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Sample Preparation for Mass-spectrometry-based Proteomics Analysis of Ocular Microvessels

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Dear Editor,

The authors thank the editorial board members and the reviewers for their kind consideration and review of our manuscript entitled "A Rapid and Robust Sample Preparation Method for Mass-Spectrometry-Based Proteomics Analysis of Ocular Microvessels" (JoVE59140) in *JoVE* as an original research article.

We appreciate the comments and points raised by the editor and reviewers to further improve the quality and content of the article. We have addressed the reviewers' and editorial board member's comments point-by-point. Our manuscript and corresponding files i.e. figures and tables have been revised accordingly.

We would like to resubmit the revised manuscript.

Thank you for considering this manuscript for publication in your journal.

Yours Sincerely,
Caroline Manicam, Ph.D.

TITLE:

Sample Preparation for Mass-spectrometry-based Proteomics Analysis of Ocular Microvessels

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KEYWORDS:

blood vessels, microvessels, short posterior ciliary arteries, proteomics, mass spectrometry, eye, pig

SUMMARY:

Proteome characterization of ocular microvascular beds is pivotal for in-depth understanding of many ocular pathologies in humans. This study demonstrates an effective, rapid, and robust method for protein extraction and sample preparation from small blood vessels employing the porcine short posterior ciliary arteries as model vessels for mass-spectrometry-based proteomics analyses.

ABSTRACT:

The use of isolated ocular blood vessels in vitro to decipher the pathophysiological state of the eye using advanced technological approaches has greatly expanded our understanding of certain diseases. Mass spectrometry (MS)-based proteomics has emerged as a powerful tool to unravel alterations in the molecular mechanisms and protein signaling pathways in the vascular beds in health and disease. However, sample preparation steps prior to MS analyses are crucial to obtain reproducible results and in-depth elucidation of the complex proteome. This is particularly important for preparation of ocular microvessels, where the amount of sample available for analyses is often limited and thus, poses a challenge for optimum protein extraction. This article endeavors to provide an efficient, rapid and robust protocol for sample

preparation from an exemplary retrobulbar ocular vascular bed employing the porcine short posterior ciliary arteries. The present method focuses on protein extraction procedures from both the supernatant and pellet of the sample following homogenization, sample cleaning with centrifugal filter devices prior to one-dimensional gel electrophoresis and peptide purification steps for label-free quantification in a liquid chromatography-electrospray ionization-linear ion trap-Orbitrap MS system. Although this method has been developed specifically for proteomics analyses of ocular microvessels, we have also provided convincing evidence that it can also be readily employed for other tissue-based samples.

INTRODUCTION:

The advancement in the field of proteomics, which permits integrated and unsurpassed data collection power, has greatly revolutionized our understanding of the molecular mechanisms underlying certain disease conditions as well as in reflecting the physiological state of a specific cell population or tissue[1-4]. Proteomics has also proved to be an important platform in ophthalmic research owing to the sensitivity and unbiased analysis of different ocular samples that facilitated identification of potential disease markers for eventual diagnosis and prognosis, as evidenced elegantly by many studies in recent years, including some of ours[1,5-10]. However, it is often difficult to obtain human samples for proteomic analyses due to ethical reasons, especially considering the need for control material from healthy individuals for reliable comparative analyses. On the other hand, it is also challenging to obtain sufficient amount of samples for optimal and reliable mass spectrometric analyses. This is particularly crucial for mass-limited biological materials such as the micro-blood vessels of the eye. One such major retrobulbar blood vessel that plays pivotal roles in the regulation of ocular blood flow is the short posterior ciliary artery (sPCA). Any perturbation or anomalies in this vascular bed may result in severe clinical repercussions, which can lead to the pathogenesis of several sight-threatening diseases such as glaucoma and nonarteritic anterior ischemic optic neuropathy (NAION)[11,12]. However, there is a lack of studies elucidating the proteome changes in this arterial bed due to the above-mentioned drawbacks. Therefore, in recent years, the house swine (*Sus scrofa* domestica Linnaeus, 1758) has emerged as a good animal model in ophthalmic research owing to the high morphologic and phylogenetic similarities between humans and pigs[13-15]. Porcine ocular samples are easily available and most importantly, are more accurate representation of human tissues.

Considering the important role of these blood vessels in the eye, as well as the dearth of methodology catered for efficient protein extraction and analyses from these microvessels, we have previously characterized the proteome of the porcine sPCA using an in-house protocol that resulted in the identification of a high number of proteins[16]. Based on this study, we have further optimized and described in-depth our methodology in this article, which allows proteome analysis from minute amounts of samples using the porcine sPCA as model tissue. Albeit the main aim of this study was to establish a MS-compatible methodology for mass-limited ocular blood vessels, we have provided substantial experimental evidence that the described workflow can also be broadly applied to various tissue-based samples.

It is envisioned that this workflow will be instrumental for preparation of high-quality MS-compatible samples from small quantities of materials for comprehensive proteome analyses.

PROTOCOL:

All experimental procedures using animal samples were performed in strict adherence to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and by institutional guidelines. This study was conducted and approved at the Department of Ophthalmology, University Medical Centre Mainz.

NOTE: Porcine eyes together with optic nerve and extraocular tissues were obtained fresh from the local abattoir immediately post-mortem. Enucleated eyes were transported to the laboratory in ice-cold phosphate buffered saline (PBS) and used immediately. The schematic overview of the workflow employed is as depicted in **Figure 1**.

1. Solutions

1.1. To prepare Krebs-Henseleit (K-H) buffer, weigh the following chemicals (27.68 g of NaCl, 8.4 g of $NaHCO_3$, 1.4 g of KCl, 1.18 g of $MgSO_4$, and 0.64 g of KH_2PO_4) into one dry and clean 50 mL conical centrifuge tube and, 1.47 g of $CaCl_2$ and 8.0 g of glucose into another tube.

NOTE: The powder mixture of these chemicals should be stored in a cool and dry place. The $CaCl_2$ and glucose powder mixture is best prepared fresh to prevent absorption of moisture and clumping.

1.2. To prepare 200 µL of extraction buffer 2A per pellet sample, mix 100 µL of extraction buffer 2 and 100 µL of reagent A from the transmembrane protein extraction kit (see B6 in the **Table of Materials**).

NOTE: Aliquot the components of the transmembrane protein extraction kit comprising extraction buffer 2, reagent A and protease inhibitor cocktail into working portions and store at -20 °C for prolonged use. Avoid repeated freezing and thawing. Thaw the reagents to room temperature (RT) prior to the start of the pellet extraction procedure.

1.3. To prepare ammonium bicarbonate (ABC, 100 mM) solution, dissolve 0.39 g of ABC in 50 mL of deionized water.

1.4. To prepare 10 mM 1, 4-dithiothreitol (DTT) solution, dissolve 30 mg of DTT in 20 mL of 100 mM ABC.

1.5. To prepare 55 mM Iodoacetamide (IAA) solution, dissolve 200 mg of IAA in 20 mL of 100 mM ABC.

NOTE: IAA must be kept in the dark. Use an aluminum foil to wrap the tubes with light-sensitive solutions.

1.6. Prepare trypsin buffer containing 10 mM ABC and 10% (vol/ vol) acetonitrile (ACN). Add 1.5 mL of trypsin buffer into one vial of 20 µg of trypsin to dissolve its content to prepare a 13 ng/µL trypsin working solution.

NOTE: Sequencing grade modified trypsin is supplied lyophilized and must be stored in -20 °C till use.

1.7. To prepare peptide extraction buffer, mix 1:2 (v/v) of 5% formic acid with ACN.

NOTE: Prepare all solutions in steps 1.3-1.7 fresh just before use and discard any unused volume.

1.8. To prepare 0.1% trifluoroacetic acid (TFA), mix 10 µL of TFA with 10 mL of deionized water.

2. Isolation of short posterior ciliary arteries

NOTE: The porcine eye is basically divided into the anterior (**Figure 2A**) and posterior sections (**Figure 2B**).

2.1. Place the eye globe in a dissection chamber containing ice-cold K-H buffer. Carefully cut away surrounding muscle and tissues with a pair of sharp Mayo scissors.

2.2. Cut the globe along the equatorial plane with a scalpel until the eye is separated into the anterior and posterior halves. Remove as much vitreous body as possible from the posterior half of the eye using a pair of standard pattern forceps.

NOTE: Separation of the eye globe into two halves facilitates the isolation of sPCA with ease without having a moving eye in the dissection dish. However, this step is not mandatory. The vessels can also be isolated without opening the eye globe.

2.3. Use dissection pins to carefully pin down the posterior half of the eye with the retina side down and the optic nerve facing up towards the experimenter.

2.4. Gently cut away the connective tissues surrounding the optic nerve to expose the underlying retrobulbar vasculature with a pair of Student Vannas spring scissors.

2.5. The sPCA can be seen as short branches of vessels (between 5-8 branches) that penetrate the sclera and circumferentially surround the optic nerve head (**Figure 2C**). Isolate the paraoptic and distal sPCA together with the surrounding connective tissues with a pair of type 5 precision tweezers and Vannas capsulotomy scissors (**Figure 2D**).

NOTE: Ensure that the K-H buffer remains ice-cold throughout the isolation procedure. It is highly recommended to change the buffer every 20-30 min, depending on the ambient temperature.

3. Sample preparation

3.1. Gently remove connective tissues from the arterial segments using extra fine-tipped type 5 precision tweezers and Vannas capsulotomy scissors under a stereomicroscope and rinse the isolated arteries in ice-cold PBS to remove contaminants and blood residues.

NOTE: If samples are not subjected to the next steps immediately, snap-freeze them in liquid nitrogen and store in -80 °C until further use.

3.2. Pool arteries from two eyes to obtain one biological replicate, transfer them into 1.5 mL microcentrifuge tubes, and weigh the samples using an analytical balance.

3.3. To each tube, add a mixture of 0.5 mm and 1.0 mm zirconium oxide beads, followed by tissue protein extraction reagent (T-PER; see B7 in the **Table of Materials**).

NOTE: The volume of T-PER is dependent on the weight of the samples in each tube, with a ratio of 1 mL of reagent to 50 mg of sample. A general rule of the thumb to follow when loading tubes for homogenization is a volume ratio of 1:1:2 = sample: beads: T-PER (**Figure 3A**). Do not use too large a volume of the extraction buffer and too little an amount of beads to prevent inefficient homogenization.

3.4. Load the sample tubes into a blender homogenizer (see D6 in the **Table of Materials**). Set the timer to 2 min and speed level 6 and, start homogenization. After running, check the samples for complete homogenization and repeat cycle until samples are completely homogenized (**Figure 3B**).

NOTE: A total of 24 sample tubes can be homogenized in one run. It is important to keep the samples cold during the homogenization procedure to minimize protein denaturation. The bullet blender homogenizer used in this study has an inherent cooling feature that keeps the samples cold. In cases where no internal cooling feature is available in the homogenizer, samples can be kept on ice in between each run.

3.5. Carefully pipette homogenate into fresh microcentrifuge tubes. Centrifuge samples at 10,000 *x g* for 20 min at 4 °C to pellet insoluble proteins and separate supernatant containing soluble proteins. Carefully pipette out the supernatant into fresh microcentrifuge tubes without touching the pellet layer (**Figure 3C**).

NOTE: Both supernatant and pellet can be stored in -80 °C until further use.

4. Pellet digestion

4.1. Add 200 µL of extraction buffer 2A and 5 µL of protease inhibitor cocktail to each pellet sample. Suspend the pellet several times with a pipette.

NOTE: Mix the extraction reagents well before adding to the pellet. Keep extraction buffer 2A on ice and the protease inhibitor at RT throughout the extraction procedure.

4.2. Use an ultrasonic homogenizer to completely homogenize the pellet.

4.2.1. Set the amplitude to 60 and cycle to 1. Use a probe made of titanium (Ø 0.5 mm, 80 mm long), which is appropriate for homogenization of samples with small volumes (10-500 µL).

4.2.2. Immerse the probe in the pellet and extraction buffer mixture and press the start button to expose the sample to ultrasonic waves.

4.2.3. Sonicate the sample until the pellet clumps are completely homogenized. Pause for a few seconds between each sonication.

4.2.4. Check for complete homogenization visually. Mix the homogenate several times with a pipette to ensure that there are no clumps.

NOTE: The pellet extraction procedure should be carried out on ice to prevent protein denaturation due to heat generated during homogenization (**Figure 4**).

5. Sample cleaning and buffer exchange

5.1. Use centrifugal filter devices with 3 kDa cutoff (see D3 in the **Table of Materials**) for this procedure according to the manufacturer's instructions with modifications where applicable. First, insert the filter unit into a microcentrifuge tube (provided in the filter pack).

5.2. Pipette 200 µL of sample homogenate to one filter device and add 200 µL of deionized water into the same filter. Cap it securely (**Figure 5A**).

5.3. Place the filter unit into a centrifuge and spin for 14,000 *x g* for 15 min at 4 °C.

5.4. Remove the device from the centrifuge and separate the filter unit containing sample concentrate from the microcentrifuge tube containing the filtrate. Discard the filtrate (**Figure 5B**).

5.5. Reconstitute the concentrate by adding 400 µL of deionized water into the filter unit. Repeat steps 5.3 and 5.4 three times. Carefully pipette the 'cleaned' sample concentrate into a clean microtube.

NOTE: Typically, a 200 µL crude sample will yield ~50-70 µL of concentrate after filtration.

5.6. Repeat steps 5.1-5.5 for the remaining sample homogenates.

6. Protein measurement

6.1. Use the bicinchoninic acid (BCA) protein assay kit (see B5 in the **Table of Materials**) to determine protein concentrations of the supernatant and pellet fractions of the sPCA samples on a plate reader.

6.2. Prepare the albumin standard (BSA) dilutions (final BSA concentrations range between 25 to 2000 µg/mL) from one 1 mL ampoule comprising 2 mg/mL BSA, according to the manufacturer's instructions.

6.3. Pipette 10 µL of each BSA standard dilution and sample replicates (both supernatant and pellet) into each well of a 96-well flat-bottom microplate.

6.4. Prepare the BCA working reagent by adding 50 parts of BCA Reagent A to 1 part of BCA Reagent B.

NOTE: Calculate the total volume of the working reagent required for each measurement prior to the preparation and freshly prepare sufficient volume based on the number of samples and standard dilutions to be assayed.

6.5. Carefully pipette 200 µL of the BCA working reagent into each well. Cover the microplate and incubate at 37 °C for 30 min. After cooling the plate to RT, measure the absorbance at 562 nm on a plate reader (see D13 in the **Table of Materials**).

6.6. Based on the standard curve generated for each BSA standard concentration (µg/mL), determine the protein concentrations of the samples.

7. One-dimensional gel electrophoresis (1DE)

7.1. Prepare the samples for 1DE as shown in **Table 1** (for one sample). Mix the sample mixture well with a pipette. Heat the samples at 70 °C for 15 min and cool to RT.

7.2. Prepare 1x Running Buffer by adding 50 mL of Running Buffer to 950 mL deionized water. Mix well.

7.3. Prepare precast 4-12% Bis-Tri gels (see B4 in the **Table of Materials**).

7.3.1. Cut open the plastic pouch to remove the gel cassette and rinse the cassette with deionized water.

7.3.2. Remove the comb out of the gel cassette in one fluid motion, taking care not to damage the wells.

7.3.3. Gently rinse the loading wells 2-3 times with 1x Running Buffer using a pipette. Invert and gently shake the gel cassette to remove the buffer, ensuring that there no air bubbles in the wells.

7.3.4. Remove the white tape at the bottom of the gel cassettes.

7.4. Insert gels (maximum two cassettes) into the gel running tank and once fully assembled, lock the gel tension wedge.

7.6. Fill the upper (cathode) buffer chamber with 200 mL of Running Buffer until the wells are completely covered. Check for any leaks.

7.7. Add 500 µL of antioxidant (see A14 in the **Table of Materials**) to the Running Buffer.

NOTE: The antioxidant is a propriety reagent that must be used with samples reduced with reducing agent (refer **Table 1**) to maintain the reducing conditions during electrophoresis and to prevent re-oxidation of sensitive amino acids such as tryptophan and methionine.

7.10. Carefully load 50 µg of sample per lane using a pipette. Then, load the pre-stained protein standard as a molecular mass marker (see A18 in the **Table of Materials**).

7.11. Fill the lower (anode) buffer chamber with 600 mL of Running Buffer.

7.12. Run the gels for ~60 min at a constant voltage of 175 V. At the end of the run, carefully remove the gel from the cassette plate using a gel knife.

7.13. Carefully transfer the gels into a gel staining box.

7.13.1. Prepare the fixing solution fresh based on the total number of gels that need to be stained, according to the manufacturer's instructions as described in **Table 2.**

7.13.2. Shake the gel(s) in the fixing solution for 10 min at RT on a rocking platform.

7.14. Discard the fixing solution and stain the gels with Colloidal Blue staining kit.

7.14.1. Prepare the staining solutions fresh based on the total number of gels that need to be stained, according to the manufacturer's instructions as described in **Table 3**.

7.14.2. First, measure the appropriate volume and mix directly the components marked with an asterisk (*) in the gel staining box. Shake the gel(s) in the staining solution without Stainer B for 10 min at RT on a rocking platform.

7.14.3. Add Stainer B directly into the staining box containing the *staining solution. Make sure to shake Stainer B well prior to use.

CAUTION: Carry out preparation steps 8.4.1 and 8.5.1 under a fume hood.

7.15. Shake the gel(s) in the staining solution overnight for best overall results.

NOTE: The stained bands will become visible within 10 min after the addition of Stainer B. Staining requires a minimum of 3 h and the intensity does not vary if left for longer hours in the staining solution.

7.17. Carefully decant the staining solution and replace with 200 mL of deionized water. Shake the gel(s) for at least 7-8 h in water to clear the background.

NOTE: Deionized water must be changed several times during the destaining procedure to better remove excessive stain. The gel(s) can also be left in water for up to 3 days without compromising the protein band intensity. If the gel(s) is not subjected to the next steps immediately, it can be stored in 20% ammonium sulfate solution at 4 °C for long-term storage.

8. In-gel tryptic digestion

NOTE: This protocol is according to the method by Shevchenko et al.[17], with slight modifications. This procedure should be carried out in a laminar flow hood and use dedicated set of pipettes, tips, tubes, and glassware specifically for this purpose. Wear gloves and appropriate lab apparel at all times to prevent keratin and other contamination. Prepare all solutions and reagents used in this procedure shortly before use.

8.1. Excise the protein bands from the gel with clean, new microtome blades. Cut the band into small pieces (approximately 1 mm x 1 mm to 2 mm x 2 mm). Carefully transfer the gel pieces into 1.5 mL microcentrifuge tubes.

NOTE: Pieces that are too small could clog pipette tips and larger pieces will reduce peptide recovery. Exercise caution when using microtome blades, which are extremely sharp.

8.2. Add 500 µL of destaining solution containing 100 mM ABC solution/ACN (1:1, v/v) and incubate samples at RT for 30 min with occasional shaking or vortexing.

8.3. Carefully pipette out the destaining solution. Check visually for any tubes with residual stain. Repeat step 8.2 if gel pieces are still stained blue.

8.4. Reduction and alkylation

8.4.1. Add ~400 µL of freshly prepared DTT solution and incubate at 56 °C for 30 min. Make sure that the solution completely covers the gel pieces. Carefully pipette out the reducing solution and discard.

8.4.2. Add ~400 µL of freshly prepared IAA solution and incubate in the dark at RT for 30 min. Remove the alkylating buffer with a pipette and discard.

8.5. Digestion

8.5.1. Add 500 µL of neat ACN at RT for 10-15 min until the gel pieces shrink and become opaque. Pipette out ACN and air-dry gel pieces for 5-10 min under the hood.

8.5.2. Pipette 50 µL of trypsin solution into each tube to completely cover the gel pieces. Incubate the tubes in 4 °C for 30 min.

8.5.3. After 30 min, check each tube if all trypsin solution has been absorbed by the gel pieces. Add sufficient volume of trypsin buffer (50-100 µL, depending on the volume in the tube) to completely cover the gel pieces, if necessary. Incubate the samples overnight at 37 °C.

8.6. Peptide extraction

8.6.1. Carefully pipette the extracted peptide solution from the tubes and transfer to clean microtubes. Dry the supernatant in a centrifugal vacuum evaporator.

NOTE: Do not discard the remaining gel pieces.

8.6.2. Add 100 µL of extraction buffer (refer to step 1.9) to each tube and incubate for 30 min at 37 °C with shaking.

8.6.3. Pipette the supernatant into the same microtubes containing the extracted peptides according to the respective bands and dry down in a vacuum centrifuge.

NOTE: If not used immediately, dried peptide extracts can be stored in -20 °C up to several months until further use.

9. Peptide purification

NOTE: This peptide sample desalting and purification procedure is carried out with the use of C_{18} pipette tips (see C15 in the **Table of Materials**). Use a new tip for each sample.

9.1. Prepare the following solutions fresh in conical centrifuge tubes and aliquot into 2 mL microcentrifuge tubes for the peptide purification procedure, as shown in **Table 4**. A summary of the tubes for peptide purification is as follows: Tube A (Sample solution), Tube B (Wetting solution), Tube C (Equilibration solution), Tube D (Washing solution), Tube E (Elution solution),

Tube F (An empty 2 mL or 5 mL microcentrifuge tube, depending on the number of samples to be cleaned, for waste disposal), and Tube G (A clean, empty microtube, capacity 0.2 mL).

9.2. Reconstitute dried peptide extracts from step 8.6.3 with 10 µL of 0.1% TFA. This tube is designated Tube A.

9.3. Sonicate tubes A in an ultrasonic bath with ice for 5 min.

9.4. Spin down the sample solution in a benchtop centrifuge at 1,000 x *g* for 1 min.

9.5. Set a P10 micropipette to the maximum volume setting of 10 µL and attach a C_{18} pipette tip securely.

9.6. Immerse the pipette tip into the wetting solution (Tube B) and carefully aspirate into the packing material. Slowly dispense the waste into Tube F. Repeat this step at least 7-8 times.

NOTE: Depress pipette plunger to a dead stop and slowly release or dispense plunger throughout the procedure. Take precaution not to introduce air bubbles into the tips during pipetting to ensure maximum peptide binding to the C18 column.

9.7. Equilibrate the tip for peptide binding by aspirating 10 µL equilibration solution in Tube C. Discard the solution and repeat this procedure 3 to 4 times.

9.8. Next, aspirate and dispense sample solution in Tube A several times (depending on the corresponding gel band thickness) to bind the peptides to tip column.

CAUTION: The aspiration and dispensing steps during the binding procedure (step 9.8) are carried out within Tube A. Do not dispense the sample solution to waste.

9.9. Wash the C_{18} pipette tip by aspirating the washing solution in Tube D and discarding it to waste (Tube F) 4 to 5 times.

9.10. Finally, elute the bound peptides by pipetting the elution solution (Tube E) and dispensing the eluant into tube G.

9.11. Repeat the entire steps 2 to 3 cycles per sample to increase sample recovery.

9.12. Discard the tip after one sample purification and use a new tip for the next sample.

9.13. Dry the purified peptide eluates in a vacuum concentrator.

NOTE: The purified samples are now ready for LC-MS/MS analyses in the LC-ESI-LTQ-Orbitrap MS system. Store the dried samples in in -20 °C until further use.

10. Liquid chromatography-electrospray ionization-MS/MS analyses

NOTE: Label-free quantitative proteomics analysis is performed on a liquid chromatography-electrospray ionization-linear ion trap-Orbitrap (LC-ESI-LTQ-Orbitrap) MS system. The LC is composed of Rheos Allegro quaternary pump equipped with an online degasser (coupled to an HTS PAL autosampler, and the system comprises a 30 mm x 0.5 mm C_{18} pre-column connected to a 150 mm x 0.5 mm C_{18} column. Use reverse phase aqueous solvent A consisting of LC-MS grade water with 0.1% (v/v) formic acid and organic solvent B consisting of LC-MS grade acetonitrile with 0.1% (v/v) formic acid. Use the gradient with a running time of 60 min per gel band, as described in detail in our previous studies[6,16].

10.1. Dissolve the purified peptide samples in 10 µL of 0.1% TFA. Sonicate the samples on ice for 5 min.

10.2. Pipette 10 µL samples into a V-bottom 96-well plate and seal with a clear, self-adhesive cover film.

10.3. Transfer the plate to the autosampler.

10.4. Use the following gradient for each running time of 60 min: 0-35 min (15-40% solvent B), 35-40 min (40-60% solvent B), 40-45 min (60-90% solvent B), 45-50 min (90% solvent B), 50-53 min (90-10% solvent B), and 53-60 min (10% solvent B).

10.5. Use the following mass spectrometric conditions of the instrument: positive ion electrospray ionization mode, spray voltage (2.15 kV), capillary temperature (220 °C), data-dependent acquisition mode, automatic acquisition switching between Orbitrap-MS and LTQ MS/MS, orbitrap resolution (30,000), m/z range (300 to 1,600), target automatic gain control (AGC, 1.0 x 10^6 ions), internal recalibration (polydimethlycyclosiloxane [PCM] at m/z 445.120025 ions in real time), lock mass option enabled in MS mode, tandem data obtained by selecting the top five most intense precursor ions, further fragmentation by collision-induced dissociation (CID), normalized collision energy (NCE, 35% with activation time of 30 ms with repeat count of 3), and dynamic exclusion duration (600 s).

NOTE: The resulting fragmented ions are recorded in the LTQ.

10.6. Run at least three biological replicates for each sample.

REPRESENTATIVE RESULTS:

Limited sample availability is one of the major drawbacks in ophthalmic research. Correspondingly, extraction methods for optimum protein yield from small amounts of samples such as ocular blood vessels are often debatable. To date, there is a paucity of methods catered particularly for protein extraction from retrobulbar blood vessels. Therefore, as a first step in method optimization and as a proof-of-principle to compare the efficacy and robustness of several commonly employed protein extraction detergents to a relatively new reagent, T-PER,

we carried out a pilot study using cardiac tissues from mice (due to easy and sufficient sample availability for optimization steps). We compared the protein yield by comparing the total protein concentrations and total proteins identified using the following reagents: T-PER, 0.02% n-dodecyl-β-D-maltoside (DDM), 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 1% amidosulfobetaine-14 (ASB-14) and a mixture of ACN and TFA (20% ACN/ 1% TFA). The total amount of proteins in samples extracted with each of these detergents is as depicted in **Figure 6A**, with the highest yield from tissues extracted with T-PER (56.4 µg/mg tissue), followed by DDM (22.88 µg/mg tissue), CHAPS (16.01 µg/mg tissue), ASB-14 (11.56 µg/mg tissue) and the lowest yield from ACN/TFA (4.38 µg/mg tissue). Consistently, the total proteins identified were also the highest in the T-PER-extracted sample (1649 proteins) > DDM (1310 proteins) > CHAPS (1319 proteins) > ASB-14 (1121 proteins) > ACN/TFA (924 proteins), as shown **Figure 6B.** Based on these results, we proceeded with the protein extraction from sPCA samples with T-PER.

Next, optimization of sample preparation protocols prior to pre-fractionation in 1DE is a very crucial step to obtain well-separated protein bands and highly reproducible results between samples and replicates. The importance of these steps is reflected and highlighted in the results of 1DE. **Figure 7** shows the comparison of the 1DE protein profiles of sPCA before and after subjected to the optimized sample preparation and cleaning steps. Overall, a high degree of smearing and poor separation of the protein bands was observed at lane 3. This profile demonstrates that the samples may contain extraction reagent and also contaminants such as lipids and cellular debris. However, the sPCA samples that were separated into supernatant and pellet and, subjected to the optimized protocol resulted in exemplary 1DE profiles, as represented in lane 1 and 2 (**Figure 7**).

In gist, based on these promising results, the optimized method for rapid, robust and efficient soluble protein extraction from ocular microvessels is employing tissue protein extraction reagent (T-PER) and using Extraction Buffer 2A (TM-PEK) to extract membrane-based proteins found in the sample pellet. Subsequently, sample homogenates (the T-PER fraction) are subjected to buffer exchange and sample cleaning with the 3 kDa centrifugal filter units prior to 1DE. The optimum sample concentration is 50 µg per well. On the other hand, it has to be highlighted here that this protocol is not only applicable for small tissues such as blood vessels, but is also feasible for other tissue-based samples. This is evidenced by the 1DE profiles of murine brain and heart tissues, which demonstrated that there are large numbers of proteins that can be extracted from both supernatant (**Figure 8A,B**) and pellet fractions (**Figure 8C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow overview. A schematic representation of the protocols employed for label-free quantitative proteome analysis of the porcine sPCA. In general, this procedure is divided into two major sections comprising microvessel sample preparation steps and MS-based proteomics approach.

Figure 2: Representative photographs of the porcine eyes. (**A**) Lateral view of the eye globe shows the cornea, which is located in the anterior part of the eye, the sclera, surrounding

muscle and optic nerve. (**B**) Posterior view of the eye shows the optic nerve. (**C**) Branches of sPCA seen at the back of the eye globe composed of the paraoptic and distal branches. (**D**) Isolated sPCA with surrounding fat and connective tissues.

Figure 3: Sample homogenization. Representative photographs showing the sample (**A**) before and (**B**) after homogenization. (**C**) The homogenized samples were further separated into supernatant containing soluble proteins and pellet containing insoluble, transmembrane-based proteins.

Figure 4: Pellet protein extraction procedure. In order to prevent protein denaturation, pellet samples are extracted on ice.

Figure 5: Sample cleaning. Buffer exchange is carried out with 3 kDa cutoff centrifugal filters to clean the samples prior to MS analysis. Representative photographs showing the homogenate (**A**) before and (**B**) after filtration.

Figure 6: Comparison of protein extraction efficacy and robustness between T-PER and four common protein extraction detergents. Bar charts depicting the (**A**) protein amounts and (**B**) total number of proteins identified using T-PER, 0.02% DDM, 1% CHAPS, 1% ASB-14 and 20% ACN/ 1% TFA.

Figure 7: Representative 1DE gel of the porcine sPCA protein profiles before and after optimization. Lane 3 depicts the 1DE profile of sample that was not subjected to any prior cleaning steps. Lane 1 and 2 show the exemplary protein profiles of sPCA supernatant and pellet, respectively, after subjecting the samples to the optimized sample preparation and cleaning steps. Gel was stained with colloidal blue staining kit. M: Marker.

Figure 8: Representative colloidal blue-stained 1DE gel of exemplary tissue-based samples. Protein profiles of supernatant (**A,B)** and pellet (**C,D)** of murine brain and cardiac tissue samples, respectively, at 50 µg per well. Supernatant and pellet proteins were extracted employing the T-PER and transmembrane protein extraction kit, respectively. M: Marker. R1-R3 represent three replicates.

Table 1: 1 Dimensional Gel Electrophoresis (1DE). The details of the components required for sample preparation to perform 1DE.

Table 2: Gel fixation. The details of the components required to prepare fixing solution for the 1DE.

Table 3: Gel staining. The details of the components for preparation of the Colloidal Blue staining solution.

Table 4: Peptide purification. The details of the components and their respective compositions for peptide purification procedure using the C_{18} pipette tips.

DISCUSSION:

Comprehensive proteome profiling of a diverse range of ocular samples is an important and indispensable first step to elucidate the molecular mechanisms and signaling pathways implicated in health and disease. In order to obtain high quality data and to ensure the reproducibility of results obtained from these analyses, the preceding sample preparation steps are crucial, as highlighted in a review by Mandal et al. that discussed in-depth the sample processing procedures for different parts of the eye employing two-dimensional gel electrophoresis and mass spectrometry strategy[1]. In line of these investigations, our current study provides an optimized step-by-step protocol for rapid, robust and highly efficient MS-compatible sample preparation using the porcine sPCA as model ocular microvessels. This investigation was initiated following the paucity of specific methodology to extract sufficient amounts of proteins from quantity-limited arterial samples to generate high quality MS data. Our method is also an endeavor to contribute to the existing body of knowledge on the use of micro-scale techniques that enable excellent proteome mapping[18].

There are several critical aspects in this experimental protocol that need to be taken into consideration for optimal performance for a quantitative proteome analysis. First, it is important that the samples, regardless of the amounts, are subjected to complete homogenization to ensure optimal protein extraction. In our methodology, the use of a mixture of different sized beads and bullet blender homogenizer was instrumental for complete tissue lysis. The type and size of beads used depend on the sample type and amount. Beads with higher densities, such as the currently utilized ZrO_2 and stainless steel, are suitable for medium-tough tissues and worked especially well for blood vessels.

Second, it is imperative to separate the supernatant from pellet and, to subject the latter to digestion and extraction using the specified kit. This step is pivotal to extract high molecular weight proteins such as transmembrane proteins, which are otherwise difficult to homogenize using mild detergents[19-21]. Precipitated pellet is best dissolved using sonication to avoid sample loss incurred by splash-up introduced during vigorous agitation or shaking methods.

Third, it is noteworthy that all sample preparation procedures are carried out at low temperature (4 °C), unless otherwise indicated in the methodology. This is to ensure minimal protein denaturation during the extraction procedures. Fourth, repeated freeze-thawing of samples should be avoided to prevent protein degradation and deterioration of sample quality.

Finally, removal of contaminants and detergents is necessary following protein extraction to prevent downstream interference during in-gel fractionation, enzymatic digestion, and MS analysis[18,22]. These contaminants often interfere with the resolution of the electrophoretic separation and correspondingly, influence the visualization of the result, as shown in the 1DE profile (**Figure 7**). To circumvent this issue, the use of centrifugal cutoff filter devices is favored for their ease of use and minimal protein loss.

Although the current experimental procedures provide an in-depth outlook into the important sample preparation steps for optimal label-free quantitative MS analyses, there are two limitations. First, sPCA samples were pooled from two porcine eyes to provide sufficient amounts of tissues for subsequent analysis. Since the eyes obtained from the local abattoir are randomized and therefore, it is not known if the blood vessels are being isolated from the eyes of the same animal, sample pooling mitigates inter-individual variations[5,6]. However, the current methodology can also be adapted for individual sample preparation depending on the amount of samples available. Second, the presented methodology has been specifically developed for 1DE gel-based fractionation. Although the compatibility of the current method for integration with top-down and other fractionation methods warrant investigation, we opted for 1DE owing to several factors ranging from good reproducibility, ease of quality control to better depth of analyses, especially for complex samples such as the currently exemplified ocular blood vessels[1,5,23].

In conclusion, despite the limitations highlighted above, the described workflow represents a simple yet robust approach to stringent sample preparation steps catered specifically for analysis of small amount of blood vessels. It is also important to highlight here that this method can be readily integrated for mass spectrometry-based proteomic analysis of other cell- and tissue-based samples.

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DISCLOSURES:

The authors have nothing to disclose.

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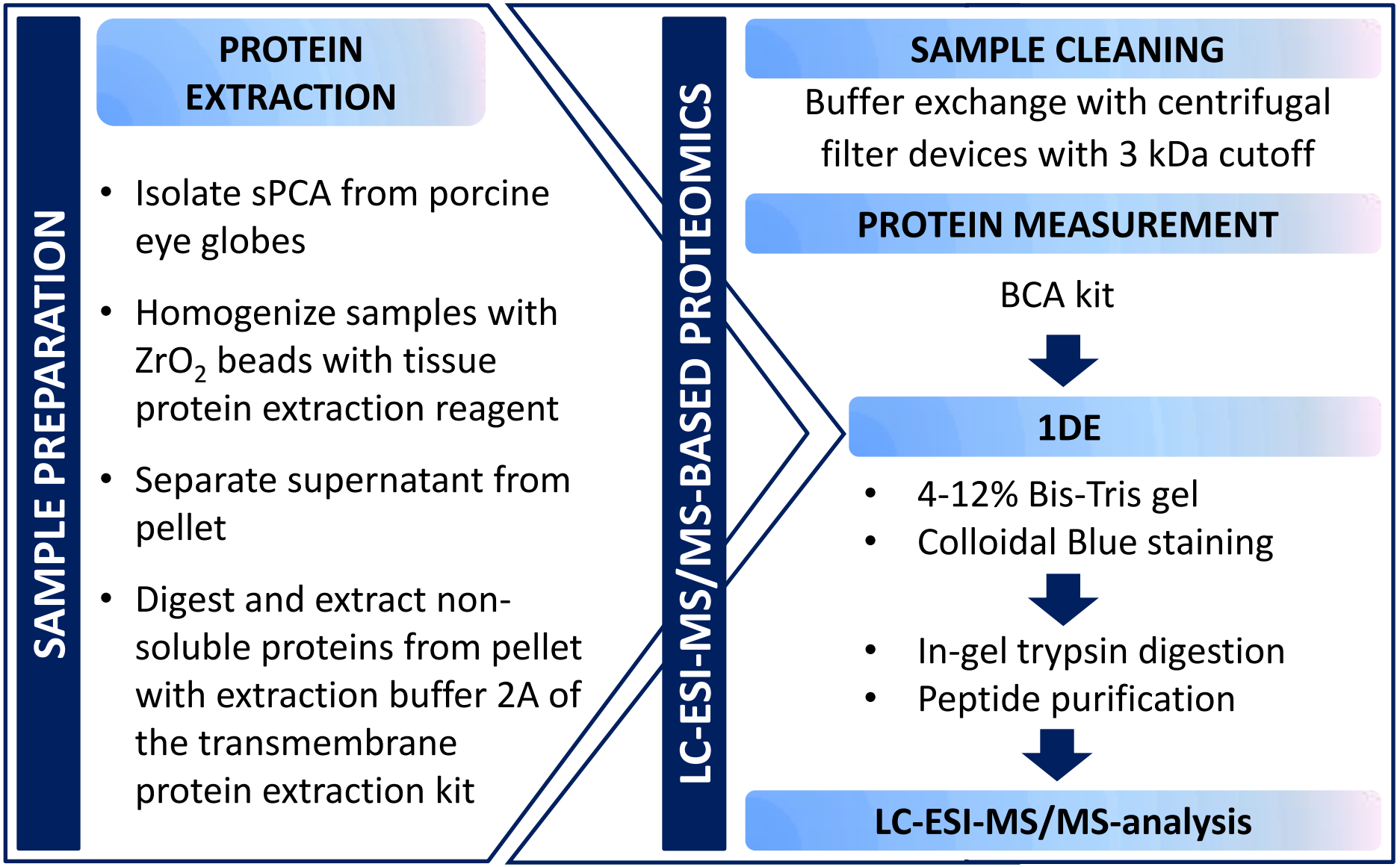

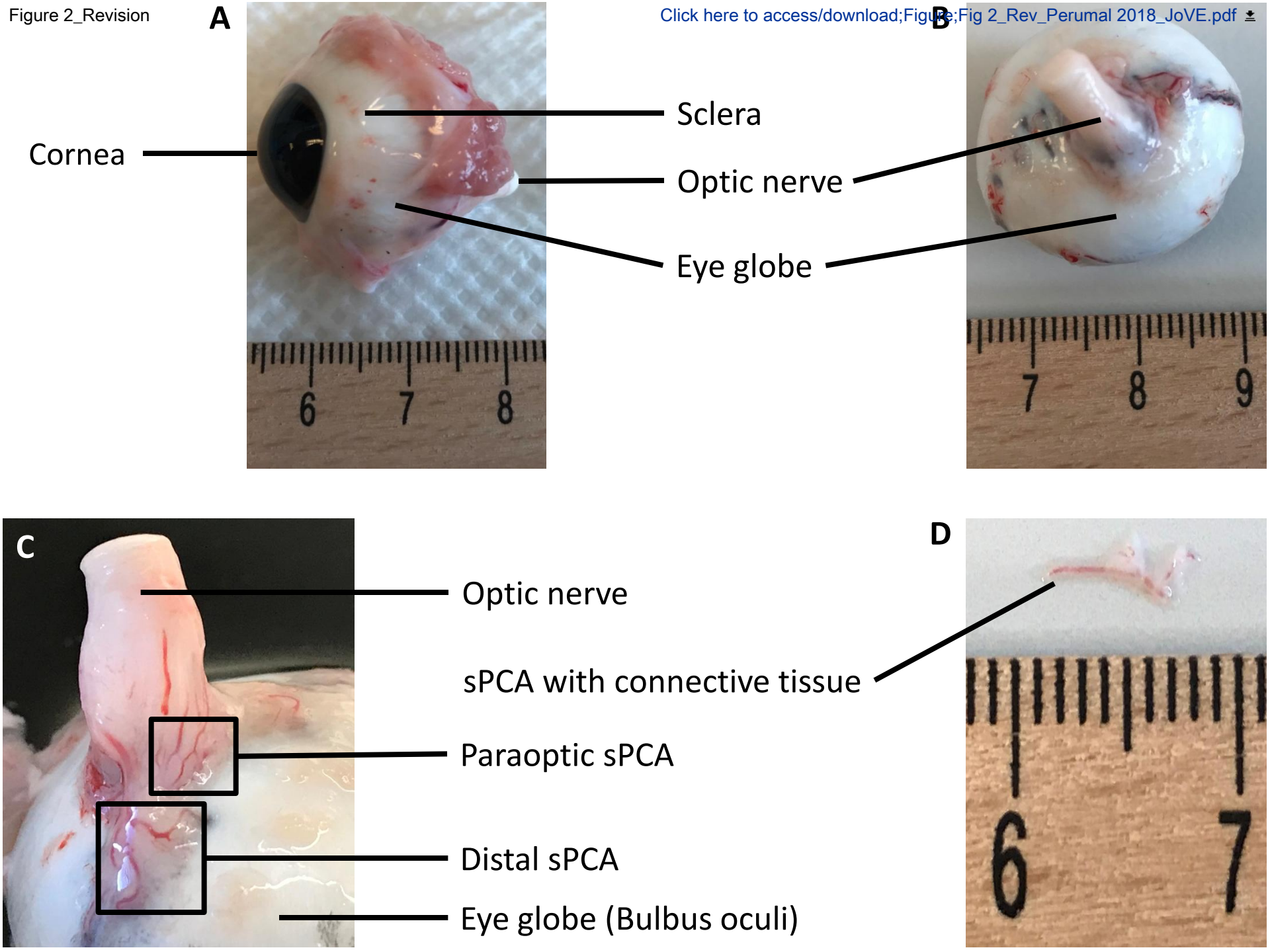

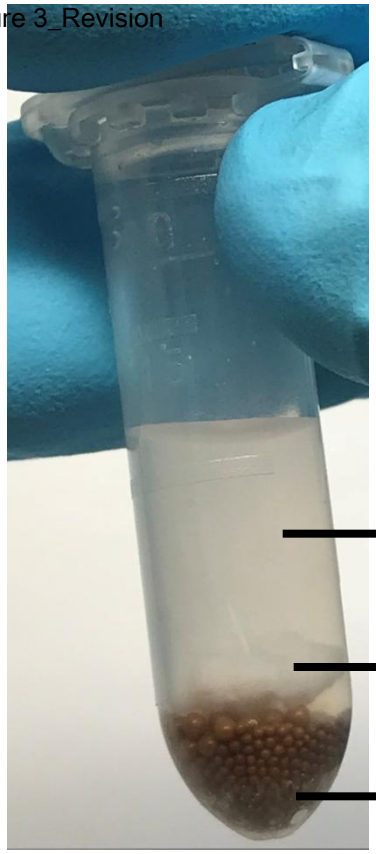

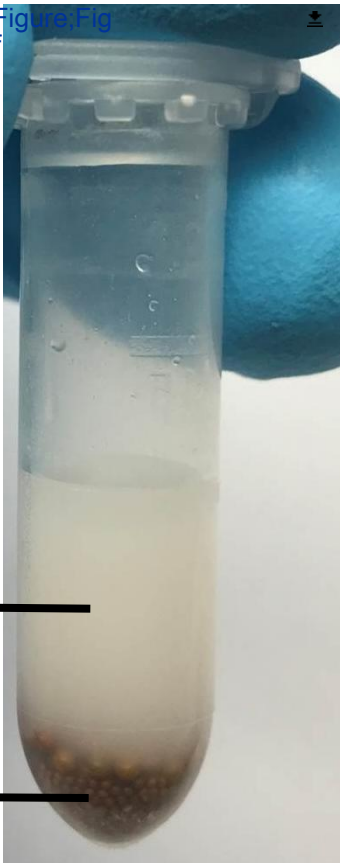

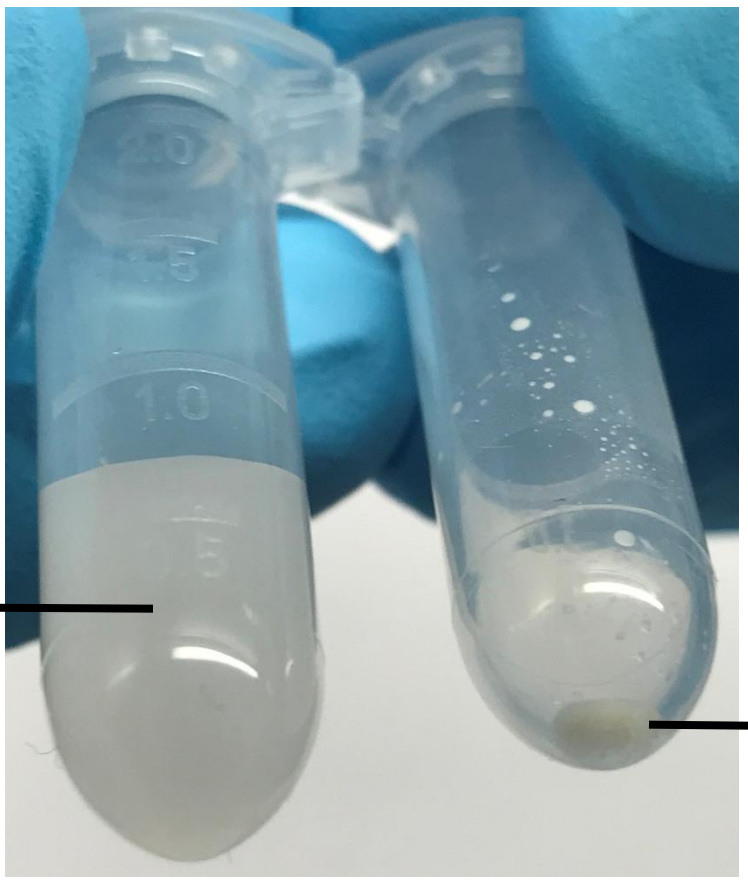

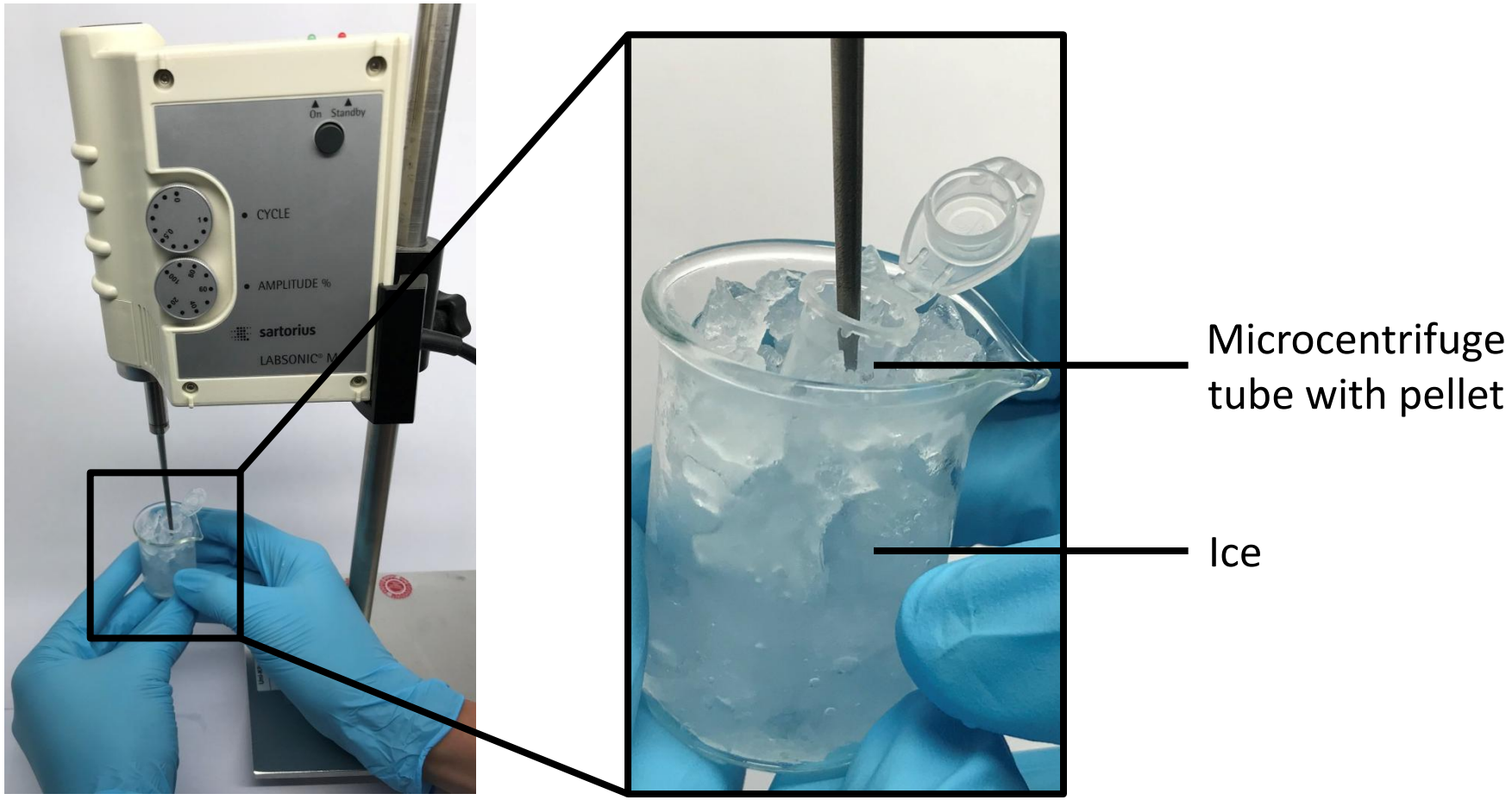

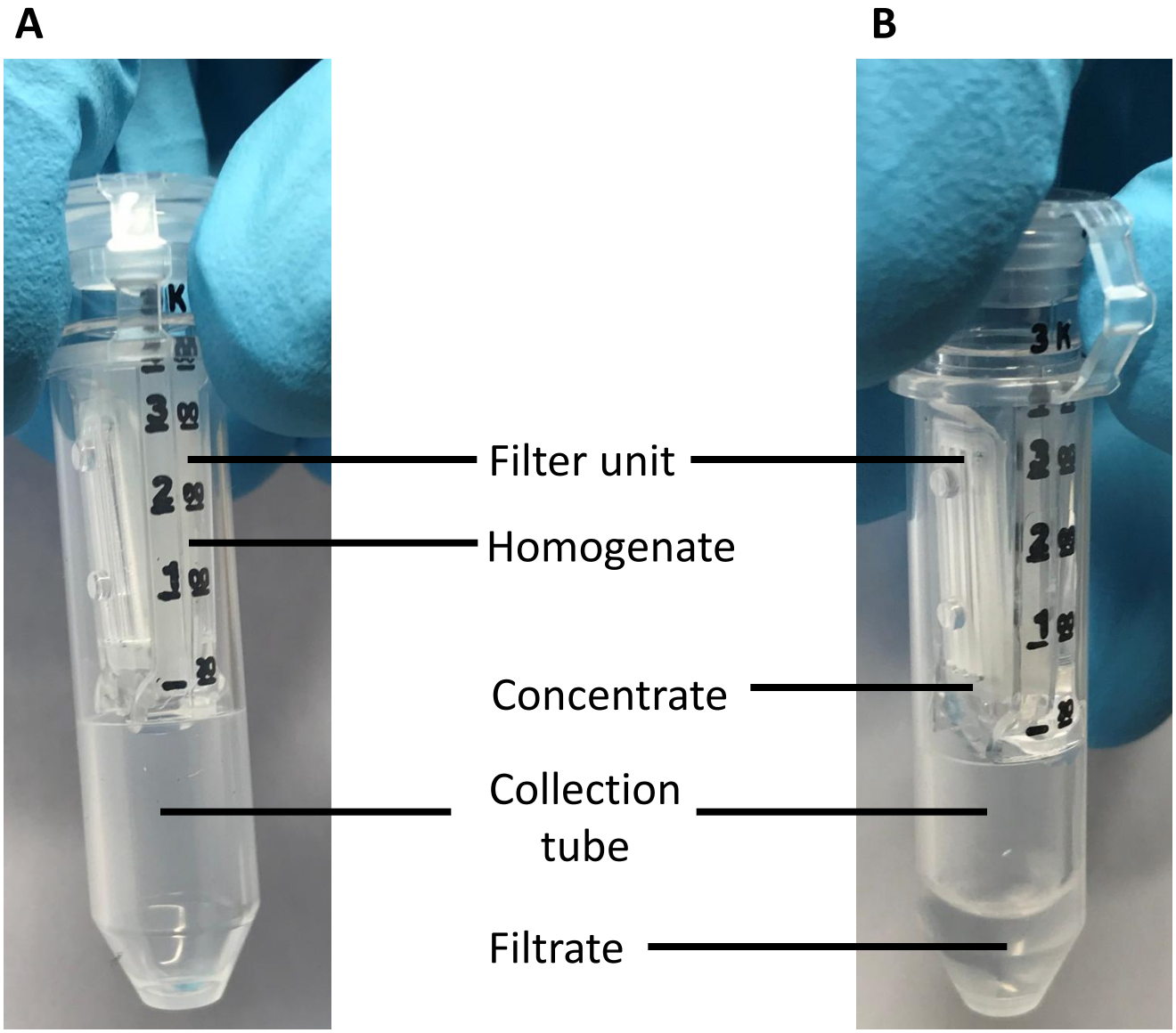

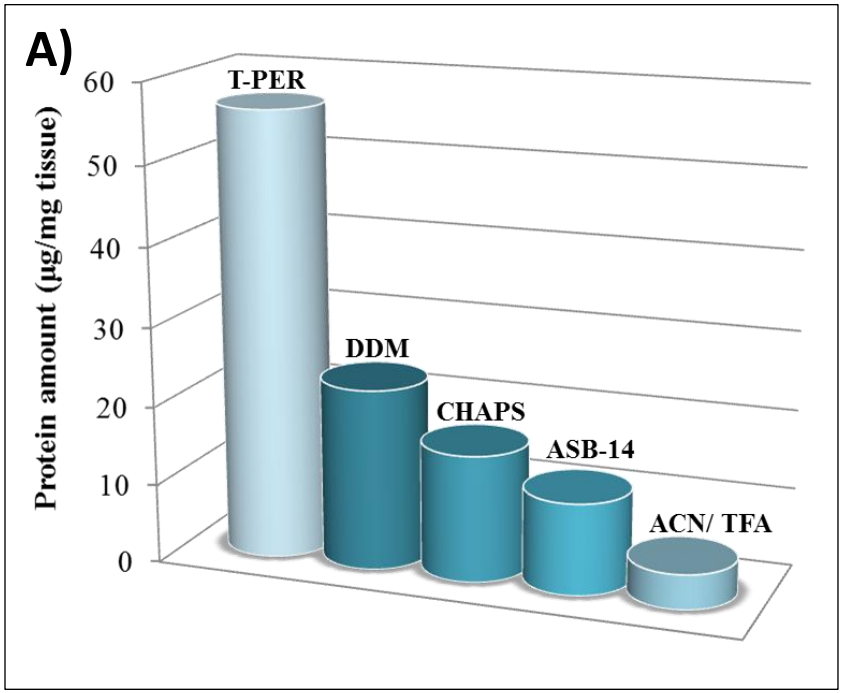

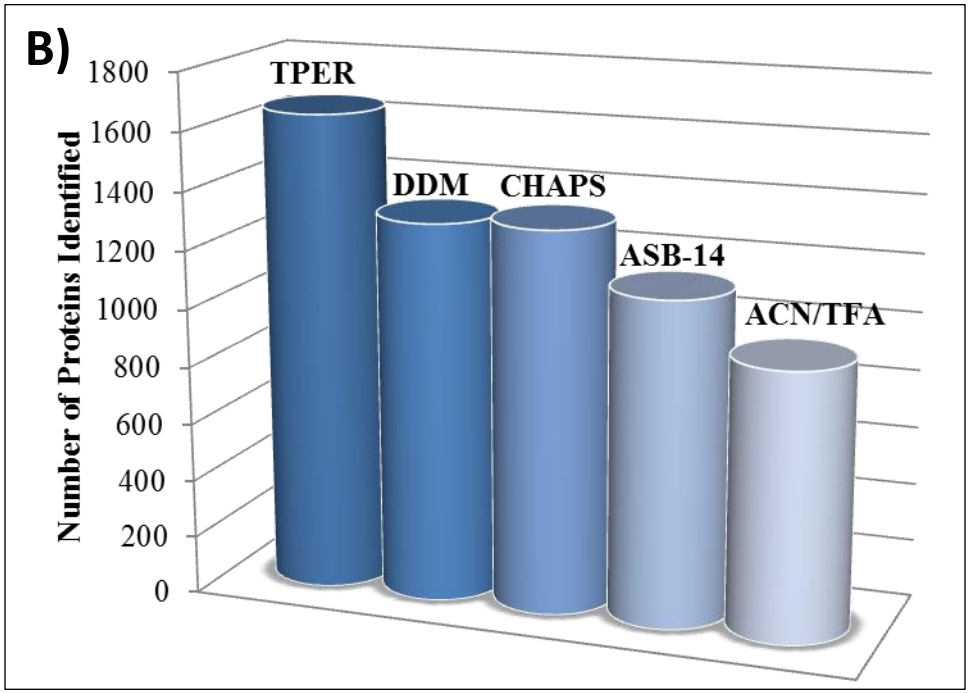

Figure 7_Revision

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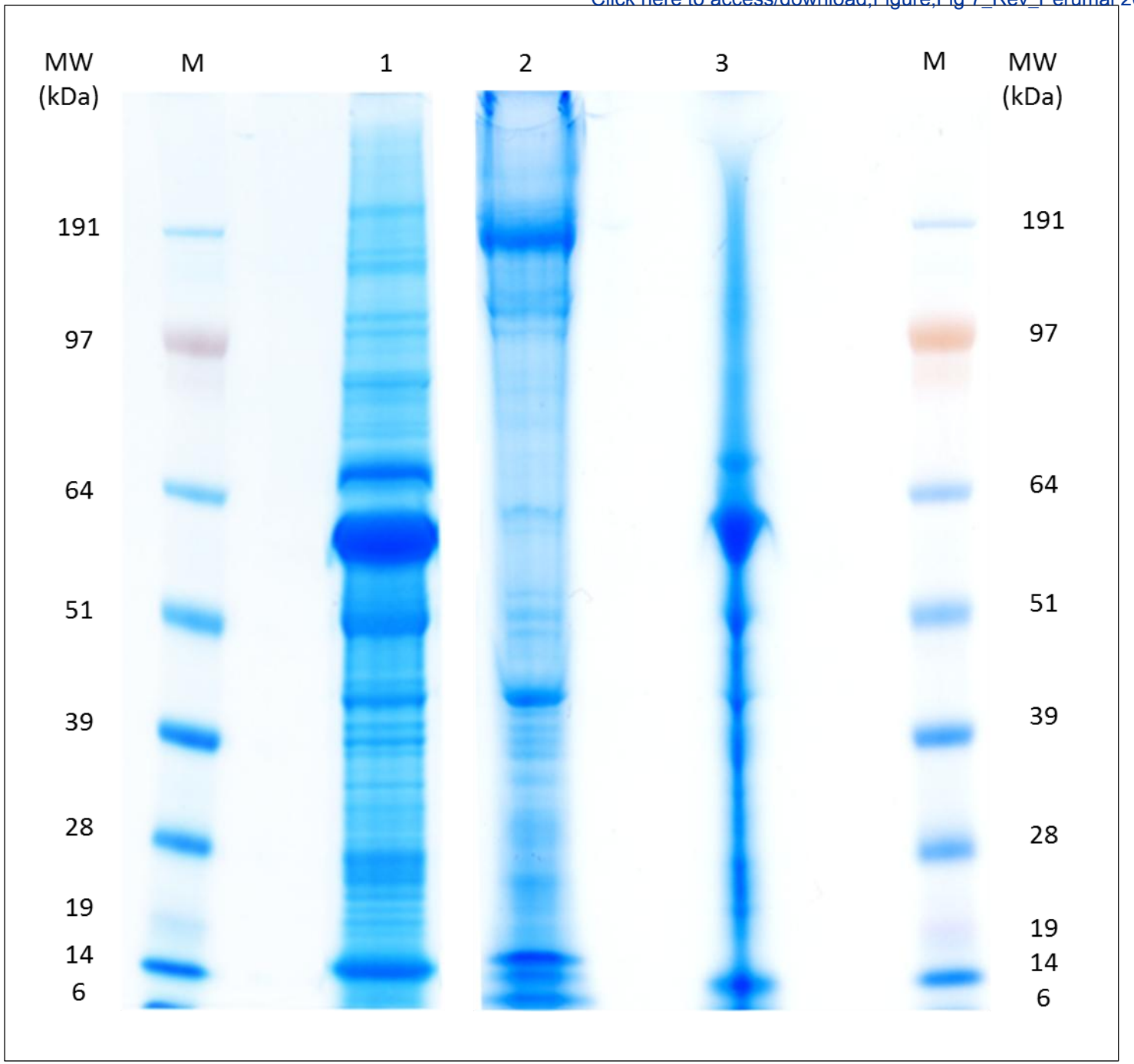

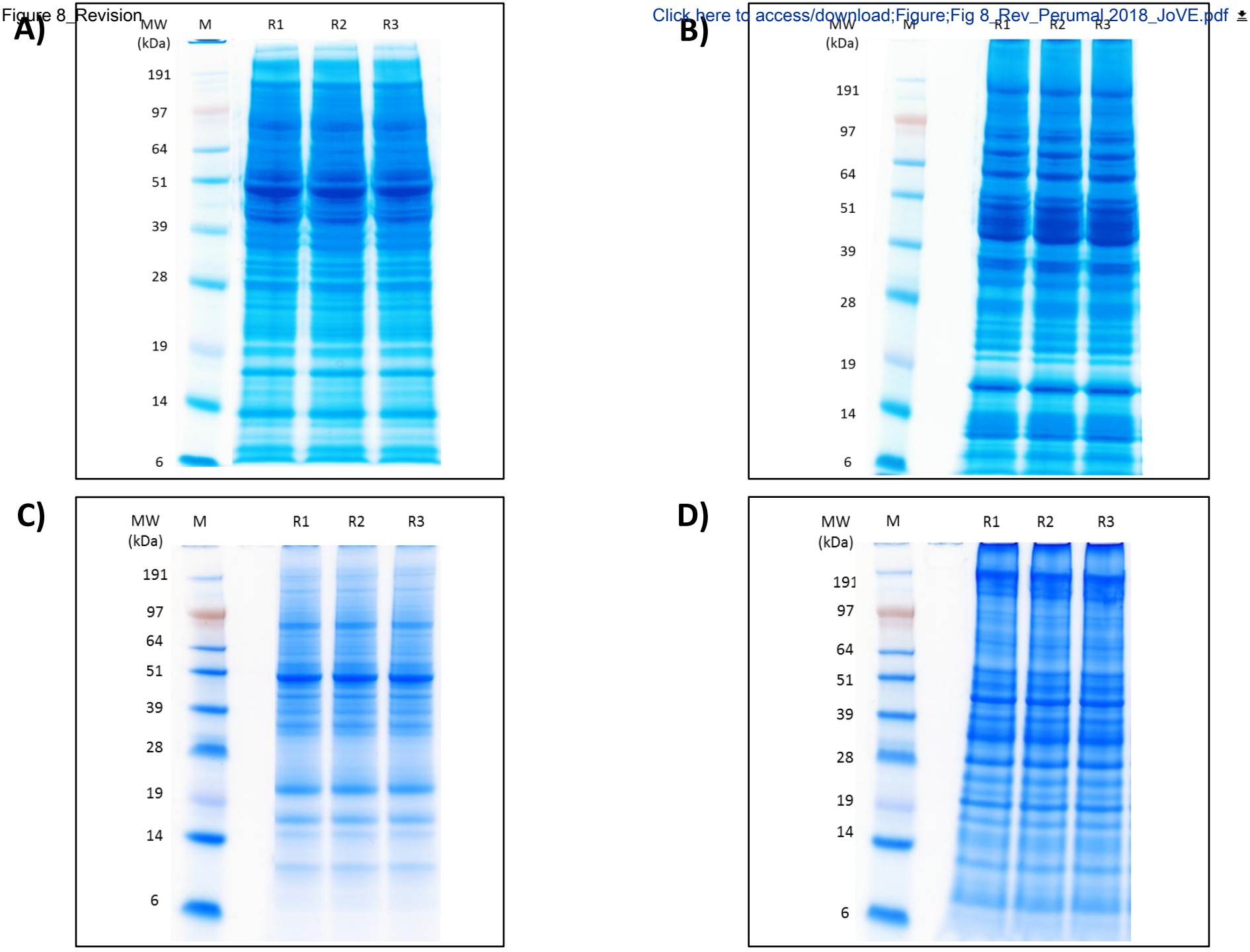

Table 1

Component	Volume (µL)
Sample (supernatant or pellet)	x
LDS Sample Buffer (4x)	2.5
Reducing agent (10x)	1
Deionized water	Up to 6.5 (depending on sample volume)
Total volume per sample	**10**

Note: x is calculated based on the protein concentration (50 µg total protein per sample).

Table 2

Solution	For 1 gel (mL)	For 2 gels (mL)	For 4 gels (mL)
Deionized water	40	80	160
Methanol	50	100	200
Acetic acid	10	20	40

Table 3

Solution	For 1 gel (mL)	For 2 gels (mL)	For 4 gels (mL)
*Deionized water	55	110	220
*Methanol	20	40	80
*Stainer A	20	40	80
Stainer B	5	10	20

Table 4

Solution (for)	Composition	ACN**	H2O**	TFA	Total volume*
Wetting	100% ACN	2 mL			2 mL
#Washing and equilibration	0.1% TFA		10 mL	10 µL	~ 10 mL
Peptide elution	0.1% TFA in 60:40= ACN: H2O	6 mL	4 mL	10 µL	~ 10 mL

Note: * The total volume should be adjusted according to the total number of samples to be subjected to Zip Tip cleaning.

** Use HPLC-grade or LC-MS-grade.

Prepare two separate Eppendorf tubes for washing and equilibration, respectively.

	Name of Material/ Equipment	Company	Catalog Number	Comments/Description
	A. Chemicals			
1	1, 4-Dithiothreitol (DTT)	Sigma-Aldrich	1.11474	
2	Ammonium bicarbonate (ABC, CH_5NO_3)	Sigma-Aldrich	5.33005	
3	Calcium chloride dihydrate ($CaCl_2$)	Carl Roth	5239.1	2.5 mM
4	Dulbecco's phosphate-buffered saline (PBS)	Thermo Fisher Scientific	14190169	
5	Formic acid (CH_2O_2)	AppliChem	A0748	
6	HPLC-grade acetonitrile (ACN, C_2H_3N)	AppliChem	A1605	
7	HPLC-grade methanol (CH_3OH)	Fisher Scientific	M/4056/17	
8	HPLC-grade water	AppliChem	A1589	
9	Iodoacetamide (IAA)	Sigma-Aldrich	I6125	
10	Kalium chloride (KCl)	Carl Roth	6781.1	4.7 mM
11	Kalium dihydrogen phosphate (KH_2PO_4)	Carl Roth	3904.2	1.2 mM
12	LC-MS-grade acetic acid	Carl Roth	AE69.1	
13	Magnesium sulphate ($MgSO_4$)	Carl Roth	261.2	1.2 mM
14	NuPAGE Antioxidant	Thermo Fisher Scientific (Invitrogen)	NP0005	
15	NuPAGE LDS Sample buffer	Thermo Fisher Scientific (Invitrogen)	NP0007	4x
16	NuPAGE MES SDS Running Buffer	Thermo Fisher Scientific (Invitrogen)	NP0002	20x
17	NuPAGE Sample reducing agent	Thermo Fisher Scientific (Invitrogen)	NP0004	10x
18	SeeBlue Plus2 pre-stained protein standard	Thermo Fisher Scientific (Invitrogen)	LC5925	
19	Sequencing grade modified trypsin	Promega	V5111	
20	Sodium chloride (NaCl)	Carl Roth	9265.2	118.3 mM
21	Sodium hydrogen carbonate ($NaHCO_3$)	Carl Roth	965.3	25 mM
22	Trifluoroacetic acid (TFA, $C_2HF_3O_2$)	Merck Millipore	108178	
23	α-(D)-(+)- Glucose monohydrate	Carl Roth	6780.1	11 mM
	B. Reagents and Kits			
1	0.5mm zirconium oxide beads	Next Advance	ZROB05	
2	1.0mm zirconium oxide beads	Next Advance	ZROB10	
3	Colloidal Blue Staining Kit	Thermo Fisher Scientific (Invitrogen)	LC6025	To stain 25 mini gels per kit
4	NuPAGE 4-12 % Bis-Tri gels	Thermo Fisher Scientific (Invitrogen)	NP0321BOX	1.0 mm, 10-well
5	Pierce Bicinchoninic Acid (BCA) Protein Assay Kit	Thermo Fisher Scientific	23227	
6	ProteoExtract Transmembrane Protein Extraction Kit, TM-PEK	Merck Millipore	71772-3	20 reactions per kit
7	Tissue Protein Extraction Reagent (T-PER)	Thermo Scientific	78510	

	Name of Material/ Equipment	Company	Catalog Number	Comments/Description
	C. Tools			
1	96-well V-bottom plates	Greiner Bio-One	651180	
2	Corning 96-well flat-bottom plates	Sigma-Aldrich	CLS3595-50EA	
3	Disposable microtome blades	pfm Medical	207500014	
4	Disposable scalpels #21	pfm Medical	200130021	
5	Dissection pins	Carl Roth	PK47.1	
6	Extra Fine Bonn Scissors	Fine Science Tools	14084-08	
7	Falcon conical centrifuge tubes (50 mL)	Fisher Scientific	14-432-22	
8	Mayo scissors, Tough cut	Fine Science Tools	14130-17	
9	Precision tweezers	Fine Science Tools	11251-10	Type 5
10	Precision tweezers, straight with extra fine tips	Carl Roth	LH53.1	Type 5
11	Self-adhesive sealing films for microplates	Ratiolab (vWR)	RATI6018412	
12	Standard pattern forceps	Fine Science Tools	11000-12	
13	Student Vannas spring scissors	Fine Science Tools	91501-09	
14	Vannas capsulotomy scissors	Geuder	19760	Straight, 77 mm
15	ZipTip$_{C18}$ pipette tips	Merck Millipore	ZTC18S096	
	D. Equipment and devices			
1	150 × 0.5 mm BioBasic C18 column	Thermo Scientific, Rockford, USA	72105-150565	
2	30 × 0.5 mm BioBasic C18 pre-column	Thermo Scientific, Rockford, USA	72105-030515	
3	Amicon Ultra-0.5 3K Centrifugal Filter Devices	Merck Millipore	UFC500396	Pack of 96.
4	Analytical balance	Sartorius	H51	
5	Autosampler	CTC Analytics AG, Zwingen, Switzerland	HTS Pal	
6	BBY24M Bullet Blender Storm	Next Advance	NA-BB-25	
7	Eppendorf concentrator, model 5301	Sigma-Aldrich	Z368172	
8	Eppendorf microcentrifuge, model 5424	Fisher Scientific	05-403-93	Non-refrigerated
9	Heraeus Primo R Centrifuge	Thermo Scientific	75005440	Refrigerated
10	Labsonic M Ultrasonic homogenizer	Sartorius	BBI-8535027	
11	LC-MS pump, model Rheos Allegro	Thermo Scientific, Rockford, USA	22080	
12	LTQ Orbitrap XL mass spectrometer	Thermo Scientific, Bremen, Germany		
13	Multiskan Ascent plate reader	Thermo Labsystems	v2.6	
14	Rotator with vortex	neoLab	7-0045	
15	Titanium probe (Ø 0.5mm, 80mm long)	Sartorius	BBI-8535612	
16	Ultrasonic bath, type RK 31	Bandelin	329	
17	Xcell Surelock Mini Cell	Life Technologies	El0001	

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Author(s): NATARAJAN PERUMAL, LARS STRAßBURGER, CARSTEN SCHMELTER, ADRIAN GERICKE, NORBERT PFEIFFER, FRANZ H. GRUS, CAROLINE MANICAM

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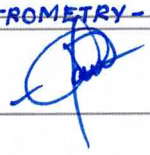
Article Title: A RAPID AND ROBUST SAMPLE PREPARATION METHOD FOR MASS-SPECTROMETRY-BASED PROTEOMICS ANALYSIS OF OCULAR MICROVESSELS

Signature: 

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Point-by-point response to reviewers' comments

A Rapid and Robust Sample Preparation Method for Mass-Spectrometry-Based Proteomics Analysis of Ocular Microvessels

Natarajan Perumal, Lars Straßburger, Carsten Schmelter, Adrian Gericke, Norbert Pfeiffer, Franz H. Grus, Caroline Manicam

Department of Ophthalmology, University Medical Centre of the Johannes Gutenberg University Mainz, Mainz, Germany.

The authors appreciate the remarks and comments of the reviewers to further improve the clarity and content of this manuscript. We have addressed the reviewers' comments point-by-point. Our manuscript and corresponding files (i.e. figures and tables) have also been revised accordingly.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1) Comment: *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
Response: The manuscript has been thoroughly proofread to ensure that the article is grammatically sound.

2) Comment: *Please revise lines 497-501 to avoid previously published text.*
Response: The highlighted lines have been revised accordingly.

3) Comment: *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ProteoExtract, Eppendorf, Bullet Blender, Amicon Ultra, Multiskan Ascent, NuPAGE, ZipTip, Thermo Scientific, CTC Analytics AG, etc.*
Response: All commercial terms have been removed and replaced with generic names, as suggested.

4) Comment: *Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*
Response: The protocol has been revised to contain imperative sentences, as required.

5) Comment: *1.2 and 1.4: The Protocol should contain only action items that direct the reader to do something. Please write the text in the imperative tense in complete sentences.*
Response: Points 1.2 and 1.4 have been revised to complete imperative sentences, as required.

6) Comment: *3.6: Please specify the ratio of 0.5 mm and 1.0 mm zirconium oxide beads.*
Response: The ratio of the zirconium oxide bead mixture used has been specified in point 3.5 as the volume ratio relative to the sample and tissue protein extraction reagent (ratio of 1:1:2 = sample: beads: T-PER).

7) Comment: *Line 199: Is this a volume ratio? Please specify.*
Response: The volume ratio of the 0.5 mm and 1.0 mm zirconium oxide beads has been specified (please refer to comment 6).

8) Comment: *Lines 298-299, 344-345, 352-353, 437-438: Please include them as tables and upload them to your Editorial Manager account as an .xls or .xlsx file.*
Response: The highlighted texts are included in table format as excel files, as requested.

9) Comment: *Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.*
Response: Shorter protocol steps have been combined, as suggested.

10) Comment: *Please include single-line spaces between all paragraphs, headings, steps, etc.*
Response: The manuscript is formatted to single-line spacing, as required.

11) Comment: *After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*
Response: The important steps in the protocol are highlighted in yellow for video purposes, according to the requirements.

12) Comment: *Figure 1: Please remove commercial language from figure (Bullet Blender, ProteoExtract, Amicon, ZipTip, etc.) and use generic terms instead.*
Response: All commercial terms have been removed and replaced with generic names in Figure 1, as suggested.

13) Comment: *Please consider combining Figure 2 and Figure 3 to reduce the total number of figures.*
Response: Figure 2 and Figure 3 have been combined (now Figure 2), as suggested.

14) Comment: *Figures 4, 5, 8: These figures of commercial products are not necessary because readers can find out the product information from the Table of Materials. Please remove them.*
Response: Figures 4, 5 and 8 have been removed, as suggested.

15) Comment*: Figure 6 and Figure 7: Please combine these figures if possible.*
Response: Figure 6 and Figure 7 have been combined (now Figure 3), as suggested.

16) Comment: *Figure 9: Please remove commercial language from figure (Eppendorf).*
Response: The commercial name has been removed and replaced with the generic name in Figure 9 (now Figure 4), as suggested.

17) Comment: *Discussion: Please discuss any limitations of the technique.*
Response: Two major limitations of the present study are discussed in the Discussion section, as suggested (page 15 line 580-592).

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript describes preparation of micro vessels from ocular tissues for mass spectrometric proteomics.

1) Major Concerns:
Comment: *There is not enough cross validation of isolated micro vessels. A combination of other methods such as DiI mediated identification of micro vessels prior to isolation has not been considered. The question is "are you isolating what you think you are isolating?". This has not been addressed in the manuscript. However, it still is a useful method for many researchers. Despite this concern manuscript still has utility in its current form and should be acceptable.*
Response: Thank you for highlighting this interesting point pertaining to vessel painting employing the lipophilic carbocyanine dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) method. In this study, we did not discuss in-depth about the anatomical aspects as well as the isolation procedures of the short posterior ciliary arteries (sPCA) owing to several factors. First, the description of the sPCA branches chosen for isolation has been provided in detail in our previous study (Manicam *et al*., 2016 DOI: 10.1038/srep38298), which is the basis of the current protocol and this has been highlighted in the Introduction section of the manuscript (page 3, line 87-92). The main focus of this article is the optimization steps for a rapid, robust and highly efficient protein extraction method from mass-limited samples and as such, the sPCA were used as exemplary ocular microvessels, as reiterated in the Discussion section (page 14 line 550-552). Second, although we did not elaborate on the verification steps of the identification of sPCA in the present study, this vascular bed was identified and isolated based on many other valid retrospective studies, which have provided excellent diagrammatic representation of the vascular bed being isolated based on detailed experimental works (Hayreh *et al.*, 2004 DOI:10.1167/iovs.03–0469; Ramirez *et al.*, 2012 DOI: 10.5772/47794; Erdogmus and Govsa, 2006 DOI: 10.1111/j.1600-0420.2006.00673.x). Third, the DiI technique is usually performed by direct manual intracardiac injection of the DiI solution followed by perfusion of fixative in rodents (Salehi *et al*., 2018 DOI: 10.1007/s12975-018-0632-0; Konno *et al*., 2017 DOI:

10.1038/s41598-017-09496-4; Li *et al*., 2008 DOI: 10.1038/nprot.2008.172; Hughes *et al*., 2014 DOI 10.1007/978-1-4939-0320-7_12). In the present study, we obtained our study samples i.e. the porcine eyes from the local abattoir and therefore, the standard method is not applicable in the current scenario. Consequently, the method may have to be modified to ensure its functionality to stain the porcine retrobulbar ocular vasculature *ex vivo*. Although we are in agreement with the reviewer that a verification stage would be better to further strengthen the identity of the isolated blood vessels, this procedure is beyond the scope of the present study and would be a highly suitable topic for another independent study. Nevertheless, we appreciate this highly useful and important suggestion and, we believe that future studies using this method would be feasible to provide such information.

2) *Minor Concerns:*
Comment: *The page 37-38 comments in pdf has gotten out of the table. Authors should reformat the original table so that there is no spill over of the information beyond page 36.*
Response: The original table has been reformatted to ensure no spill over to the next page, as requested.

Reviewer #2:
Manuscript Summary:
Well presented and useful method for ocular proteomic investigation.

1) Minor Concerns:
Comment: *two earlier and very relevant articles addressing proteomic methodology require brief discussion and reference. In order of importance:*
1. Ocular proteomics with emphasis on two-dimensional gel electrophoresis and mass spectrometry. Mandal et al. Biol Proced Online. 2009 Dec 24;12(1):56-88.
2. Analytical platforms in vitreoretinal proteomics. Cehofski et al. Bioanalysis. 2014;6(22):3051-66.

Response: Thank you for highlighting these highly relevant and interesting articles. As suggested by the reviewer, we have included these two papers in the reference list (page 16 reference 1 and 5) and, discussed several important points emerging from them in the Introduction (page 3 line 65-71) and Discussion sections (page 14, line 546-550; page 15, 583-585; page 15, line 588-592).