

STANDARD OPERATING PROCEDURE

Title: Trypsin Digestion of Human Plasma

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Author: Paulovich Lab

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Purpose

The purpose of this document is to describe the enzymatic digestion of human plasma for protein and peptide analysis by mass spectrometry. The procedure has been previously described by Schoenherr, R. M., *et al.*¹.

Scope

This procedure may be used to denature, reduce, alkylate, and proteolyze human plasma samples.

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

Equipment

- Incubator and shaker (Innova 40, New Brunswick Scientific/Eppendorf)
- Extraction manifold, 20 position (Waters)
- Savant™ SPD111V SpeedVac (Thermo Scientific)

Materials

- 50 mL conical centrifuge tubes (352070, Corning Life Sciences)
- 15 mL conical centrifuge tubes (352196, Corning Life Sciences)
- 1.7 mL microcentrifuge tubes (24-282, Genesee Scientific)
- Desalting columns, Discovery® DSC-18 SPE Tube (52607-U, Supelco, Sigma)
- Human plasma (P9523-5 mL, Sigma)
- Distilled water

- Urea (U0631, Sigma)
- 1 M Trizma hydrochloride buffer, Tris (T2694, Sigma)
- Dithiothreitol, DTT (20291, Pierce)
- Iodoacetamide, IAM (90034, Pierce)
- Trypsin (T1426-250MG, Sigma)
- Formic acid (1.11670.1000, EMD Millipore)
- Water, Optima® LC/MS, suitable for UHPLC-UV (W6-4, Fisher Scientific)
- Acetonitrile, Optima® LC/MS, suitable for UHPLC-UV (A955-4, Fisher Scientific)
- 10X Phosphate buffered saline (PBS) (BP39920, Fisher Scientific)

Solutions

- Prepare 1 M DTT fresh by adding 50 μ L of distilled water to a tube of 7.7 mg DTT. For 1.0 mL of plasma, 30 μ L of 1 M DTT is needed.
- Prepare 1 M IAM fresh by adding 50.3 μ L of distilled water to a tube of 9.3 mg of IAM. For 1.0 mL of plasma, 45 μ L of 1 M IAM is needed. Prepare this IAM solution toward the end of the plasma incubation with DTT, then keep the IAM solution away from light until use.
- Prepare a 0.15 mg/ μ L trypsin solution by adding slightly more than 15 mg of trypsin to a 1.6 mL microcentrifuge tube, then adding distilled water to yield a 0.15 mg/ μ L concentration.
- Prepare 0.1% formic acid in 80% acetonitrile in Optima® water by adding 200 mL of Optima® water to a 1 L volumetric flask, adding ~500 mL of acetonitrile, then 1.0 mL of neat formic acid, and finally adding more acetonitrile to the 1 L mark.
- Prepare 0.1% formic acid in 5% acetonitrile in Optima® water by adding ~500 mL of Optima® water to a 1 L volumetric flask, adding 50 mL of acetonitrile, then 1.0 mL of neat formic acid, and finally adding more Optima® water to the 1 L mark.
- Prepare 1X PBS from 10X PBS in distilled and deionized water.

Procedure

Reconstitution of lyophilized human plasma

1. Add 5.0 mL of distilled water to a vial of lyophilized human plasma and gently mix to resuspend the pellet. Let the vial sit at 4 °C for one hour.

Denaturation, reduction, and alkylation

2. Aliquot 5 x 1.0 mL of resuspended human plasma into five 50 mL conical centrifuge tubes. The following steps are based on reagent additions to one tube containing 1.0 mL of resuspended plasma.
3. Add 400 μ L of distilled water.

4. Add 1.44 g urea (final concentration will be 8 M urea; the urea does not dissolve immediately, but will dissolve once incubation at 60 °C is started; of note is that the urea adds considerably to the volume of the solution, and the final volume after the IAM addition (below) will be approximately 3.0 mL).
5. Add 150 μ L of 1 M Tris, pH 8.0 (final concentration will be 50 mM Tris).
6. Add 30 μ L of 1 M DTT (final concentration will be 10 mM DTT).
7. Incubate at 60 °C for 1 hour with shaking.
8. Add 45 μ L of freshly-prepared 1 M IAM (final concentration will be 15 mM IAM).
9. Incubate at room temperature in the dark for 30 minutes.

Proteolysis using Trypsin

10. Dilute the urea concentration to 1 M by adding 21 mL of 20 mM Tris, pH 8.0.
11. Add 20 μ L of 0.15 mg/ μ L trypsin to achieve a 1:20 (w/w) enzyme:protein ratio, with the human plasma having approximately a 60 mg/mL concentration (previously determined using a bicinchoninic acid (BCA) assay).
12. Incubate at 37 °C overnight with shaking.

Desalting the urea-containing digested plasma samples

Each Discovery® DSC-18 SPE desalting column (catalog # 52607-U) has a bed weight of 2 g and a capacity of 5%, and is therefore enough for 100 mg of protein. Given a 60 mg/mL protein concentration, one column is sufficient per 1 mL of plasma. Liquids can be passed through the columns at a rate of approximately 5 mL/minute. A suitably-sized syringe plunger can help push liquid through the columns in case the vacuum is not sufficient. Care should be taken to not let the top of the column beds become dry.

13. Attach the columns to the extraction manifold.
14. Place 15 mL conical tubes underneath each column inside the manifold to capture any wash or flow-through solutions.
15. Wash each column by passing 2 x 5 mL of 0.1 % formic acid in 80% acetonitrile through it using vacuum.
16. Condition each column by passing 2 x 5 mL of 0.1% formic acid in 5% acetonitrile through it using vacuum.
17. Add digested plasma sample to each column, ~5 mL at a time, and let most of the 5 mL pass through the column using vacuum or positive pressure before adding the next 5 mL, until the ~24 mL sample (for each 1 mL starting plasma) has passed through.
18. Wash each column by passing 2 x 5 mL of 0.1% formic acid in 5% acetonitrile through it using vacuum or positive pressure.
19. Place a clean 15 mL conical tube underneath each column inside the manifold to capture the eluted peptide sample.

20. Elute the peptides from each column by passing 2 x 5 mL of 0.1 % formic acid in 80% acetonitrile through it using vacuum or positive pressure.

Drying, resuspending, and freezing the desalted plasma digest samples

21. Mix the 10 mL eluted samples briefly in the 15 mL conical tubes, then aliquot 1.0 mL into ten 1.7 mL microcentrifuge tubes.
22. Place the microcentrifuge tubes in a SpeedVac and dry the samples (the samples might not dry completely, but rather 5-10 μ L of a yellowish, gel-like concentrate might be left).
23. Add 100 μ L of 1X PBS, pH 7.4 to each microcentrifuge tube (or slightly less, depending on whether complete dryness was achieved) and vortex to aid in the resuspension. Samples might be slightly cloudy after resuspension.
24. Pool all samples into a 15 mL conical tube and mix the pooled sample before aliquoting 1.0 mL into 1.7 mL microcentrifuge tubes.
25. Freeze the 1.0 mL aliquots at -80 $^{\circ}$ C until use.

Referenced Documents

1. Schoenherr, R. M., Zhao, L., Whiteaker, J. R., Feng, L. C., Li, L., Liu, L., Liu, X., and Paulovich, A. G. (2010) Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-selected reaction monitoring-mass spectrometry. *J Immunol Methods* 353, 49-61.