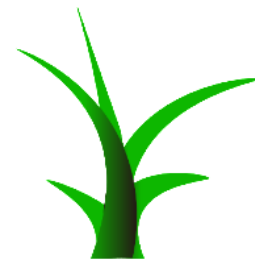


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Proteomic protocol for Tibetan hulless barley under osmotic stress

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We use this protocol and it's working

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Abstract

This protocol describes the complete procedures for performing data-independent acquisition-based analysis from Tibetan hulless barley samples under osmotic stress.

Troubleshooting

Safety warnings


- ⚠ Wear labcoat and gloves at every step of the protocol. Formic acid and acetonitrile can be harmful, handle with care.


Before start


Prepare solutions as described in the steps.


Protein extraction


- 1 Weigh 1-g subsample and homogenized by grinding in liquid nitrogen, moved the powdered samples to 50 cm³ tubes;
- 2 Add 25 cm³ precooled acetone (-20 °C) containing 10% (v/v) trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT);


 25 mL precooled acetone (-20 °C)
- 3 Mix for 2 min, the homogenate was precipitated overnight at -20 °C and then centrifuged (20,000×g, 4 °C) for 30 min;


 Overnight homogenate at -20 °C


 20000 x g, 4°C, 00:30:00
- 4 Wash the pellet with 20 cm³ chilled acetone (-20 °C) and left at -20 °C for 30 min followed by centrifugation (20,000×g, 4 °C) for 30 min, repeat this step for twice;

 00:30:00

 20000 x g, 4°C, 00:30:00
















 20000 x g, 4°C, 00:30:00
- 5 Remove the supernatant and add lysis buffer (4% SDS, 100 mM Tris-HCl, 10 mM DTT, pH 8.0) in the precipitate;
- 6 Sonicate for 5 min at 60 W (5 s sonication followed by 10 s break);

 00:05:00 sonicate at 60 W
- 7 Centrifuge for 30 min (20,000×g, 20 °C) and collect the supernatant;

 20000 x g, 20°C, 00:30:00
- 8 Estimate the protein concentration using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, China)



Protein digestion using the FASP (filter-aided sample preparation) procedure

- 9 Mix up to protein extract with 200 mm³ UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) and centrifuged at 14,000 g at 20°C for 40 min.
 -  200 µL UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0)
 -  14000 x g, 20°C, 00:40:00
- 10 Add 200 mm³ of UA to the filter unit and centrifuge at 14,000 x g for 40 min. Discard the flow-through form the collection tube. Repeat this step twice.
 -  200 µL UA
 -  14000 x g, 00:40:00
- 11 Add 100 mm³ IAM solution (10 mM IAM in UA buffer) and incubate for 30 min.
 -  100 µL IAM solution (10 mM IAM in UA buffer)
 -  00:30:00 incubate
- 12 Centrifuge the filter units at 14,000 x g for 30 min.
 -  14000 x g, 00:30:00
- 13 Add 100 mm³ of UA to the filter unit and centrifuge at 14,000 x g for 40 min. Repeat this step twice.
 -  100 µL UA
 -  14000 x g, 00:40:00
 -  14000 x g, 00:40:00
- 14 Add 100 mm³ of ABC (0.05 M NH₄HCO₃ in water) and centrifuged at 14,000 g. Repeat this step twice.
 -  100 µL ABC (0.05 M NH₄HCO₃ in water)
 -  14000 x g, 00:40:00
 -  14000 x g, 00:40:00
- 15 Add 40 mm³ ABC with trypsin (Promega, USA) and incubated for 18 h at 37°C;
 -  40 µL ABC with trypsin (Promega, USA)
 -  18:00:00 incubate at 37°C
- 16 Transfer the filter units to new collection tubes.



- 17 Centrifuge the filter units at 14,000 x g for 10 min and collect the filtration.

 14000 x g, 00:10:00

Peptide fractionation by high-pH RP

- 18 Separate peptides using an LC-20AB HPLC system (Shimadzu, Kyoto, Japan) with a high-pH reversed-phase (high-pH RP) column (Phenomenon, Torrance, CA). Peptides were eluted at a flow rate of 0.8 cm³/min. Buffer A consisted of 10 mM ammonium acetate (pH 10.0), and buffer B consisted of 10 mM ammonium acetate and 90% v/v acetonitrile (pH 10.0). The following gradient was applied to perform separation: 100% buffer A for 40 min, 0-5% buffer B for 3 min, 5-35% buffer B for 30 min, 35-70% buffer B for 10 min, 70-75% buffer B for 10 min, 75-100% buffer B for 7 min, 100% buffer B for 15 min and, finally, 100% buffer A for 15 min. The elution process was monitored by measuring absorbance at 214 nm, and fractions were collected every 75 s. Finally, collected fractions (approximately 40) were combined into 12 pools. Each fraction was concentrated via vacuum centrifugation and reconstituted in 40 mm³ of 0.1% v/v formic acid.
- 19 Peptides were separated with a Dionex UltiMate 3000 RSLCnano system with an Acclaim PepMap C18 (3 μm, 100 Å, 75 μm x 50 cm) and emitted into a Thermo Q-Exactive HF tandem mass spectrometer. Solvent A was 0.1% formic acid in water, while solvent B was 0.1% formic acid in 98% acetonitrile. For each injection, 3 mm³ (approximately 3 μg) was loaded and eluted using a 90-minute gradient from 5 to 35% B followed by a 40 min washing gradient. Data were acquired using either data-dependent acquisition (DDA) or data-independent acquisition (DIA).