# STANDARD OPERATING PROCEDURE

## Title: Preparation of Peptides from Tumor Lysates

## SOP#: WU-SOP- TD1-01

**Version #: 2**

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### 1. PURPOSE

This document describes the procedure for the digestion (i.e. trypsin) of proteins that are extracted from breast cancer patient-derived xenografts. The preparation of a desalted complex peptide mixture for utilization as a matrix for MS-based assay development is detailed.

### 2. SCOPE

This procedure is used to reduce, alkylate, and digest proteins as an unfractionated mixture for LC-MS analysis. A method for rapid, high-pressure trypsin digestion is provided.

### 3. 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.

### 4. EQUIPMENT

Barocycler NEP3229, Pressure Biosciences Inc.

Biomek NXp, Beckman Coulter

Speedvac SC250 Express, Thermo Savant

Sorval RC6 Plus, Thermo Scientific

Vantage Lyophilizer, SP Scientific

## 5. MATERIALS

| Item | Vendor | Catalog # | Unit |
|---|---|---|---|
| Advanced Protein Assay Reagent | Cytoskeleton | ADV01 | 500ml |
| DTT, No-Weigh Format, 7.7mg DTT per microtube | Thermo Scientific | 20291 | 48 microtubes |
| 2D clean up kit | GE Healthcare | 80-6484-51 | |
| Urea | GE Healthcare | 17-1319-01 | 500g |
| Tris | BioRad | 161-0719 | 1kg |
| TCEP Bond-breaker (tris (2-carboxyethyl) phosphine) | Thermo Scientific | 77720 | 5mL |
| Iodoacetamide | Sigma | A3221-10VL | 56mg per vial |
| Trypsin | Sigma | T6567-5x20µg | 20µg per vial |
| PCT microtubes | Pressure Bioscience | MT-96 | 96 per pack |
| PCT MicroCaps, 50µL and 100 µL | Pressure BioScience | MC50-96, MC100-96 | 96 per pack |
| Chromasolv Acetonitrile, LC-MS grade | Fluka | 34967 | 1L |
| Chromasolv Water, LC-MS grade | Fluka | 39253-1L-R | 1L |
| 1.7mL Eppendorf tubes | Axygen | MC-175-C | 500 per pack |
| Horseradish Peroxidase | Sigma | P-6782 | |
| Autosampler vials, 250 µL polypropylene crimptop | VWR | 200 046 | 1000/case |
| Snap caps for autosampler vials, 11mm, PTFE/DK blue silicone | SUN SRI | 501 390 | 1000/case |
| 15mL conical tubes | Fisher Scientific | 05-539-12 | 50 per pack |
| Sep-Pak Vac 3cc (200mg) tC18 Cartridges | Waters | WAT054925 | 50 per box |
| Ultracel YM-30 centrifugal filter | Millipore | MRCF0R030 | 100 per pack |
| Glass vial, 4mL | Fisher Scientific | 03-339-22B | 144 per pack |

## 6. REAGENTS

Preparation of reagent solutions for samples containing ~ **100 µg** of protein (up to 30 samples at a time)

**Solutions**:

**A.** 100 mM Tris Buffer, pH 8.5.

a) Add 0.61 g of Tris base into 40 mL of DI water. Stir until Tris is dissolved.
b) Adjust pH to 8.5 with concentrated hydrochloric acid.
c) Fill graduated cylinder to final volume of 50 mL with DI water.
d) Mix well and store at 4°C for up to 2 weeks.

**B.** 8M Urea, Tris Buffer

a) Weigh 480 mg of urea into a 1.7 mL Eppendorf tube.
b) Add 100 mM Tris buffer to the 1mL mark (~680 µL).
c) Vortex until urea is dissolved.
d) Mix well and discard at the end of the day.

**C.** 400 mM Iodoacetamide (alkylating reagent)
a) Add 760 µL of DI water to each vial of Iodoacetamide.
b) Vortex and dissolve.
c) Discard after IAM has been added to all samples.

**D.** 50 mM TCEP (reducing reagent)
a) Dilute TCEP 1:10 with DI water to prepare 500 µL (50 mM).
b) Make diluted solution just prior to reduction step.
c) Discard after TCEP has been added to all samples.

**E.** 200 mM DTT quench
a) Add 250 µl DI water to the DTT (7.7mg pre-weighed) in the microtube.
b) Mix well to dissolve.
c) Discard after DTT has been added to all samples.

**F.** Preparation of the internal standard solution
a) Weigh ~ 10mg of horseradish peroxidase into a 1.7mL Eppendorf tube.
b) Add 1 mL DI water to make a 10 mg/mL solution.
c) Vortex until the protein is dissolved.
d) Dilute to 0.25 µg/µL with DI water. Prepare 500 µL. add 12.5 µL of 10 mg/mL HRP solution to 487.5 µL of DI water.
e) Store 20 µL aliquots of diluted HRP solution at -20$^{O}$C.

**G.** Preparation of Trypsin. Must be prepared fresh just prior to usage.
a) Add 20 µL of Tris Buffer to the vendor vial of trypsin (20 µg).
b) Each vial is sufficient for four samples.

## 7. PROCEDURE

### A. INSTRUMENT SETUP FOR ENDOPROTEASE DIGESTION IN THE PRESSURE BIOSCIENCE BAROCYCLER

*NOTE: TURN ON THE BAROCYCLER AFTER ADDITION OF THE REDUCING REAGENT. BY THE TIME SAMPLES ARE READY FOR ADDITION OF TRYPSIN, THE BAROCYCLER SHOULD BE READY.*

1) Turn on the barocycler and test the barocycler with method 25 (35,000 psi, 90 sec duration, 5 sec at ambient, 2 cycles). The unit should come up to 35,000 psi and should not lose more than 1000 psi pressure for the 90 sec hold time of the method (see user manual for additional details).

2) Turn on water chiller and set to 37°C.

3) Allow 30 min for barocycler to reach set temperature.

### B. Digestion of solubilized tumor tissue with trypsin using high-pressure.

1) Prepare protein pellets (24 x 200 µg) in 1.7 mL tubes using the 2D clean-up kit and vendor instructions.

2) Add 40 µL of 8M urea in Tris Buffer to solubilize protein pellets.
3) Add 4 µL of the internal standard protein solution to each vial of solubilized protein.
4) Add 4.0 µL of the TCEP solution and incubate for 30 min at room temperature.
5) Add 4.4 µL of the IAM solution to alkylate reduced proteins. Incubate in the dark for 30 min at room temperature.
6) Quench the reaction by adding 2.2 µL of the DTT solution.
7) Incubate for 15 min at room temperature.
8) Add 10 µL of Trypsin to each vial followed by ~190 µL of the Tris Buffer to a final volume of 200 µL.
9) Using gel loading pipette tips, transfer 100 µL of sample to each PCT-Microtube and cap with "100 µL" caps.
10) Load them into the microtube carousel (for loading and operation details, see Section VIII).
11) Load carousel with samples into the barocycler and select digest method “Trypsin30” (25,000 psi for 20 sec, 5 sec at ambient pressure, 90 cycles, 30 min at high pressure, 60 min total run time).
12) Once digest program has finished, remove carousel from barocycler, unload microtubes and uncap.
13) Add 5 µL of trypsin to each sample.
14) Cap vials with "100 µL" caps and load them into the microtube carousel.
15) Load carousel into the barocycler and select digest method “Trypsin30” (as described above).
16) Once digest method is completed, remove microtube carousel from barocycler, unload and uncap microtubes.
17) Transfer samples to 15 mL conical tubes.
18) Add 10 µL of 10% trifluoroacetic acid to final concentration of 1%.
19) Filter samples through a 30K MWCO filter.

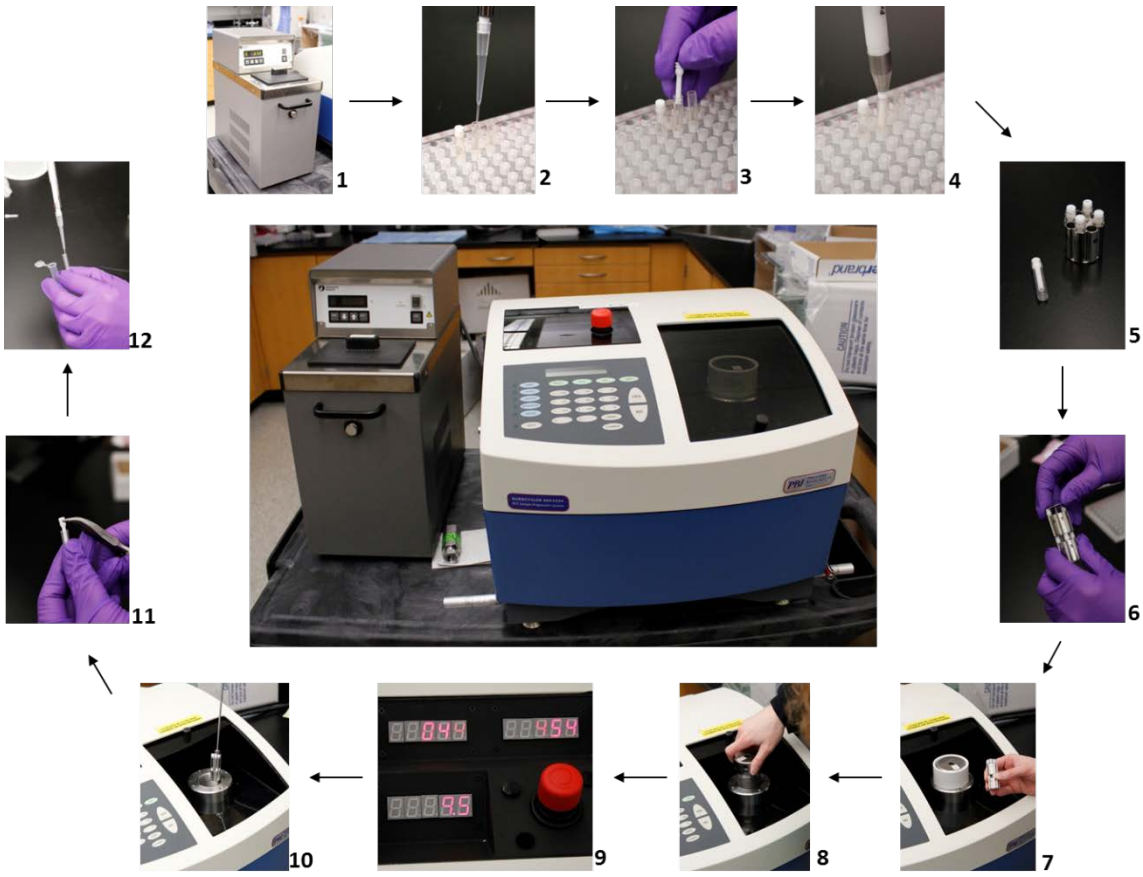
**C. <u>Solid phase extraction of peptides</u>**

1) Label 15mL conical tubes with the following labels: methanol, wash, equilibrate, desalt, 40% Elution, 70% elution. One of each is required per SepPak.
2) Wet SepPak with 6mL of 100% methanol. Collect flow-through in conical tube marked “methanol” and discard. Wash SepPak with 3mL of 80% Acetonitrile. Collect flow-through in conical tube and discard.
3) Equilibrate SepPak with 6mL of 1% Trifluoroacetic acid. Collect flow-through in conical tube and discard.

4) Load sample on SepPak using gravity flow. Collect flow through in a 15mL conical and re-load the sample onto the same SepPak.
5) Desalt SepPak with 6mL 2%Acetonitrile in 0.1% Trifluoroacetic acid. Collect flow-through and discard.
6) Elute peptides with 1mL 40% Acetonitrile in 0.1% Trifluoroacetic acid and collect flow-through in 4mL glass vial for lyophilization. Repeat for a total of 2mL elution volume.
7) Elute peptides with 2mL of 70% Acetonitrile in 0.1% Trifluoroacetic acid and collect flow-through in a second 4mL glass vial for lyophilization.
8) Freeze eluates at -80°C for 30minutes or until the lyophilizer shelf temperature has reached -60°C.
9) Turn on Vantage lyophilizer and set shelf temperature to -60°C. it will take 30-45 minutes to reach temperature.
10) Place frozen samples on the pre-chilled shelf with the cap half a turn open.
11) Turn on the condenser and set temperature to -70°C. Once temperature is reached, turn on the vacuum. Vaccuum is set to 200mTor.
12) Once vacuum is reached, bring shelf temperature to 0°C and let the samples lyophilize over night (approximately 16 hours).
13) Next day, break vacuum, turn off condenser and take lyophilized samples out when atmospheric pressure has been reached.
14) Solubilize lyophilized peptides in 1% formic acid solution containing 1% Acetonitrile to a theoretical concentration of 10µg/µL based on total protein input.
15) Freeze aliquots at -80°C for analysis use as a matrix for peptide assay development.

## 8. BAROCYCLER SETUP (SEE SECTION VII-A) AND OPERATION

**A.** Barocycler Set-up and Operation (abridged from PBI User Manual for NEP 3229)




1) Turning on chiller to equilibrate at 37°C
2) Loading the PCT micro-tubes with gel loading pipette tips
3) Placing the cap into the PCT microtube
4) Capping with the high-pressure cap-insert tool
5) Loading and balancing the carousel
6) Assembling the carousels
7) Loading the carousel assembly
8) Capping the high pressure chamber
9) Running a cycle
10) Removing the carousel assembly with the magnetic wand
11) Removing the cap with vendor supplied pliers
12) Transferring sample with a gel loading pipette tip.

## 9. REFERENCED DOCUMENTS


**A.** Lopez-Ferrer, D., Petritis, K., Robinson, E.W., Hixson, K.K., Tian, Z., Lee, J.H., Lee, S.W., Tolic, N., Weitz, K.K., Belov, M.E., et al. (2011). Pressurized pepsin digestion in proteomics: an automatable alternative to trypsin for integrated top-down bottom-up proteomics. Mol Cell Proteomics 10, M110 001479.

**B.** Sun, B., Ranish, J.A., Utleg, A.G., White, J.T., Yan, X., Lin, B., and Hood, L. (2007). Shotgun glycopeptide capture approach coupled with mass spectrometry for comprehensive glycoproteomics. Mol Cell Proteomics 6, 141-149.

**C.** Washburn, M.P. (2008). Sample preparation and in-solution protease digestion of proteins for chromatography-based proteomic analysis. Curr Protoc Protein Sci Chapter 23, Unit 23 26 21-23 26 11.

**D.** Zybailov, B., Coleman, M.K., Florens, L., and Washburn, M.P. (2005). Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. Anal Chem 77, 6218-6224.