

STANDARD OPERATING PROCEDURE

Title: Preparation of Peptides from Tumor Lysates

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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.

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.

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

- Barocycler NEP3229, Pressure Biosciences Inc.
- Biomek NXp, Beckman Coulter
- Speedvac SC250 Express, Thermo Savant
- Sorval RC6 Plus, Thermo Scientific
- Vantage Lyophilizer, SP Scientific.

Materials

Item	Vendor	Catalog #	Unit
2D clean up kit	GE Healthcare	80-6484-51	
PCT microtubes	Pressure Bioscience	MT-96	96 per pack
PCT MicroCaps, 50µL and 100 µL	Pressure BioScience	MC50-96, MC100-96	96 per pack
1.7mL Eppendorf tubes	Axygen	MC-175-C	500 per pack
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

Reagents

Item	Vendor	Catalog #	Unit
Advanced Protein Assay Reagent	Cytoskeleton	ADV01	500ml
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
Chromasolv Acetonitrile, LC-MS grade	Fluka	34967	1L
Chromasolv Water, LC-MS grade	Fluka	39253-1L-R	1L
Horseradish Peroxidase	Sigma	P-6782	
DTT, No-Weigh Format, 7.7mg DTT per microtube	Thermo Scientific	20291	48 microtubes

Solutions

100 mM Tris Buffer, pH 8.5.

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

8M Urea, Tris Buffer

- Weigh 480 mg of urea into a 1.7 mL Eppendorf tube.
- 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.

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.

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.

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.

Preparation of the internal standard solution

- a. Weigh $\sim 10\text{mg}$ 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 $\mu\text{g}/\mu\text{L}$ 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°C .

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.

Procedure

1. 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.

- a. 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).

- b. Turn on water chiller and set to 37°C.
- c. Allow 30 min for barocycler to reach set temperature.

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

- a. Prepare protein pellets (24 x 200 µg) in 1.7 mL tubes using the 2D clean-up kit and vendor instructions.
- b. Add 40 µL of 8M urea in Tris Buffer to solubilize protein pellets.
- c. Add 4 µL of the internal standard protein solution to each vial of solubilized protein.
- d. Add 4.0 µL of the TCEP solution and incubate for 30 min at room temperature.
- e. Add 4.4 µL of the IAM solution to alkylate reduced proteins. Incubate in the dark for 30 min at room temperature.
- f. Quench the reaction by adding 2.2 µL of the DTT solution.
- g. Incubate for 15 min at room temperature.
- h. Add 5 µL of Trypsin to each vial followed by ~130 µL of the Tris Buffer to a final volume of 200 µL.
- i. Using gel loading pipette tips, transfer 100 µL of sample to each PCT-Microtube and cap with "100 µL" caps.
- j. Load them into the microtube carousel (for loading and operation details, see Section VIII).
- k. 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).
- l. Once digest program has finished, remove carousel from barocycler, unload microtubes and uncap.
- m. Add 5 µL of trypsin to each sample.
- n. Cap vials with "100 µL" caps and load them into the microtube carousel.
- o. Load carousel into the barocycler and select digest method "Trypsin30" (as described above).
- p. Once digest method is completed, remove microtube carousel from barocycler, unload and uncap microtubes.
- q. Transfer samples to one 15 mL conical tube.
- r. Add 10 µL of 10% trifluoroacetic acid to final concentration of 1%.
- s. Filter samples through a 30K MWCO filter.

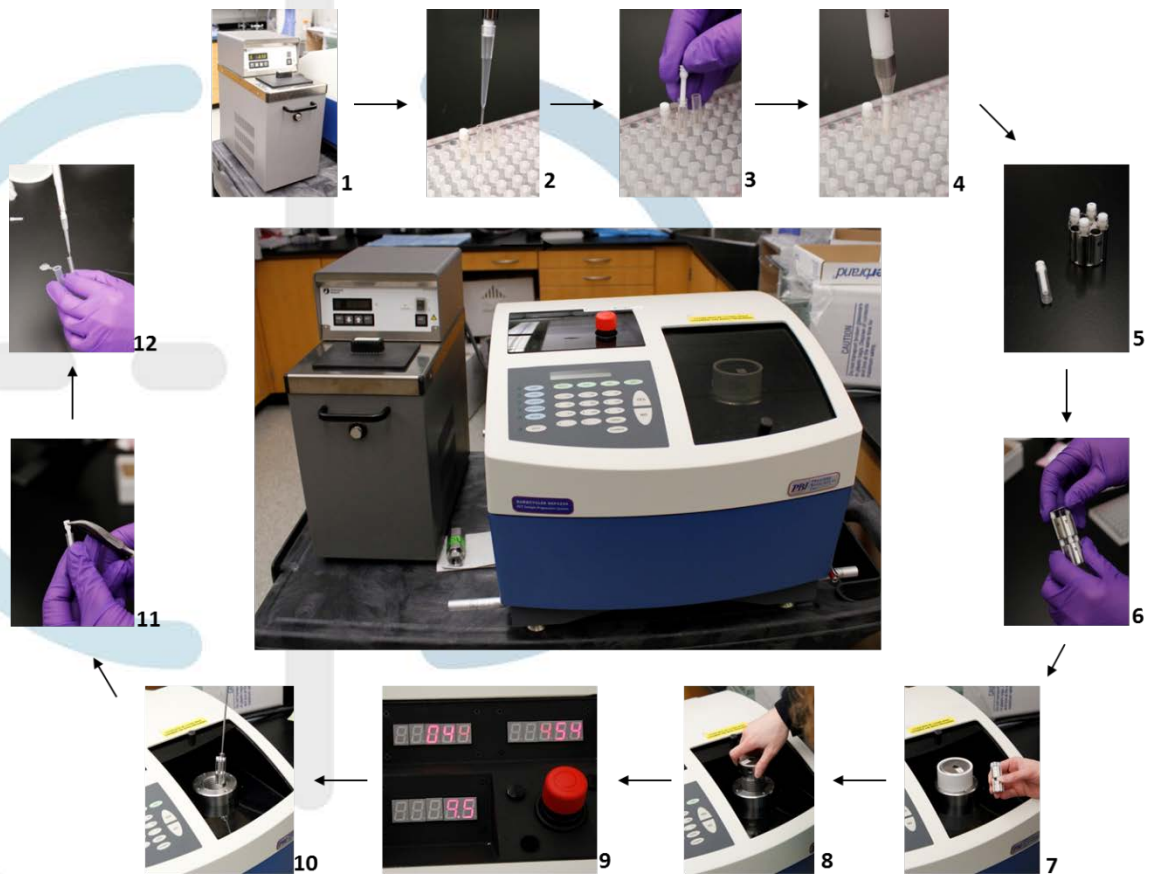
3. Solid phase extraction of peptides

- a. Label 15mL conical tubes with the following labels: methanol, wash, equilibrate, desalt, 40% Elution, 70% elution. One of each is required per SepPak.
- b. 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.

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

4. Barocycler Setup (see section VII-A from PBI User Manual) 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.

Referenced Documents

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