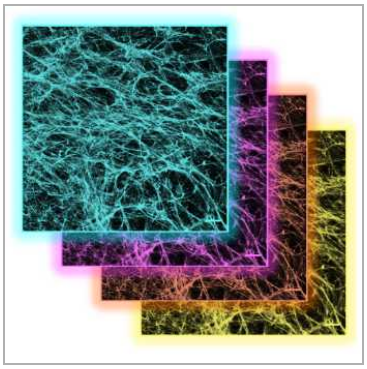

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In-solution Digestion of ECM-Enriched Proteins Samples for Mass Spectrometry Analysis

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ABSTRACT

The pellet obtained after the decellularization procedure and removal of SDS is highly enriched in insoluble ECM proteins. For further analysis by mass spectrometry, these proteins need to be digested into peptides. Note that, as a consequence of ECM protein insolubility, it is not possible at this step to measure the protein concentration of the sample. We thus provide volumes of reagents to digest the ECM-enriched samples into peptides based on the size (mm) or dry weight of the ECM-enriched pellet (Table 1).

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MATERIALS

Reagents:

1. HPLC-grade water **Thermofisher Catalog #51140**
2. Urea **Merck MilliporeSigma (Sigma-Aldrich) Catalog #U1250-1KG**
3. Dithiotreitol (DTT) **Thermofisher Catalog #A39255** *
4. Ammonium bicarbonate (NH4HCO3) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #AC393212500**
5. Iodoacetamide **Thermofisher Catalog #A39271** *
6. Peptide -N-Glycosidase F (PNGaseF) **New England Biolabs Catalog #P0704S**
7. LysC mass spectrometry-grade **Thermofisher Catalog #90307** §
8. Trypsin mass spectrometry-grade **Thermo Fisher Catalog #90058** §
9. Trifluoro-acetic Acid **Thermo Fisher Catalog #85183**

10. Acetonitrile mass spectrometry-grade **Thermo Scientific Catalog #51101**

* Reagent is reconstituted in ammonium bicarbonate

§ Reagent is reconstituted in HPLC grade water to its concentration

Table 1: Volume of reagents to digest ECM-enriched samples into peptides

Reagents	**Preparation**	**Final Concentration / Amount for 1mm-thick pellet (~5-10mg dry weight)**	**Volume for 1mm-thick pellet (~5-10mg dry weight)**
Ammonium bicarbonate (NH4HCO3)	100mM solution in HPLC-grade water	-	-
Urea	8M solution in 100mM ammonium bicarbonate	8M	50µL
Dithiotreitol	Reconstitute in HPLC-grade water at 500mM	10mM	1µL
Iodoacetamide	Reconstitute in HPLC-grade water at 250mM	25mM	5µL
Peptide -N-Glycosidase F (PNGaseF)	Commercial solution at 500U/µL	1000U	2µL
Endoproteinase LysC, mass spectrometry-grade	Reconstitute in HPLC-grade water at 0.5 µg/µL	1µg	2µL
Trypsin, mass spectrometry-grade *(round 1)*	Commercial solution at 0.5 µg/µl	3µg	6µL
Trypsin, mass spectrometry-grade *(round 2)*	Commercial solution at 0.5 µg/µl	1.5µg	3µL
Trifluoro-acetic acid (TFA)	50% solution in HPLC-grade water	-	2 – 5µL
Acetonitrile *(elution)*	50% solution with 0.1% TFA in HPLC-grade water	-	500 µL
Acetonitrile *(reconstitution)*	5% solution with 0.1% Formic acid in HPLC-grade water	-	100 µL

BEFORE START INSTRUCTIONS

- The solutions of ammonium bicarbonate, urea, DTT, iodoacetamide and trifluoroacetic acid all need to be freshly prepared.
- We recommend using low retention microcentrifuge tubes and tips to maximize recovery

Protein resuspension and reduction

2h

1 Resuspend the ECM-enriched sample by adding the appropriate volume of **8 M *urea*** (**urea** is dissolved in **100 mM ammonium bicarbonate**) to the ECM-enriched pellet and add **Dithiothreitol(*DTT*)** at a final concentration of **10 mM** (see Table 1). Incubate with continuous agitation at 1400 rpm for 02:00:00 at 37 °C. 2h

Note

- The 8M urea solution needs to be prepared fresh.
- At this point the ECM proteins will not be fully dissolved and the visibly large protein "particles" should not be discarded by centrifugation or filtration. The suspension will clear upon deglycosylation and digestion (see video: https://www.jove.com/t/53057/enrichment-extracellular-matrix-proteins-from-tissues-digestion-into).

Protein alkylation

30m

2 Protein alkylation

2.1 Prepare a **250mM *iodoacetamide*** solution in **100 mM ammonium bicarbonate**.

2.2 Cool the sample to Room temperature and add the ***iodoacetamide*** to a final concentration of **25 mM**. For complete alkylation, the DTT:iodoacetamide ratio should be between 1:2.5 and 1:3.

2.3 Incubate in the dark for 00:30:00 at Room temperature. 30m

Protein deglycosylation

2h

3 Deglycosylation is needed to remove carbohydrate side chains that interfere with identification of peptides modified by N-linked glycosylation.

3.1 Dilute to **2 M *urea*** with **100 mM *ammonium bicarbonate*** and add the appropriate amount of ***PNGaseF*** (see Table 1). Incubate with continuous agitation at 1400 rpm for 02:00:00 at 37 °C. 2h

Digestion - Day 1

4h

4 Digestion - Day 1

4.1 Add ***Lys-C*** *(refer to Table 1 for volume amounts)* and incubate with continuous agitation at 1400 rpm for 02:00:00 at 37 °C. 2h

4.2 Add ***trypsin*** *(refer to Table 1 for volume amounts)* and incubate with continuous agitation at 1400 rpm Overnight at 37 °C 2h

Note

- The ECM-rich suspension that began cloudy upon initial reconstitution in 8M urea appears clear after overnight digestion (see video).

Digestion - Day 2

2h

5 Digestion - Day 2

5.1 Add a second aliquot of ***trypsin*** *(refer to Table 1 for volume amounts)* to the sample and 2h

incubate with continuous agitation at 1400 rpm for an additional 02:00:00 at 37 °C.

Acidification

5m

6 Acidification

6.1 Upon completion of the digestion, the ***trypsin*** is inactivated by acidifying the sample with freshly prepared ***50% trifluoro-acetic acid (TFA) in HPLC-grade water***. The sample should reach < pH 2

Note

- We suggest adding 1 – 1.5µL of 50% TFA at a time and using 1µL of the peptide solution to measure the pH of the solution using pH paper (see video: https://www.jove.com/t/53057/enrichment-extracellular-matrix-proteins-from-tissues-digestion-into).).

6.2 5m

Centrifuge the acidified sample at 16000 x g for 00:05:00 at Room temperature. Collect the supernatant in a clean low-retention tube. At this point, the peptide solution can be stored at -20 °C prior to desalting:

Protocol

NAME

Desalting of Peptides to Prepare for Mass Spectrometry Analysis

CREATED BY

Ikram Isa

PREVIEW

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