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# An optimized protocol for high-resolution complexome profiling by cryo-slicing BN-MS analysis

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**TITLE:**

# High-Resolution Complexome Profiling by Cryoslicing BN-MS Analysis

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**KEYWORDS:**

complexome profiling, protein complex, protein-protein interactions, native gel electrophoresis, functional proteomics, membrane proteins

**SUMMARY:**

A versatile cryoslicing BN-MS protocol using a microtome is presented for high-resolution complexome profiling.

**ABSTRACT:**

Proteins generally exert biological functions through interactions with other proteins, either in dynamic protein assemblies or as a part of stably formed complexes. The latter can be elegantly resolved according to molecular size using native polyacrylamide gel electrophoresis (BN-PAGE). Coupling of such separations to sensitive mass spectrometry (BN-MS) has been well-established and theoretically allows for exhaustive assessment of the extractable complexome in biological samples. However, this approach is rather laborious and provides limited complex size resolution and sensitivity. Also, its application has remained restricted to abundant mitochondrial and plastid proteins. Thus, for a majority of proteins, information regarding integration into stable protein complexes is still lacking. Presented here is an optimized approach for complexome profiling comprising preparative-scale BN-PAGE separation, sub-millimeter sampling of broad gel lanes by cryomicrotome slicing, and mass spectrometric analysis with label-free protein quantification. The procedures and tools for critical steps are

described in detail. As an application, the report describes complexome analysis of a solubilized endosome-enriched membrane fraction from mouse kidneys, with 2,545 proteins profiled in total. The results demonstrate identification of uniform, low-abundance membrane proteins such as intracellular ion channels as well as high resolution, complex protein assembly patterns, including glycosylation isoforms. The results are in agreement with independent biochemical analyses. In summary, this methodology allows for comprehensive and unbiased identification of protein (super)complexes and their subunit composition, providing a basis for investigating stoichiometry, assembly, and interaction dynamics of protein complexes in any biological system.

**INTRODUCTION:**

BN-PAGE separation was first directly coupled to LC-MS analysis (BN-MS) by the Majeran[1] and Wessels[2] research groups using manual slicing of BN-PAGE gel lanes. Their analyses identified a number of abundant membrane protein complexes with known subunit composition from plant plastids and HEK cell mitochondria, respectively. However, these analyses were far from comprehensive and did not allow for unbiased identification of novel assemblies. Performance of mass spectrometers and label-free quantification methods has considerably improved since then, which has enabled comprehensive BN-MS analyses. This has coined the term "complexome profiling". For example, Heide and coworkers analyzed rat heart mitochondria identifying and clustering 464 mitochondrial proteins, thereby confirming many known assemblies. In addition, they found TMEM126B to be a novel and crucial subunit of a specific assembly complex[3]. Comparable results (with 437 mitochondrial protein profiles) were obtained in a parallel study of HEK cell mitochondria[4].

Despite these improvements, several issues have remained that restrain the full potential of BN-MS for complexome profiling. A major limitation is the effective size resolution of complexes that is determined by two factors: the (i) quality of the BN-PAGE separation, which depends on the uniformity of the gel matrix pore gradient as well as the stability/solubility of the sample complexes, and (ii) step size of gel sampling, which is at best 1 mm when using conventional manual slicing[5,6]. Poor size resolution not only misses subtle complex isoforms and heterogeneities, but it also negatively impacts the dynamic range and confidence of unbiased, de novo subunit assignment and quantification.

Other challenges include the precision of protein quantification and coverage of the actual dynamic range of protein abundances in the sample by mass spectrometric analysis. Therefore, application of BN-MS complexome profiling has remained largely restricted to biological samples with lower complexity, high expression of target complexes, and favorable solubilization properties (i.e., plastids, mitochondria, and microorganisms)[6-10].

We recently introduced cryomicrotome slicing-assisted BN-MS (csBN-MS), which combines precise sub-millimeter sampling of BN-PAGE gel lanes with comprehensive MS analysis and elaborate MS data processing for determination of protein profiles with high confidence[11]. Application to a mitochondrial membrane preparation from rat brains demonstrated previously

unmet effective complex size resolution and maximum coverage of oxidative respiratory chain complex (OXPHOS) subunits (i.e., 90 of 90 MS-accessible). This example also identified a number of novel protein assemblies.

Described here are optimized procedures for preparative scale BN-PAGE separation of protein complexes (not restricted to a particular biological source), casting of large preparative BN-PAGE gels, cryomicrotome slicing of broad gel lanes, and MS data processing. Performance of high resolution profiling is demonstrated for a protein complex preparation from mouse kidney endosome-enriched membranes. Finally, the benefits from increasing resolution and precision of mass spectrometric quantification are discussed.

**PROTOCOL:**

## 1 Preparative BN-PAGE

### 1.1 Gel preparation:

1.1.1 Use a middle-to-large format vertical gel electrophoresis system (>10 cm gel separation distance; 14 cm x 11 cm, 1.5 mm spacer) with effective cooling set to 10 °C.

1.1.2 Cast linear or hyperbolic pore gradient gel (1.5–3.0 mm spacers) using a stirring two-chamber gradient mixer driven by a pump (see **Table of Materials and Reagents**). In the presented example (linear gradient gel 1%–13%):

1.1.2.1 Produce a 13 mL solution for the front (mixing) chamber consisting of: 13% acrylamide (from 30% stock solution, 37.5:1.0 acrylamide:bisacrylamide), 0.75 M aminocaproic acid, 50 mM Bis-Tris (pH = 7.0), and 10% glycerol.

1.1.2.2 Produce a 10 mL solution for the reservoir chamber consisting of: 1% acrylamide (from 30% stock solution, 37.5:1.0 acrylamide:bisacrylamide), 0.75 M aminocaproic acid, 50 mM Bis-Tris (pH = 7.0), and 0.2% CL-47 detergent.

1.1.3 Start the stirrer and add 30 µL of APS (ammonium peroxo disulfate, 10% stock solution) and 2.5 µL of TEMED (N,N,N',N'-tetramethyl ethylenediamine) to the solution in the front chamber, and 90 µL of APS and 5 µL of TEMED to the solution in the reservoir chamber. Then, open the front valve, start the pump (flow should be adjusted to complete casting in 10 min), and open the chamber connection.

1.1.4 Allow the gel to polymerize slowly but thoroughly for at least 24 h at room temperature (RT) to generate a homogenous pore size gradient. When kept moist, the polymerized gel can be stored upright at 4 °C for up to 1 week.

NOTE: Intentionally, the top of the gel will have a soft/slimy consistency. This will later be removed but allows for smooth entry of proteins into the gel, with minimal risk of protein

precipitation that may otherwise lead to migration artifacts (i.e., streaking or protein precipitation).

1.2 Sample preparation and loading:

1.2.1 Prepare loading slots by inserting appropriate spacers (e.g., silicon tubes) between the glass plates for separating 0.5–2.0 mg of protein. The slots should be made at least 3 cm wide (or better, 5–6 cm wide).

1.2.2 Solubilize ~2.5 mg of membrane (mouse kidney endosome-enriched preparation) in 2 mL of solubilization buffer containing 1% (w/v) non-denaturing detergent (ComplexioLyte CL-47) for 30 min on ice. Ultracentrifugate (sedimentation cut-off = 200 S or less; 120,000 *x g*/10 min is used here).

1.2.3 Concentrate the solubilisate on a short 50%/20% (w/v, 0.3 ml each) sucrose step gradient by ultracentrifugation for 1 h at 400,000 *x g*. The final protein yield should be at least 1 mg.

1.2.4 Add 0.05% (w/v) Coomassie G-250 to the solubilisate and load the sample on the gel. Limit the protein load to a 10–15 µg/mm$^2$ gel lane cross-section to obtain high resolution and avoid artifacts resulting from protein precipitation.

1.3 BN-PAGE running conditions:

1.3.1 For running buffers, prepare a standard cathode buffer consisting of 50 mM tricine, 15 mM Bis-Tris, and 0.01% Coomassie G-250. Prepare a standard anode buffer consisting of 50 mM Bis-Tris (pH = 7.0).

1.3.2 Run a preparative BN-PAGE at 10 °C overnight using a three-step voltage protocol[13] consisting of: an equilibration phase for 30 min at 100 V, then a slow (3 h) ramp to maximum voltage (40–50 V/cm gel length) that is finally maintained for at least 6 h for endpoint focusing of proteins.

NOTE: It is recommended to pause electrophoresis when the migration front has reached the middle of the gel and exchange the cathode buffer for fresh buffer without Coomassie G250. This helps to avoid precipitation artifacts in the gel resulting from local collapse of the matrix pore structure.

## 2 Gel sampling and digestion

2.1 Excision of gel lanes:

2.1.1 After the run, scan the gels for documentation purposes while keeping it between the glass plates.

2.1.2 Disassemble the plates and excise the lane section(s) of interest. Fix them for at least 30 min (twice) with 30% (v/v) ethanol and 15% (v/v) acetic acid.

2.1.3 Take a sample strip of the lane and analyze by 2D BN/SDS-PAGE and protein staining or western blotting (as shown in **Figure 1B**) to determine regions of interest, effective complex size resolution, and protein abundance.

2.1.4 Transfer the sample to embedding medium and allow it to soak and equilibrate for at least 2 h at 4 °C, while keeping the gel slab in slow motion on an orbital shaker.

NOTE: The gel separation should be carefully inspected for overall separation quality and migration artifacts. Gel bands representing dominant proteins should be distortion-free and homogeneous in intensity. Local artifacts on the gel should be excised or left out of analysis.

2.2 Embedding and cryomicrotome slicing

NOTE: This is an improved version of the embedding procedure described and photo-documented previously that allows for embedding and slicing of broader gel lanes of up to 8 cm[11].

2.2.1 First, cut fixed gel lanes into sections (here, 3 cm) exactly parallel to the protein migration front/band pattern. For easier handling, place each section on a plastic film support with equal dimensions.

2.2.2 Transfer the lanes into an open tube with stoppers (closed on the bottom, centrally perforated on the top, both precisely aligned with the upper and lower ends of gel section).

2.2.3 Dip the cylinder briefly into liquid nitrogen to rapidly initiate the solidification. The transparent embedding medium solidifies within seconds and becomes white in color.

2.2.4 Fill the cavity with embedding medium and freeze the cylinder at -20 °C for several hours.

NOTE: Cooling the cylinder rapidly by dipping it into liquid nitrogen helps to avoid displacement of the gel slab within the tube. Distortion should be avoided to ensure high resolution in the following MS analysis.

2.2.5 After disassembly, remove the plastic film and transfer the block with the embedded gel section to a cooled, larger in diameter, metal cylinder placed on a flat support (i.e., Petri dish) and sealed with embedding medium on the outside of the cylinder. Fill the cylinder with embedding medium and freeze thoroughly.

2.2.6 Repeat this procedure with the other side of the cylinder to obtain a solid block with a

coplanar bottom surface.

2.2.7 Remove the block from the cylinder, glue it with embedding medium on a precooled metal holder, and insert the holder into the cryoslicing machine (cryotome) that is carefully aligned with respect to the slicing plane. Allow it to equilibrate at the optimum temperature for the slicing process (here, 13 °C).

NOTE: Use a slowly progressing manual slicing cycle of 0.1 mm step size until hitting the surface of the embedded gel section to ensure correct positioning.

2.2.8 Harvest the gel slices one after another, with a final desired thickness of 0.25 mm step size, and transfer them individually to reaction tubes with low protein binding properties.

NOTE: In this set-up, uniform gel slices can be readily obtained as thin as 0.1 mm and as thick as 0.5 mm.

2.3 Tryptic digestion:

2.3.1 Perform in-gel tryptic digestion after extensive washing of the gel slices (at least three additional rounds of washing are recommended to remove polymeric components of the embedding medium) following a standard procedure[11].

2.3.2 Vacuum-dry eluted peptides and redissolve in 0.5% (v/v) trifluoroacetic acid by shaking at 37 °C (10 min) followed by bath sonication (5 min).

## 3 Mass spectrometry

3.1 nanoHPLC and MS set-up

3.1.1 Load digested samples onto a C18 precolumn (particle size = 5 µm; diameter = 300 µm) with 0.05% (v/v) trifluoroacetic acid using a (split-free) nano-HPLC coupled to a mass spectrometer with high resolution.

3.1.2 Elute captured peptides with an aqueous-organic gradient (eluent A): 5 min 3% B, 120 min from 3% B to 30% B, 20 min from 30% B to 99% B, 5 min 99% B, 5 min from 99% B to 3% B, 15 min 3% B (flow rate = 300 nL/min).

NOTE: csBN-MS gel slices typically result in samples with low to intermediate peptide abundance and a limited degree of complexity. nanoLC-MS/MS analysis should therefore be performed with a set-up providing reasonable sensitivity and sequencing speed, high mass resolution (>100,000) and maximum dynamic range (effectively 3–4 orders of magnitude). However, it does not require long column dimensions or elution gradients extended beyond 3 h.

3.1.3 Separate eluted peptides in an emitter (i.d. 75 µm; tip = 8 µm) manually packed approximately 10 cm with C18 material (particle size = 3 µm). Electrospray the samples at 2.3 kV (positive ion mode) into the heated transfer capillary (250 °C) of the mass spectrometer.

3.1.4 Perform analyses with the following instrument settings[11]: maximum MS/MS injection time = 400 ms; exclusion duration = 60 s; minimum signal threshold = 5,000 counts, top 10 precursors fragmented; isolation width = 1.0 m/z).

NOTE: To facilitate calibration of mass, retention time, and assignment of peptide signals in a large number of datasets or measurements, it is recommended to perform respective MS measurement series without interruptions or changes in parameters and hardware (i.e., on the same C18 column/emitter).

3.2 Protein identification (MS data evaluated as described previously[11])

3.2.1 Extract peak lists from fragment ion spectra using the "msconvert.exe" tool (part of ProteoWizard).

3.2.2 Shift all precursor m/z values for each dataset by the median m/z offset of all peptides assigned to proteins in a preliminary database search with 50 ppm peptide mass tolerance.

3.2.3 Search the corrected peak lists with a suitable search engine (here, Mascot 2.6.2) against all mouse entries of the UniProtKB/Swiss-Prot database (release 2018_11).

3.2.4 Select "Acetyl (Protein N-term)", "Carbamidomethyl (C)", "Gln | pyro-Glu (N-term Q), Glu | pyro-Glu (N-term E)", "Oxidation (M)", and "Propionamide (C)" as variable modifications.

3.2.5 Set peptide and fragment mass tolerance to ± 5 ppm and ± 0.8 Da, respectively, and allow one missed tryptic cleavage. Set the expect value cut-off for peptide identification to 0.5 or less. Use a decoy database search to determine the false positive discovery rate (FDR). Set the FDR to 1% or apply additional quality criteria to ensure reliable identification.

NOTE: The presented experiment identified more than 3,500 proteins, with an average peptide FDR of 4.4 ± 0.77% (n = 101 slice samples), or 3,000 proteins when peptide FDR was set to 1%. Importantly, more stringent criteria were used for the selection of profiled proteins (2,568). It included all proteins that were identified with at least two peptides, at least one of them being protein-specific, in at least one of the 101 slice samples.

3.3 Protein quantification

3.3.1 Use peptide signal intensities (peak volumes [PVs]) for protein quantification that are obtained from FT full scans and correct for retention time and mass shifts using appropriate software (here, MaxQuant v1.6.3).

3.3.2 Align MS datasets one-by-one to reference (total average) peptide elution times using LOESS regression. Assign PVs to peptides either directly (MS/MS-based identification) or indirectly (i.e., based on their matching m/z and elution time within very narrow tolerances).

NOTE: This protocol uses in-house software for assignment of the "inserted" termed peptides. Set parameters result in effective m/z and elution time matching tolerances of ± 2 ppm and ± 1 min, respectively (see **Figure 2A,B**).

3.3.3 Correct for systematic run-to-run variations in peptide load and ionization efficiency by peptide intensity rescaling calculated from the median differences of relative peptide intensities between neighboring slice samples (**Figure 2C**).

3.3.4 Filter PV data for outliers and remaining false-positive assignments identified by internal PV consistency analysis.

3.3.5 Normalize PVs of each peptide to their maximum values over all slice datasets yielding relative peptide profiles.

3.3.6 Finally, calculate relative protein abundance profiles as averages of at least two (and up to six or 50%, whichever value is greater) of the best correlating peptide profiles over a window of three consecutive slices. This allows bridging of missing PV values and reducing noise.

NOTE: This finally resulted in 2,545 (of 2,568 preselected) protein profiles (**Figure 2D**).

3.4 Characterization of protein complexes

3.4.1 Analyze protein profiles by first performing peak detection using the local maxima method, and consecutively fit normal distributions to these peaks, yielding the position (i.e., slice index or apparent complex size) of their maxima and FWHM (full width at half-maximal intensity) values (inset of **Figure 4**).

NOTE: In the dataset, profiles are analyzed automatically using custom scripts. The smallest FWHM values are indicative of the effective size resolution of the approach (here, 6 x 0.25 = 1.5 mm).

3.4.2 Use reference protein complex peaks with defined molecular mass (as reported in the UniProtKB/Swiss-Prot database) for linear regression analysis of the log10(predicted molecular mass) values to convert slice number indices to apparent molecular sizes (i.e., apparent complex size in kDa).

NOTE: 23 marker complexes in the sample were selected in this study (**Figure 4**) based on (i) monodisperse shapes of profile peaks, (ii) experimental support of molecular weights, and (iii) distributions along the investigated BN-PAGE gel sections.

**REPRESENTATIVE RESULTS:**

The vast majority of conventional BN-MS studies as well as the recently established high-resolution csBN-MS approach have been applied to mitochondrial and plastid preparations that are (i) readily available, (ii) have limited complexity, and (iii) express target (membrane) protein complexes at high densities. This protocol extends the application of high-resolution complexome profiling to non-mitochondrial membranes expressing low-abundant proteins, about which little information on their integration into complexes is available. For demonstration purposes, we chose an endosome-enriched membrane preparation from mouse kidney obtained by density gradient centrifugation.

Optimization of this preparation has been guided by the marker protein TPC1 that forms intracellular ion channels predominantly localized to early and recycling endosomes[12]. It is also highly expressed in renal proximal tubular cells, as shown by immunohistochemical analysis of kidney tissue sections (**Figure 1A**). These membranes were gently solubilized (ComplexioLyte 47 at a low protein:detergent ratio of 1:8) and concentrated on a sucrose cushion by ultracentrifugation. The latter turned out to be an important step for removing excess lower molecular weight components (i.e., detergents, lipids, salts, organic polymers, and metabolites) that tend to negatively impact on resolution of preparative BN-PAGE separations.

Complex separation on a native 1%–13% (w/v) polyacrylamide gradient gel (**Figure 1B**, middle panel) showed strongly stained protein bands with very little migration artifacts. SDS-PAGE separation of a narrow BN-PAGE gel strip (**Figure 1B**, frame boxed in red) as a second dimension followed by western blot analysis showed a well-resolved pattern of distinct TPC1-associated complex populations (**Figure 1B**, upper panel, marked by red arrows), most likely resulting from association with additional protein subunits and/or posttranslational modifications (such as glycosylation[12]). A 3 cm section of interest was excised, fixed, and processed for cryomicrotome slicing as described[11]. The individual steps of this procedure (in particular, the precise alignment of the broad gel section), which is of critical importance for preserving resolution during sampling, are documented in the accompanying video. The embedded gel section was finally cut into 101 gel slices with a uniform thickness of 0.25 mm (**Figure 1B**, lower panel), which were separately digested and analyzed by high performance LC-coupled mass spectrometry.

In addition to size resolution, the quality of protein quantification is key for successful complexome profiling. With the MS set-up and settings used, analysis of the samples was quite comprehensive, resulting in an average identification of more than 1,000 proteins and 10,000 peptides (8,200 of which were protein-specific) per slice, and around 3,000 proteins and 43,000 peptides (38,500 of which were protein-specific) in total. Nevertheless, due to the stochastic nature of data-dependent MS/MS sequencing and its limitations in dynamic range, intensity information was still fragmentary for less abundant proteins. Therefore, an elaborate MS data processing procedure[11] was performed that is based on precise assignment of peptide signals (peak volumes [PVs] = peptide-related signal intensities integrated over m/z and time) over the entire series of datasets.

As shown in **Figure 2A,B**, deviations of peptide signals in mass and retention time that remained after calibration were identical for MS sequenced and for indirectly assigned PVs (with very narrow tolerances of <1 ppm and <0.5 min for 95% of the PVs), indicative of a very low rate for false-positive PV assignment. Remaining outliers were filtered based on their consistency with other related PVs. Since all MS measurements were performed consecutively on the same LC-MS set-up without changes in parameters or hardware components, run-to-run variations (determined as the median of all PV intensities in a sample relative to those in the neighboring slices) were small and easily eliminated by rescaling of the PV datasets (**Figure 2C**). The resulting peptide intensity information was then used to reconstruct 2,545 protein relative abundance profiles. As shown in **Figure 2D**, more than 75% of these protein profiles were based on at least three independent protein-specific peptides.

Next, the protocol assessed relevance of the step size of BN-PAGE gel sampling for the resolution of protein complexes. For this purpose, slice datasets were joined by summing the PV information from two, three, or four consecutive slices, thus simulating the outcome for step sizes of 0.5 mm, 0.75 mm, and 1 mm (compared to the original sampling of 0.25 mm). **Figure 3** illustrates the resulting abundance profiles for protein TPC1 as an example (A–D). At 0.25 mm, relative intensities and size separation of TPC1-associated complex populations (**Figure 3A**) were nicely in agreement with the results from western blot analysis (**Figure 1B,** upper panel); although, the profile showed some noise, mostly resulting from missing values ("gaps") in the PV matrix used for quantification.

Joining of two slices corresponding to 0.5 mm maintained the correct intensities and separation of the TPC1-associated complexes and removed quantification noise (**Figure 3B**). In contrast, larger step sizes of 0.75 mm and 1 mm (**Figure 3C,D**) led to a loss of size resolution and abolished discrimination of TPC1 complex subpopulations. It should be noted that the vast majority of published conventional BN-MS analyses use manually cut 2 mm slices (around 60 to cover the entire gel lane)[7-10].

Conversion of migration distance or slice index to molecular size is generally based on markers, either commercially available native standard proteins or well-characterized endogenous protein complexes with known subunit composition (mostly [super]complexes of the mitochondrial oxidative respiratory chain [OXPHOS])[13]. However, since BN-PAGE separation is based on the effective molecular cross-section that is determined not only by the molecular mass but also by the 3D structure and number of associated lipids, detergent, and Coomassie molecules, individual proteins may show larger deviations. Therefore, it was chosen to use larger sets of protein complexes as markers[11]. The plot in **Figure 4** shows 23 selected markers with a representative subunit shown as a black circle, indicating the log10 values of its predicted molecular mass (according to the UniProtKB/Swiss-Prot database) *vs*. the slice index of the corresponding profile peak maximum. The latter were obtained from automatized Gaussian fits to the relative abundance data, as shown in the inset of **Figure 4** showing the example with chaperone BCS1. Linear regression (red line) provided a function to convert slice index values to apparent molecular sizes, which ranged from 160–630 kDa, along the investigated gel section.

Finally, the analysis provided information on well-characterized complexes and demonstrated the existence of novel subunits and complex assemblies. Examples highlighting different aspects of the complexome are shown in **Figure 5** (A–C: proteins expressed or preferably located in endosomal compartments; D–F: complexes from other subcellular localizations). The iron-transporting protein ferritin is known to form complexes from 24 light (FRIL1) and/or heavy (FRIH) subunits, with a total molecular weight of 440 kDa[14] (**Figure 5A**, filled arrow). The subunit profiles (**Figure 5A**) suggest the existence of at least two smaller forms of the complex (with an apparent mass of 360 kDa and 340 kDa; open arrows) with distinct heavy/light chain stoichiometries (better visible after rescaling, inset of **Figure 5A**) that are abundantly present in endosomes.

In contrast, nicalin-nomo1 complexes[15] (**Figure 5D**), the gamma-secretase core complex[16] (**Figure 5B**), and the GPI-transamidase machinery[17] (**Figure 5E**) show fixed abundance ratios of their core subunits over the entire size range and independent from association with additional proteins. This indicates that their subunits are exclusive to each other. Vacuolar $H^+$-ATPases are multiprotein complexes assembled from a pool of more than 20 subunits in a modular manner with a total molecular weight of around 900 kDa. **Figure 5C** reveals sub-complexes with distinct compositions from at least 17 subunits, either representing biological (dis)assembly intermediates or subcomplexes generated by the experimental conditions, some of which have also been observed in a recent BN-MS study[18]. Another multi-protein complex example is the proteasome[19] (**Figure 5F**). Close inspection of the abundance profiles of the alpha and beta subunits forming the 20S proteasome core suggests two major complex populations with subtle differences in size (590 kDa and 575 kDa, indicated by grey arrows) and integration of three beta subunits.

In summary, csBN-MS complexome profiling of endosome-enriched kidney membranes provide comprehensive and detailed results regarding the (i) integration of uniform, low-abundance target proteins into complexes, (ii) general complex subunit composition and stoichiometry, and (iii) complex heterogeneities, substructures, and (dis)assembly intermediates.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Preparative BN-PAGE separation of solubilized endosome-enriched membranes from mouse kidney using intracellular channel TPC1 as a marker.** (**A**) Immunohistochemical localization of TPC1 protein in renal proximal tubules by confocal microscopy. Green: anti-TPC1 antibody[12] staining visualized with secondary Cy3-biotinylated goat anti-rabbit IgG; red: biotinylated Lotus tetragonolobus lectin (LTL, 10 µg/mL, FITC-conjugated) marking the luminal surface of proximal tubulus cells. The inset shows staining of a corresponding section from a TPC1-KO kidney as a negative control. White scale bars are 20 µm. Also of note is strong TPC1 expression in intracellular vesicles, known from independent experiments to represent early and recycling endosomes[12]. (**B**) Preparative BN-PAGE separation of 2.5 mg of solubilized endosome-enriched membranes on a 1%–13% (w/v) polyacrylamide gradient gel. A narrow lane (framed in red) was cut for subsequent SDS-PAGE/western blot analysis (upper panel), resolving

different TPC1-associated complexes and glycosylation patterns (red arrows: anti-TPC1/anti-rabbit HRP/ECL prime; green: positions and predicted masses [MDa] of marker protein complexes identified by total protein staining [SYPRO Ruby blot stain]) of the blot. From right to left: Na+/K+-transporting ATPase, Cytochrome b-c1 complex dimer, ATP synthase, NADH:ubiquinone oxidoreductase. A 3 cm section of interest from the gel lane was excised, embedded in tissue embedding medium, mounted, and sliced into 101 sections (0.25 mm) along the protein migration front using a cryomicrotome (lower panel; see video link).

**Figure 2: Key parameters determining accuracy of MS signal assignment and quantification as well as depth of analysis.** (**A**) Distribution of relative mass errors (in ppm) after m/z calibration of MS/MS sequenced (red bars) and indirectly assigned (i.e., based on closely matching mass and retention times, see protocol; blue bars) peptide signals. This suggests a final mass error of <1 ppm (for 95% of the signals/assigned PVs) and very low rate of false positive assignments. (**B**) Distribution of nano-HPLC retention time deviations from total average after elution time alignment of peptide signals, using Loess regression (see protocol) and color coding as used in (A). Time error is less than 30 s for >95% of peptide signals/assigned PVs. (**C**) Run-to-run variation of total MS intensities plotted relative to the average of the two neighboring samples. These scale factors were applied to the raw PV tables to minimize systematic technical errors. (**D**) Peptide information used for calculating relative abundance profiles of proteins. After filtering protein-specific peptides for outliers, poorly scored, or single identifications (see protocol), 2,545 protein abundance profiles were determined, >75% of which were based on at least three peptides with reasonable confidence.

**Figure 3: Critical impact of step size in gel sampling on complexome resolution of TPC1.** Datasets were joined by summing the signal intensities in groups of 1, 2, 3, and 4 consecutive slices (A–D, respectively) and processed identically to simulate different step sizes in gel slicing as indicated. The TPC1 profile shows some (oversampling) noise at 0.25 mm but good size resolution of three complex populations (see also **Figure 1B**), which is largely preserved at a 0.5 mm step width. Discrimination of these populations becomes lost as 1 mm is approached.

**Figure 4: Determination of apparent molecular weight**. 23 marker complexes with defined molecular composition (as indicated, according to UniProtKB/Swiss-Prot) were used as size markers. Logarithmic values of their expected molecular weights (in kDa) were plotted *vs*. the profile peak maximum slice index of the indicated representative protein subunit (filled circles in black). Linear regression fitting to this data (red line) provided a function converting slice index values to apparent molecular weights. Peak maxima were determined by automatized Gaussian fits to protein profile peaks as shown in the inset (right) for the chaperone protein BCS1 (primary data in blue, fit boundaries indicated by orange lines, fit function in red). In addition, these fits determined peak half-maximal widths (green line, 6.5 slices, or 1.6 mm for the example shown) with the sharpest focusing complexes spanning around a 1.5 mm gel.

**Figure 5: Examples of protein complex subunit profiles**. Relative protein abundance *vs*. apparent molecular weight plotted for heavy and light chain of ferritin (**A**) revealing molecular heterogeneity of ferritin subunit stoichiometry, more clearly visible after rescaling (inset). Filled

arrow and open arrows denote the full complex (440 kDa) and two subcomplexes, respectively. The gamma-secretase (**B**) subunits quantitatively integrated into a single-core complex population. The subcomplexes of vacuolar $H^+$-ATPases (**C**) exhibited multiple assemblies with distinct subunit composition, all expressed in endosomes. The nomo1 and nicalin proteins (**D**) formed an exclusive complex (the GPI-transamidase), which is a multi-subunit enzymatic machinery forming several complexes. (**E**) The 20S proteasome core complex showing (**F**) a subtle subcomplex pattern with two populations indicated by arrows in grey, all originating from other subcellular localizations.

**DISCUSSION:**

The presented study built on the csBN-MS technique previously benchmarked with a mitochondrial preparation[11] and incorporated improvements in sample preparation, gel processing, and MS data evaluation. Focused analysis of a section of the large-scale separation BN-PAGE gel provided a comprehensive set of data showing quality measures comparable to the study with mitochondrial membranes. Mass and retention time errors as well as run-to-run variations were kept very low and provided the basis for determining reliable protein abundance profiles. Size resolution appeared to be good, with half-maximal peak widths as low as six slices (corresponding to 1.5 mm, **Figure 4**) and relative size differences of less than 10% resolved (**Figure 3, Figure 5A**). These values did not fully meet the size resolution quality of the previous csBN-MS analysis of mitochondria (despite the smaller gel sampling step size chosen), but they are significantly better than the performance of conventional BN-MS or size-exclusion MS approaches[20] that have recently become popular.

The importance of a high effective complex size resolution is underlined by the simulation experiment in **Figure 3** (using the TPC1-associated complexes) that can hardly be resolved by 2D BN/SDS-PAGE western blot analysis (**Figure 1B**). These results suggest that the 0.25 mm slicing in this case resulted in some oversampling, but it this still proved to be useful for elimination of “quantification noise” without compromising effective size resolution. Thus, in line with previous results[11], a sampling step size of ~0.3 mm is generally recommendable.

Notably, discrimination of TPC1-associated complexes is completely lost with 1 mm gel sampling, which is the smallest step size provided by manual slicing in conventional BN-MS[5,6]. This may explain the fact that despite powerful MS technologies being available, very few protein complexes and subunits have been identified de novo by complexome profiling. Besides its good resolving power, csBN-MS offers high versatility. Membrane-bound complexes and soluble protein complexes ranging from 50 kDa to several MDa can be effectively resolved in a single experiment with minimal bias[11]. This contrasts with alternative separation techniques used for complexome profiling like size exclusion or ion exchange chromatography, which operate with subsets of soluble proteins with certain size ranges or charge properties. On the downside, csBN-MS is less scalable (maximum load of ~3 mg protein per gel), may be technically challenging, and cannot be automatized.

Overall, the results demonstrate that csBN-MS-based complexome profiling can be successfully

applied to non-mitochondrial targets but also indicate some associated challenges. Thus, efficient extraction and biochemical stability of protein complexes require more optimization, and cleaning steps and may still be limited. Within the investigated size window, the number of well-focused, monodisperse protein complexes was indeed considerably lower (data not shown) compared to a mitochondrial sample. It is also recommended to lower BN-PAGE sample loads to obtain acceptable gel separation. Higher loads may require broader gel lanes that are more difficult to process properly for slicing (see accompanying video). Furthermore, protein complexity of the samples was higher (around two-fold) than the mitochondria-derived slice digests, leading to more missing PV values and a reduced dynamic range. In fact, some small proteins expected to be part of the complexes shown in **Figure 5** were missing in the analyses. These problems can be resolved in the future by using faster and more sensitive MS instruments or data-independent acquisition modes.

Sample preparation is highly critical for protein complex retrieval, stability, and gel separation quality. Parameters and procedures should be optimized for each source tissue, cell lysate, membrane (fraction), and protein complex of interest. The following general recommendations are provided that may help extend applications of csBN-MS:

(i) Preparing samples fresh and avoiding warming/freezing, strong dilutions, changes in buffer conditions, and unnecessary delays;
(ii) Using buffers that are essentially devoid of salts (replace with 500–750 mM betaine or aminocaproic acid), about a neutral pH, and containing up to 1% (w/v) of non-denaturing detergent (protein:detergent ratio between 1:4–1:10 for solubilization of membrane protein complexes, no detergent required for soluble protein complexes);
(iii) Careful testing and adjusting of detergent conditions by analytical BN-PAGE, since these may strongly impact efficiency of complex solubilization, representation of membrane protein complexes in the sample, stability, and homogeneity of protein-detergent micelles. The latter are prerequisites for proteins to focus as distinct bands/complex populations on BN-PAGE gels. Previous literature offers a broad range of neutral detergents. However, DDM (n-dodecyl β-d-maltoside)[1,2,4-6] and digitonin[3,5,7-10,13,18] have been the most popular choices for BN-MS analyses so far. It must be emphasized that any detergent condition necessarily represents a compromise between efficiency and preservation of protein interactions and may not be equally suited for all types of target protein and source material;
(iv) Removing charged polymers like fibrils, filaments, polylysine, DNA, and abundant lower molecular weight components (i.e., metabolites, lipids, or peptides). This may be accomplished by ultracentrifugation, gel filtration, or dialysis. This is particularly important for total cell or tissue lysates;
(v) Adding Coomassie G-250 (final concentration 0.05%–0.1%) and sucrose (to increase density for loading, final concentration 10%–20% [w/v]) to the sample just prior to loading, to clear by short ultracentrifugation, load the sample without perturbation, and start the run immediately thereafter.

As a future perspective, csBN-MS-based complexome profiling offers options for multiplexing to study protein complex dynamics or changes related to specific biological conditions. Combined

separation of metabolically labelled samples as proposed for size-exclusion based profiling[21] appears straightforward, but it may be hampered by spontaneous subunit exchange in complexes occurring independently of the used separation method. Alternatively, labelled samples can be resolved in neighboring gel lanes, which can then be co-sliced or combined post-digesting for differential analysis with high sensitivity and robustness.

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**DISCLOSURES:**

The author Uwe Schulte is an employee and shareholder of Logopharm GmbH that produces ComplexioLyte 47 used in this study. The company provides ComplexioLyte reagents to academic institutions on a non-profit basis.

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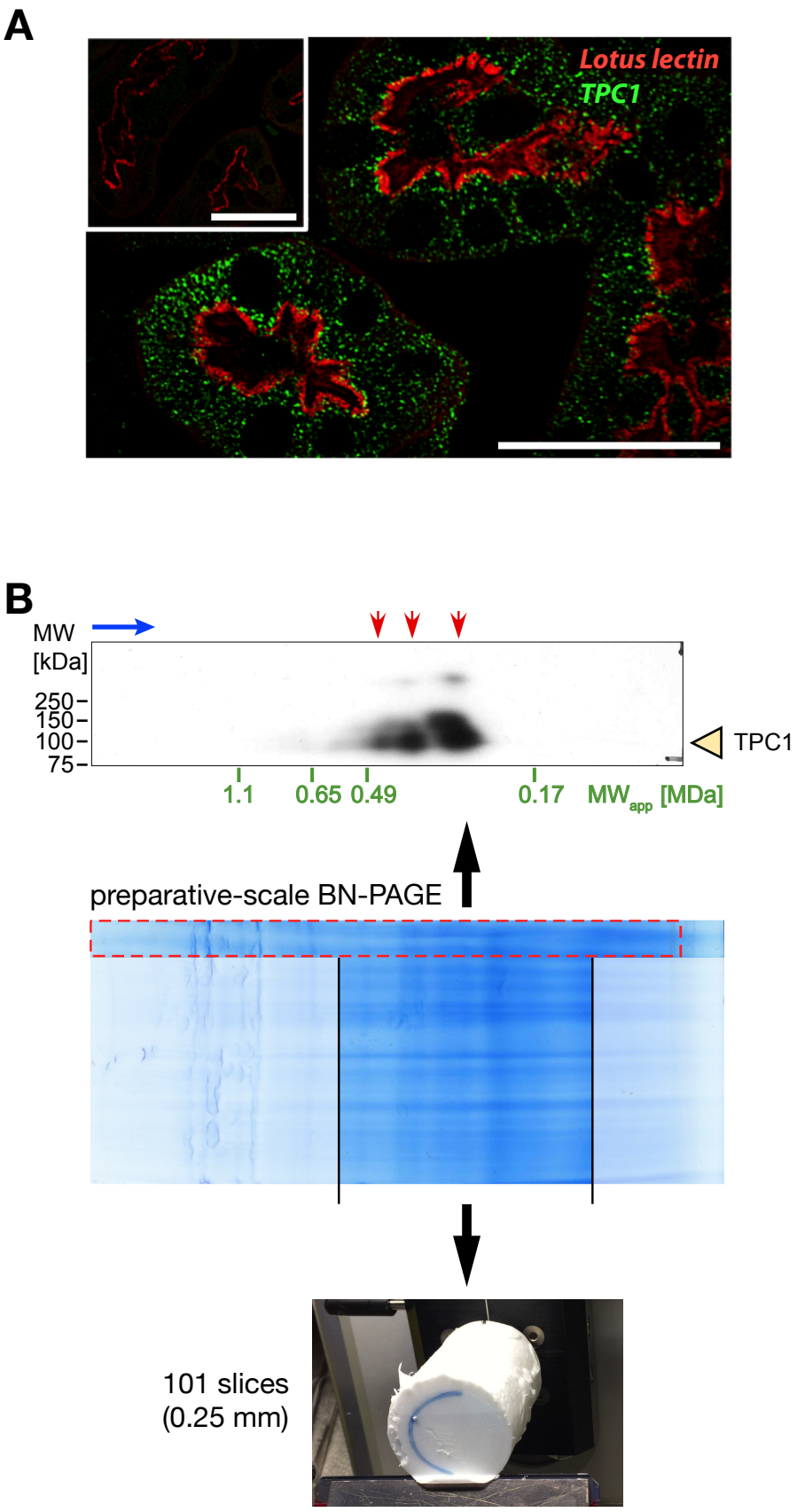


Figure 1

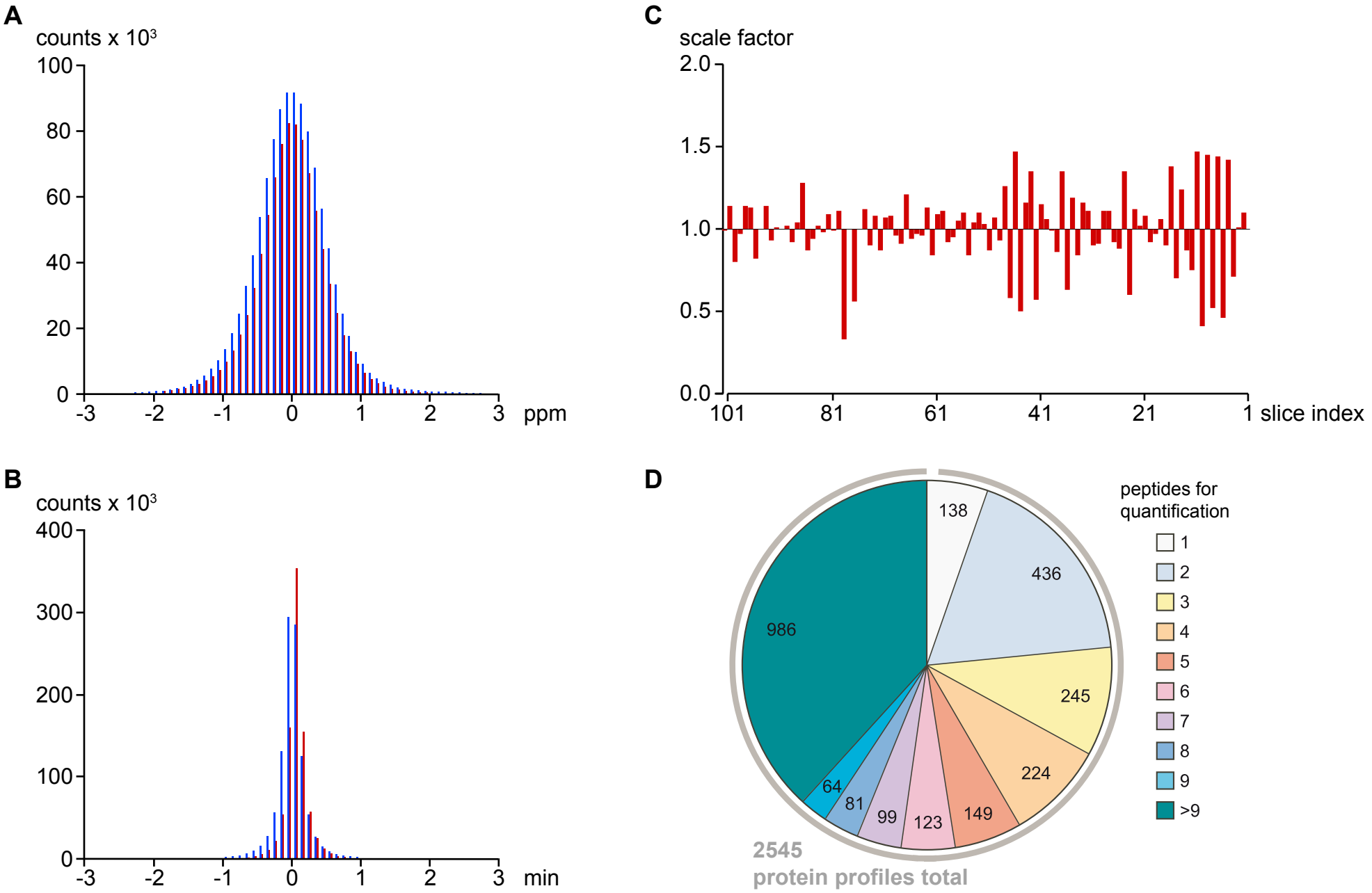


Figure 2

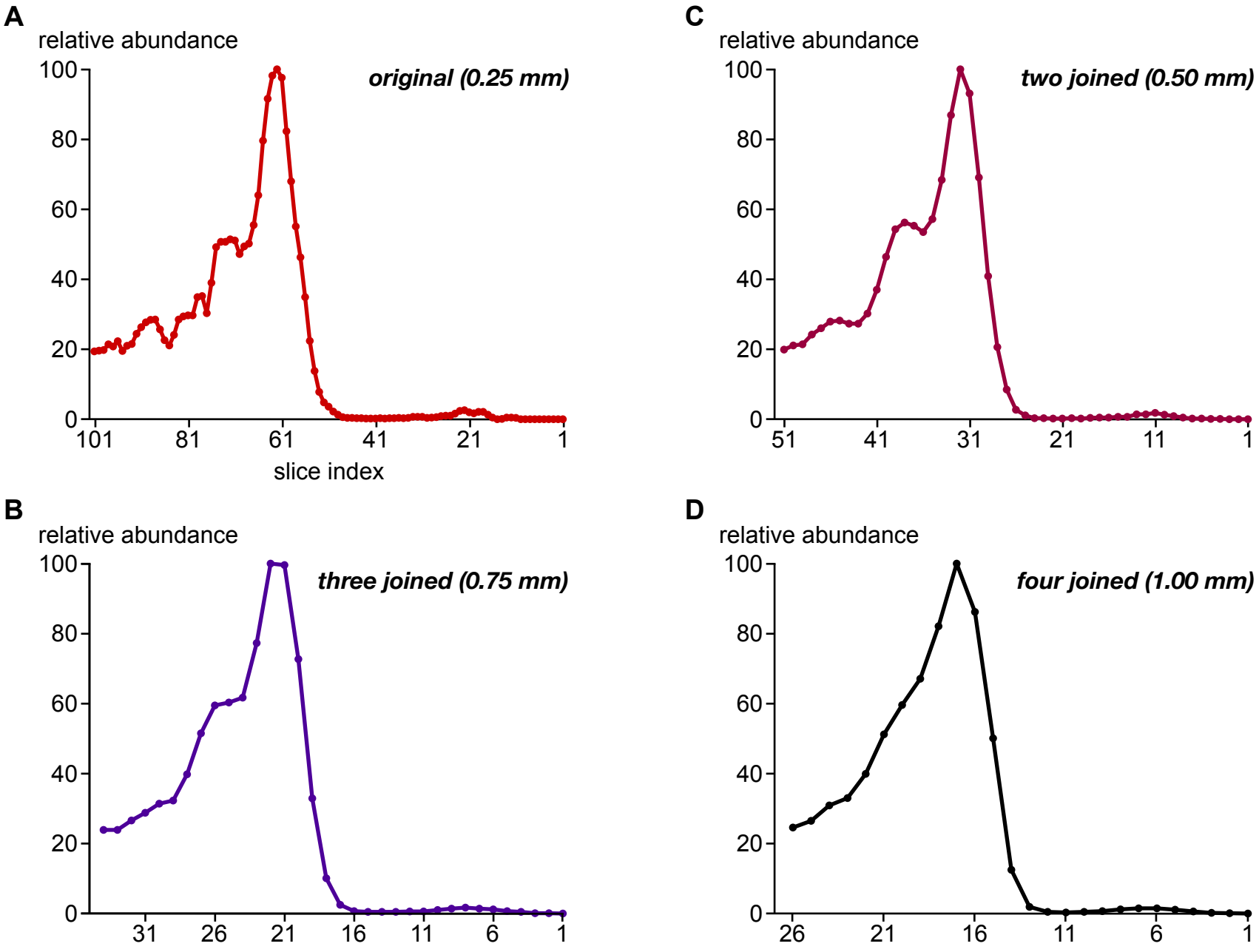


Figure 3

Figure

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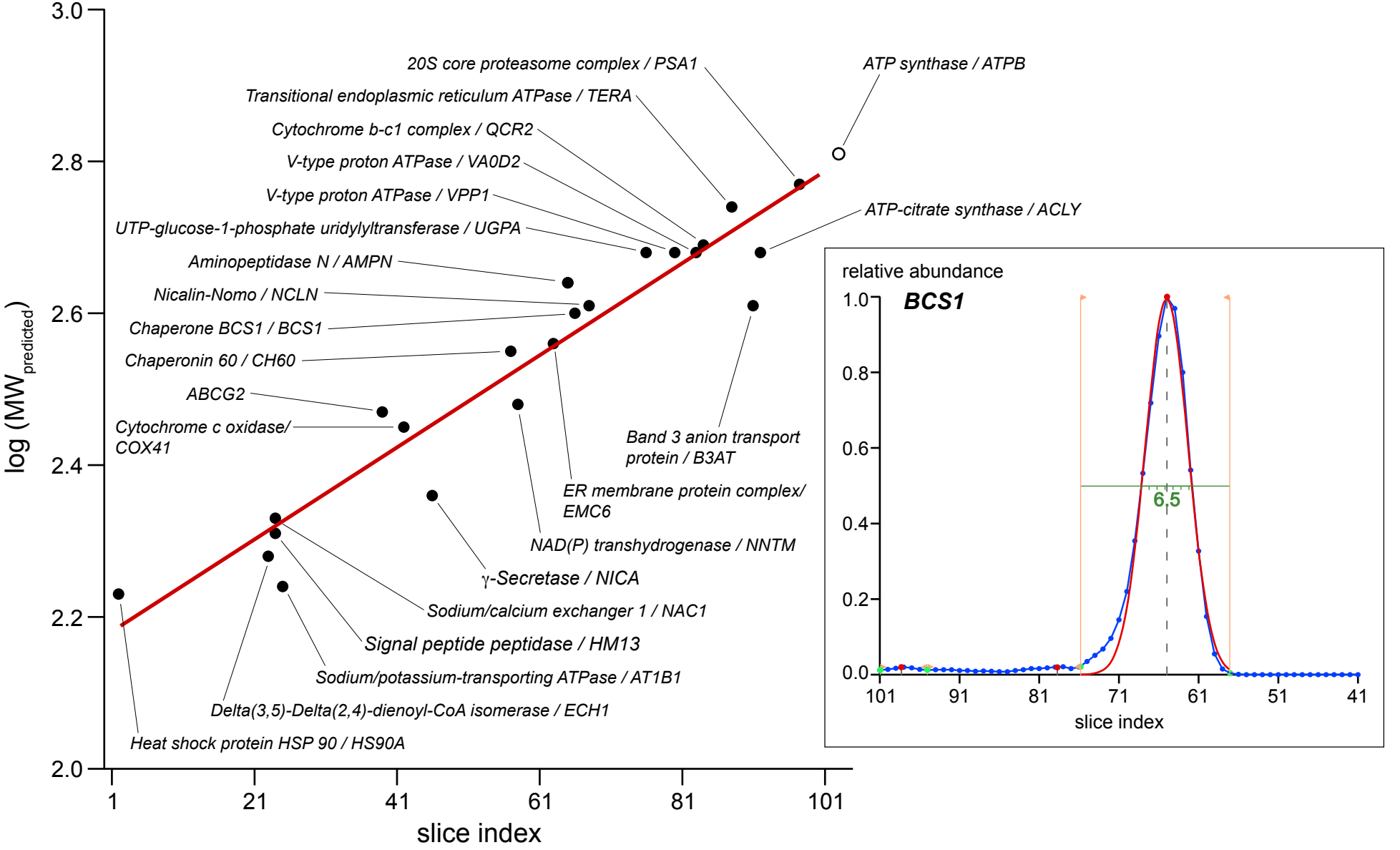


Figure 4

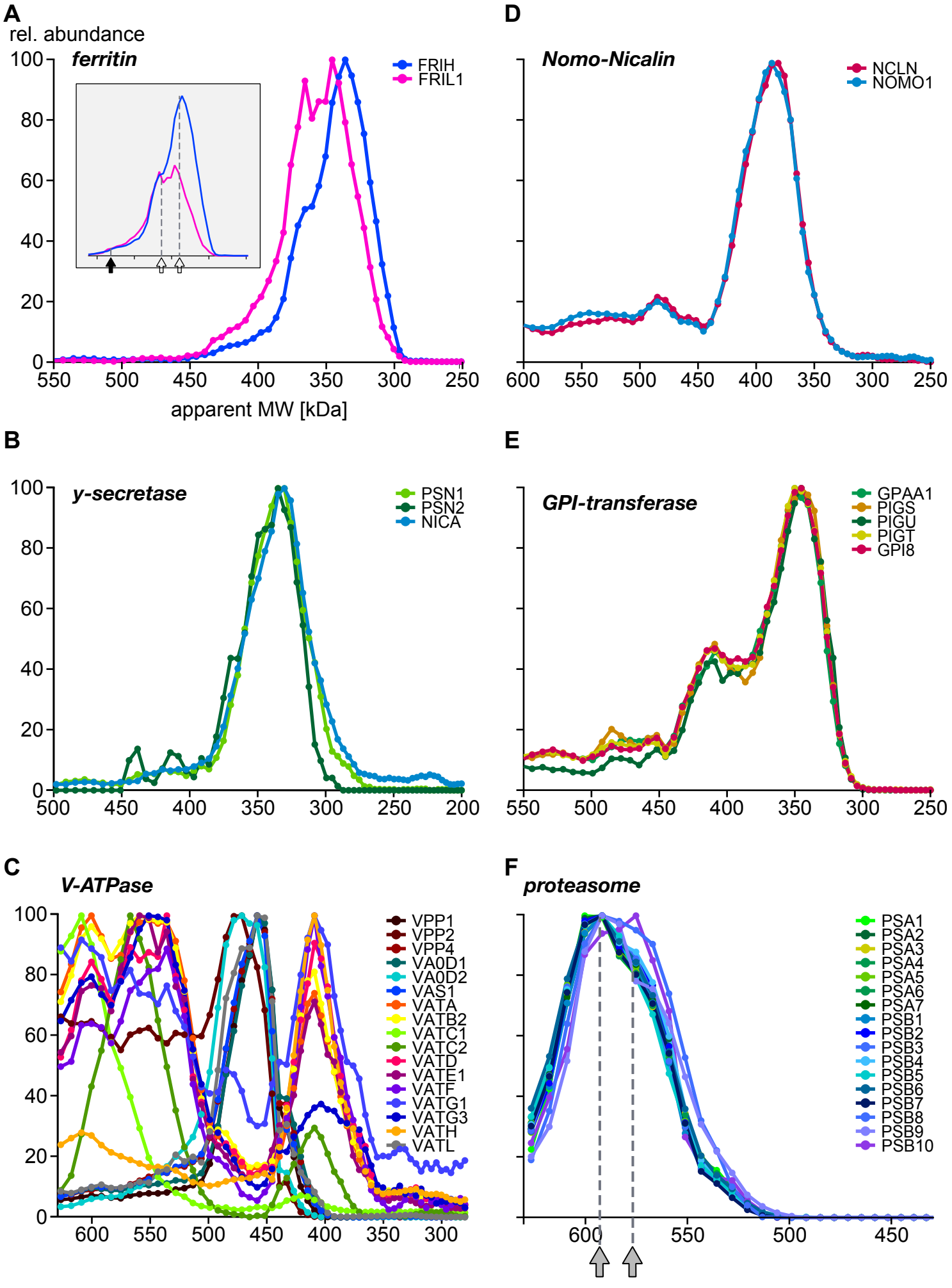


Figure 5

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 30% Acrylamide/Bis Solution, 37.5:1 | Bio Rad | #1610158 | Recommended for acrylamide gradient gel solutions up to 13% |
| 30% Acrylamide/Bis Solution, 19:1 | Bio Rad | #1610154 | Recommended for acrylamide gradient gel solutions >13% |
| SYPRO Ruby Protein Blot Stain | Bio Rad | #1703127 | Total protein stain on blot membranes; sensitive and compatible with immunodetection |
| Coomassie Brilliant Blue G-250 | Serva | no. 35050 | Centrifugate stock solutions prior to use |
| ComplexioLyte 47 | Logopharm | CL-47-01 | Ready-to-use detergent buffer (1%) for mild solubilization of membrane |
| Embedding Medium / Tissue Freezing Medium | Leica Biosystems | 14020108926 | Embedding medium for gel sections to be sliced by a cryo-microtome |
| Immobilon-P Membrane, PVDF, 0,45 µm | Merck | IPVH00010 | |
| ECL Prime Western Blotting Detection Reagent | GE Healthcare | RPN2232 | |
| Plastic syringe with rubber stopper, 20-30 ml | n.a. | n.a. | any supplier, important for making gel section embedding tool |
| broad razor blade | n.a. | n.a. | any supplier, for BN-PAGE gel trimming / excision of lanes |
| metal tube / cylinder, ca. 4 cm long | n.a. | n.a. | mold for embedding and freezing of gel samples |
| Protein LoBind Tubes, 1.5 ml | Eppendorf | Nr. 0030108116 | highly recommended to minimize protein/peptide loss due to |
| sequencing-grade modified trypsin | Promega | V5111 | |
| C18 PepMap100 precolumn, particle size 5 | Dionex / Thermo Scientific | P/N 160454 | |
| PicoTip emitter (i.d. 75 µm; tip 8 µm) | New Objective | FS360-75-8 | |
| ReproSil-Pur 120 ODS-3 (C18, 3 µm) | Dr. Maisch GmbH | r13.93. | columns packed manually |
| rabbit anti-TPC1 antibody | Gramsch Laboratories | custom production | described in Castonguay, et al., 2017 (Reference 12) |
| Cy3-biotinylated goat anti-rabbit IgG | Vector Laboratories | CY-1300 | described in Castonguay, et al., 2017 (Reference 12) |
| biotinylated Lotus tetragonolobus lectin, FITC- | Vector Laboratories | #B1325 | described in Castonguay, et al., 2017 (Reference 12) |
| cryo-microtome Leica CM1950 | Leica Biosystems | 14047743905 | |
| Mini Protean II Cell with wetblot unit | Bio Rad | n.a. | for SDS-PAGE and Westernblot (not sold any more) |
| Penguin Midi Gel Electrophoresis System | PeqLab | n.a. | for BN-PAGE (not sold any more) |
| Zeiss Axiovert 200 M microscope + Photometrics | Zeiss / Photometrics | n.a. | |
| peristaltic pump (IP high precision multichannel) | Ismatec | ISM940 | for casting of gradient polyacrylamide gels |
| gradient mixer with stirring (two chambers) | selfmade, alternatively Bio Rad | 1652000 or 1652001 | for casting of gradient polyacrylamide gels, manual provides instructions to cast linear or hyperbolic gradient gels (http://www.bio- |
| ultracentrifuge Sorvall M120 with S80 AT3 rotor | Sorvall / Thermo Scientific | n.a. | for sample preparation (not sold any more) |
| UltiMate 3000 RSLCnano HPLC | Dionex / Thermo Scientific | ULTIM3000RS LCNANO | |
| Orbitrap Elite mass spectrometer | Thermo Scientific | IQLAAEGAAPF ADBMAZQ | |

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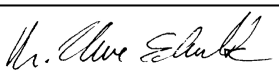
| Signature: |  |
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# Responses to the reviewers' comments

## Reviewer #1

We thank the reviewer for the positive comments on our work and his suggestions for further improvement that were incorporated into the revised manuscript.

*The description of the procedure to embed the gel is rather vague at the moment and difficult to follow. The accompanying video for this part is absolutely needed and will be very useful. If it is the intention that this manuscript can stand alone without the video, then this part should be improved somewhat. Maybe some pictures/figures could be added.*

We agree that video documentation of gel embedding will be important to comprehend the procedure and reproduce it in your own lab. Key steps of the original gel embedding and slicing procedure have been described and photo-documented in (Müller et al., 2016) now referenced in the revised manuscript (line 511).

*While size exclusion chromatography profiling can be used to separate protein complexes in a single sample, some of the earlier versions aimed at assessing differences between two samples/conditions. This was obtained by metabolic labeling (SILAC) and can also be obtained by TMT labeling approaches. It would be good if the authors could comment on the usefulness of such a labeling approach with their separation approach? Such labeling could compensate for technical variation and make the method particularly useful to detect (subtle) differences in the complexes.*

In theory, multiplexed csBN-MS analysis could be realized by mixing metabolically labelled protein samples prior to separation. However, in preliminary experiments with solubilized membrane proteins we observed dynamic exchange of subunits even in tightly assembled complexes which would hamper interpretation of results independent of the separation method used. A workaround for csBN-MS could be to run differentially labelled samples on neighbouring lanes followed by combined slicing or by TMT-labelling of digests of matched slices. This aspect has been added to Discussion (last section of the revised manuscript).

**Reviewer #2**

We thank the reviewer for the positive comments on our work and his constructive criticism that led to further improvements that were incorporated into the revised manuscript.

*1. Please briefly mention and contrast csBN-MS with other protein complex separations like ion exchange and size exclusion chromatography, particularly in terms of resolution anisotropy, separation range and sample amount.*

With experiments not yet in hand that would allow to directly compare performance of different complexome profiling separation techniques, such contrasting may appear somewhat preliminary.
Clearly, BN-PAGE provides the best size resolution of all protein complex fractionation techniques published to far. In addition, it is highly versatile (i.e. it effectively resolves solubilized membrane protein complexes as well as soluble protein complexes evenly over a large size range). Disadvantages may be the limited separation capacity (approximately 3 mg protein per gel), low throughput and incompatibility with automatization as compared to chromatographic methods.
These aspects were added to the Discussion section (lines 1157-1164).

*2. Please indicate if a decoy database was used in the spectral match search and, if so, please indicate the false discovery rate corresponding to the suggested expected value cutoff for peptide identifications.*

A decoy database was indeed used as part of the Mascot spectral match search. The average peptide FDR in slice samples was 4.4±0.7%. This information, together with additional details on further filtering of results to obtain reliable protein abundance profiles were added to Protocol section 3.2.5.

*3. The table of materials formatting makes it hard to match a certain material/equipment with the Catalogue Number and Comments/Description. Please reformat the table of materials to fit all columns in the page width.*

The table was reformatted accordingly.

*4. Please speculate about what adaptations would deem the protocol suitable for further extension to either soluble complexes or even whole cell lysates.*

We refrain from speculations, but the presented protocol can be indeed used for analysis soluble protein complexes (which are well resolved here and in Müller et al., 2016). It should also be generally applicable to whole tissue or cell lysates when following the guidelines in the extended general introduction of protocol section 1.2.

*Minor concerns*

- The acronym PV is now explained in Protocol section 3.3.1 and in the main text lines 962-963.
- Units (w/v and v/v) were added throughout the manuscript.

- A commercial source for a two-chamber gradient mixer was added to the Table of Materials and Reagents. The accompanying manual also describes casting of hyperbolic pore gradient gels.
- The terms "heavy" and "light" were replaced with "concentrated" and "diluted".
- Maximum storage time and condition for polymerized BN-PAGE gels were added to Protocol section 1.1.4.
- “embedding medium” is Tissue Freezing Medium from Leica, listed in the Table of Materials and Reagents.
- Recommended manual slicing speed has been added to the Protocol (note of section 2.3.7).
- The maximum number of precursors picked for fragmentation per full scan cycle (Top N) has been added to the Protocol (section 3.1.4).

**Reviewer #3**

We thank the reviewer for the positive comments on our work and his constructive criticism that led to further improvements that were incorporated into the revised manuscript.

*1) On line 101 the authors describe making a gradient gel "using a stirring gradient mixer driven by a pump". Is this imperative to making good quality gels or is there an alternative method which can be used for laboratories that do not possess this equipment? I also noticed that this set-up was self-made - could the authors recommended where to buy the components of this equipment and describe how to successfully assemble them for this protocol?*

A gradient mixer is mandatory for casting BN-PAGE gels whereas the pump just allows for better control and reproducibility of the gel casting process. We added commercial sources for a two-chamber gradient mixer and a peristaltic pump to the Table of Materials and Reagents.

*2) On line 105 the authors recommended using 2 x CMC detergent. However, they do not state what detergent. Later in the manuscript they state that they use CL-47 detergent, is this what is recommended? If so, could they elaborate on why this detergent and whether others can be used (or may even be more advantageous). For membrane proteins especially, the choice of detergent is essential for the maintenance of non-covalent interactions within protein complexes and there needs to be a larger discussion of the choice of detergent (along with some reference to relevant literature - DDM detergent is a common choice for BN-PAGE, see: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2648668/ and http://www.jbc.org/content/288/30/22163.full.html). Other developments in the field have also shown that using polymers for solubilization can maintain membrane protein complexes during BN-PAGE (https://www.sciencedirect.com/science/article/pii/S0005273619301087), could the authors comments on this also?*

We agree with the reviewer that the detergent condition used for membrane protein solubilization is an important factor determining BN-PAGE separation quality and complexome comprehensiveness. Given the large number of neutral detergents available, the protein-specific differences in complex stability and distinct types of biological source materials, however, an elaborate discussion of detergents is clearly beyond the scope of this manuscript. Furthermore, any detergent condition necessarily represents a compromise between efficiency and preservation of protein interactions and has therefore to be tested / selected according to the specific requirements of each project. For BN-MS experiments with membrane proteins, DDM (n-dodecyl β-d-maltoside) and digitonin have so far been the most popular choices. A respective paragraph has been added to the general introduction of Protocol section 1.2.

*3) On line 164 embedding medium is mentioned but it is unclear what the embedding medium is and how to prepare it, or even where to purchase it from. Could the authors expand on this?*

“embedding medium” is Tissue Freezing Medium from Leica, listed in the Table of Materials and Reagents.

*4) On line 178 the authors say the embedding medium was cooled briefly for solidification. To what temperature was this cooled and for how long? Again, this links with the lack of information on the embedding procedure and the medium, which needs to be expanded upon.*

The requested information has been added to Protocol section 2.3 together with further experimental details. Key steps of the original gel embedding and slicing procedure have been described and photo-documented in (Müller et al., 2016) which is now referenced in the revised manuscript (line 518).

*Minor concerns*

*1) On line 134 the authors mention that they add sucrose. Why is this? To increase sample density for loading? Please elaborate on this.*

Sucrose was indeed added to the sample to increase its density for loading (now explicitly stated in the general introduction of Protocol section 1.2 (v).

*2) On line 222 the authors state a "NOTE" that all measurements were carried out on the day and same columns without any wash procedures. Indeed, they state that this important for good quality results. One concern I have for this set-up is the existence of peptide carry over (especially with membrane protein samples) - do the authors experience this and does it greatly influence your results? If so, how can this be avoided in their set-up or in another's?.*

As stated in Protocol section 3.1.4, LC-MS/MS measurements were carried out consecutively on the same column. The gradient protocol (detailed in section 3.1.2) included two short wash steps (5 + 15 min). However, we did not separate sample measurements by empty gradients. Carry-over in the range of a few percent may therefore occur for sticky peptides, but this would not have a significant impact on our results (due to the high-resolution sampling protein abundances change by less than an order of magnitude from slice to slice). If carry-over is still a concern, it could be easily reduced by separating sample runs by empty gradients.

*3) On line 267 the authors say they use custom scripts for performing peak detection using the local maxima method - are these widely available? If not, they can they be made so? As without them if becomes very difficult to reproduce the authors entire workflow and result when benchmarking the produce in your own laboratory.*

The custom script for performing peak detection and fitting is a preliminary tool that we did not publish yet but could provide upon request. Software solutions performing similar functions (and more) have also been developed and made available by other groups (e.g. Heusel et al., (2019). Mol Syst Biol. 15: e8438).

*4) On line 427 the authors say they used 23 marker complexes. It is unclear but were these in the samples already or were they performed separately to produce a calibration curve? Also, why 23 and not say 20?*

These markers were endogenous to the sample and selected based on (i) monodisperse shape of their profile peak, (ii) experimental support of their molecular weight and (iii) distribution along the investigated BN-PAGE gel section. This has been clarified in the revised manuscript (note in Protocol section 3.4.2).

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 30% Acrylamide/Bis Solution, 37.5:1 | Bio Rad | #1610158 | Recommended for acrylamide gradient gel solutions up to 13% |
| 30% Acrylamide/Bis Solution, 19:1 | Bio Rad | #1610154 | Recommended for acrylamide gradient gel solutions >13% |
| SYPRO Ruby Protein Blot Stain | Bio Rad | #1703127 | Total protein stain on blot membranes; sensitive and compatible with immunodetection |
| Coomassie Brilliant Blue G-250 | Serva | no. 35050 | Centrifugate stock solutions prior to use |
| ComplexioLyte 47 | Logopharm | CL-47-01 | Ready-to-use detergent buffer (1%) for mild solubilization of membrane proteins |
| Embedding Medium / Tissue Freezing Medium | Leica Biosystems | 14020108926 | Embedding medium for gel sections to be sliced by a cryo-microtome |
| Immobilon-P Membrane, PVDF, 0,45 µm | Merck | IPVH00010 | |
| ECL Prime Western Blotting Detection Reagent | GE Healthcare | RPN2232 | |
| Plastic syringe with rubber stopper, 20-30 ml | n.a. | n.a. | any supplier, important for making gel section embedding tool |
| broad razor blade | n.a. | n.a. | any supplier, for BN-PAGE gel trimming / excision of lanes |
| metal tube / cylinder, ca. 4 cm long | n.a. | n.a. | mold for embedding and freezing of gel samples |
| Protein LoBind Tubes, 1.5 ml | Eppendorf | Nr. 0030108116 | highly recommended to minimize protein/peptide loss due to absorption |
| sequencing-grade modified trypsin | Promega | V5111 | |
| C18 PepMap100 precolumn, particle size 5 µm | Dionex / Thermo Scientific | P/N 160454 | |
| PicoTip emitter (i.d. 75 µm; tip 8 µm) | New Objective | FS360-75-8 | |
| ReproSil-Pur 120 ODS-3 (C18, 3 µm) | Dr. Maisch GmbH | r13.93. | columns packed manually |
| rabbit anti-TPC1 antibody | Gramsch Laboratories | custom production | described in Castonguay, et al., 2017 (Reference 12) |
| Cy3-biotinylated goat anti-rabbit IgG | Vector Laboratories | CY-1300 | described in Castonguay, et al., 2017 (Reference 12) |
| biotinylated Lotus tetragonolobus lectin, FITC- | Vector Laboratories | #B1325 | described in Castonguay, et al., 2017 (Reference 12) |
| cryo-microtome Leica CM1950 | Leica Biosystems | 14047743905 | |
| Mini Protean II Cell with wetblot unit | Bio Rad | n.a. | for SDS-PAGE and Westernblot (not sold any more) |
| Penguin Midi Gel Electrophoresis System | PeqLab | n.a. | for BN-PAGE (not sold any more) |
| Zeiss Axiovert 200 M microscope + Photometrics | Zeiss / Photometrics | n.a. | |
| peristaltic pump (IP high precision multichannel) | Ismatec | ISM940 | for casting of gradient polyacrylamide gels |
| gradient mixer with stirring (two chambers) | selfmade, alternatively Bio Rad | 1652000 or 1652001 | for casting of gradient polyacrylamide gels, manual provides instructions to cast linear or hyperbolic gradient gels (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1652000.pdf) |
| ultracentrifuge Sorvall M120 with S80 AT3 rotor | Sorvall / Thermo Scientific | n.a. | for sample preparation (not sold any more) |
| UltiMate 3000 RSLCnano HPLC | Dionex / Thermo Scientific | ULTIM3000RSLCNANO | |
| Orbitrap Elite mass spectrometer | Thermo Scientific | IQLAAEGAAPFADBMAZQ | |