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Preparation of Proteins from Whole Body Larval Zebrafish Tissue for Proteomics V.1

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Proteomics Protocols



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We use this protocol and it's working

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Abstract

This protocol describes the steps needed to prepare proteins from whole body larval zebrafish tissue for bottom-up shotgun proteomics via high resolution liquid chromatography mass spectrometry. Steps include solution preparation, sample collection, protein extraction, filter aided sample preparation, reduction, alkylation, enzymatic digestion via trypsin, and desalting steps using filter tips prepared in-house. This protocol results in a 1 μ g/ μ L solution of purified peptide samples.

Image Attribution

Protocol images attributed to Abigail N. Henke

Guidelines

This protocol is for the extraction, filter-aided sample preparation (FASP), reduction, alkylation, digestion, acidification, and desalting of proteins from whole body larval zebrafish (*Danio rerio*) tissue for bottom-up shotgun data-independent acquisition (DIA) proteomics.

Materials

sodium chloride (NaCl)
potassium chloride (KCl)
disodium phosphate (Na_2HPO_4)
potassium phosphate (KH_2PO_4)
tris-hydrochloride (Tris-HCl)
dithiothreitol (DTT, $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$)
sodium dodecyl sulfate (SDS, $\text{CH}_3(\text{CH}_3)_{11}\text{OSO}_3\text{Na}$)
Roche cOmplete protease inhibitor tablet
Pierce (TM) BCA Protein Assay kit (23227)
liquid nitrogen (1L)
ammonium bicarbonate (ABC, HCO_3)
urea (NH_2CONH_2)
iodoacetamide (IAA, $\text{C}_2\text{H}_4\text{INO}$)
methanol (CH_3OH)
sodium chloride (NaCl)
sodium deoxycholate (DOC, $\text{C}_{24}\text{H}_{39}\text{NaO}_4$)
Trifluoroacetic acid (TFA, $\text{CF}_3\text{CO}_2\text{H}$)
sequencing grade modified porcine trypsin (Promega, frozen, 5/20ug, cat#V5113)
HPLC-grade Methanol (CH_3OH)
HPLC-grade Acetonitrile (CH_3CN)
HPLC-grade Formic Acid (HCOOH)
Waters MassPREP™ Enolase digestion standard (SKU: 186002325)

1L, 100mL glass media bottles
1000mL, 500mL, 100mL, 10mL graduated cylinders
2mL, 1.5mL lo bind microcentrifuge tubes
15mL polypropylene falcon tubes
disposable plastic dropper pipettes
1000-, 200-, 100-, 20-, and 10 μL pipettes with appropriate tips
30kD Amicon Centrifugal filter units
ice bucket with wet ice
glass autosampler vials with re-sealing lids
Empore 2215 C-18 filters
18-gauge blundt tip needle
small screwdriver or awl (~.25cm diameter)

mass balance capable handling milligram scale measurements
pH probe and pH balancing solutions (standard 1M HCl and NaOH)
ultrapure water source

heat block
microcentrifuge
Scilogex (110-120V-60Hz, Speed = 8000-3000r/min, watts= 160W) homogenizer
probe sonicator
Thermomixer
Speed-Vap solvent evaporation apparatus or equivalent instrument
Nanodrop or peptide quantification kit
-80C freezer
ring stand with crimp attachment
dewar for liquid nitrogen use, transport, and storage
cold protection gloves
cold-resistant forceps
Kim wipes
waste beaker
tin foil
spray bottle of 70% ethanol
canned air (for office cleaning applications)
personal protective equipment

Safety warnings

- ❗ Follow institutional environmental health and safety guidelines while working with liquid nitrogen, sonication equipment, and trifluoroacetic acid.

Ethics statement

Humane animal handling techniques outlined by the AVMA allow for the euthanasia of larval Zebrafish on ice.

Follow all institutional animal handling and care guidelines.

Before start

Reserve any community equipment needed, perform equipment calibrations, and verify operational status of equipment prior to commencement of procedure. Pre-cool centrifuges and pre-heat heat blocks.

Read the full procedure and determine pause points before starting.

Solution Preparation For Protein Extraction Steps

1h 30m

1 Phosphate Buffered Saline (PBS, 1X, 1L)

30m

Obtain a clean 1L glass media bottle, 1000mL graduated cylinder, sodium chloride (NaCl), potassium chloride (KCl), disodium phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), a mass balance capable handling milligram scale measurements, a pH probe and pH balancing solutions (standard 1M HCl and NaOH), and an ultrapure water source.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 1L glass media bottle:

 8.0 g NaCl. Record actual mass.

 0.200 g KCl. Record actual mass.

 1.44 g Na_2HPO_4 . Record actual mass.

 0.245 g KH_2PO_4 . Record actual mass.

Record the model and identification of the balance used.

Record source and purity of ultrapure water. Measure  900 mL of ultrapure water using the graduated cylinder and add to the 1L bottle. Mix to dissolve.

Measure the pH of the solution with the pH probe and adjust to  7.4 \pm 0.1 with 1M HCl or 1M NaOH. Record pH. Record model and identification of the pH probe used.

Bring solution up to  1000 mL using a graduated cylinder. Return solution to 1L media bottle.

Label: Phosphate buffered saline, [Initials], date of preparation, date of expiration (2 years from preparation date at ambient room temperature), storage location, and storage temperature.

2 Tris-HCl Stock, (0.1M, pH 7.8, 50mL)

20m

Obtain a clean 100mL glass media bottle, 100mL graduated cylinder, tris-hydrochloride (Tris-HCl) a mass balance capable handling milligram scale measurements, a pH probe and pH balancing solutions (standard 1M HCl and NaOH), and an ultrapure water source.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 1L glass media bottle:

⚠ 0.788 g of Tris-HCl. Record actual mass.

Record the model and identification of the balance used.

Record source and purity of ultrapure water. Measure ⚗ 40 mL of ultrapure water using the graduated cylinder and add to the 100mL bottle. Mix to dissolve.

Measure the pH of the solution with the pH probe and adjust to ⌂ pH 7.8 ± 0.1 with 1M HCl or 1M NaOH. Record pH. Record model and identification of the pH probe used.

Bring solution up to ⚗ 50 mL using a graduated cylinder. Return solution to 100mL media bottle.

Label: Tris-HCl 0.1M Stock, pH:[actual], [Initials], date of preparation, date of expiration (6mo from preparation date at ambient room temperature), storage location, and storage temperature.

3 Dithiothreitol (DTT) Stock, (1M, 10 mL)

20m

Obtain a clean 15mL polypropylene falcon tube, 10mL graduated cylinder, dithiothreitol (DTT, C₄H₁₀O₂S₂), a mass balance capable of handling milligram scale measurements, an ultrapure water source, 16 1.5mL microcentrifuge tubes, 1000µL pipette and appropriate tips.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

⚠ 1.5425 g of DTT. Record actual mass.

Record the model and identification of the balance used.

Record source and purity of ultrapure water. Measure ⚗ 10 mL of ultrapure water using the graduated cylinder and add to the tube. Cap and mix to dissolve.

Open the 1.5mL microcentrifuge tubes and aliquot ⚗ 600 µL of DTT into each. Cap.

Label and store in the dark at -80C.

Note

DTT is light sensitive and will degrade. Thaw individual aliquots of DTT by wrapping in tinfoil and allowing to come to ambient room temperature prior to preparation of Lysis Buffer.

Label: Dithiothreitol (DTT) Stock, 1M, [Initials], date of preparation, date of expiration (3mo from preparation date at -80C), storage location, and storage temperature.

4 **Lysis Buffer (0.05M Tris-HCl, 0.05M DTT, Protease Inhibitors, 10mL, enough to prepare 24 samples)**

15m

Obtain a clean 15mL polypropylene falcon tube, 10mL graduated cylinder, **Tris-HCl Stock**, Dithiothreitol (DTT) Stock aliquot, Roche cOmplete Protease Inhibitor Tablet (04693116001), an ultrapure water source, and 1000 μ L pipette and appropriate tips.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 5 mL of 0.1M Tris-HCl stock reagent

 500 μ L of DTT Stock, thawed in the dark

 1 Tablet of Roche cOmplete Protease Inhibitor.

Record source and purity of ultrapure water. Measure  10 mL of ultrapure water using the graduated cylinder and add to the tube. Cap and mix vigorously with a vortex to dissolve. Place solution  On ice, do not store past 24hrs.

Label: Lysis Buffer Working Solution, [Initials], date of preparation, date of expiration (24hr from preparation date).

5 **Sodium Dodecyl Sulfate (SDS) Stock, (10%, 10mL)**

10m



Obtain a clean 15mL polypropylene falcon tube, sodium dodecyl sulfate (SDS, CH₃(CH₃)₁₁OSO₃Na), a mass balance capable handling milligram scale measurements 10mL graduated cylinder, and an ultrapure water source.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 1.0 g of SDS reagent. Record actual mass.

Record the model and identification of the balance used.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 1.0 g of SDS reagent. Record actual mass.

Record the model and identification of the balance used.

Record source and purity of ultrapure water. Bring SDS up to  10 mL with ultrapure water using the graduated cylinder and add to the tube. Cap and mix gently with a vortex to dissolve, but avoid frothing. Store solution at  Room temperature .

Note

Dissolution may be improved by gently heating the mixture below its boiling point using a heat block until dissolved. Following heating, allow to return to ambient room temperature. Do not place on ice as SDS will salt out of solution.

Label: SDS Stock, [Initials], date of preparation, date of expiration (6mo from preparation date at ambient room temperature), storage location, and storage temperature.

Tissue Collection

1h 30m

- 6 Obtain 2mL low protein binding (lo bind) microcentrifuge tubes, an ice bucket with crushed ice, 200 μ L pipette and appropriate tips, plastic dropper pipettes, a dewar of liquid nitrogen, and liquid nitrogen personal protective equipment (protective cold gloves, eye protection, lab coat, forceps).

Safety information

Liquid nitrogen can be dangerous! Always follow recommended institutional environmental health and safety practices and utilize personal protective equipment. Work in a well ventilated environment.

Note

While samples will fit in a smaller volume tube, 2mL lo bind tubes are recommended due to the diameter of homogenizer probes utilized in the extraction procedure and the volume of solution used.

Place the container of larval fish into the ice bucket for  00:05:00 minutes to slow fish swimming speeds.

Note

This procedure is for the collection of 25 pooled larval (3-45 days post fertilization) zebrafish per sample. Animal alternative studies examining larval fish tissue via proteomics suggest as few as 5 pooled larval zebrafish may be sufficient for proteomic analysis. However, in this case 25 pooled larvae were selected as to have excess tissue available for repeating subsequent preparation steps or re-running samples via mass spectrometry as needed.

Humane animal handling techniques outlined by the AVMA allow for the euthanasia of larval and adult Zebrafish on ice.

Citation

Langan LM, Brooks BW (2022)

. Exploratory analysis of the application of animal reduction approaches in proteomics: How much is enough?.

<https://doi.org/10.14573/altex.2107212>

LINK

Citation

Wallace CK, Bright LA, Marx JO, Andersen RP, Mullins MC, Carty AJ (2018)

. Effectiveness of Rapid Cooling as a Method of Euthanasia for Young Zebrafish (*Danio rerio*)..

<https://doi.org/>

LINK

- 7 Count and individually collect larval fish with a disposable dropper pipette, placing fish in the 1.5mL lo-bind tube. Cap tube and place on ice until all fish gravitationally pellet at the bottom. Remove as much water as possible with the dropper pipette.
- 8 Depress 200 μ L pipette to the first stop. Place the tip at the bottom of the fish pellet and slowly release plunger to remove all of the water without disturbing the fish. Cap tube and label.

10m

- 9 Gently lower the tube into the liquid nitrogen with forceps to snap freeze the sample. Repeat steps 7 and 8 until all samples are collected.
- 10 Remove sample collection tubes from the liquid nitrogen using forceps. Place in a sample box and store samples between  -20 °C and  -80 °C until extraction. 

Protein Extraction

5h 30m

- 11 Obtain wet ice, 1000-, 200-, 20-, and 10 μ L pipettes with appropriate tips, a mass balance capable of making milligram scale measurements, a heat block, ultrapure water source, benchtop centrifuge capable of accommodating at least 24 samples, prepared **Lysis Buffer**, prepared **SDS Stock**, 1 spare 2mL lo bind microcentrifuge tube, 1.5mL lo bind microcentrifuge tubes, a Scilogex (110-120V-60Hz, Speed = 8000-3000r/min, watts= 160W) homogenizer, a probe sonicator, ring stand with crimp attachment, Kimwipes, a waste beaker, and a spray bottle of 70% ethanol.

Record equipment identification data, reagent preparation dates, and reagent expiration dates.

Cool benchtop centrifuge down to  4 °C prior to use.

Turn on heat block to  90 °C. Record actual temperature.

- 12 Remove collected tissue samples from storage and thaw on wet ice. This procedure can accommodate 24 samples / day. 

Place lysis buffer on ice.

- 13 Wash thawed tissue samples with  500 μ L 1X PBS. Centrifuge samples for 10 sec at 4C using the "quick spin" function.
Gently pipette off supernatant without disturbing the tissue pellet or losing tissue. It is not critical to remove all buffer at this step.

- 14 Repeat wash step. Pipette off as much supernatant as possible without disturbing the pellet. Return samples to ice. 

- 15 Tare balance with an empty 2mL tube from the same lot used to collect samples. Measure and record the approximate mass of protein samples in each tube. 

16 Perform a 1:20 dilution of tissue with Lysis Buffer.

15m

Volume of Lysis Buffer to add (uL) = (wet weight of tissue (mg) × 20)

If samples + Lysis Buffer are below the 200uL marking on the 2mL tube, add lysis buffer to 200uL final volume.

Record volume added to each sample tube.

17 Clean Silogex homogenizer with 70% ethanol and assemble.

45m

Place homogenizer probe 1-2mm from the bottom of the sample tube.

Homogenize samples with a homogenizer on level 4 speed for 30 seconds moving the probe up and down slowly to thoroughly homogenize sample. Promptly return each sample to ice.

Rinse probe in ultrapure water, spray with 70% ethanol, and wipe with a Kim wipe.

Repeat for all samples.

Clean homogenizer by disassembling and cleaning parts in 70% ethanol. Allow to dry and reassemble.

18 Add 10% SDS stock to each tube until it comprises 2%.

10m

Volume of SDS Stock to add (uL) = (Volume of lysis buffer added (uL) × 2) ÷ 10

Do not place samples on ice as SDS will precipitate out of solution.

19 Place samples in a  heat block for  with weighted top to prevent samples from popping their lids.

5m

20 Clean sonicator instrument and secure microtip probe. Program sonicator to operate in 15 second bursts with 15 seconds of delay for a total of 1 minute sonication at 60hz.

1h

Set up ring stand and crimp holder capable of securely holding sample tubes inside the sonication cabinet.

Safety information

Follow institutional environmental health and safety recommendations for working with sonic equipment. Hearing protection is recommended.

Secure a sample tube in the crimp holder. Lower the sonicator probe 1-2mm below the surface of the sample without touching the tube itself. Close door and run sonication program.

Wipe probe with 70% ethanol and a Kim wipe between samples.

Repeat for all samples.

- 21 Return samples to a  90 °C heat block for  00:05:00, then cool for  00:15:00 at ambient room temperature. 20m
- 22 Centrifuge samples at  14000 rpm, 4°C, 00:45:00. 45m
- 23 Remove samples from centrifuge and return to ice without disturbing the dark pellet (unlysed cells, insoluble protein, pigments, cell membranes, waste). 15m

Pipette off supernatant (no portion of dark pellet) and place in a labeled 1.5mL lo-bind tube. This is the extracted protein and should be kept on ice while in use and stored at  -80 °C following BCA protein quantitation.
- 24 (Optional steps 24-27 to increase total protein yield)
Resuspend the un-lysed pellet in each tube with  96 µL Lysis Buffer and  24 µL of 10% SDS stock. Vortex well to resuspend. 10m

- 25 Return samples to a  90 °C heat block for  00:05:00, then cool for  00:15:00 at ambient room temperature. 20m
- 26 Remove samples from heat block (turn off heat block) and place in centrifuge at  14000 rpm, 4°C, 00:15:00. 15m
- 27 Pipette off supernatant and add to the labeled protein tube without disturbing or sucking up remaining dark pellet.
Discard pellet and raw tissue storage tube. 15m


Immediately store extracted protein samples at  -80 °C or proceed with BCA quantitation.

Solution Preparation for Filter-Aided Sample Preparation, Reduction, Alkylation, Digestion, and Acidification

28 ABC Buffer (50mM Ammonium Bicarbonate, 100mL)

10m

Obtain a clean 100mL glass media bottle, 500mL graduated cylinder, ammonium bicarbonate (ABC, HCO_3), a mass balance capable handling milligram scale measurements, a pH probe and pH balancing solutions (standard 1M HCl and NaOH), and an ultrapure water source.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 100mL media bottle

 1.9764 g of ABC. Record actual mass.

Record the model and identification of the balance used.

Record source and purity of ultrapure water. Measure  400 mL of ultrapure water using the graduated cylinder and add to the tube. Cap and mix to dissolve.

Measure the pH of the solution with the pH probe and adjust to  8.5 ± 0.1 with 1M HCl or 1M NaOH. Record actual pH. Record model and identification of the pH probe used.

Bring solution up to 500 mL with ultrapure water using the graduated cylinder. Return to media bottle.

Label: ABC Buffer 50mM, [Initials], date of preparation, date of expiration (2yr from preparation date at ambient room temperature), storage location, and storage temperature.

29 Urea (UA) Buffer, (0.01M Tris-HCl, 2M Urea, pH 8.5, 25mL)

20m

Obtain a clean 50mL polypropylene falcon tube, 50mL graduated cylinder, Urea (NH_2CONH_2), prepared **Tris-HCl Stock**, a mass balance capable of handling milligram scale measurements, an ultrapure water source, a pH probe and pH balancing solutions (standard 1M HCl and NaOH), 1.5mL microcentrifuge tubes, 1000 μ L pipette and appropriate tips.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 3.003 g of Urea. Record actual mass.

Record the model and identification of the balance used.

Add  2.5 mL of 0.1M Tris-HCl stock reagent. Cap and Vortex well to mix.

Record source and purity of ultrapure water. Measure  15 mL of ultrapure water using the graduated cylinder and add to the tube. Cap and vortex vigorously to dissolve.

Measure the pH of the solution with the pH probe and adjust to  8.5 ± 0.1 with 1M HCl or 1M NaOH. Record pH. Record model and identification of the pH probe used.

Bring solution up to 25mL with Ultra-Pure water using the graduated cylinder. Return to falcon tube and mix well.

Label: Urea (UA) Buffer, [Initials], date of preparation, date of expiration (24hr from preparation date at room temperature), storage location, and storage temperature.

Note

Dissolution may be improved by gently heating the mixture below its boiling point using a heat block until dissolved. Following heating, allow to return to ambient room temperature. Do not place on ice as urea will salt out of solution.

Urea degradation products carbamylate lysine residues and protein N-termini, which reduces digestion efficiency and should be accounted for as variable modifications in analysis. This can be mitigated by preparing UA buffer fresh for each use.

30 Iodoacetamide (IAA) Stock (1M, 0.01M Tris-HCl, 2M Urea, 1mL)

10m

Obtain iodoacetamide (IAA, C₂H₄INO), prepared **Urea (UA) Buffer**, a mass balance capable of handling milligram scale measurements, an ultrapure water source, 1.5mL microcentrifuge tubes, 0.6mL microtubes, 1000- and 100µL pipettes with appropriate tips.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to a 1.5mL microcentrifuge tube:

 0.18496 g of IAA. Record actual mass.

Record the model and identification of the balance used.

Add 1 mL of UA buffer to the tube. Vortex until mixed.

Aliquot solution into 0.6mL tubes, $\text{60 } \mu\text{L}$ in each tube.

Label: IAA Stock, [Initials], date of preparation, date of expiration (1yr from preparation date at $\text{-80 } ^\circ\text{C}$), storage location, and storage temperature.

Note

IAA is light sensitive and will degrade. Thaw individual aliquots of IAA by wrapping in tinfoil and allowing to come to ambient room temperature prior to use.

31 Iodoacetamide (IAA) Working Buffer, (0.05M IAA, 0.01M Tris-HCl, 8M Urea, 1mL)

5m

Obtain a 1.5mL microcentrifuge tube, prepared **UA buffer**, prepared **IAA Stock**, 1000- and 100 μL pipettes with appropriate tips.

Record the date opened and date of expiration for all reagents.

Thaw aliquot of IAA by wrapping in tinfoil and allowing to come to ambient room temperature prior to use.

Add $\text{50 } \mu\text{L}$ of IAA Stock to a 1.5mL microcentrifuge tube. Discard remaining stock.

Add $\text{950 } \mu\text{L}$ of UA buffer to the tube, vortex to mix. Wrap in foil until use.

Note

IAA is light sensitive and will degrade. Thaw individual aliquots of IAA by wrapping in tinfoil and allowing to come to ambient room temperature prior to use.

Label: IAA Working Buffer, [Initials], date of preparation, date of expiration (24hr from preparation date at ambient room temperature).

32 Priming Solution (50:50 Methanol: Water, 50mL)

10m

Obtain a 50mL falcon tube, 100mL graduated cylinder, Methanol (CH_3OH), and an ultrapure water source.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure  25 mL of Methanol.

Record source and purity of ultrapure water. Measure  25 mL of ultrapure water using the graduated cylinder and add to the tube. Cap and mix.

Label: Priming Solution, [Initials], date of preparation, date of expiration (1yr from preparation date at ambient room temperature), storage location, and storage temperature.

33 **Sodium Deoxycholate Solution (1% DOC, 0.01M Tris-HCl, 10mL)**

5m

Obtain a 15mL falcon tube, sodium deoxycholate (DOC, $\text{C}_{24}\text{H}_{39}\text{NaO}_4$), prepared **Tris-HCl Stock**, a mass balance capable of handling milligram scale measurements, 10mL graduated cylinder, 1.5mL microcentrifuge tubes, and an ultrapure water source.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to a 1.5mL microcentrifuge tube:

 0.1 g of DOC. Record actual mass.

Record the model and identification of the balance used.

Record source and purity of ultrapure water. Measure  9 mL of ultrapure water using the graduated cylinder and add to the tube. Cap and mix to dissolve.

Add  1 mL of **Tris-HCl Stock** to the tube. Vortex until mixed.

Aliquot solution into 1.5mL tubes,  1 mL in each tube.

Label: 1% DOC Solution, [Initials], date of preparation, date of expiration (6mo. from preparation date at  -80 °C), storage location, and storage temperature.

Note

DOC is light sensitive and will degrade. Thaw individual aliquots of IAA by wrapping in tinfoil and allowing to come to ambient room temperature prior to use.

34 Trifluoroacetic acid (TFA) Buffer (5% TFA, 20mL)

10m

Obtain a 100mL glass media bottle, 50mL graduated cylinder, 1000 μ L pipette with appropriate tips, 1mL glass Trifluoroacetic acid (TFA, CF₃CO₂H) ampule, and an ultrapure water source.



Safety information

TFA is corrosive, can cause acute inhalation toxicity, serious eye damage, and is hazardous to the environment. Consult SDS sheet before use. Use extreme caution and appropriate personal protective equipment including a ventilation hood or respirator, eye protection/ face shield, and suitable gloves.

Exercise caution when working with glass ampules. Dispose of remaining TFA as hazardous waste.

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Prepare this solution inside of a ventilation hood.

Gently break TFA ampule and pipette 1 mL contents into the 100mL glass media bottle.

Record source and purity of ultrapure water. Measure 19 mL of ultrapure water using the graduated cylinder and add to the bottle. Cap and mix.

Label: TFA 5% Solution, [Initials], date of preparation, date of expiration (1yr from preparation date at ambient room temperature), storage location [inside of an acid storage cabinet], and storage temperature.

Filter-Aided Sample Preparation

1h 20m

- 35 Obtain 30kD Amicon Centrifugal filter units, microcentrifuge, thermomixer fitting 24 samples, ultrapure water source, ice bucket with ice, 1000-, 200-, 100-, 20-, and 10 μ L pipettes and appropriate tips, 15mL falcon tube, 1.5mL lo bind microcentrifuge tube, sequencing grade modified porcine trypsin (Promega, frozen, 5-20ug, cat#V5113), prepared **DOC Solution**, prepared **DTT stock**, prepared **IAA working solution**, prepared **priming solution**, prepared **UA buffer**, prepared **NaCl solution**, prepared **TFA Buffer**, and prepared **ABC buffer**.

Preheat thermomixer to 50 °C .

- 36 Thaw samples on wet ice. Thaw frozen **Trypsin** on wet ice. 30m

Record source and purity of ultrapure water. Collect  10 mL into a 15mL falcon tube.

Label 30kD Amicon Centrifugal filter units and accompanying 2mL tubes with sample names. Pipette  200 µL of ultrapure water into each filter unit. Visually inspect each filter unit for leaks or damage.

Spin filter units at  14000 x g, 25°C, 00:15:00 .

Visually inspect filter units to ensure liquid is passivating through. If solution does not passivate through the filter, discard and prime another filter unit before proceeding.

- 37 Pipette  200 µL of **Priming Solution** into each filter unit. 20m

Spin filter units at  14000 x g, 25°C, 00:15:00 .

Decant remaining solution from filter, but do not allow filters to dry out.

- 38 Using calculations from previously performed BCA assay, add  50 µg of protein sample to each respectively labeled filter unit. 45m

Add  200 µL of **UA buffer** to each unit.

Pipette to mix 15-20 times.

Spin samples at  14000 x g, 25°C, 00:30:00 .

Expected result

Liquid level in the filter should passivate down to roughly ~40µL.

- 39 Add  100 µL of **ABC Buffer** to each filter unit. 25m

Spin samples at  14000 rpm, 25°C, 00:20:00 .

Expected result

Liquid level in the filter should passivate down to roughly 20-50µL.

- 40 Repeat Step 38. Discard eluate.

25m

Protein Reduction

31m

- 41 Transfer samples to the pre-warmed thermomixer.

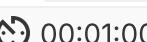
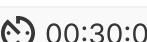
35m

Add  20 µL of **DTT stock** to each unit and shake for  00:01:00 on thermomixer at  600 rpm .

Incubate in the dark for  00:30:00 at  50 °C .

Protein Alkylation

31m

- 42 Add  11 µL of **IAA Working Buffer** to each filter unit. Shake at  600 rpm for  00:01:00 and incubate samples in the dark for  00:30:00 at  50 °C .

35m

Pre-heat thermomixer to  37 °C .

- 43 Spin samples at  14.000 x g, 25°C, 00:20:00 .

20m

Protein Digestion

18h 59m

- 44 Wash filter units with 100mL ABC buffer.

35m

Spin samples at  14.000 x g, 25°C, 00:30:00 .

Discard eluate.

- 45 Transfer samples back to the thermomixer. Using the volume markings on the side of the filter unit, add ~  30 µL of prepared **DOC Solution** to each filter unit until you reach a total volume of 60µL.

15m

Add  3 μL of Trypsin to each tube (a total of 1ug Trypsin, Ratio of 1:50). Mark trypsin usage on the lid, thawing no more than 3 times total before disposal.

- 46 Shake samples at  600 rpm for  00:01:00 and incubate samples at  37 °C for  15h 1m 

- 47 Add  1.5 μL **Trypsin** to each tube and mix at  600 rpm for  00:01:00 . 
- Incubate for  03:00:00 at  37 °C in the thermomixer shaded with a heated top.

- 48 Spin samples  14000 rpm, 25°C, 00:07:00 . 
- Add  100 μL of **NaCl solution**, and mix in thermomixer at  600 rpm for  00:15:00 .

- 49 Transfer filter units to a clean collection tube. 
- Invert filters and collect supernatant by spinning at  4.000 x g, 25°C, 00:05:00 .

Protein Acidification

20m

- 50 Acidify samples by adding  80 μL of **5% TFA Solution** (to a final concentration of 2% TFA) to each sample. Vortex to mix. 
- 51 Spin samples at  4000 rpm, 25°C, 00:05:00 and transfer the supernatant to labeled 1.5mL lo bind microcentrifuge tubes. 

Store samples at  -80 °C or immediately proceed with sample desalting.

Solution Preparation for Peptide Sample Desalting

25m

- 52 **Solution A** (100% Methanol, 5mL) 
- Obtain a 15mL falcon tube, 5mL serological pipette, pipette aid, and HPLC-grade Methanol (CH_3OH).

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 5 mL HPLC grade methanol. Cap tube.

Label: Solution A, [Initials], date of preparation, date of expiration (1 week at ambient room temperature), storage location [flammables cabinet], and storage temperature.

53 **Solution B** (60% Acetonitrile, 39.9% Water, .1% Formic Acid, 5mL)

5m

Obtain a 15mL falcon tube, 5mL serological pipettes, pipette aid, 10 μ L pipette and appropriate tips, HPLC-grade Acetonitrile (ACN, CH₃CN), HPLC-grade water, and HPLC-grade Formic Acid (or >95% purity, HCOOH).

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 3 mL HPLC-grade acetonitrile.

 2 mL HPLC-grade water. Use a 10 μ L pipette to remove  5 μ L and add

 5 μ L Formic acid.

Cap and vortex to mix.

Label: Solution B, [Initials], date of preparation, date of expiration (1 week at ambient room temperature), storage location (flammables cabinet), and storage temperature.

54 **Solution C** (.1% Formic Acid in Water, 8mL)

5m

Obtain a 15mL falcon tube, 10mL serological pipettes, pipette aid, 10 μ L pipette and appropriate tips, HPLC-grade water, and HPLC-grade Formic Acid (or >95% purity, HCOOH).

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 8 mL HPLC-grade water. Use a 10 μ L pipette to remove  5 μ L and add

 5 μ L Formic acid.

Cap and vortex to mix.

Label: Solution C, [Initials], date of preparation, date of expiration (1 week at ambient room temperature), storage location, and storage temperature.

55 **Solution D** (80% Acetonitrile, 19.9% Water, .1% Formic Acid, 8mL)

5m

Obtain a 15mL falcon tube, 10mL serological pipettes, pipette aid, 10 μ L pipette and appropriate tips, HPLC-grade Acetonitrile (ACN, CH₃CN), HPLC-grade water, and HPLC-grade Formic Acid (or >95% purity, HCOOH).

Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 15mL falcon tube:

 6.4 mL HPLC-grade acetonitrile.

 1.5 mL HPLC-grade water. Use a 10 μ L pipette to remove  5 μ L and add

 5 μ L Formic acid.

Cap and vortex to mix.

Label: Solution D, [Initials], date of preparation, date of expiration (1 week at ambient room temperature), storage location (flammables cabinet), and storage temperature.

56 **Formic Acid Solution** (0.1% Formic Acid in Methanol)

5m

Obtain a clean 100mL glass media bottle, 100mL graduated cylinder, 200 μ L pipette and appropriate tips, HPLC-grade Methanol (CH₃OH), and HPLC-grade Formic Acid (or >95% purity, HCOOH).



Record the manufacturer, lot number, date opened, and date of expiration for all reagents.

Measure out the following and add to the 100mL glass media bottle:

 99.9 mL HPLC-grade Methanol.

 100 μ L Formic acid.

Cap and mix.

Label: Formic Acid Solution, [Initials], date of preparation, date of expiration (1 week at ambient room temperature), storage location (flammables cabinet), and storage temperature.

Filter Tip Preparation

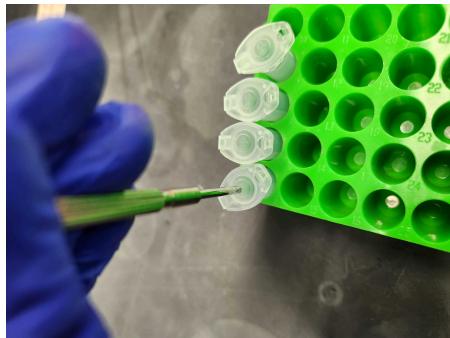
15m

- 57 Obtain 3M Empore 2240 High performance Extraction Disks, 200 μ L unfiltered pipette tips, 10 μ L unfiltered pipette tips, 2.0mL lo-bind tubes, 1.5mL lo-bind tubes, an 18 gauge flat tip syringe needle, compressed air duster with fine tip, and a 3mm Philips head screwdriver.

- 58 Label and prepare a 2.0mL and 1.5mL tube for each sample by puncturing a hole in the center of each tube cap with the 3mm Philips head screwdriver.

15m





Widen hole as necessary until a 200 μ L unfiltered pipette tip can be fitted snugly into each tube with the tip end inserted to approximately the 1.0mL volume line marked on side of each tube.



The 2.0mL tube will serve as the wash collection tube.

The 1.5mL tube will serve as the sample collection tube.

The inserted 200 μ L tip in each will serve as the filter tip protector sheath.

- 59 Prepare the filter tip by placing a 10 μ L unfiltered pipette tip in the protector sheath of the wash collection tube.

Using the 18 gauge flat tipped syringe needle, punch 3 holes through the Empore filter disk, collecting the punched circles inside the hollow syringe needle. Place the end of the needle in the 10 μ L tip and with the fine applicator of compressed canned air, release a puff down the needle to dislodge the filter paper punches and deposit them snugly in the 10 μ L tip to create the filter column.





Peptide Sample Desalting

2h 45m

- 60 Obtain ice bucket with ice, prepared **filter tips**, prepared **Solution A**, prepared **Solution B**, prepared **Solution C**, prepared **Solution D**, prepared **Formic Acid Solution**, Waters MassPREP™ Enolase digestion standard (SKU: 186002325), a microcentrifuge (capable of accommodating 24 samples simultaneously), a Speed-Vap solvent evaporation apparatus (capable of accommodating 24 samples simultaneously) or equivalent instrument, Nanodrop or peptide quantification kit, glass autosampler tubes with re-sealing caps (compatible with liquid chromatography autosampler to be used), 200-, 100-, 20-, and 10 μ L pipettes with appropriate tips.

Record date of preparation, date of expiration, and lot number of all reagents where possible.

Note

According to the manufacturer, "Waters MassPREP™ Enolase digestion standards is prepared by digesting Yeast Enolase (SwissProt P00924) with sequencing grade trypsin. The resulting protein digest contain no undigested standard protein, trypsin, or other hydrophilic components." <https://www.waters.com/nextgen/us/en/shop/standards-reagents/186002325-massprep-enolase-digestion-standard.html#:~:text=The%20Waters%20MassPREP%20Enolase%20Digestion%20Standard%20is%20prepared%20by%20digesting,trypsin%2C%20or%20other%20hydrophilic%20components.>

Pre-heat Speed-Vap to  65 °C and turn on vacuum pumps.

- 61 Thaw samples on ice.

Determine peptide concentration via NanoDrop. Record peptide concentrations and determine the volume of each sample needed for $\text{30 } \mu\text{g}$ of peptide.

20m

- 62 Activate column by adding $\text{75 } \mu\text{L}$ of **Buffer A** to the $10\mu\text{L}$ filter tip seated in the filter protection sheath of the wash tube. Spin at $4000 \times g, 25^\circ\text{C}, 00:00:30$. Liquid should be passivated through the filter, but the filter should not dry between steps.

5m

- 63 Activate column by adding $\text{75 } \mu\text{L}$ of **Buffer D**. Spin at $4000 \times g, 25^\circ\text{C}, 00:00:30$. Liquid should be passivated through the filter, but the filter should not dry between steps.

5m

- 64 Activate column by adding $\text{75 } \mu\text{L}$ of **Buffer C**. Spin at $4000 \times g, 25^\circ\text{C}, 00:00:30$. Liquid should be passivated through the filter, but the filter should not dry between steps.

5m

- 65 Repeat column activation steps.

20m

Visually examine each filter tip and collection tube to ensure that filter layers have not been lost and that solvent is passivating through. If a tip is defective, discard it, prepare a new one, and activate as described above.

Decant solution from the wash tube and discard. Place column back in the wash tube sheath protector.

Label each filter tip and tube for its respective peptide sample.

- 66 Based on calculations from the BCA protein quantification in step 27, add $\text{30 } \mu\text{g}$ of peptide to each respectively labeled filter tip.

10m

Spin at $4000 \times g, 25^\circ\text{C}, 00:02:00$ to bind protein to the filter.

- 67 Draw up eluate from the collection tube and reapply to the filter tip.

10m

Spin at $4000 \times g, 25^\circ\text{C}, 00:02:00$ to bind more protein to the filter.

- 68 Repeat step.

10m

- 69 Wash tip with $\text{75 } \mu\text{L}$ of **Buffer C** to remove salts from filter-bound proteins.

2m

Spin at $4000 \times g, 25^\circ\text{C}, 00:01:00$.

- 70 Transfer filter tip to the protector sheath of the collection tube. Discard wash tubes. 5m
- 71 Add  50 µL of **Buffer B** to the column and spin at  4000 x g, 25°C, 00:01:00 . 1m
Keep eluate.
- 72 Add  50 µL of **Buffer D** to the column and spin at  4000 x g, 25°C, 00:01:00 . 1m
Keep eluate.
- 73 Repeat step 70. Discard filter tips and keep eluate in labeled collection tubes. 1m
- 74 Cap collection tubes and place into the pre-warmed Speed-Vap (the hole in the lid where the filter tip was previously placed will allow solvent evaporation). 15m 

Run the Speed-Vap in 5-10 minute intervals closely monitoring the volume of each sample until each is reduced from 150µL to a final volume of 5-10µL.

Remove samples from speed-vap and turn off apparatus.

Note

If samples are accidentally completely dried, add 30µL of **Formic Acid Solution** to each dried out tube and place on a thermomixer for  00:30:00 at  1000 rpm, 30°C to resuspend protein in solution. Proceed with following step.

- 75 Individually, use a 10µL pipette with appropriate tips to draw up sample from the lo bind collection tube, estimate the volume of peptide, and transfer to a labeled glass autosampler vial. 30m

Bring total volume in the autosampler vial up to 29µL with **Formic Acid Solution**.

- 76 Add 1uL of Waters MassPREP™ Enolase digestion standard to each sample. 5m
Cap and vortex well to mix.

Note

Final concentration each sample should be 1 µg/µL of sample peptides and 25 fmol/µL of Enolase digest for our mass spectrometry instrumentation.

Final volume, peptide, and standard concentrations can be adjusted for different instrumentation needs at steps 8, 13, 23, and 24.

- 77 Prepare an intrastudy quality control (QC) sample by transferring  1 µL from each sample into a single fresh autosampler vial. Label as intrastudy QC with date of preparation.

15m

Cap and mix.

- 78 Prepare an enolase intra/inter laboratory standard at 25 fmol/µL.

5m

- 79 Immediately proceed with liquid chromatography tandem mass spectrometry data acquisition or store samples at  -80 °C until analysis.

II

Protocol references

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