## Depletion of High-Molecular-Mass Proteins for the Identification of Small Proteins and Short Open Reading Frame Encoded Peptides in Cellular Proteomes

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**Samples**

A simple protein mixture consisting of six proteins (bovine insulin, bovine serum albumin (BSA), lactoglobulin and casein from bovine milk, equine cytochrome *c*, and ovalbumin) was utilized for method optimization. For the initial experiments, the six-protein mixture was solubilized in 100 mM triethylammonium bicarbonate (TEAB) buffer (pH 8.5).

Cultivation of *M. mazei* Gö1 under nitrogen-limiting conditions was performed as previously described on minimal media in an anaerobic environment until an optical density (OD) at 600 nm of 0.6 was reached.[(6)](javascript:void(0);) The cells were concentrated via centrifugation, washed in 100 mM TEAB, and then disrupted via freeze–thaw cycling and homogenization in a mixing mill with glass beads in lysis buffer (100 mM TEAB, 1× EDTA-free cOmplete protease inhibitor cocktail (Roche, Germany), pH 7.4). The protein concentration was determined via BCA protein assay, and aliquots of the samples were stored at −20 °C until required.

**Depletion of the High-Molecular-Mass Proteome, SDS-PAGE, and Protein Digestion**

Following optimization of the parameters for the depletion of the HMWPs, the following standardized procedure was performed for all samples. During the optimization process, minor alterations to the procedure were employed. These are stated where appropriate.

Aliquots of 100 μg of total protein lysate were concentrated to near (but not complete) dryness via vacuum evaporation on a Concentrator Plus (Eppendorf, Germany). The samples were then made up to a volume of 20 μL of either 210 mM NaCl (acidic depletion mixture with final concentrations of 50 mM NaCl/0.1% trifluoroacetic acid (TFA)) or 420 mM TEAB (basic depletion mixture with a final concentration of 100 mM TEAB). The samples were vortexed to ensure solubilization in the solution prior to the addition of 64 μL of ACN plus 0.1% TFA. The samples were vigorously vortexed for 30 s before incubation at 20 °C on a temperature-controlled shaker (1300 rpm) for 1 h. Following incubation, the samples were centrifuged (21 000*g* for 20 min at 20 °C), and the supernatants were transferred to new Eppendorf tubes. The samples were dried down via vacuum evaporation prior to further analysis.

For visualization of the isolated proteins, SDS-PAGE (16% T) was performed, and the gels were stained with either Coomassie Brilliant Blue (CBB) or silver staining.

For the analysis of proteins via LC–MS, protein samples were suspended in 100 mM TEAB and reduced with dithiothreitol (DTT) (10 mM, 56 °C, 1 h) before alkylation was performed with iodoacetamide (IAA) (50 mM, RT, 30 min). Enzymatic digestion was performed with sequencing-grade trypsin (Promega, Germany) (enzyme to protein ratio of 1:50, 37 °C, overnight). All samples were then cleaned on a single-use solid phase extraction cartridge (as per manufacturer’s protocol) before the peptides were eluted (60% ACN, 0.1% TFA) and dried down via vacuum evaporation.

**Depletion of High-Molecular-Weight Proteins Using the Different Solubility Method**

To evaluate our high mass protein depletion procedure, we also performed and established a DS procedure that is based on precipitation under denaturing conditions, followed by resolubilization.[(9)](javascript:void(0);) An aliquot of our sample was diluted 1:2 with 20 μL of denaturating solution (7 M urea, 2 M thiourea, and 20 mM DTT), slowly dropped into 900 μL of ice-cold acetone, and immediately stirred at 4 °C for 1 h, followed by centrifugation at 19 000*g* for 15 min at 4 °C. The precipitate was taken up in 200 μL of 70% ACN containing 12 mM HCl and mixed at 4 °C for 1 h, then centrifuged again at 19 000*g* for 15 min at 4 °C. The low-molecular-mass proteins/peptides were extracted into the supernatant.

## Improved Identification and Analysis of Small Open Reading Frame Encoded Polypeptides

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**Cell Culture**

K562 and A549 cells were maintained in RPMI and F-12K media, respectively. HeLa and HEK293 cells were cultured using DMEM. The media contained 10% fetal bovine serum (FBS). Cells were grown under an atmosphere of 5% CO2 at 37 °C until confluent. Before cells lysis and enrichment of SEPs, the media was removed from adherent cells by aspiration (A549, HeLa, HEK293) or nonadherent cells (K562) by centrifugation. HEPES-buffered saline (pH 7.5) was used to wash the cells to remove residual media and FBS.

**SEP Enrichment Methods**

We tested three conditions for SEP enrichment: (1) acid precipitation, (2) 30-kDa molecular weight cut off (MWCO) filter, and (3) reverse-phase (C8) cartridge enrichment. Cellular proteomes from 4 × 107 cells were extracted by lysis with boiling water. After cooling the samples on ice, the cells were sonicated for 20 bursts at output level 2 with a 30% duty cycle (Branson Sonifier 250; Ultrasonic Convertor). For the acid precipitation, the addition of acetic acid (to a final concentration of 0.25% by volume) was followed by centrifugation at 14 000*g* for 20 min at 4 °C. This step precipitates larger proteins to reduce the complexity of the supernatant and enriches lower molecular weight proteins that are then analyzed by LC–MS/MS proteomics for SEPs. For the 30-kDa MWCO, the addition of acetic acid (to a final concentration of 0.25% by volume) was followed by centrifugation at 14 000*g* for 20 min at 4 °C. The supernatant is then passed through a 30-kDa MWCO filter and the flow through is analyzed for SEPs. Lastly, the reverse phase enrichment, the cellular extracts are centrifuged at 25 000*g* for 30 min and supernatants removed and filtered through 5 μM syringe filters followed by enrichment of SEPs using Bond Elute C8 silica cartridges (Agilent Technologies, Santa Clara, CA). Approximately 100 mg sorbent was used per 10 mg total lysate protein. Cartridges were prepared with one column volume methanol and then equilibrated with two-column volumes triethylammonium formate (TEAF) buffer, pH 3.0 before the sample was applied. The cartridges were then washed with two column volumes TEAF and the SEP enriched fraction eluted by the addition of acetonitrile:TEAF pH 3.0 (3:1) and lyophilized using a Savant Speed-Vac concentrator. BCA protein assay (Thermo Scientific) was used to measure protein concentration of each sample after extraction and enrichment.

**SEP Extraction Methods**

Four different methods were compared for extraction of SEPs from 4 × 107 total cells: (1) 50 mM HCl, 0.1% β-mercaptoethanol (β-ME); 0.05% Triton X-100 at room temperature (lysis buffer); (2) 1 N acetic acid/0.1 N HCl at room temperature; (3) boiling in water; or (4) boiling in lysis buffer. After extraction using these four methods, the extracts were centrifuged at 25 000*g* for 30 min, and supernatants filtered through 5 μM syringe filters. The flow through was then enriched for SEPs by binding and elution using Bond Elute C8 silica cartridges (Agilent Technologies, Santa Clara, CA). Approximately 100 mg sorbent was used per 10 mg total lysate protein. Cartridges were prepared with one column volume methanol and equilibrated with two-column volumes triethylammonium formate (TEAF) buffer, pH 3.0 before the sample was applied. The cartridges were then washed with two column volumes TEAF and the SEP enriched fraction eluted by the addition of acetonitrile:TEAF pH 3.0 (3:1) and lyophilized using a Savant Speed-Vac concentrator. BCA protein assay (Thermo Scientific) was used to measure protein concentration of each sample after extraction and enrichment.

**Digestion and Sample Preparation for LC–MS/MS**

An aliquot of 100 μg of enriched samples was precipitated with chloroform/methanol extraction. Dried pellets were dissolved in 8 M urea/100 mM TEAB, pH 8.5. Proteins were reduced with 5 mM tris 2-carboxyethylphosphine hydrochloride (TCEP, Sigma-Aldrich) and alkylated with 10 mM iodoacetamide (Sigma-Aldrich). Proteins were digested overnight at 37 °C in 2 M urea/100 mM TEAB, pH 8.5, with trypsin (Promega). Digestion was stopped with formic acid, 5% final concentration.

Comparative Proteomics Enables Identification of Nonannotated Cold Shock Proteins in E. coli

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**Cell Lysis and Protein Size Selection**

Lysis and size selection were adapted from Ma et al.[(5)](javascript:void(0);) as follows: Frozen cells from the stress conditions were resuspended in lysis buffer (50 mM HCl and 0.1% β-mercaptoethanol). The resuspension was sonicated at 35% amplitude with eighteen 10 s bursts with a 20 s rest on a Fisher Scientific model 120 sonic dismembrator. Triton X-100 was added to the sample to a final concentration of 0.05%. The sample was heated for 10 min at greater than 95 °C, allowed to cool on ice for 10 min, and then pelleted by centrifugation for 30 min at 21 100*g* at 4 °C. The supernatant was removed, and the pellet was discarded. The supernatant was filtered through a 5 μm filter.

A Bond Elut C8 column (Agilent) preconditioned with 1 column volume of methanol followed by 2 column volumes of triethylammonium formate (TEAF) pH 3.0 was loaded with approximately 10 mg of protein per 100 mg of bed resin and washed with 2 column volumes of TEAF pH 3.0. Size-selected proteins were eluted with two column volumes of 3:1 acetonitrile/TEAF pH 3.0 and concentrated on a Savant SPD10 SpeedVac concentrator (Thermo Scientific).

## Optimized Sample Preparation Workflow for Improved Identification of Ghost Proteins

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**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovin serum (FBS), l-glutamine, penicillin, streptomycin, and phosphate-buffered saline (PBS) were obtained from Thermo Fisher Scientific (Les Ulis, France). Formic acid (FA), HPLC grade water, trifluoroacetic acid (TFA), acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), acetone, and trichloroacetic acid (TCA) were purchased from Biosolve BV (Dieuze, France). d,l-Dithiothreitol (DTT), iodoacetamide (IAA), chloroform, dimethylsulfoxide (DMSO), and amonium bicarbonate (AmBic) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Tris and SDS were purchased from Bio-Rad (Steenvoorde, France). Trypsin was obtained by Promega (Charbonnières-les-Bains, France).

**Cell Culture**

Human NCH82 stage IV glioma cells stage IV were supplied by obtained from Régnier Vigouroux and grown at 37 °C under an atmosphere of 5% CO2. The cells were grown in high glucose Dulbecco’s Modified Eagle’s Medium (D-MEM, Thermofisher), supplemented with 10% fetal bovine serum and 100 U/mL penicilin/streptomicin antibiotic. For AltProts extraction, the cells were grown in cell culture plate with 6 wells. Two wells were used for protein extraction.

**Protein Extraction Methods**

Prior to cell lysis and enrichment of AltProts, culture plates were placed on ice, media were removed from all the plates, and the cells were washed twice with Dulbecco’s phosphate-buffered saline DPBS 1× Mg2+ and Ca2+. The AltProt extraction was performed scraping the cells from the wells with 150 μL of extraction buffer. The extracts were then collected into 1.5 mL low binding tubes. . Four different extraction buffers were compared from 1.8 × 106 cells/mL. Three different methods were used for peptides and proteins extraction: (1) SDS 4% buffer (SDS 4%, Tris-HCl 0.1 M, DTT 0.1 M, pH: 7.6), (2) RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 50 mM EGTA, 2 mM EDTA, NP40, 100 mM sodium pyrophosphate, IGEPAL 1%), (3) MeOH acid buffer (1:9:90 acetic acid/H2O/MeOH). Samples were incubated 30s in liquid nitrogen, and then 30 s in boiling water. This was repeated 4 times. Samples were then sonicated at level 3 for 20 bursts and placed on ice between every 5 pulses for 30 s. The homogenate was then centrifuged at 17 000 g for 20 min at 4 °C The supernatant was collected and directly used or dried under vacuum and stored at −80 °C. The fourth method was investigated only for peptides extraction: (4) Boiling Water. The extracts were washed three times with PBS by centrifugation at 1000*g* for 1 min, pelleted and then stored at −80 °C. Boiling water (500 μL) was directly added to the frozen pellet and the sample was then boiled for 20 min to stop the proteolytic activity and maintain the integrity of the peptidome. Once the samples were brought to room temperature (RT), they were sonicated on ice for 20 bursts. Acetic acid in water (1:4, v/v) was added to the cell lysate and the samples were centrifuged at 17 000*g* for 20 min at 4 °C. The supernatant was directly used or dried under nitrogen and stored at −80 °C.

**AltProts Enrichment Methods**

Three AltProts enrichment methods were compared: (1) gel fractionation, (2) acetic acid precipitation, and (3) trichloroacetic acid precipitation. (1) Gel fractionation: Stored protein pellets were solubilized in Laemmli 1X buffer and then loaded onto a 4–12% acrylamide of SDS-PAGE gel. Proteins were separated at 70 V for 20 min and at 170 V for 60 min. The gel was stained with Instant blue (Expedeon) and cut into 5 pieces below the 50 kDa marker as follows: 50–40, 40–25, 25–15, 15–10, 10–1 kDa. (2) Acetic acid precipitation (AA): AA in water (1:4, v/v) was added to the supernatant followed by the centrifugation at 15 000*g* for 20 min at 4 °C. This step precipitates larger proteins to reduce the complexity of the supernatant and enriches low molecular weight proteins. The supernatant was then collected. (3) Trichloroacetic acid (TCA) precipitation: 10 volumes of cold acetone/TCA. (9:1, v/v) were added to the supernatant. The solution was mixed and stored overnight at −20 °C followed by a centrifugation at 15 000*g* for 10 min. The supernatant was removed, and 1 volume of acetone was added to the pellet, mixed, and then stored for 10 min at −20 °C. After the additional centrifugation for 5 min at 15 000*g* the pellets and the supernatant were split in two samples and dried under vacuum.