Recommended protocol

LCMS peptidomics

Introduction

Mass spectrometric analysis of endogenous peptides and neuropeptides in biological tissues is easily compromised by a rapidly emerging background of peptides from ex vivo degrading proteins. However, when stabilizing the samples with Stabilizor™ system, the active enzymes causing protein degradation is halted and thereby the protein/peptide content is unaltered. When working with stabilized samples, a few aspects should be especially considered:

- Perform homogenization thoroughly
- Consideration must be taken to the selection of appropriate extraction buffer and the buffer-to-sample ratio

High-quality peptidomics results are found when extraction is performed in water-based buffers, and the buffer-to-sample ratio is greater than 5, *i.e.* at least 5 µl of buffer for each mg of sample.

The protocol is primarily written with brain tissue samples in mind; however a majority of the steps is also valid for other sample types. Other protocols may also be applicable but should be tested for compatibility with Stabilizor system.

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 T1 instrument using the appropriate program mode. Alternatively, the sample can be frozen and stabilized at a later stage.
4. If you do not intend to analyze the samples directly, samples should be stored in a freezer.
5. If necessary, sub-dissect samples after stabilization but before freezing.
6. Weigh samples using pre-weighed sample containers, e.g. 1.5 ml centrifugation tubes. If you intend to use the Microcon spin filter device (Peptide Extraction Kit, Denator) for peptide separation, avoid collecting samples smaller than 20 mg because of the small sample loss in the spin filter.
   If the samples are frozen, work efficiently and keep samples on dry ice while weighing the centrifugation tubes.
7. Calculate weight of the sample.

Extraction buffer

Peptides in stabilized samples can be extracted in water based buffers, *e.g.* 0.25 % acetic acid in H2O, pH 3.1 or similar. However, we recommend using Stabilizor peptide extraction kit (DST0001) developed for stabilized tissue samples. It is important to add an appropriate amount of buffer, at least 5 µl of buffer for each mg of sample.

Homogenization

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

Ball mill

1. Homogenization is preferably done frozen. The tube, tube holder and homogenization balls should be pre-cooled in liquid nitrogen. The balls should be placed in the tube prior to adding the sample.
2. Homogenize frozen sample for 1 min at maximum frequency.
3. Add extraction buffer (≥5 µl of buffer for each mg of sample) to the homogenized sample, while balls are still in tube. Mix while sample thaws and remove balls using a magnet.
4. An additional sonication step is recommended to ensure complete solubilization of proteins.
5. Centrifuge sample for 25 min at top speed to sediment cellular debris.
6. Carefully recover supernatant into a new tube without disturbing the pellet.
   Protein quantification (Optional for semi-quantitative measurements)
   Measure protein concentration in sample using a protein measurement kit, e.g. BCA kit (Pierce).

Microtip sonication

Homogenization may be done in frozen state as well as in room temperature.

1. Add extraction buffer to the sample (≥5 µl of buffer for each mg of sample)
2. Homogenize the sample at high enough amplitude to disintegrate the tissue, for approximately 30 s.
3. Centrifuge sample for 25 min at top speed to sediment cellular debris.
4. Carefully recover supernatant into a new tube without disturbing the pellet.

Peptide separation

1. Add extraction buffer to level out any concentration differences according to the results from the protein concentration measurement.
2. Centrifuge the extract at maximum speed (20,000 x g) for 30 min.
3. Re-centrifuge the supernatant using a 10 kDa Microcon cut-off filter device (Peptide Extraction kit, Denator) or similar.

Subsequent steps in the peptidomics work flow are not affected by the Stabilizor system and traditional LCMS protocols can be followed without alterations.