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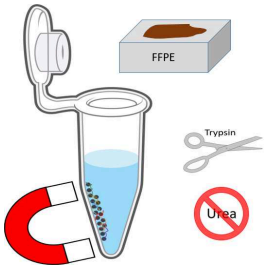
FFPE sections SP3 digestion -rapizyme and ACN



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Main workhorse protocol used in our proteomics lab for FFPE samples since 2023.

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Abstract

This procedure takes as input Formalin-Fixed Paraffin-Embedded (FFPE) samples, typically 2 to 5 slices of 5µmx5mmx5mm. It begins with lysis/deparaffinization of the samples including an ultrasonication step, followed by protein clean-up with sp3-beads, denaturation, reduction and alkylation of cysteines, followed by digestion of proteins to peptides by trypsin (rapizyme from Waters), while using 10% ACN instead of urea during digestion, and ends with peptide level sp3 cleanup.

Materials

- **Reagents and solvents:**

Trizma® base **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T1503

TRIS Buffer, 1.0M, pH 8.0, Molecular Biology Grade **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**648314-100ML

Hydrochloric acid 1.000 l **Merck Millipore (EMD Millipore) Catalog #**109911

SDS 20% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030-500ML-F

Sodium n-dodecyl sulfate (SDS), 20% aq. soln.

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43815

DL-Dithiothreitol, threo-1,4-Dimercapto-2,3-butanediol
Product number: 43815-1G
Molecular Weight: 154.25 g/mol

- Use the BioUltra grade powder to prepare 1M aqueous stock solution; aliquots can be prepared and frozen.

2-Chloroacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C0267

2-Chloroacetamide
CAS No.: 79-07-2
Molecular Weight: 93.51 g/mol

- Use crystalline powder (e.g., Sigma-Aldrich Cat. No. **C0267**, ≥98%) to prepare fresh aqueous solution immediately before use

Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic, 65152105050250 **Fisher Scientific Catalog #**11819912

(Sigma-Aldrich P/N: GE65152105050250)

Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic, 45152105050250 **Fisher Scientific Catalog #**11548692

(Sigma-Aldrich P/N: GE45152105050250)

Gibco™ HEPES (1M) **Fisher Scientific Catalog #**15-630-080

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
CAS No.: 7365-45-9
Molecular Weight: 238.30 g/mol

- Use commercially available 1M aqueous stock solution (e.g., Gibco, Fisher Scientific Cat No. **15-630-080**; 100 mL)

Calcium chloride hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**21108-500G

Water, Optima™ LC/MS Grade, Fisher Chemical™ **Thermo Fisher Scientific Catalog #**AAB-W6-4

- Use ultra-pure water - we recommend Fisher Optima LCMS grade (Fisher Scientific P/N **10505904**)

Acetonitrile, Optima LC/MS grade, Fisher Chemical **Fisher Scientific Catalog #**A955212

CAS No.: 75-05-8

- Use LCMS-grade, e.g. Acetonitrile Optima LCMS grade (Fisher Scientific P/N **10055454**) or HPLC grade S (Rathburn)

Ethanol, 99.8%, for HPLC, absolute, Thermo Scientific Chemicals **Fisher Scientific Catalog #** 12347163

RapiZyme Trypsin, MS Grade, 4/pk (100ug) **Waters Catalog #**186010108

- **Plastics and equipment:**

Eppendorf Safe-Lock micro test tubes 2mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030123344

Eppendorf Safe-Lock micro test tubes 1.5mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030123328

Eppendorf® Protein LoBind Tubes, 1.5mL **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030108442-1EA

FiveEasy Plus pH meter, METTLER TOLEDO.

Ultrasonic processor (Twitter), **UP200ST**, Hielscher.

Vortex-Genie® 2 mixer, AC/DC input 220 V AC, Schuko plug, Merck, cat Number: **Z258423-1EA**.

VWR® MiniStar/MiniStar blueline, Spin-down/Microcentrifuge, VWR art.nr: **521-2161P**, VWR.

Eppendorf® ThermoMixer® C, Product Number: **EP5382000015-1EA**, Merck.

ELMI Intelli-Mixer™ , **RM-2S**, ELMI tech.

Centrifuge, **5430/ 5430 R**, eppenorf.

Reagent preparation

1h 10m

1 **Preparing 1M Tris-HCl pH8** 20m

Prepare 10 mL of [M] 1 Molar (M) aqueous solution of Tris-base.

- Weigh approximately 1.211 g of Trizma® base powder put in 10 mL Volumetric flask.
- Fill the flask up to the etched ring mark.
- Mix thoroughly until dissolved.
- Calibrate pH meter.
- Adjust the pH of the solution to pH 8 using HCl solution.

Alternatively, use premade TRIS buffer pH 8.0 [M] 1 Molar (M) from Merck.

2 **Lysis Buffer preparation** 5m

Final buffer concentration: 4%SDS, [M] 0.2 Molar (M) Tris-HCL pH 8, [M] 0.2 Molar (M) DTT

To have the above final concentration in 1 mL volume, use the following volumes and fill up to 1 mL with water:

200 µL SDS 20%

200 µL DTT 1M

200 µL Tris-HCl 1M pH 8

3 **Alkylation reagent preparation** 5m

Prepare 5 mL of [M] 750 millimolar (mM) aqueous solution of chloroacetamide (CAA).

- weigh approximately 350.6 mg of the microspatula of CAA powder.
- add the exact volume of water to a final concentration of [M] 750 millimolar (mM)
- mix thoroughly (vortex) until dissolved
- pulse-spin in a microcentrifuge

Note

Example
$n = 350.662mg/93.51mg/mmol \approx 3.75mmol$
dissolving in 5 ml of water gives the desired 750 mM concentration.

Note

The volumes of reagents can be adjusted to the number of samples.

4 **Preparing 1M CaCl2** 5m

Prepare 1 mL of [M] 1 Molar (M) aqueous solution of calcium chloride hexahydrate (CaCl2).

- weigh approximately 219 mg of calcium chloride hexahydrate (CaCl2) powder.
- add the exact volume of water to a final concentration of [M] 1 Molar (M)
- mix thoroughly (vortex) until dissolved
- pulse-spin in a microcentrifuge

Note

You can aliquot the solution and keep them in freezer.

5 **Digestion Buffer preparation** 5m

Final buffer concentration: [M] 50 millimolar (mM) HEPES pH 7.8 , [M] 10 millimolar (mM) CaCl2 , [M] 10 % (v/v) ACN)

To have the above final concentration in 1 mL volume, use the following volumes and fill up to 1 mL with water:

50 µL HEPES 1M pH 7,8

100 µL ACN

10 µL 1M CaCl2

6 **SP3-bead slurry preparation** 15m

(sufficient for 10 samples)

6.1 Take out the two bottles with Sera-Mag speed beads from the fridge and keep them at room temperature for 10 minutes. 00:10:00 10m

6.2 Shake the two SP3 bead bottles gently (without vortex, until all beads are suspended in liquid, takes about 5 minutes). 00:05:00 5m

6.3 Combine 50 µL of each bead type in a clean Safe-lock Eppendorf tube (1.5mL or 2mL).

6.4 Wash the beads:

- Place the tube with the bead mixture on a magnetic rack and let the beads settle for 2 minutes. 00:02:00
- Remove and discard the supernatant.
- Rinse the beads with 500 µL water by gentle pipette mixing (off the magnetic rack).
- Repeat wash steps two more times.
- Re-suspend and store the beads in 500 µL water in 4 °C

Note

The washed beads can be stored in +4°C up to two weeks. Beads can handle low and high pH, and can be safely heated up to 60°C.
!!! Never freeze the beads.
!!! Never sonicate the beads.

Deparaffinization - Protein extraction from FFPE tissue sections in tubes 1h 34m 30s

7 Add 200 µL of lysis buffer to each FFPE sample. 5m

Note

This protocol is originally written for use with the Twitter Ultrasonic processor from Hielscher, **UP200ST**. If a Covaris AFA ultrasonicator is available that is also a good option.

Bear in mind that optimal sample capacities are different:
Covaris sonication will have a capacity of 100µl of lysate maximum.
Hielscher sonication can take up to 1mL if necessary. However, 200µl is recommended as maximum due to volume constraints in subsequent steps of the protocol. (see steps 16 and 18 below).

As such, for Covaris, a good size of FFPE sample input is 2 slices of 5µmx5mmx5mm. (about 10µg to 20µg of protein).
For Hielscher, sample input of 5 such slices is recommended. (about 25µg to 50µg of protein).

8 Be sure every sample is submerged in the lysis buffer, otherwise vortex or shake them.

9 Heat the samples in the heating block at 95 °C for 00:30:00 (30min) in agitation, 400 rpm. 30m

10 Remove the samples from the heating block and let it cool down for some minutes at Room temperature . 2m

11 Spin down the samples and Sonicate the samples with Twitter sonicator with the following settings: 15m

- Set the Amplitude **(A)** to 100%
- Set the pulsation mode **(C)** to 100%
- **Period Clock**: On
- **On Time**: 60s
- **Off Time**: 30s
- **Limit Type**: Time
- **Limit Value**: To perform 10 cycles you need to set 00:15:00 (15min) .

Note

Calculating Limit Value:
(60s On Time + 30 Off Time) * Number of Cycles

For example:
*For 10 Cycles: (60+30)*10= 900s = You should set 15 min on instrument.*

- **IMPORTANT: Ensure all positions on the Twitter sonicator are occupied. If you do not have enough samples to fill the positions, use tubes filled with water to balance the instrument.**

12 Heat the samples in the heating block at 95 °C for 00:15:00 (15min) in agitation, 400 rpm. 15m

13 Spin down the samples and Sonicate the samples with twitter with the following settings: 7m 30s

- Set the Amplitude **(A)** to 100%
- Set the pulsation mode **(C)** to 100%
- **Period Clock**: On
- **On Time**: 60s
- **Off Time**: 30s
- **Limit Type**: Time

- **Limit Value**: To perform 5 cycles you need to set 00:07:30 .

Note

IMPORTANT: Ensure all positions on the Twitter sonicator are occupied. If you do not have enough samples to fill the positions, use tubes filled with water to balance the instrument.

14 Centrifuge at 13000 x g, 23°C for 00:10:00 (10 min) at Room temperature . 10m

15 Recover the supernatant and put in a new Safe-lock 2mL Eppendorf tube. 10m

SP3 digestion of FFPE samples 17h 14m

16 Add 300 µL 750 mM chloroacetamide to each sample, vortex, and incubate for 00:10:00 (10min) at Room temperature , keeping the samples in the fume hood. 15m

Note

The volume of 300 µl here is expecting the recommended lysate volume of 200µl (see step 7 above). If the lysate volume is different, the needed volume of chloroacetamide solution to be added should be adjusted accordingly.

µµ

17 Add 50 µL of beads suspension to each sample and pipette gently to resuspend them. 10m

18 Add 1500 µL ACN to the samples (acetonitrile concentration of 73%). 2m

Note

Concerning volumes, given that the maximum volume capacity of the Eppendorf tube is 2mL, consider the volumes used in steps 7 and 16.

19 Incubate the suspension in a rotating rack for 00:20:00 (20 min) at Room temperature . 20m

20 At the end of the incubation remove the tubes from the rack, shake them to detach the beads from the lid and incubate in a magnetic rack for 00:02:00 (2 min). 5m

21 Aspirate and discard the supernatant, without removing the tube from the rack. 1m

22 While maintaining the tubes on the magnetic rack, flush the beads with 500 µL Ethanol 70% and incubate 00:00:30 (30s), then aspirate the liquid phase and discard it. 2m

23 Repeat the previous step. 3m

24 While maintaining the tubes on the magnetic rack, flush the beads with 500 µL ACN , incubate 00:00:30 (30s), aspirate and discard the supernatant. 3m

25 Let the beads dry for 00:00:20 (20s), then close the lid to avoid excessive drying. 1m

26 Dissolve RAPIZYME powder (100 µg of trypsin) in 100 µL of Fisher Optima water (quick vortex or pipette up and down). Add the 100 µL of trypsin solution to 1900 µL of digestion buffer. 2m

Note

If the entire 100 µg of trypsin are to be used up in one experiment, then one can directly dissolve in the digestion buffer.
Otherwise, leftover Rapizyme dissolved in water can be kept in the -20C freezer and be used later (up to a month). Beware of activity degradation due to freeze/thaw cycles, so ideally plan ahead and freeze in aliquots to be used up in single occasions.

27 Add 100 µL from the trypsin containing digestion buffer to each sample (i.e. onto the dry beads).

28 Incubate for 16:00:00 (16h) at 37 °C with mild shaking, using an oven type incubator (i.e. avoid temperature gradient between the bottom and the top of the tube). 16h

SP3 peptide clean-up, peptide concentration 52m

29 After overnight incubation, add 2 mL acetonitrile to reach a concentration of more than [M] 95 % (v/v) ACN. 2m

> Note
>
> In order guarantee the binding of all peptides to the SP3 beads, the organic content composition of the liquid must be >95%.

30 Incubate the tubes in a gently rotating rack for 20min 00:20:00 at Room temperature . 20m

31 After incubation, shake the tubes to recover the beads stuck on the lid, then incubate the tubes on a magnetic rack for 00:02:00 (2 min). 5m

32 While maintaining the tubes on the magnetic rack, aspirate and discard the liquid phase, then flush the beads with 500 µL acetonitrile and wait for 00:00:30 (30s). 2m

33 Repeat the previous step. 3m

34 While maintaining the tubes on the magnetic rack, aspirate and discard the liquid phase, then let it dry a few seconds. 10m

35 Take the tubes off the magnetic rack, and resuspend in 50 µL of MS grade water. Mix gently by brief vortex or pipetting and spin down.

36 Incubate the tube on the magnetic rack for 00:02:00 (2 min), then recover the liquid phase and put it in a new Low-bind 1.5mL Eppendorf tube. 5m

37 Repeat the previous step, to be sure of removing all the beads. 5m

38 Perform the peptide concentration measurement, using Optima LCMS grade water as blank.

Protocol references

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