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Protein extraction of zebrafish and fathead minnow embryos

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Funders Acknowledgement:

Abstract

This protocol describes an optimized protein extraction method for proteomic analyses for zebrafish and fathead minnow embryo's aged ~96 hpf/dpf (species dependent), and wouldn't have been possible without the following papers

References:

Masuda, T., Tomita, M., Ishihama, Y., 2008. Phase Transfer Surfactant-Aided Trypsin Digestion for Membrane Proteome Analysis. J. Proteome Res. 7, 731–740. https://doi.org/10.1021/pr700658q

Lin Y, Lin H, Liu Z, Wang K, Yan Y. Improvement of a sample preparation method assisted by sodium deoxycholate for mass-spectrometry-based shotgun membrane proteomics. J Sep Sci. 2014; 37(22): 3321-9. doi: 10.1002/jssc.201400569

Rappsilber, J., Ishihama, Y., Mann, M., 2003. Stop And Go Extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75, 663–670. https://doi.org/10.1021/ac026117i

Wiśniewski, J.R., 2016. Quantitative Evaluation of Filter Aided Sample Preparation (FASP) and Multienzyme Digestion FASP Protocols. Analytical Chemistry 88, 5438–5443. https://doi.org/10.1021/acs.analchem.6b00859

Wiśniewski, J.R., Zougman, A., Nagaraj, N., Mann, M., 2009. Universal sample preparation method for proteome analysis. Nat. Methods 6, 359–362. https://doi.org/10.1038/nmeth.1322

Guidelines

Wear appropriate safety clothing (lab coat, safety goggles, gloves, mask) at all times. Wear mask especially when handling DTT, SDS and SDC.

Pelleted SDS is safer and more convenient to handle than SDS powder. Please also refer to the respective MSDS sheets.

Before start

Before starting

- Prepare stock solutions
- Samples can be lysed and quantified in advance and stored at -20/-80 ° C, therefore sample preparation and peptide digestion are separated into two categories. If samples are prepared in advance, then only PBS wash solution, lysis buffer and 10 % SDS stock need to be made in advance.
- Defrost and store samples on ice
- Pre-heat thermo-shaker (95 °C)

	A	B	C
	Item	Retailer	Catalogue
	Phosphate buffered saline (PBS)	Sigma-Aldrich	P4417
	HPLC water	Sigma-Aldrich	34877
	Trizma hydrochloride (Tris-HCL)	Sigma-Aldrich	T3253
	Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	71726
	cOmplete Ultra tablets (Protease inhibitor cocktail)	Sigma-Aldrich	5892970001
	Methanol	Sigma-Aldrich	A456-4
	Ammonium acetate	Sigma-Aldrich	A7262
	Acetone	Sigma-Aldrich	179124
	Pierce BCA protein assay kit	Thermo Fisher Scientific	23225
	Bovine serum albumin (BSA; 1mg/mL)	Fisher Scientific	AAJ60205AD
	Amicon Ultra-0.5 Centrifugal Filter Unit (3K)	Sigma-Aldrich	UFC5003BK
	Urea	Sigma-Aldrich	U5128
	Ammonium Bicarbonate Buffer (abc)	Sigma-Aldrich	S2454
	Dithiothreitol (DTT)	Sigma-Aldrich	646563
	Iodoacetamide (IAA)	Sigma-Aldrich	I1149
	Sodium deoxycholate (SDC)	Sigma-Aldrich	D6750
	Sodium chloride solution	Sigma-Aldrich	S5150
	Trypsin (frozen)	Promega	V5113
	Trifluoroacetic acid (TFA)	ProteoChem	LC6203
	Pierce C18 tips	Thermo Fisher Scientific	87782
	Acetonitrile	Fisher Scientific	A955
	Formic acid	Sigma-Aldrich	F0507

Stock solutions

PBS wash solution
- 1 tablet/100 mL of water

Lysis buffer
- 0.1 M Tris-HCL (pH 7.8)
- 0.05 M DTT
- cOmplete Ultra tablets (Protease inhibitor cocktail) [1 tablet/10 mL buffer]

10 % SDS stock solution (w/v) [HPLC water]

UA buffer
- 2 M Urea (pH 8.5; 0.1 M Tris-HCL)

Digestion buffer (DB)
- ABC buffer (50 mM)

DTT buffer
45 μL 1 M stock to 955μL HPLC water

IAA stock (1M)
- 189 mg/1 mL ABC buffer

Sodium deoxycholate (SDC), 10 % (w/v)

Elution buffer
- 70 % NaCL [1 M] + 30 % methanol
- ABC buffer (sodium bicarbonate)

5 % TFA (v/v)

Equipment
- Micro-centrifuge
- Tissue homogenizer (e.g. Scilogex D160 homogenizer + saw tooth probe)
- Sonicating probe (Misonix sonicator; ultrasonic liquid processor with micro-tip)
- ThermoMixer C eppendorf + smartblock and/or heating block
- Speedvac

Materials

1. Laboratory equipment + buffer preparation

*See guidelines and warnings

2. Tissue lysis and total protein extraction

- Defrost samples on ice
- Add 200 μL PBS, and centrifuge to pellet samples (2000 g; 1 min X room temperature (RT))
- Repeat three times ensuring the removal of as much solution as possible
- Weigh sample to determine lysis buffer volume (ratio of 1:10 used, 1 mg : 10 μL lysis buffer)

** For fathead minnow, RIPA buffer was used due to consistency in the literature*

- Homogenize at 30,000 RPM for 20 sec, and add 2 % SDS. Vortex to mix, and heat at 95 $^\circ$ C for 5 min. Place on ice.
- Sonicate samples in three rounds of 15 sec on and 15 sec off (30 amplitude)
- Heat at 95 $^\circ$ C for 15 min, and centrifuge at 16,000 g for 15-30 min at 4° C to remove debri. Transfer supernatant to a new eppendorf (Low-bind protein)
- To precipitate proteins, to 100 μL sample, add 600 μL 0.1 M ammonium acetate/methanol (5 volumes) and incubate at -20° C for 1 h, vortexing every 15 minutes

  ** for fathead minnow, 200 μL was used; for higher protein yield, use all lysis buffer yield*
- Pellet protein through centrifugation (18,000 G x 20 mins, 4 $^\circ$ C) and discard supernatant
- Wash pellet (2x) with 0.1 M Ammoniuim Acetate in Methanol (Resuspend for 15 min at -20 $^\circ$ C and then 10 min centrifugation >14,000G, 4 $^\circ$ C)
- Wash pellet (2x) with acetone (Resuspend for 15 min at -20 $^\circ$ C and then 10 min centrifugation >14,000G, 4 $^\circ$ C)
- Wash pellet (2x) with 70 % ethanol (Resuspend for 15 min at -20 $^\circ$ C and then 10 min centrifugation >14,000G, 4 $^\circ$ C)
- Dry pellet under vacuum and resuspend in100 μL 0.1 M Tris-HCL (without SDS or DTT)

  **or whatever resuspension volume you want to use. Optimization may be necessary.*
- Quantify protein using BCA assay/nanodrop and calculate volume required for FASP method (50 µg)

**A protein standard curve is typically run on a BCA assay. It is important for the standard to be made in the resuspension buffer as the samples for accuracy. For zebrafish and fathead minnow, dependent on pool size, dilution maybe necessary.*

**Do not forget to account for any dilution in protein concentration*

**to calculate final protein, display as µg/µL per sample and divide by protein concentration required (e.g. sample = 6 µg/µL, 50 µg required, 50/6 = 8.3 µL of stock required for a total protein content of 50 µg). Alternatively, use C1V1 formula.*

3. Filter aided FASP preparation (modified)

Label **30 kD Amicon Ultra-centrifugal filter unit**

- *Assemble filters and prepare by washing with water, methanol and then water over 15 minutes*
- Decant any excess solution but do not allow filter to dry out

1. Add **protein lysate (**50 µg or whatever protein volume required **)** and top up volume to 200 µl of **UA** buffer (2 M Urea; pH 8.5) in the filter unit
   **15-20 pipette mixes.*
   ** UA buffer must be prepared fresh each day.*
   **Some ultrafilters have a maximum protein loading concentration. Please check before using.*

2. Sample cleanup

- Centrifuge at 14,000 g/RCF for 25-30 min
- Volume in filter should reduce to ~40 µl
- Add 100 µl of ABC buffer (50 mM) and centrifuge for 20 min
- ~ 20 -50 µl left in filter
- Repeat

3. Reduction

- Add 20 µl DTT stock to sample.
- Pipette mix, shake at 600 rpm for 1 min and then incubate at RT for 30 min at 50 °C

4. Alkylation

- Add 11 µl of **IAA** stock (final concentration 90.9 mM) to filter unit and pipette mix
- Shake at 600 rpm (1 min) prior to 30 min incubation in the dark
- Add 11 µl DTT for 30 minutes before centrifugation (14,000 RCF for 20 mins)
- Discard flow-through

5. In solution digestion

- Wash through with ABC buffer (100 µl) and centrifuge (14,000 RCF for 30mins)
- Repeat
- Volume should be reduced to 45-47 µl
- Add 1 % SDC solution to ~ 60 µl (should be adding ~ 20-30 µl)
- Add 1 µg trypsin : 50 µg protein [1:50 ratio; ~*0.02* µg/ µL required)
  Trypsin stock 0.4 µg/ µL
  Add 3 µl trypsin stock to 60 µL sample

- Mix at 600 rpm for 1 min and then incubate overnight in humid chamber at 37 °C for 16-18 hours

6. Sample collection

- Transfer the filter units to a new collection tube, and add 50 μL NaCL/methanol solution. Pipette mix and shake at 600 rpm for 10 min at room temperature
- Invert to collect supernatant and centrifuge at 1,000 g for 5 min

**Can repeat elution if you feel like proteins may have adhered to filter. Alternatively, some studies have found improvement through prewashing filters to reduce unspecific binding , see Erde et al. 2017* https://doi.org/10.1021/pr4010019

- Transfer supernatant to low bind Eppendorf and add 2 % TFA to remove SDS (forms precipitate which can be removed through centrifugation)
- Centrifuge 13,000 G for 5 min and transfer supernatant to a new collection tube. Quantify peptides using nanodrop (A250) or through other methods

7. Sample clean up

- In desalting samples (Pierce C18 tip or Rapseiller et al (2003)), ensure loading concentration is proportionate to maximum loading volume of stage tip. For consistency, injection volume was adjusted to ensure loading of 1 μg per sample.

**If you are making your own stage tips, we have observed that dependent on Empore filters (2215, 2240), and using two filters to bind 10 µg of protein (~assumed binding capacity of 2-4 µg per filter), an average recovery of ~27 % for zebrafish and 38 % recovery for fathead minnow was observed with elution of peptides from filter repeated three times. It is critical that the filter at no point dries out as this can significantly impact recovery. Centrifugation timing as such may need to be adjusted dependent on wash volumes etc.*
We have not confirmed what the pierce recovery is, although the manual reports recovery of > 85%, but may be as low as 35% for hydrophilic peptides. It is possible recovery is linked to protein type and so care should be taken in quantifying recovery/loss at each step of the methodology. (For example, we quantify at initial lysis, post protein digestion and post stage tipping. Nanodrop and A250 nm can be used following post digestion, or other methods such as the tryptophan method proposed by ***Wiśniewski*** *and Gaugaz (2015).*

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Files

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