

Standard Operating Procedure

Collection and Preparation of Human Saliva Samples for Protein Quantification by LC-MRM-MS

Version # 1, Creation Date: February 2, 2017

1. Materials

- 1.1. The commercial kit
 - 1.1.1. Protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA)
 - 1.1.2. BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA)
- 1.2. Solid-phase extraction (SPE) kit (Waters, Milford, MA, USA)
 - 1.2.1. Oasis HLB μ Elution Plate
 - 1.2.2. μ Elution spacer
 - 1.2.3. 350 μ L 96-well ACQUITY collection plate
 - 1.2.4. 10 mL 24-well collection plate (used as a waste tray)
 - 1.2.5. Polypropylene mat cap for 96-well collection plate
- 1.3. Modified sequencing-grade trypsin (Promega, Madison, WI, USA)
- 1.4. Stable Isotope-labeled Standard (SIS) peptide standards
- 1.5. Ammonium bicarbonate
- 1.6. Sodium deoxycholate (DOC)
- 1.7. Tris(2-carboxyethyl) phosphine (TCEP)
- 1.8. Iodoacetamide
- 1.9. Formic acid
- 1.10. Trifluoroacetic acid (TFA)
- 1.11. Acetonitrile
- 1.12. Distilled deionized water
- 1.13. Safety Equipment
 - 1.13.1. Lab Gloves
 - 1.13.2. Lab Coat
 - 1.13.3. Lab Safety Glasses
- 1.14. Pipettes and pipette tips
- 1.15. Eppendorf microcentrifuge tubes (1.5 mL)
- 1.16. Lab trays for 1.5 mL tubes
- 1.17. Freeze- and solvent- resistant labels and markers

2. Equipment

- 2.1. Centrifuge
- 2.2. -80 °C freezer
- 2.3. -20 °C freezer
- 2.4. Vacuum concentrator

Standard Operating Procedure

Collection and Preparation of Human Saliva Samples for Protein Quantification by LC-MRM-MS

Version # 1, Creation Date: February 2, 2017

- 2.5. Shaking incubator
- 2.6. Positive pressure-96 processor (Waters)
- 2.7. Nitrogen gas

3. Safety

- 3.1. Appropriate safety apparel, including safety glasses, lab coat, and lab gloves.

4. Collection of Clinical Saliva Samples

- 4.1. The donors are asked to avoid eating, drinking, smoking, and using oral hygiene products for at least 1 hour prior to collection.
- 4.2. Saliva samples are collected only from participants who have reviewed and signed the appropriate Research Information and Participants' Consent Form.
- 4.3. The participant should rinse his/her mouth with water first. Then 3~4 mL unstimulated whole saliva is collected into the collection tube.
- 4.4. The collected saliva samples are centrifuged at 3000 x g for 15 minutes at 4°C to remove the pellet.
- 4.5. The resulting cleared supernatant is treated with a protease inhibitor cocktail and then aliquoted for storage at -80°C until use. The freeze thaw cycles before sample preparation should not be more than two times.
- 4.6. Each sample is assigned a sample ID.

5. Processing the Saliva Samples for Protein Enrichment and Digestion

- 5.1. The frozen saliva samples are thawed on ice.
- 5.2. The protein concentration in the saliva sample is measured using a BCA Protein Assay Kit. The saliva samples are aliquoted as 15 µg protein per tube for lyophilization.
- 5.3. The dried sample (15 µg protein) is dissolved in 15 µL of 25 mM ammonium bicarbonate.
- 5.4. After addition of 15 µL 10% sodium deoxycholate (DOC) to achieve complete dissolution, 81.4 µL of 25 mM ammonium is added to dilute the final DOC concentration.
- 5.5. The sample is reduced by incubating with 12.4 µL of 50 mM tris (2-carboxyethyl) phosphine (TCEP) at 60°C for 30 minutes.
- 5.6. The sample is alkylated by incubating with 13.8 µL of 100 mM iodoacetamide at 37°C for 30 minutes.
- 5.7. The modified sequencing-grade trypsin (0.75 µL, 1 µg/µL) is added to the reduced and alkylated protein samples at a 20:1 protein/enzyme ratio, and the sample is digested at 37°C for 9 hours.
- 5.8. The tryptic digestion is stopped by acidifying the sample with 6 µL of 10% formic acid and 1.5 µL of 10% trifluoroacetic acid (TFA) solution which contains a cocktail of 56 SIS peptide standards.

Standard Operating Procedure

Collection and Preparation of Human Saliva Samples for Protein Quantification by LC-MRM-MS

Version # 1, Creation Date: February 2, 2017

- 5.9. The digested sample is centrifuged at room temperature for 10 minutes at $16,000 \times g$ and 4°C . After effectively pelleting DOC and removing it from the digest, the sample is stored at -20°C before subsequent processing.
- 5.10. Samples are desalted and concentrated by solid-phase extraction using a Waters Oasis HLB μ Elution Plate.
 - 5.10.1. The resin in each well is rinsed with acetonitrile and equilibrated with $200 \mu\text{L}$ equilibration buffer (0.1% TFA and 0.1% formic acid).
 - 5.10.2. The salivary protein digest is loaded onto the plate, washed with water, and then eluted twice with $25 \mu\text{L}$ of 70% acetonitrile.
 - 5.10.3. Eluted samples are lyophilized to dryness. Samples are rehydrated with 0.1% formic acid (v/v) to produce a $0.25 \mu\text{g}/\mu\text{L}$ concentration for LC-MRM/MS analysis.

6. Storage of Saliva Samples

- 6.1. The frozen and lyophilized saliva samples are stored at -80°C . Make sure to clearly label each vial with the sample code.

7. Responsibility.

This SOP is written by: Hsiao-Wei Chen

This SOP is reviewed by: Yi-Ting Chen

This SOP is authorized by: Yi-Ting Chen

Institution: Proteomic core laboratory, Molecular Medicine Research Center, Chang Gung University, Taiwan.

