Recommended protocol

LCMS shotgun proteomics for tissue samples

Introduction

Shotgun proteomics, or bottom up, starts with total enzymatic digestion of the proteome usually with trypsin. The resulting peptides are then separated and finally identified and quantified using mass spectrometry. Differences in protein abundance and levels of post-translations modification (PTMs), such as phosphorylations, are based on peptide intensities. There are several shotgun strategies that enable quantification of protein levels between samples as well as more targeted approaches focusing on specific PTMs.

Shotgun proteomics in biological matrices is, just like other protein analysis techniques, sometimes compromised by sample degradation. When using the Stabilizor™ system, active enzymes causing protein degradation is halted and thereby the protein content is unaltered. However, when working with stabilized samples it is important to homogenize thoroughly. Consideration must also be taken with regards to the selection of extraction buffer and the buffer-to-sample ratio. The best results are found when extraction is performed in denaturing buffers and the buffer-to-sample ratio is greater than 10, *i.e.* at least 10 µl of buffer for each mg of sample.

The following protocol have been tested to work well together with stabilized samples; however, other protocols may also be applicable but should be tested for compatibility with the Stabilizor system and downstream assays.

Specific protocol

Sample collection

1. Make sure that everything is ready for stabilization prior to sampling.
2. Extract sample and place in Maintainor® Tissue card.
3. Immediately stabilize in Stabilizor instrument according to Stabilizor manual using the appropriate program mode.
   Alternatively, the sample can be frozen and stabilized at a later stage.
4. If necessary, sub-dissect samples after stabilization but before freezing.
5. If you do not intend to analyze the samples directly, store samples at -80°C.
6. Weigh samples together with pre-weighed centrifugation tubes. If you intend to use micro pestles and grinding for sample homogenization, avoid collecting samples larger than 50 mg per 1.5 ml tube, because of the risk of sample loss during open-tube homogenization.
7. Calculate sample weight.

Protein extraction

Transfer the stabilized tissue to buffer A (6 M urea/2 M thiourea in 10 mM HEPES (pH 8.0)). Add at least 10 times the amount of sample, i.e. 10 µl of buffer for each mg of sample. For samples less than 10 mg use at least 100 µl of buffer. (See buffer composition on last page).

1. Homogenize
2. Clear the homogenate by centrifugation 25 min at top speed (20,000xg) for 4°C
3. Collect supernatant, without disturbing the pellet, into a new tube (store sample in -80°C freezer or continue with digestion)
4. Measure protein concentration (see below)

Homogenization

Stabilized samples generally homogenize well but it is very important that the samples are thoroughly homogenized to facilitate solubilization of proteins. Soft tissue such as brain can be homogenized using just microtip sonication rod whereas firmer tissues such as liver or heart need a more rigid homogenization method such as grinding or ball mill pulverization before sonication. The combination of of physical homogenization followed by rod sonication is recommended for best extraction.

Sample grinding using pestle for 1.5 ml tube

1. Use a pestle directly in an ordinary 1.5 ml centrifugation tube without the abrasive material (if any). It is commonly included abrasive material to sample grinding kits; however, this does not increase extraction efficiency for stabilized tissue samples.
2. Crush sample coarsely prior to adding extraction buffer.
3. Add 10 µl of extraction buffer for each mg of sample. Add the buffer in two steps and homogenize after each addition. Start by adding 30% of intended buffer and after initial homogenization, add the remaining buffer and continue homogenization.
4. Homogenize for at least 2 min using pestle; continue even when no traces of coarse material remain.
5. An additional sonication step is recommended to ensure complete solubilization of proteins.
6. Centrifuge at top speed for 25 min to sediment cellular debris.
7. Carefully recover supernatant into a new tube without disturbing the pellet.
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Ball mill

1. Homogenization is preferentially done frozen, with tissue pieces smaller than 1 mm3. The tube holder and the sample tube, together with balls, should be cooled in liquid nitrogen prior to adding the sample.
2. Homogenize frozen sample for 1 min at maximum frequency or until fully pulverized.
3. Add 10 µl of extraction buffer for each mg homogenized sample, while balls are still in tube. Mix while sample thaws and remove balls using a magnet when they are no longer covered with sample.
4. An additional sonication step is recommended to ensure complete solubilization of proteins.
5. Centrifuge at top speed for 25 min to sediment cellular debris.
6. Carefully recover supernatant into a new tube without disturbing the pellet.

Sonication

1. The combination of physical homogenization followed by rod sonication is recommended for best extraction.
2. Using a microtip, sonicate at 40 W output for at least 20 s or until no traces of coarse material remain, preferably in pulses of 2 s.
3. Centrifuge at top speed for 25 min to sediment cellular debris.
4. Carefully recover supernatant into a new tube without disturbing the pellet.

Protein concentration measurement

Measure protein concentration in sample using a urea-compatible protein measurement kit, *e.g.* 2-D Quant kit (GE Healthcare) or dilute sample fourfold and measure using BCA kit, *e.g.* BCA Protein Assay Kit (Pierce).

Subsequent steps in the shotgun work flow, such as reduction/alkylation, proteolytic digestion, pre-fractionation using ion exchange and mass spectrometric analysis are not affected by the Stabilizor T1 system and traditional

protocols can be followed without alterations as long as they are compatible with the sample buffer (6 M urea/2 M thiourea or 8M urea). However, Denator has successfully tested the following protocol.

Safety Information

When working with chemicals, always wear suitable protective clothing, disposable gloves, and protective goggles. For more information please consult the appropriate material safety data sheets (MSDS).

Recommended digestion protocol

Digestion

5. Add 1 µl Reduction buffer C/10 µl cleared supernatant
6. Incubate for 30 min. at room temp
7. Add 1 µl Alkylation buffer D/10 µl solution
8. Mix well and, incubate for 20 min. at room temp. (keep dark)
9. Measure protein concentration (see below)
10. Add 1 µl LysC solution buffer E /200 µg protein (if protein concentration is too high it is advisable to adjust to a smaller volume ending with approx 10 mg protein)
11. Incubate for at least 3 h at room temp
12. Dilute sample 4-fold to a final concentration of less than 2M urea with Digestion buffer B
13. Remove a small aliquot, e.g. 30 µl, for SDS-PAGE analysis.
14. Add 1 µl Trypsin solution buffer F /50 µg protein
15. Incubate over night at room temp
16. Add TFA to reduce pH ≈ 2
17. Centrifuge (20 min, 20.000 x g) and collect supernatant into clean tube
18. Analyze the sample before and after digestion on a polyacrylamide gel to check for incomplete digestion

Buffers

A. Extraction buffer: 6M Urea, 2M Thiourea, 40mM HEPES, 40ml

	Final concentration	Amount
Urea (FW 60.06)	6M	14.41 g
Thiourea (FW 76.12)	2M	6.09 g
HEPES (FW 238,3)	10mM	95.3 mg
Double distilled H2O		To 40 ml

B. Digestion buffer: 50 mM ammonium bi-carbonate, 10ml

	Final concentration	Amount
Ammonium bi-carbonate* (FW 79.06)	50mM	39.5 mg
Double distilled H2O		To 10 ml

*Adjust buffer to (pH 8.0)

C. Reduction buffer: 10 mM dithiothreitol (DTT) in 50 mM ammonium bi-carbonate, 1ml

	Final concentration	Amount
Dithiothreitol (FW 154.24)	10 mM	1.5 mg
Digestion buffer (buffer B)		To 1000 µl

D. Alkylation buffer: 55 mM chloroacetamide in 50mM ammonium bi-carbonate, x ml

	Final concentration	Amount
Chloroacetamide (FW 93,52)	55 mM	5.14 mg
Digestion buffer (buffer B)		To 1000 µl

E. LysC Solution: 0.5 µg/µL LysC (Lys-C 0.5 µg/µl WAKO #129-02541 Lysyl Endopeptidase) in 50 mM ammonium bi-carbonate, x ml

	Final concentration	Amount
Lysyl Endopeptidase	0,5 µg/µl	500 µg
Digestion buffer (buffer B)		To 1000 µl

F. Trypsin solution: (0.4 µg/µL trypsin (modified, sequencing grade, Promega) in 50 mM ammonium bi-carbonate, 1000 µl

	Final concentration	Amount
Trypsin	0,4 µg/µl	400 µg
Digestion buffer (buffer B)		To 1000 µl