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SP3 protocol for proteomic analysis of tendon cryosections

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protocol .

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This protocol details the preparation of cryosection samples and laser capture microdissection (LCM) samples collected with a MMI Laser-Microdissection device for MS/MS analysis. The sample preparation includes tissue lysis, Sp3 technology (single-pot, solid-phase-enhanced sample preparation*)*, trypsin digestion, C18-clean-up and peptide resuspension.

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Stefania Wunderli, Jess Snedeker, The Tendon Seed Network 2022. SP3 protocol for proteomic analysis of tendon cryosections. **protocols.io** https://protocols.io/view/sp3-protocol-for-proteomic-analysis-of-tendon-cryo-bzndp5a6

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Reagents:

- Needle (1.2x40mm)
- RIPA buffer (Sigma)
- [M]**100 millimolar (mM)** TCEP
- [M]**500 millimolar (mM)** IAA
- MS water
- 80 % EtOH
- 100 % EtOH
- Carboxylated Magnetic Beads (hydrophilic and hydrophobic)

- SpeedBead Magnetic Carboxylated, modified particles, Ref: 65152105050250 (**15 mL** , azide 0.05%, Sigma (?))

- SpeedBead Magnetic Carboxylated, modified particles, Ref: 45152105050250 (**15 mL** , azide 0.05%, Sigma (?))

- Ammonium bicarbonate (ABC) [M]**500 millimolar (mM)**

  100 µL of [M]**500 millimolar (mM)** ABC + **900 µL** MilliQ
- Trypsin ([M]**0.1 µg/µL** in [M]**50 millimolar (mM)** ABC) -> dissolved in **200 µL**
- 5% TFA
- MS buffer (3% ACN, 0.1% formic acid)
- iRT peptides (Biognosys) (**4 °C**)
- Waters glass vial

Preparing cryosection samples 1h 11m

1 Fill a **200 µL** PCR tube with **100 µL** 100% EtOH.

2 Wetten the dry tissue section on the glass slide with ca. **50 µL** 100% EtOH.

3 Use a 1.2x40mm needle (18Gx1.5) to gently scrape off the tissue section

4 Transfer the scraped off tissue to EtOH in PCR tube.

5 

1m

Spin down **00:01:00** at **12000 x g** .

6 

Remove 100% EtOH supernatant from tubes by using a **200 µL** pipette.

7 Heat the tubes on thermocycler at **50 °C** for ca. **00:10:00** .

10m

8 Continue with A) or B).
Step 8 includes a Step case.
A) Tissue lysis LCM samples
B) Tissue lysis cryosection samples

step case

A) Tissue lysis LCM samples

9 

Add **20 µL** RIPA lysis buffer to each sample: Add to tube, close tubes and shake down the liquid onto cap.

9.1

10

Preparing cryosection samples 1h 21m 30s

11 

1h

Incubate samples at **95 °C** on thermocycler for **01:00:00** .

11.1 LCM samples: upside-down, using magnetic rack for SP3 as a holder and stick tubes to rack with a tape.

12  10m

Centrifuge LCM samples for **00:10:00** at **2000 x g** .

13 

Take **5 µL** of RIPA in each sample tube, pipet onto cap and scrape off remaining tissue from cap. Pipet RIPA back into tube. (LCM samples only).

14  30s

Spin down for **00:00:30** at **2000 x g** .

15 

Check under microscope, whether tissue pieces are still sticking to the cap.

16 Pool all the blank samples into one tube (ca. **40 µL** in total).

Reduction and alkylation 30m

17

Final volume/concentrations:

- **2 millimolar (mM)** TCEP: Add **0.4 µL** of **100 millimolar (mM)** TCEP solution to **20 µL** of sample.

- **15.5 millimolar (mM)** IAA: Add **0.622 µL** of **500 millimolar (mM)** IAA

solution to **20 µL** of sample.

Prepare a reduction/alkylation stock solution for TCEP and IAA with the final concentrations from above.

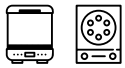
8.8 µL TCEP + **13.684 µL** IAA

18 

Add **1.022 µL** of reduction/alkylation stock solution to each sample (if volume is **20 µL**),
add **2.044 µL** to blank.

19 

Vortex

20  30m

Incubate samples at **60 °C** for **00:30:00** at **600 rpm** .

21 Spin down samples quickly.

Sp3 assisted protein capture and clean-up 45m

22

Hydrophilic and hydrophobic beads come at a stock concentration of **50 µg/µL** . Use **1 µL** of stock solution (**10 µg/µL**) for each µg of protein.

LCM samples: assuming a protein amount of **10 µg** per sample we need **10 µL** stock solution per sample: 15(+1) samples x **10 µL** = **160 µL** stock.

Cryosection samples: ca. **60 µL** total protein per sample, so we need **60 µL** stock solution: 4 samples (+1) x **40 µL** = **200 µL** .

Prepare: **360 µL** at **10 µg/µL** = **3600 µg** beads in total (**1800 µg**

hydrophilic beads + **1800 µg** hydrophobic beads).

Prepare the Carboxylated Magnetic Beads stock solution (**360 µL**) with a concentration of **10 µg/µL** .

22.1 Gently shake flasks with beads to resuspend bottom layer.

22.2 

Mix hydrophilic and hydrophobic beads at a 1:1 ratio: **36 µL** hydrophilic and **36 µL** hydrophobic beads (conc: **50 µg/µL**).

22.3 Put beads on rack and remove liquid.

22.4 

Wash the beads 3 times with water at a concentration of **50 µg/µL** :
3600 µg beads
at **5 µg/µL** = **720 µL** total volume.

22.5 Resuspend the beads in water at a working concentration of **10 µg/µL** :
3600 µg beads
at **10 µg/µL** = **360 µL** water total.

23 Protein binding to beads.

23.1 

Add 100% EtOH to the sample to reach 80% EtOH (v/v).

- LCM samples: add **80 µL**
- Cryosection samples: add **400 µL**

23.2

Add magnetic beads stock to samples (stock concentration: [M] **10 µg/µL** , **100 µg** or **600 µg** beads)

- LCM samples: add **10 µL**
- Cryosection samples: **60 µL**

23.3 45m

Incubate for **00:45:00** at **Room temperature** and **800 rpm** .

23.4 Spin down quickly.

24

Wash beads.

24.1 Insert the tubes into magnetic stand to collect the magnetic beads.

24.2 Remove supernatant and discard.

24.3

Add 80% EtOH (2 times the sample volume):

- LCM samples: use **200 µL**
- Cryosection samples: use **960 µL**

24.4 3m

Shake **00:03:00** at **Room temperature** , **800 rpm** .

24.5

Collect beads in-between washed on magnetic stand, discard wash solution.

24.6 Repeat in total 3x.

Trypsin Digestion

25

1. Make a trypsin stock solution in **[M]50 millimolar (mM)** ABC for LCM and cryosection samples separately:

- **LCM samples:** final concentration = **[M]0.005 µg/µL** , final volume = 15(+1) * **20 µL** = **320 µL**

  Mix **304 µL** ABC (**[M]50 millimolar (mM)**) with **16 µL** trypsin (at **[M]0.1 µg/µL** in **[M]500 millimolar (mM)** ABC).

- **Cryosection samples:** final concentration = **[M]0.025 µg/µL** , final volume = 4 * **60 µL** (+ **10 µL**) = **250 µL**

  Mix **187.5 µL** ABC (**[M]50 millimolar (mM)**) with **62.5 µL** trypsin (at **[M]0.1 µg/µL** in **[M]500 millimolar (mM)** ABC).

26

Add trypsin stock solution to each sample:

- **LCM samples:** add **20 µL** to LCM samples (contains ca. **0.1 µg** trypsin).
- **Cryosection samples:** add **60 µL** l to reference samples (contains **1.2 µg** trypsin).

27

Digest overnight at **37 °C** .

Peptide Extraction 15m

28 Spin the tubes down quickly.

29 Place tubes onto magnetic stage and transfer supernatant into a new **1.5 mL** Eppendorf tube.

30 

Add ddH2O to magnetic beads

- LCM samples: add **30 µL**
- Cryosection samples: add **70 µL**

31 Sonicate for **00:15:00** . 15m

32 Spin down quickly.

33 Combine supernatant in the same Eppendorf tube as in step 1 above.

34 Quench digestion by adding 5% TFA

- LCM samples: add **10 µL**
- Cryosection samples: add **26 µL**

35 Check pH.

Drying and resuspension 10m

36 Snap freeze samples and place open collection tube in a vacuum evaporator (until completely dry).

37 Freeze samples or resuspend.

38 For resuspension: Prepare iRTs in MS buffer at a ratio of 1:20.

39 Resuspend samples in **10 µL** (LCM) or **20 µL** (cryo) MS buffer + iRT.

40 Sonicate for **00:10:00** at **Room temperature** . 10m

41 Spin down quickly.

42 Transfer the whole sample into a Waters glass vial.