 25 mM solution Dissolve 2 mg of the cross-linker in 140 µl of 20 mM HEPES-NaOH (pH 8.0). ▲ CRITICAL Use freshly prepared solutions and discard them after use. Do not keep stock solutions.

Tris-HCl, 1 M, pH 8.0 Filter Tris-HCl with a 0.2-µm nylon filter, and store it at 4 °C for up to 6 months.

CH_3CN in Tris-HCl Prepare CH_3CN in 30 mM Tris-HCl, (20% vol/vol; pH 8.0). ▲ CRITICAL Freshly prepare the solution and discard the unused portion.

Alkylation solution (55 mM IAA) Mix 0.11 g of IAA and 10 ml of digestion buffer. ▲ CRITICAL Freshly prepare the solution, protect it from light and discard after use.

Digestion buffer (50 mM NH_4HCO_3, pH 8.5) Add 0.7906 g of NH_4HCO_3 to 200 ml of water. ▲ CRITICAL This buffer can be stored for 2–3 d at 4 °C. After 2–3 d, discard any remaining buffer and prepare a fresh batch.

Digestion solution (12.5 ng µl^{-1} trypsin) Add 20 µg of trypsin to 1,600 µl of digestion buffer. ▲ CRITICAL Use freshly thawed aliquots of trypsin. Discard both unused, thawed trypsin and digestion solution after use.

Ethanol wash (50% ethanol, 50% water (vol/vol)) Mix 125 ml of ethanol and 125 ml of water. Freshly prepare the solution before use; store the solution at RT and discard the unused portion.

Extraction solution Extraction solution is 100% CH_3CN. Prepare an aliquot of the volume required for the experiment. Store the solution at RT and discard the unused portion.

Reduction stock (500 mM DTT) Add 0.3855 g of DTT to 5 ml of water. Store the stock at −20 °C. ▲ CRITICAL Do not refreeze thawed aliquots. Discard the aliquots after use.

Reduction solution (10 mM DTT) Mix 200 µl of reduction stock and 9.8 ml of digestion buffer. ▲ CRITICAL Freshly prepare the solution before use. Discard the unused portion.

Wash solution (50% ethanol, 50% digestion buffer (vol/vol)) Mix 25 ml of ethanol and 25 ml of digestion buffer. Freshly prepare the solution; store it at RT and discard the unused portion.

Alkylation solution (1 M IAA) Add 0.037 g of IAA to 10 µl of water.
▲ CRITICAL Prepare the solution freshly and protect it from light; discard after use.

Reduction solution (500 mM DTT) Add 0.3855 g of DTT to 5 ml of water. Store the aliquots at −20 °C. ▲ CRITICAL Do not reuse thawed aliquots; discard after use.
Formic acid, 5% (vol/vol) Mix 95 ml of water and 5 ml of formic acid. The solution can be stored as aliquots at 4 °C for several months.
▲ CRITICAL Do not use plastic tips to pipette 100% acid; always use glass to prevent acid extraction of plasticizers.
TFA, 30% (vol/vol) Mix 70 ml of water and 30 ml of TFA. The solution can be stored in aliquots at 4 °C for several months.
Stage tip solution (0.4% (vol/vol) formic acid, 2% (vol/vol) TFA) Mix 48.8 ml of water, 1 ml of TFA and 200 µl of formic acid. The solution can be stored in aliquots at 4 °C for several months.
Stage tip elution (0.4% (vol/vol) formic acid, 90% (vol/vol) CH_3CN) Mix 45 ml of CH_3CN, 4.8 ml of water and 200 µl of formic acid. The solution can be stored in aliquots at 4 °C for several months.
2D Sample buffer Mix 7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 1 mM EDTA, 30 mM Tris-HCl (pH 8.5), 1 tablet of complete mini protease inhibitor per 100 ml and water. Aliquots can be stored at −80 °C for a maximum of 6 months.
Rehydration buffer Mix 5 M urea, 0.5% (wt/vol) CHAPS, 0.5% (vol/vol) Pharmalyte, 12 µl ml^{-1} of DeStreak reagent and water. Aliquots can be stored without DeStreak reagent at −80 °C for a maximum of 6 months.
Equilibration buffer stock solution Mix 375 mM Tris-HCl (pH 8.8), 6 M urea, 2% (wt/vol) SDS and 5 mg of bromophenol blue per 100 ml to visualize the run in the second-dimension SDS-PAGE gel. Aliquots can be stored at −20 °C for a maximum of 6 months.
Equilibration buffer I Add 100 mg of DTT to 10 ml of equilibration buffer stock solution. ▲ CRITICAL Freshly prepare the buffer and discard it after use.
Equilibration buffer II Add 480 mg of DTT to 10 ml of equilibration buffer stock solution. ▲ CRITICAL Freshly prepare the buffer and discard it after use.
TFA stock (1% TFA, vol/wt) for HPLC Mix 990 g of water and 14.8 g of TFA. The solution can be stored at ambient temperature in amber glass for 1 month. ▲ CRITICAL Do not use plastic tips to pipette 100% acid; always use glass in order to prevent acid extraction of plasticizers.
HPLC: binary pump mobile phase A (0.1% TFA, vol/wt) pH 2.5–3 Mix 900 g of water and 100.48 g of 1% (vol/wt) TFA stock. Store the solution at 4 °C for up to 1 week.
HPLC: binary pump mobile phase B (70% CH_3OH, 20% isopropanol, 0.1% TFA, wt/wt/wt), pH 2.5–3 Mix 554 g of CH_3OH, 157 g of isopropanol and 100.48 g of 1% (vol/wt) TFA stock. Store the solution at 4 °C for up to 1 week.
HPLC: nanopump mobile phase A (0.4% formic acid, wt/wt), pH 2.5–3 Mix 996 g of water and 4.88 g of formic acid. Store the solution at 4 °C for up to 1 week.
HPLC: nanopump mobile phase B (70% CH_3OH, 20% isopropanol, 0.4% formic acid, wt/wt/wt), pH 2.5–3 Mix 554 g of CH_3OH, 157 g of isopropanol, 96 g of water and 4.88 g of formic acid. Store the solution at 4 °C for up to 1 week.

EQUIPMENT SETUP

Stage tip purification Excise small discs of C18 reversed-phase beads embedded in Teflon mesh (Empore discs) using a 17-G metal needle. Insert the metal needle into a pipette tip (200-µl tips are preferred). Release the excised disc into the tapered end of the tip by pushing the insert to produce a completed stage tip[51].
Liquid chromatography mass spectrometry We use Xcalibur mass spectrometry software (ThermoFisher Scientific) for acquisition of the mass spectrometric data. The peptides are eluted from the analytical/separation column with a 27-min gradient ranging from 3 to 30% solvent B, followed by a 25-min gradient from 30 to 70% solvent B and, finally, a 7-min gradient from 70 to 100% solvent B at a constant flow rate of 100 nl min^{-1} (tabulated below). The analyses are performed in a data-dependent acquisition mode using a 'top 15' collision-induced dissociation (CID) method for peptide identification. Dynamic exclusion for selected ions is 60 s. We use a single lock mass at *m/z* 445.120024 to recalibrate the *m/z* range as the data are acquired. Maximal ion accumulation time allowed on the LTQ Orbitrap in CID mode is 150 ms for MS^n in the LTQ and 1,000 ms in the C-trap. Automatic gain control is used to prevent overfilling of the ion traps and was set to 5,000 (CID) in MS^n mode for the LTQ, and 10^6 ions for a full Fourier transform MS (FTMS) scan. Intact peptides are detected in the LTQ Orbitrap Velos at 60,000 resolution. The threshold for switching from MS to tandem MS (MS/MS) is 2,000 counts.

Time (min)	Mobile phase B (%)
3.00	3.0
5.00	13.0
30.00	30.0
55.00	70.0
62.00	100.0
77.00	100.0
80.00	3.0

PROCEDURE

Preparation of HEK293 cell lysate ● TIMING 60 min

▲ CRITICAL To minimize protein degradation, all steps must be performed on ice with precooled reagents and materials.
▲ CRITICAL To ensure that the integrity of the noncovalently interacting complex is maintained, perform purifications from freshly lysed cells.

1| Thaw the cells on ice for approximately 20 min.

2| Resuspend the cells in 5 ml of lysis buffer (equivalent to 1 ml per 15-cm dish) and incubate them on ice for 20 min.
▲ CRITICAL STEP For a one-step affinity purification (followed by chemical cross-linking; 'B' in **Fig. 1**) it is very important to include avidin in the lysis buffer. Avidin preclears biotinylated carboxylases that can bind to the StrepTactin beads in the first step of the purification. If the carboxylases are not precleared, they result in a high-background contamination when the samples are analyzed by LC-MS. Although this step can be omitted if you are proceeding to a two-step purification (without chemical cross-linking; 'A' in **Fig. 1**), preclearing of the lysates is still highly recommended.

3| Centrifuge the sample at 20,000*g* for 15 min at 4 °C.

4| Transfer the supernatant containing the cell extract to a dust-free 50-ml conical tube. Remove an aliquot (50 µl) for immunoblotting analysis, freeze it in liquid N_2 and store it at −80 °C until required.

5| Determine the protein concentration of the supernatant by Bradford assay. The minimum quantity required per purification is 40 mg (five cell culture dishes, each 15 cm in diameter). At this point, the sample can be used to either identify the constituents of noncovalently interacting functional protein complexes to build interactome networks (Steps 6–46 and 127–128; marked A in **Fig. 1**) and/or trap multiple, concurrently formed, heterogeneous subcomplex species via the introduction of a cross-linking agent to further dissect the multiple layers of the interactome (Steps 47–126 plus 127–128; marked B in **Fig. 1**).
▲ **CRITICAL STEP** Calculations of protein quantity are based on the use of BSA as the protein standard. Other proteins can be used as standards; however, different values will be obtained.
▲ **CRITICAL STEP** Protein concentration must be determined at this point for cross-linking, as the one-step affinity purification does not yield material of sufficient concentration for precise protein determination by standard protein assay kits available on the market.
? TROUBLESHOOTING

Two-step affinity purification without chemical cross-linking ● TIMING 3 h
▲ **CRITICAL** This is the beginning of the procedure marked A in **Figure 1**. Retain all beads, eluates, column flow-through and washes to assess the loss of the HA-tagged protein throughout the purification.

6| *First step*. Add 400 µl of StrepTactin Sepharose bead slurry (200 µl bed volume) to a fresh, dust-free BioSpin column.

7| Wash the StrepTactin Sepharose beads in the column with 1 ml of buffer I (2×).

8| Place a fresh, dust-free 15-ml conical tube under the column and slowly apply the cell extract to the beads without disturbing the bed. Allow the lysate to pass through the resin by gravity flow. No incubation time is required.

9| Wash the StrepTactin Sepharose beads with 1 ml of buffer I (4×).

10| Elute the bound proteins from the StrepTactin Sepharose beads with 3 × 300 µl of biotin elution buffer (2.5 mM biotin) into a 1.5-ml Eppendorf tube. Remove an aliquot (2–20 µl) of the eluate for immunoblotting analysis, freeze it in liquid N_2 and store it at −80 °C until required.
? TROUBLESHOOTING

11| Retain the StrepTactin Sepharose beads, freeze them in liquid N_2 and store them at −80 °C until required. If necessary, any bound (un-eluted) protein can be recovered by resuspension in Laemmli buffer and assessed by immunoblotting.

12| *Second step*. Add 200 µl of anti-HA agarose bead slurry (100 µl bed volume) to a 1.5-ml Eppendorf tube.

13| Centrifuge the mixture at 100*g* for 1 min at 4 °C and wash it with 1 ml of buffer I (1×).

14| Centrifuge the mixture again at 100*g* for 1 min at 4 °C. Remove the supernatant and resuspend the pellet in 100 µl of buffer I.

15| Add the biotin eluate to the anti-HA agarose beads and incubate the mixture on a rotating wheel at 4 °C for 1 h.
▲ **CRITICAL STEP** Do not incubate lysates in the BioSpin columns. The residual NP-40 that coats the column is efficiently eluted with 100 mM formic acid and results in a major polymer contamination of the sample observed by mass spectrometry.
▲ **CRITICAL STEP** Ensure that the tube is firmly closed and seal it with Parafilm if required. This is vital to prevent loss of the sample during rotation.

16| Centrifuge the mixture at 100*g* for 1 min at 4 °C.

17| Remove all the supernatant (i.e., unbound material after incubation with anti-HA agarose) with a pipette and retain it for immunoblotting analysis. Freeze the sample in liquid N_2 and store it at −80 °C until required.
▲ **CRITICAL STEP** It is important to remove all biotinylated carboxylases, as these proteins result in a high background contamination when the samples are analyzed by LC-MS. If the lysate has been precleared with avidin, however, removing every drop of the supernatant is not as important, because the majority of the carboxylases have been previously removed (see Step 2).

18| Resuspend the beads in 0.5 ml of buffer I and transfer the suspension to a BioSpin column. Transfer the remainder of the beads to the column with another 0.5 ml of buffer I. Allow them to drain by gravity.
▲ **CRITICAL STEP** Use a fresh dust-free Biospin column.

19| Wash the column with 1 ml of buffer I (3×).
▲ **CRITICAL STEP** To prevent resuspending any buffer containing NP-40 in the following wash step, consider washing with 500 µl of buffer I (6×).

20| Wash the column with 1 ml of 2× buffer II (without NP-40, without inhibitors). Apply buffer II to the walls of the BioSpin column from the 'top' (level of previous buffer) in a circular manner to wash the NP-40 contamination from the walls of the column. Allow the first 1 ml of buffer II to drain before applying the second 1-ml wash. Wash the outside of the column tip with buffer II to remove any residual NP-40.
▲ **CRITICAL STEP** This step is the key to producing samples that are polymer-free for subsequent analysis by LC-MS. Without extreme care at this point, protein samples will be contaminated with NP-40. Detergents and plasticizers are scavengers of charge. Thus, during analysis by mass spectrometry, these components are preferentially ionized at the expense of the peptide samples. Ultimately, weak signals or no peptide signals are observed by mass spectrometry.
▲ **CRITICAL STEP** Do not let the Biospin column stand dry. Elute the sample immediately.

21| Elute proteins from the BioSpin column directly into an amber HPLC glass vial (containing 125 µl of 1 M TEAB buffer) with 500 µl of 100 mM formic acid stored in a glass vial. If the acid does not elute from the column, it might be necessary to apply a small amount of pressure to initiate the flow of liquid and/or to flush out the remaining eluate.

22| Gently mix the sample in the vial, but do not invert the vial.

23| Remove 200 µl of eluate. This can be used for, e.g., silver stain gel and/or immunoblotting. These samples can be aliquotted into Eppendorf tubes, lyophilized and stored at −80 °C until required.

24| Analyze aliquots by 1D SDS-PAGE and immunoblotting with anti-HA-7 antibody (or an alternative anti-HA antibody) according to standard laboratory practices.
? TROUBLESHOOTING
■ **PAUSE POINT** Freeze the remaining 425 µl and store it at −80 °C until required.

Solution tryptic digest of two-step affinity purification ● TIMING ~4 h (dependent on the number of samples)
25| Reduce the 425-µl neutralized sample by adding 8.5 µl of reduction solution (500 mM DTT, final concentration ~10 mM).

26| Incubate the solution for 1 h at 56 °C.

27| Cool the solution to RT.

28| Alkylate free cysteine residues by adding 24 µl of alkylation solution (1 M IAA, to a final concentration of ~55 mM).

29| Incubate the solution in the dark for 30 min at RT.

30| Add 142 µl of 1 M TEAB to the reduced and alkylated sample containing the acid-eluted proteins (final volume = 600 µl).

31| Check the pH. Take 1 µl of the sample and pipette it onto a piece of pH paper. The pH should be between 7.5 and 8.5. If pH is less than 7, add an additional 1 M TEAB buffer (1 µl at a time) until the pH is within the correct range. Ensure consistency with each batch of samples; always remove and add the same volumes of liquid.
▲ **CRITICAL STEP** The pH must be correct for digestion with trypsin.

32| Add 2.5 µg of thawed, undiluted trypsin to the sample. Mix it gently, centrifuge briefly at 100*g* for 1 min at RT, and then incubate at 37 °C overnight.
▲ **CRITICAL STEP** Do not quench the reaction with acid.
■ **PAUSE POINT** Freeze the digested sample and store it at −80 °C until required.

Stage tip purification and concentration of two-step affinity purification samples ● TIMING ~4 h (dependent on the number of samples)

33| Prepare three stage tips per affinity purification. This is sufficient for triplicate LC-MS analyses.

34| Activate the stage tips with 50 µl of CH_3OH. Check each tip and discard any tips that leak.

35| Centrifuge the stage tips at RT for 2–3 min at a maximum of 420*g*. Ensure that the CH_3OH is completely removed from the stage tips. If the liquid does not flow through completely, push it through manually with a syringe.

36| Add 50 µl of stage tip solution to equilibrate the stage tips.

37| Centrifuge the stage tips at RT for 2–3 min at a maximum of 420*g*. If the liquid does not flow through completely, push it through manually with a syringe.

38| Add a plug of 50 µl of stage tip solution to each stage tip (do not push it through).

39| To acidify the tryptically digested proteins, pipette 30 µl of the sample (equivalent to 5% (vol/vol) of the digest and ~3% (vol/vol) of the total eluate) into the plug of the stage tip solution added to each stage tip. Look for bubbles indicating the release of CO_2 and acidification of the sample.
▲ **CRITICAL STEP** It is imperative that the digested sample is acidified in order to ensure efficient binding to the stage tip material.
▲ **CRITICAL STEP** Before analysis of the tryptically digested proteins by LC-MS, it is not possible to determine whether this sample quantity is suitable for all SH-tagged protein complexes. If weak signals are observed by LC-MS (data generated at Steps 127 and 128), the researcher can return to the original digested sample and use a larger volume. If strong signals consistent with analytical column overloading are observed by LC-MS, a lower volume of the digested proteins can be repurified and analyzed.
▲ **CRITICAL STEP** To load larger volumes of the buffered digest onto a stage tip, it is necessary to add additional volumes of 30% (vol/vol) TFA to ensure complete acidification of the sample, e.g., 50 µl of stage tip solution plus 180 µl of digested sample plus 10 µl of 30% (vol/vol) TFA gives a volume of 240 µl and a final TFA concentration of ~1% (vol/vol).

40| Centrifuge the stage tips gently for 2–3 min at RT at a maximum of 420*g*.
▲ **CRITICAL STEP** If the stage tips are centrifuged too fast, the peptides do not bind efficiently to the material and are lost in the flow-through. If the liquid does not flow through completely, push it through manually with a syringe.

41| Add 50 µl of stage tip solution to wash the bound peptides. Gently centrifuge the tips at RT for 2–3 min at a maximum of 420*g* to ensure that no peptides are lost in the flow-through. If the liquid does not flow through completely, push through manually with a syringe.

42| Ensure that all liquid has been removed from the tips.

43| Elute the bound peptides with 50 µl of stage tip elution into clear HPLC vials with a glass insert, and pool the three stage tip extractions per affinity purification.

44| Remove the CH_3CN in the stage tip elution in the vacuum concentrator (30 °C for 15 min) until ~2 µl final volume remains.
▲ **CRITICAL STEP** Do not dry to completeness, as loss of peptides via adsorption to glass surfaces is increased.

45| Reconstitute the samples with ~8 µl of 5% (vol/vol) formic acid to a final volume of ~10 µl.

46| Add multiples of 8 µl of 5% (vol/vol) formic acid depending on how many analyses will be performed by LC-MS (default = sufficient for three injections, i.e., 26 µl).
▲ **CRITICAL STEP** Volumes are dependent on the HPLC sample loop and can be adjusted accordingly.
■ **PAUSE POINT** Store the extracted samples at −20 °C until analysis by LC-MS. Store the remaining digested sample at −80 °C for re-extraction of different quantities with stage tips, peptide separation into additional fractions, iTRAQ or TMT labeling, as required.

One-step affinity purification with chemical cross-linking ● **TIMING 4 h**

▲ **CRITICAL** This section is the beginning of the procedure marked B in **Figure 1**. Retain all beads, eluates, column flow-through and washes in order to assess the loss of the HA-tagged protein throughout the purification.

47| Add 400 µl of StrepTactin Sepharose bead slurry (200 µl bed volume) to a fresh, dust-free BioSpin column.

48| Wash the StrepTactin Sepharose beads in the column with 1 ml of buffer I (2×).

49| Place a fresh, dust-free 15-ml conical tube under the column and slowly apply the cell extract to the beads without disturbing the bed. Allow the lysate to pass through the resin by gravity flow. No incubation time is required.

50| Wash the StrepTactin Sepharose beads with 1 ml of buffer I (1×).

51| Wash the StrepTactin Sepharose beads with 1 ml of buffer III (without NP-40) (1×).

52| Wash the StrepTactin Sepharose beads with 1 ml of buffer IV (with 0.02% (wt/vol) DDM instead of NP-40) (2×).

! CAUTION Changing the detergent may alter the effectiveness of the elution, thereby resulting in changes in the yield of the protein complex.

▲ **CRITICAL STEP** This exchange of NP-40 for DDM is important to remove the detergent from the sample before 2D SDS-PAGE. DDM does not disrupt the noncovalent protein interactions of the complex; however, the micelles have a lower M_r and can be removed by ultrafiltration.

53| Elute the bound proteins from the StrepTactin Sepharose beads with three washes of 300 µl of biotin elution buffer (2.5 mM biotin) prepared in buffer IV (0.02% (wt/vol) DDM instead of NP-40) into a 1.5-ml Eppendorf tube. Remove an aliquot (2–20 µl) of the eluate for immunoblot analysis.

▲ **CRITICAL STEP** Do not freeze the samples at this point unless the stability of the frozen protein complex has been assessed.

54| Retain the StrepTactin Sepharose beads. If required, any bound (uneluted) protein can be recovered by resuspension in Laemmli buffer.

55| Add the BS^3 stock solution to the affinity-purified complexes to a final concentration of 0.01–5 mM.

▲ **CRITICAL STEP** It may be necessary to prepare a serial dilution of the cross-linker to determine the optimal concentration for the specific complex under investigation. In parallel, prepare the control, non-cross-linked sample. Add the same volume of 20 mM HEPES-NaOH to the control sample as was used for the BS^3 stock solution.

▲ **CRITICAL STEP** Keep all final volumes identical. This is important to maintain the same ratio of protein to cross-linker in all samples.

▲ **CRITICAL STEP** It is not possible to measure the protein concentration at this stage, as the sample is too dilute for any protein assay. It is possible to cross-link after concentrating the sample; however, there is a risk of creating nonspecific protein aggregates that could lead to artificial subcomplex formation.

▲ **CRITICAL STEP** If a lysine-specific cross-linker such as BS^3 is used, do not use Tris buffer or any other free amine–containing reagents during protein purification. Free amines quench the cross-linker.

56| Briefly vortex the samples.

57| Incubate the samples on ice for 2 h.

58| Quench the samples for 15 min at RT with Tris-HCl (pH 8.0) at a final concentration of 50 mM.

59| Prepare the samples for 1D SDS-PAGE in LDS sample buffer supplemented with DTT at a final concentration of 50 mM. Incubate the samples at 70 °C for 10 min.

60| Analyze the aliquots by 1D SDS-PAGE[52] and immunoblotting with anti-HA antibody according to standard laboratory practices.

? TROUBLESHOOTING

■ **PAUSE POINT** Cross-linked samples can be frozen and stored at −80 °C until required.

Buffer exchange for 2D SDS-PAGE ● TIMING ~1.5–3 h (dependent on the quantity and volume of the samples)
▲ **CRITICAL** Retain all flow-through material and washes to assess loss of the sample throughout buffer exchange. Perform all steps at 4 °C.

61| Pipette the cross-linked sample into an Amicon ultrafiltration device with a 100-kDa cutoff. Centrifuge the sample at 14,000*g* for 3 min.
▲ **CRITICAL STEP** This step removes a major proportion of the DDM without loss of the protein complexes.

62| Pipette the concentrated sample into an Amicon ultrafiltration device with a 10-kDa cutoff.

63| Wash the 100-kDa filter with 200 µl of 20% (vol/vol) CH_3CN in 30 mM Tris-HCl (pH 8.0), and add to the sample in the 10-kDa Amicon ultrafiltration device.
▲ **CRITICAL STEP** This step is necessary to recover any residual protein.

64| Fill the 10-kDa Amicon ultrafiltration device to a maximum level with 20% (vol/vol) CH_3CN in 30 mM Tris-HCl (pH 8.0) and mix gently. Centrifuge for 5 min at 14,000*g*.
▲ **CRITICAL STEP** For high-quality buffer exchange, the volume of the samples should be reduced to ~100 µl or less.

65| Resuspend the concentrated sample to the maximum allowed in the filter with 20% (vol/vol) CH_3CN in 30 mM Tris-HCl (pH 8.0). Centrifuge the sample for 5 min at 14,000*g* in the same tube (2×).
▲ **CRITICAL STEP** Three volume exchanges should be performed with the new buffer.

66| Collect the concentrated sample from the filter (approximately 30–50 µl). Wash the filter with 50 µl of 20% (vol/vol) CH_3CN in 30 mM Tris-HCl (pH 8.0) to recover any residual sample and pool it with the first collection.

67| Lyophilize the concentrated sample to remove all the CH_3CN.
▲ **CRITICAL STEP** For this volume of CH_3CN, approximately 1–2 h is required.
■ **PAUSE POINT** Cross-linked samples can be frozen and stored at −80 °C until required.

2D SDS-PAGE and silver staining ● TIMING 2 d
▲ **CRITICAL** At a minimum, prepare two gels simultaneously, one for silver staining and one for immunoblotting. Preparation of the immunoblot in parallel is important to orient the pattern of the 2D gel after separation and to locate spots or regions containing the protein of interest.

68| Prepare and store the voltage protocol in the IEF device.

69| After the end of the rehydration phase of the IEF, prepare two electrode wicks wetted with water per IPG strip.

70| At the end of the IEF, prepare a plastic cover sheet by cutting the sides open in a dust-free environment (e.g., a hood).

71| Re-dissolve the cross-linked samples in 100 µl of 2D sample buffer. Assess the samples and the buffer exchange procedure for protein recovery by 1D SDS-PAGE and/or immunoblotting.
! **CAUTION** Re-freezing the samples stored in buffers containing urea reduce protein recovery owing to precipitation.
▲ **CRITICAL STEP** After re-dissolving the samples, continue immediately with 2D SDS-PAGE[53–55].

72| Bring each sample to a final volume of 200 µl (for 11-cm IPG strips) using rehydration buffer and vortex briefly.

73| Pipette the obtained rehydration mix into the sample groove of the focusing tray.
▲ **CRITICAL STEP** The sample should not be distributed over the complete length but approximately over 80% of the IPG strip length.

74| Remove the cover film from the IPG strip and apply the strip gel-side-down into the sample groove using forceps.
▲ **CRITICAL STEP** Ensure that the entire strip is wetted by the sample and that both ends of the strip make contact with the appropriate electrode. Avoid trapping air bubbles underneath the IPG strip. Check that the polarity of the IPG strip matches the IEF device.

75| Cover the IPG strip with mineral oil to prevent drying and crystallization of urea during rehydration and IEF.
▲ **CRITICAL STEP** Apply the mineral oil starting from the ends of the IPG strip to prevent the sample from being pushed out.

76| Start the rehydration protocol of the IEF device (active rehydration at 50 V and 20 °C, minimum 12 h, maximum 20 h).

77| After the rehydration phase is completed, open the cover and lift each end of each IPG strip to insert an electrode wick wetted with water.
▲ **CRITICAL STEP** The electrode wicks must be moist but must not carry excess of water. By using forceps, apply gentle pressure to the ends of the IPG strip to ensure that contact to the electrode is established.

78| Close the cover and start the focusing protocol stored in the IEF device during setup (focusing temperature 20 °C, maximum current per strip 30 μA).

Phase	Voltage	Gradient setting	Phase duration (h)	Total duration (h)
1	100	Rapid	3	3
2	500	Linear	1	4
3	500	Rapid	0.5	4.5
4	1,500	Linear	1	5.5
5	1,500	Rapid	0.5	6
6	3,500	Linear	1.5	7.5
7	3,500	Rapid	1	8.5
8	8,000	Rapid	32,000 Vh	12.5

79| After the focusing is completed, remove the IPG strip using forceps, drain excess mineral oil and store the focused strip between plastic cover sheets at −80 °C until further use.
■ **PAUSE POINT** Store the focused IPG strips at −80 °C for a maximum of 3 months.

80| Allow the IPG strip to thaw for a few minutes before removing the plastic cover.

81| Transfer the IPG strip into a 15-ml conical tube containing 10 ml of equilibration buffer I and agitate it for 15 min on a roller mixer.

82| Remove the IPG strip, drain excess buffer, transfer the IPG strip into a 15-ml conical tube containing 10 ml of equilibration buffer II and agitate it for 15 min on a roller mixer.

83| Remove the IPG strip, briefly dip it in Laemmli buffer and insert it into the Criterion gel cassette using forceps. Push the IPG strip down to the surface of the Criterion gel using a spatula.
▲ **CRITICAL STEP** Be careful not to damage the IPG strip; only push the plastic backing, not the gel. Ensure that the plus end of the IPG strip is orientated toward the pocket for the molecular-weight standard to facilitate interpretation of the gel and blot images.

84| Fill the Criterion cell with Laemmli buffer to the indicated mark.
▲ **CRITICAL STEP** Avoid trapping air bubbles under the IPG strip. If air is trapped, again gently push the backing of the IPG strip using a spatula.

85| Pipette 3 μl of molecular weight standard into the dedicated pocket next to the plus end of the IPG strip.

86| Connect the Criterion cell to the power supply and start the run at 20 mA per gel.

87| Stop the run when the bromophenol blue front has reached the lower end of the gel.
▲ **CRITICAL STEP** Depending on the proteins of interest and the acrylamide concentration of the used Criterion gel, it may be necessary to add extra run time after the bromophenol blue front has left the gel.

88| Remove the sealed gel cassette from the Criterion cell and open by breaking the side in a dust-free environment.

89| Proceed immediately with silver staining and immunoblotting.

90| Stain one gel with silver according to the method of Shevchenko *et al.*[52].
? TROUBLESHOOTING

91| Immunoblot one gel with anti–HA-7 antibody according to standard laboratory practices.
▲ **CRITICAL STEP** It is advisable to proceed immediately to gel band excision and digestion. The silver stain continues to slowly develop when stored. Proteins become irreversibly fixed in the gel matrix, and thus peptide recovery from the gel plugs after digestion is reduced.
? TROUBLESHOOTING
■ **PAUSE POINT** Store silver-stained gel(s) in a dust-free Criterion stain/blotting tray with water for a maximum of 2–3 d at 4 °C, but be aware that storage will reduce recovery from the gel.

Gel plug excision and *in situ* tryptic digestion ● TIMING 1.5 d
92| Place 150 µl of wash solution in a sufficient number of low-binding Eppendorf tubes required for the experiment (dependent on the number of plugs excised from the gel).
▲ **CRITICAL STEP** Ensure that the Eppendorf tubes remain closed as much as possible in order to avoid excessive contamination by keratin.

93| Clean the gel grid cutter before initial use with the ethanol wash and a toothbrush.
▲ **CRITICAL STEP** Use a new grid for each region of the gel excised or thoroughly clean the grid between excisions with the ethanol wash and the toothbrush.
▲ **CRITICAL STEP** Thorough cleaning of the gel grid cutter is important to remove tiny pieces of gel (containing protein), thus avoiding cross-contamination of other gel regions.

94| Remove water from the silver-stained gel stored in a Criterion stain/blotting tray.

95| Excise the gel plugs, cut them further into 1-mm square pieces and place three or four plugs in individual Eppendorf tubes containing 150 µl of wash solution.
▲ **CRITICAL STEP** When combining several gel plugs, ensure that the regions excised belong to the same subcomplex species.
▲ **CRITICAL STEP** For replicate LC-MS analyses from two gels, excise the regions as similarly as possible.
▲ **CRITICAL STEP** For this step and all later steps, ensure that the gel pieces are always suspended in the solution for effective washing, buffer exchanges and prevention from desiccation.

96| Thaw the reduction solution (10 mM DTT).

97| Remove and discard the liquid from the gel plugs with a pipette.

98| Dehydrate the gel plugs with 150 µl of ethanol for 10 min at RT. Remove and discard the liquid with a pipette. Extensive washing is not necessary for silver-stained plugs.

99| Reduce disulfide bonds by adding 50 µl of reduction solution. Incubate the sample for 45 min at 56 °C. Remove and discard the liquid with a pipette.

100| Dehydrate the gel plugs with 150 µl of ethanol for 10 min at RT. Remove and discard the liquid with a pipette.

101| Alkylate the cysteine residues by adding 50 µl of alkylation solution. Protect from light. Incubate at RT for 30 min. Remove and discard the liquid with a pipette.

102| Wash the gel plugs with 150 µl of digestion buffer for 10 min. Remove and discard the liquid with a pipette.

103| Dehydrate the gel plugs with 150 µl of ethanol for 10 min. Remove and discard the liquid with a pipette.

104| Repeat the last two steps.

105| Allow the gel plugs to dry in a fume hood for 5 min to ensure that all ethanol has evaporated.

106| Add 10 µl of digestion solution (trypsin) and incubate the plugs for 10 min at RT.
▲ **CRITICAL STEP** Ensure that the gel plugs are completely covered by the liquid to allow the trypsin to enter.

107| Add 20 µl of digestion buffer to keep the gel plugs moist.
▲ **CRITICAL STEP** Ensure that the gel plugs are covered with liquid and are not attached to the side of the Eppendorf tubes.

108| Incubate the gel plugs at 37 °C for 4 h or overnight.
▲ **CRITICAL STEP** Check the digestion after ~2 h to ensure that all the gel plugs are still moist. If some of the plugs have absorbed all the liquid, add more digestion buffer.

109| Remove the liquid from each tube and transfer the liquid to fresh, individual dust-free low-binding Eppendorf tubes.
▲ **CRITICAL STEP** Do not discard the solution, as this contains the tryptic peptides.

110| Add 50 µl of 100% CH_3CN to the gel plugs to extract peptides. Wait until the plugs are completely dehydrated (gel plugs should shrink and become opaque).
▲ **CRITICAL STEP** Cross-linked peptides are often quite large, and thus the peptide recovery from the plugs is increased by this step.

111| Remove the liquid containing the extracted peptides and add it to the individual tubes containing tryptic peptides from Step 109.

112| Rehydrate the gel plugs with a sufficient volume of digestion buffer to return the gel plugs to the original size and repeat the CH_3CN extraction.

113| Reduce the volume of the combined extracts in a vacuum concentrator to ~2 µl, and reconstitute with 50 µl of stage tip solution.
▲ **CRITICAL STEP** Do not dry the gel plugs to completeness, as loss of peptides via adsorption is increased.

114| Proceed immediately to stage tip purification and concentration.

Stage tip purification and concentration for *in situ*–digested samples ● **TIMING ~4 h**
115| Activate the stage tips with 50 µl of CH_3OH. Check each tip and discard any tips that leak.

116| Centrifuge the stage tips at RT for 2–3 min at a maximum of 420*g*.
▲ **CRITICAL STEP** Ensure that the CH_3OH is completely removed from the stage tips. If the liquid does not flow through completely, push it through manually with a syringe.

117| Add 50 µl of stage tip solution to equilibrate the stage tips.

118| Centrifuge the stage tips at RT for 2–3 min at a maximum of 420*g*. If the liquid does not flow through completely, push through manually with a syringe.

119| Load the acidified peptides from each Eppendorf tube onto a separate stage tip.

120| Centrifuge gently at RT for 2–3 min at a maximum of 420*g*.
▲ **CRITICAL STEP** If the stage tips are centrifuged too fast, the peptides do not bind efficiently to the material and are lost in the flow-through. If the liquid does not flow through completely, push through manually with a syringe.

121| Add 50 µl of stage tip solution to wash the bound peptides.

122| Gently centrifuge at RT for 2–3 min at a maximum of 420*g* to ensure that no peptides are lost in the flow-through. If the liquid does not flow through completely, push it through manually with a syringe.

123| Ensure that all liquid has been removed from the tips.

Box 3 | Data conversion and identification of proteins via database search algorithms

1. Convert the acquired mass spectrometry file (.raw extension) into individual tandem mass spectra (MS/MS) files (.dta extension) using msconvert (ProteoWizard Library, http://proteowizard.sourceforge.net/), and concatenate the individual files into a single, Mascot generic file (.mgf extension). An internally developed Perl script is used at our institute to perform this file merge; however, commercially available software can be substituted, e.g., *merge.pl* (MatrixScience).
2. Use the .mgf file to perform a search against a protein database (e.g., human SwissProt) with the two search engines Mascot (MatrixScience) and Phenyx (GeneBio)[58].
3. Before submission of the .mgf file to Mascot, set the digest enzyme to trypsin and allow one missed cleavage site.
4. Set carbamidomethyl cysteine as a fixed modification, and oxidized methionine as a variable modification.
5. Submit the .mgf file to Mascot with mass tolerances on both the precursor and fragment ions of ±10 p.p.m. and ±0.6 Da, respectively.
6. Recalibrate all precursor and fragment ion masses before a second search with narrower mass tolerances (±4 p.p.m. and ±0.3 Da). An internally developed Perl script is used at our institute to perform the recalibration; however, freeware software can be substituted, e.g., multiplierz[59], mMass (http://www.mmass.org/).
7. By using identical search parameters settings as for the initial search (steps 3 and 4), submit the recalibrated .mgf file to a second pass search with both Mascot and Phenyx.
8. Following the second-pass search, select the proteins identified by Mascot that have two unique peptides with an ion score >18 (plus additional peptides from proteins fulfilling the criteria with an ion score >10); and the proteins identified with Phenyx that have two unique peptides with a *z*-score >4.5 and a *P* value <0.001 (plus additional peptides from proteins fulfilling the criteria with a *z*-score >3.5 and a *P* value <0.001).
9. Merge the proteins retrieved by the two search algorithms that fulfill the conditions in step 8, and discard any tandem mass spectra that conflict owing to a match to different proteins. Group the remaining proteins that contain identical peptides and cannot be differentiated on the basis of the tandem mass spectral data. We use an internally developed software program at our institute to perform these functions; however, commercially available software can be substituted, e.g., Proteome Discoverer (ThermoFisher Scientific) or Scaffold (Proteome Software).
10. By using an identical procedure as described from steps 2 to 9, repeat the search against the same protein sequence database where the sequences have been reversed or randomized[60] to obtain a false-positive detection rate of <1 and <0.1% (including the peptides exported with lower scores) for proteins and peptides, respectively.

124| Elute the bound peptides with 50 µl of stage tip elution into clear HPLC vials with a glass insert.

125| Remove the CH_3CN in the stage tip elution in the vacuum concentrator (30 °C for 15 min) until ~2 µl final volume remains.
▲ **CRITICAL STEP** Do not dry to completeness, as the loss of peptides via adsorption to glass surfaces is increased.

126| Reconstitute the samples with ~8 µl of 5% (vol/vol) formic acid to a final volume of ~10 µl (or multiples of 8 µl, depending on the number of injections required or the intensity of the stained region).
▲ **CRITICAL STEP** Volumes are dependent on the HPLC sample loop and can be adjusted accordingly.
■ **PAUSE POINT** Samples can be frozen at −20 °C until required for analysis by LC-MS.

Nano-HPLC-MS/MS analysis of the digested sample ● **TIMING 100 min per injected sample**
127| Inject the acidified sample (8 µl) onto the reversed-phase trap column and wash it with 0.1% (vol/wt) TFA.

128| Elute the *in situ*–tryptically digested sample from the trap column onto the separation column and detect the peptides with the mass spectrometer. Use the HPLC gradient and MS method described in Equipment Setup. Guidelines for MS data conversion, analysis and protein identification are outlined in **Box 3**.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
No HA-tagged bait protein evident in the lysate or present at a low level			
5	Expression of the bait is absent or low	Bait protein is not expressed or expressed at low level	Check the cell line, the construct of the recombinant protein and the cell culturing conditions. Check the induction conditions (next point)
		Expression of the bait was not induced or it rapidly degraded after induction (if an inducible cell line was used)	Optimize the induction of the bait expression. Test different times of induction (6–48 h) and/or inducer concentration (lower expression levels may help if the bait protein is toxic or levels are tightly regulated in the cell)
	Bait protein is not extracted from the cells	Lysis buffer is not efficient enough or bait protein is insoluble	Check the presence of the bait in the pellet after extraction. Revise/reconsider the composition of the lysis buffer and modify according to the properties of the bait (e.g., cell localization, soluble/membrane)
No HA-tagged bait protein evident in the eluate from StrepTactin beads (as observed by immunoblot of the Step 10 sample)			
10	Bait protein did not bind to the StepTactin beads	Tag is not accessible	Prepare an alternative expression construct with the tag at the different terminus of the protein
		Binding of the tag to the beads is competed with presence of biotin in the sample (e.g., carboxylases are natural biotin-containing proteins)	Add more avidin to the sample to neutralize biotin-containing contaminants
		Binding conditions are not correct	Check buffers: salt concentration, detergent, pH. Optimize if required
		Too stringent washing conditions	Concentrate sample for loading. Reduce washing steps
	Bait protein did not elute from the StepTactin beads	Protein precipitated on the column	Reduce salt concentration or increase detergent, check pH—it should not be near the pI of the protein
		Problem with biotin	Always use freshly prepared biotin
No HA-tagged bait protein evident in the eluate from HA-agarose (as observed by immunoblot of the Step 24 sample step)			
24	Bait protein did not bind to the HA-agarose	Amount of bait protein is too low to be detected	In situations where there is a low level of bait expression, perform a one-step purification only
		Binding conditions are not correct	Check buffers: salt concentration, detergent, pH. Optimize if required
	Bait protein did not elute from the HA-agarose	Protein aggregation or precipitation on the HA-agarose	Use alternative elution buffers that are compatible with gel-free LC-MS (see INTRODUCTION) Verify that all buffers have the correct composition and pH. Prepare fresh buffers if any problems are suspected
	Sample degradation	Insufficient or inappropriate protease inhibitors	Reconsider the quantity of protease inhibitors used and complement the inhibitor cocktail with appropriate, specific protease inhibitors
		Incorrect conditions for the purification procedure	Protein can be more sensitive to temperature conditions. Take care that all solutions are kept cool during the purification. Consider performing all steps of the purification in a cold room

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
Cross-linking reaction failure			
60	No high-molecular-mass protein complex(es) detected with SDS-PAGE after the cross-linking reaction	Protein complex was disassembled by denaturing condition of elution	Only use detergents that are non-denaturing; avoid chaotropic agents
		Cross-linking reaction conditions were disrupted	Check the pH of the solutions. There is a narrow pH range where the cross-linker is active and reacts appropriately to conjugate lysine residues
		Reaction was quenched	Determine that there are no components in the buffers that can quench the cross-linking reaction
		BS^3 degraded	Ensure that buffers are freshly prepared immediately before use as BS^3 is unstable and rapidly hydrolyses in aqueous solutions
		Cross-linker concentration is too low	Titrate different concentrations of the cross-linker to optimize
		Formed cross-linked complexes are too large and did not enter the gel	Use another cross-linker concentration. Estimate the potential size of the protein complex(es). Use a gel with a lower percentage of acrylamide to favor proteins of high molecular mass
Unsatisfactory quality of 2D gels and/or blots			
90, 91	Burning of IPG strip	High salt concentration in the sample leads to high current and resulting high temperature in the first dimension	Perform additional buffer exchange steps before lyophilization and rehydration of the sample for 2D gel analysis
			Lengthen the desalting step of the IEF protocol (i.e., phase 1 at 100 V)
			Limit the voltage per IPG strip to 30 μA; if focusing different samples with varying salt concentrations perform individual focusing of each strip
	Horizontal streaking	Horizontal smear around spots results from incomplete focusing or oxidation of proteins during first dimension	Ensure that the rehydration mix contains DeStreak reagent or, if unavailable, DTT or a similar reducing agent. However, DeStreak is preferred due to the electrochemical stability of the reagent over the entire pH range
			Increase the focusing time, especially in the plateau phases 3, 5 and 7 of the IEF protocol for better equilibration of slower-migrating proteins
	Vertical streaking	Vertical smear results from funnels in the upper gel surface or trapped air bubbles between the IPG strip and the gel	Check the quality of the ready-cast gel by visual inspection. In case of irregularities in the gel or especially near the upper surface, discard and replace the gel
			Ensure that any air trapped between the IPG strip and the upper surface of the gel is removed by gentle tipping with a spatula, without harming the IPG gel
	Incomplete transfer of proteins onto the blotting membrane	High-molecular-weight proteins may incompletely migrate onto the blotting membrane because of decreased electrophoretic mobility	Increase the incubation time in transfer buffer before assembly of the blotting sandwich

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
90, 91			Increase the blotting time for complete transfer of high-molecular-weight proteins
			Use a PVDF membrane and/or a smaller pore size instead of a nitrocellulose membrane
	Staining artifacts in silver-stained gel	Silver staining might stain residual impurities of the sample	Try alternative high-sensitivity protein staining (e.g., fluorescent staining by SYPRO Ruby)
Contamination in the MS/MS analysis			
128	Polymeric contamination, detergent is still present in the sample analyzed by LCMS	Inefficient washing of the HA-agarose in the second step of the purification	Perform the washing at the second step of purification with extreme care; introduce an additional washing step, although not desired, as it can cause additional loss of protein in its turn; check composition of buffer II
		Wrong buffer used to wash the HA-agarose column	If detergent contamination is suspected, discard old solutions and prepare fresh solutions
		Pipettes are contaminated with detergent (NP-40)	Check pipettes regularly for contamination with detergent that can cause an introduction of polymers into the solutions or sample
		Some of the solutions used in downstream procedures after cell lysis were contaminated with polymer	Take more care during preparation for the experiment in general; check that all glassware does not contain traces of soap or other detergent/polymer; keep detergent-containing solutions away from wash solutions; keep all solutions and sample-containing vessels closed during the purification procedure to avoid accidental cross contamination with the drops of undesired components (this care also prevents cross contamination between samples)
	High levels of keratin in the obtained MS/MS data	Relaxation of the precautions against keratin contamination	Follow the recommended protocol: wear gloves; use dust-free cabinets when required; keep all containers, solutions, boxes closed at all times; use dust-free consumables
No bait protein detected by MS/MS analysis (HA-tagged protein is detected by immunoblot in the final eluate)			
128	No or low level of the bait protein detected by MS/MS analysis in the presence of other co-purified proteins	Size of the majority of the tryptic peptides generated from the bait protein during digestion are outside the *m/z* range of the mass spectrometer used for the analyses	Use an alternative enzyme for protein digestion
	No or low levels of the bait protein and other proteins detected by MS/MS analysis	Digestion of the proteins failed	Check the digestion conditions with particular attention to the pH conditions, and extraction of the peptides from the gel plugs
		Stage tip purification failed	Check buffer composition and pH. Do not allow the C18 material in the stage tip dry between the purification steps
	Absence of the expected interacting proteins in the affinity purification; very poor quality of the purification (a generally low number of identified proteins)	Appropriate physiological pathways were not active at cell collection	Use an appropriate and competent type of cells to express the recombinant bait protein. Induce an appropriate signaling pathway if required

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
128		Bait protein loses activity during lysis and purification (and thus some interaction partners)	Complement purification solutions with components maintaining desired activity of the protein (e.g., cofactors, metal ions, phosphatase inhibitors) that can be important for interaction with other proteins

● TIMING

Steps 1–5, preparation of HEK293 cell lysate: 60 min
Steps 6–24, two-step affinity purification without chemical cross-linking: 3 h
Steps 25–32, solution tryptic digest of two-step affinity purification: ~4 h (dependent on the number of samples)
Steps 33–46, stage tip purification and concentration of two-step affinity purification samples: ~4 h (dependent on the number of samples)
Steps 47–60, one-step affinity purification with chemical cross-linking: 4 h
Steps 61–67, buffer exchange for 2D SDS-PAGE: ~1.5–3 h (dependent on the quantity and volume of the samples)
Steps 68–91, 2D SDS-PAGE and silver staining: 2 d
Steps 92–114, gel plug excision and *in situ* tryptic digestion: 1.5 d
Steps 115–126, stage tip purification and concentration for *in situ*–digested samples: ~4 h
Steps 127 and 128, nano-HPLC-MS/MS analysis of the digested sample: 100 min per injected sample
Box 1, 1–3 d (depending on the length of the HPLC gradient for LCMS analysis)
Box 2, ~4 h

ANTICIPATED RESULTS

To assess the utility and practicality of the protocol detailed in this manuscript, N-terminally tagged SH-TBK1 was used as an example. Previous TAP-MS experiments[18,26] have suggested that TBK1 forms a multiprotein complex in which the adaptor proteins—TRAF family member associated NF-κB activator (TANK), TBK-binding protein 1 (TBKBP1, or SINTBAD) and AZI2 (NAP1 or TBKBP2)—are obligatory components[18,56,57]. Evidence now indicates that TBK1 does not form a single core complex containing the three proteins. Instead, TBK1 forms alternative complexes with each of the adaptor proteins that are localized to specific subcellular compartments and associate with distinct cellular binding partners[21].

Cross-linking of affinity-purified TBK1 complexes with BS^3 resulted in the formation of a series of high-M_r species. Partial separation of the protein species (M_r range 180–460 kDa) was feasible by 1D SDS-PAGE (**Fig. 2**, lane 2), and the presence of TBK1 was confirmed by anti-HA immunoblotting (**Fig. 2**, lane 3). Non-cross-linked SH-TBK1 is included as a control (**Fig. 2**, lane 1). LC-MS/MS analysis of *in situ* tryptically digested cross-linked proteins revealed the presence of a number of TBK1 subcomplexes. From the 1D SDS-PAGE, however, it was not possible to confirm the composition of similar-molecular-mass,

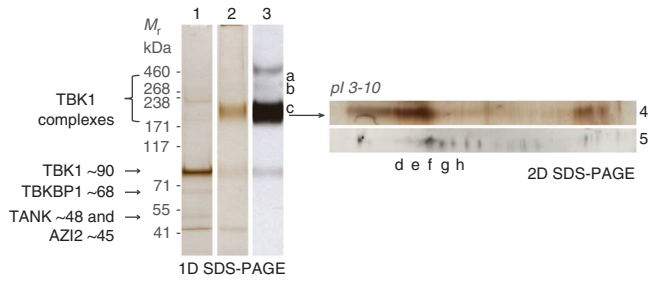

Figure 2 | SH-tagged TBK1 protein complexes separated by 1D (lanes 1–3) and 2D (regions 4 and 5) SDS-PAGE) on 3–8% (wt/vol) Tris-acetate gradient gels. Lane 1, silver stain: non-cross-linked SH-tagged TBK1 protein complex. Bands indicative of SH-TBK1 (~90 kDa), the interactors TBKBP1 (~68 kDa), TANK (~48 kDa) and AZI2 (~45 kDa) are shown. Note that the unannotated band at ~42 kDa is actin. Lane 2, silver stain: SH-TBK1 protein complexes cross-linked at a BS^3 concentration of 0.25 mM. Bands attributable to SH-TBK1, TBKBP1, TANK and AZI2 are weak or absent. The appearance of a larger region from ~180 to 460 kDa is due to the SH-TBK1-cross-linked protein complex(es). Note that the quantity of monomeric SH-TBK1 is reduced compared with lane 1. Lane 3, anti-HA-7 immunoblot: the formation of TBK1 protein complex(es) was confirmed by primary anti-TBK1 and HRP-bound secondary anti-rabbit antibodies. Region 4, silver stain: the cross-linked TBK1 complexes of similar molecular mass at ~180 to 230 kDa (lane 2) are delineated and further separated by 2D SDS-PAGE. Region 5, anti-HA-7 immunoblot: SH-tagged TBK1 protein complex(es) were confirmed by anti-HA-11 (against SH-tagged TBK1) and HRP-bound secondary anti-rabbit antibodies. The quantity of protein loaded on the gels was ~1% and 99% of the one-step affinity purification eluate (total protein input of 40 mg) for the 1D and 2D gel, respectively. The location of different TBK1 subcomplexes and interacting proteins are indicated as follows: (a) SH-TBK1_M + TBK1_H, HS90A, HS90B, AZI2, TANK; (b) SH-TBK1_M + TBK1_H, TANK; (c) SH-TBK1_M + TBK1_H, HS90A, HS90B; (d) HS90A, HS90B; (e) HS90A, HS90B, TBKBP1; (f) SH-TBK1_M, TBK1_H, HS90A, HS90B; (g) SH-TBK1_M, TBK1_H, TBKBP1; (h) SH-TBK1_M, TBK1_H. Note that SH-TBK1_M refers to the SH-tagged mouse TBK1, whereas TBK1_H refers to the human endogenous TBK1.

cross-linked subcomplexes of SH-tagged TBK1. Thus, the protein complexes were also separated by 2D SDS-PAGE (**Fig. 2**, region 4). We observed cross-linked TBK1 complexes of variable pI and M_r and confirmed them by anti-HA immunoblotting (**Fig. 2**, region 5). LC-MS analysis of *in situ*–digested cross-linked proteins (**Supplementary Table 1**) revealed differences in the composition of the TBK1 subcomplexes. Overall, the data provide evidence that TBK1 does not form a single, static core complex with TANK, TBKBP1 and AZI2. Rather, a series of heterogeneous, simultaneously formed subcomplexes are generated, captured by the cross-linker chemistry and visualized by gel electrophoresis. This approach is inherently simple, and thus the methodology can be extended to the investigation of a range of affinity-purified protein complexes.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS E.L.R. developed the protocol, conducted the experiments, interpreted the data and drafted the manuscript. R.S. developed sections of the protocol, participated in optimizing the tandem affinity purification methodology, wrote sections of the manuscript and provided valuable feedback in the review process. K.K. provided intellectual and technical expertise in optimization of the 2D SDS-PAGE, performed the 2D SDS-PAGE experiments, interpreted the gel images and wrote the 2D SDS-PAGE sections of the manuscript. M.L.H. provided technical support with 1D SDS-PAGE and sample preparation for LC-MS, and assisted in writing sections of the manuscript and proofreading of the revised versions. M.G. and G.S.-F. provided intellectual expertise on tandem affinity purifications and valuable feedback during the revision process. K.L.B. and E.L.R. conceived the notion of the protocol. K.L.B. was responsible for project supervision, data interpretation, manuscript writing and providing grant support.

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