# Title: Trypsin Digestion for Mass Spectrometry Analysis of Protein Samples

## SOP#: M-143

**Version #: 1**

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## 1. PURPOSE

This procedure will describe how to perform a trypsin digestion on protein samples destined for analysis on HPLC-ESI-MS

## 2. SCOPE

This procedure applies to all protein samples (with MW above 3 KDa) being processed for analysis by mass spectrometry.

## 3. RESPONSIBILITIES

It is the responsibility of the person(s) executing this procedure to be familiar with laboratory safety procedures and to have basic laboratory skills. It is the responsibility of the analyst to follow the procedure and document any deviations and all observations in the laboratory notebook.

## 4. EQUIPMENT

4.1. Thermo LP Vortex Mixer (Thermo Scientific, 88880017) or equivalent

4.2. Spectrafuge Mini Centrifuge, Labnet International Inc. (Millipore-Sigma, S6941) or equivalent

4.3. Eppendorf Centrifuge 5810R with 1.5 mL tube rotor (Eppendorf, 5382000023) or equivalent

4.4. Eppendorf ThermoMixer C (Eppendorf, 022627040) or equivalent

4.5. Mettler AJ100 Analytical Balance (Mettler Toledo, discontinued) or equivalent

4.6. MyTemp Mini Digital Incubator, (Benchmark Scientific, H2200-HC) set at 37°C

4.7. Waters Positive Pressure-96 Processor (Waters, 186006961)

## 5. MATERIALS

5.1. Weighing Paper, 3” x 3”

5.2. Falcon BLUE MAX Jr. 15 ml Polypropylene Conical Tube

5.3. Falcon BLUE MAX Jr. 50 ml Polypropylene Conical Tube

5.4. Pipet-Lite LTS Pipette L-200XLS+ (or equivalent), adjustable 20-200 µL

5.5. Pipet-Lite LTS Pipette L-1000XLS+ (or equivalent), adjustable 100-1000 µL

5.6. Pipet-Lite LTS Pipette L-20XLS+ (or equivalent), adjustable 0.2-20 µL

5.7. Pipet-Lite LTS Pipette L-2XLS+ (or equivalent), adjustable 0.1-2 uL

5.8. Pipet-Lite Multi Pipette L12-300XLS+, adjustable 20-300 µL

5.9. Rainin LTS 20 tips, 0.1-20 uL (or equivalent)

5.10. Rainin LTS 250 tips, 20-250 µL (or equivalent)

5.11. Rainin LTS 300 tips, 20-300 uL (or equivalent)

5.12. Rainin LTS 1000, 100-1000 µL (or equivalent)

5.13. 5 mL serological pipettes

5.14. 50 mL serological pipettes

5.15. Amicon Ultra 0.5 mL 3000 MWCO Centrifugal Filter Unit (Millipore Sigma UFC5003BK)

5.16. SOLAµ SPE HRP (Reverse phase for hydrophobic retention, 2 mg/mL) Plates, Thermo Scientific 60209-001

5.17. Eppendorf Centrifuge Tubes, 1.5 ml

5.18. Corning 50 ml Reagent Reservoir, Polystyrene

## 6. REAGENTS

6.1. Seppro Ammonium Bicarbonate (ABC) Buffer 2M, Millipore Sigma S2454-200ML

6.2. 3 -[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), VWR 0465-500G

6.3. 20X ABC/CHAPS (50 mM ABC/1 % CHAPS)

6.3.1. Store at RT for up to 3 months

6.4. Urea, Millipore Sigma U5378-500G

6.5. Trizma hydrochloride solution pH 8.0 1 M, Millipore Sigma T2694-100MLTris

6.6. Iodacetamide, Single-Use, Thermo Scientific A39271

6.7. Dithiothreitol, Bio-Rad 1610611

6.8. Acetonitrile LC-MS grade, OmniSolv Cat., Number AX0156-1

6.9. Formic acid, LC-MS grade, Thermo Scientific TS-28905

6.10. Trypsin/Lys-C Mix, Mass Spec Grade, Promega V5073

## 7. DEFINITIONS

TUB – Tris Urea Buffer, Denaturing Buffer

ACN – Acetonitrile

FA – Formic Acid

DTT – Dithiothreitol

IAM – Iodoacetamide

HPLC - High-performance liquid chromatography

ESI – Electrospray Ionization

MS – Mass Spectrometry

## 8. PROCEDURE

### 8.1. Preparation of Denaturing Buffer

8.1.1. Fresh Tris Urea buffer (TUB) must be prepared for each experiment

8.1.2. Final buffer concentration is 8 M Urea, 0.1 M DTT, and 0.05 M Tris-HCl in water

8.1.2.1. Calculate the final volume needed for each experiment

8.1.2.2. Calculate required mass in grams for Urea and DTT and required volume in milliliters for Tris-HCl based on final volume from above

8.1.3. Weigh out Urea and DTT and add to an appropriately sized sample tube

8.1.4. Add half of the final volume of water and all of the required volume of Tris-HCl to the tube

8.1.5. Mix thoroughly until Urea has mostly dissolved

8.1.6. QS to final volume with water and mix until Urea/DTT has completely dissolved

**Preparation of Wash Buffer**

8.1.7. Prepare fresh 1X ABC/CHAPS buffer from 20X stock (1000 mM ABC/1 % CHAPS) and store for up to 1 month at room temperature (RT)

8.1.7.1. Label a 50 mL conical tube 1X ABC/CHAPS

8.1.7.2. Add 47.5 mL of DI water

8.1.7.3. Add 2.5 mL 20X ABC/CHAPS stock

8.1.7.4. Invert tube several times until well combined

8.2. **Denaturation & Reduction**

8.2.1. Dilute or resuspend protein samples in desired volume of denaturing (TUB) buffer (200 uL)

8.2.2. Incubate at room temperature with gentle shaking for 1 hour to reduce disulfide bonds

8.3. **Alkylation**

8.3.1. Prepare 500 mM IAM stock by adding 100 uL water to pre-weighed dried pellet as provided by the vendor and mixing until dissolved

8.3.2. Add sufficient volume of IAM stock to protein samples (from 8.2.2) for a final concentration of 50 mM IAM (do not exceed 500 uL)

8.3.3. Incubate at room temperature with gentle shaking while protected from light for 45 minutes.

8.4. **Buffer Exchange**

8.4.1. Transfer protein samples (from 8.3.3) from current tube to a 3000 MWCO 0.5 mL Ultracel filter cup

8.4.1.1. Adjust (qs) sample volume to 500 uL in ABC/CHAPS if necessary

8.4.2. Buffer exchange samples with 1X ABC/CHAPS until nominal Urea concentration is ≤ 1 M

8.4.2.1. **Spin** samples at 14,000 xg for 5 minutes to reduce volume in filter cup by ½

8.4.2.2. **Add** 250 uL of 1X ABC/CHAPS to samples in filter cup and gently mix with pipette

8.4.2.3. Discard flow through

8.4.2.4. Repeat **Spin** and **Add** steps until urea is sufficiently diluted

8.4.2.4.1. Determine what the appropriate number of 2-fold dilutions is to achieve the desired final concentration from the starting concentration (8 M Urea). Each spin/add step is a 2-fold dilution.

8.4.3. QS samples to 500 uL as needed after buffer exchange

8.4.4. Transfer filter cup containing sample to a fresh collection tube (supplied)

8.5. **Digestion**

8.5.1. Prepare Trypsin/LysC at 1 ug/mL from lyophilized stock using manufacturer provided buffer

8.5.2. One 20 ug aliquot of Trypsin/LysC is sufficient to digest 1 mg of total protein at a ratio of 1:50. Remaining Trypsin/LysC can be aliquoted and frozen at -80 C for 1 month. Spike in Trypsin/LysC to samples in filter cup (from 8.4.4) at a ratio of 1:50

8.5.3. Incubate overnight at 37°C with gentle shaking in saturated humidity to prevent evaporation.

8.6. **Stop Digestion & Collect Peptides**

8.6.1. After overnight incubation, spin filter cup (from 8.5.2) at 14,000 x g for 15 minutes to separate peptides from trypsin and undigested material

8.6.2. Collect flow through for analysis and discard remaining material in filter cup

8.7. **Reverse Phase SPE Clean up Using Positive Pressure Manifold**

8.7.1. Prepare SOLAμ HRP plate with adapter and flow through waste plate

8.7.2. Select and mark as many columns as needed for experiment, then process the samples using the following steps

8.7.2.1. Wet columns 1X with 200 uL 100% ACN

8.7.2.2. Switch on nitrogen to 10-15 psi on low flow setting or up to 20 psi on max flow setting until volume passes at 1 drop per second; flow until volume passes through

8.7.2.3. Condition columns 2X with 200 uL 0.1% FA in water

8.7.2.4. Switch on nitrogen to 10-15 psi on low flow setting or up to 20 psi on max flow setting until volume passes at 1 drop per second; flow until volume passes through

8.7.2.5. Load samples on columns to a maximum volume of 500 uL per column

8.7.2.6. Switch on nitrogen to 10-15 psi on low flow setting or up to 20 psi on max flow setting until volume passes at 1 drop per second; flow until volume passes through

8.7.2.7. Wash samples 2X with 500 uL 0.1% FA in water

8.7.2.8. Switch on nitrogen to 10-15 psi on low flow setting or up to 20 psi on max flow setting until volume passes at 1 drop per second; flow until volume (500 uL) passes through

8.7.2.9. Switch waste plate to collection plate

8.7.2.10. Elute samples 2X with 25 uL 70% ACN

8.7.2.11. Switch on nitrogen to 10-15 psi on low flow setting or up to 20 psi on max flow setting until volume passes at 1 drop per second; flow until volume passes through

8.7.3. Transfer eluates to 1.5 mL microcentrifuge tubes

8.7.4. Dry eluates in SpeedVac at 40°C

8.7.5. Store dry at least -20°C and reconstitute in 50 uL 0.1% FA when ready to analyze on the MS.

## 9. REFERENCED DOCUMENTS

Preparation of samples for intact protein immuno-precipitation (SOP, M-142)