Title:
Preparation of protein lysates from frozen tumor samples**.**

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Purpose: Generate mass spec compatible protein lysates from frozen tumor samples.
Background and notes:

- Protein yields are tumor type dependent but typically rage from 1% to 10% of tumor mass
- We adjust lysate buffer volume based on tumor type and size to achieve a lysate protein concentration between 1 and 5 mg/mL. Lysates at higher or lower protein concentrations are still acceptable.
- Tumor samples should be stored at -80 ^{o}C or in the vapor phase of Liquid Nitrogen (LN2) dewar.
- We process up to 16 tumor samples at a time.

Revision history

Revision date	Revision Author	Revision notes

A. Preparation:

- ☐ Dissolve urea in HPLC water to generate 7.5M Urea (see below).
- ☐ Turn on refrigerated microcentrifuge and cool to 4°C.
- ☐ Turn on refrigerated benchtop centrifuge and cool to 4°C.
- ☐ Thaw phosphatase and protease inhibitors.
- ☐ Prepare 1 bucket of dry ice per 8 tissue samples.
- ☐ Prepare 1 bucket of wet ice per 8 tissue samples.
- ☐ Prepare 1 tray of dry ice per 16 tissue samples to pre-cool tissue tubes and tissue bags.
- ☐ Prepare 1 tray of dry ice per 16 tissue samples to pre-cool microfuge tubes.
- ☐ Label, assemble, and pre-cool glass tissue tubes with tissue bags (see figure 1 below).
- ☐ Label and pre-cool two 1.7 mL snap-cap micro-centrifuge tubes per tissue sample (see figure 2 below).
- ☐ Label and pre-cool one 1.7 mL screw-top micro-centrifuge tubes per tissue sample.
- ☐ Label and pre-cool one Cryo-vial per tissue sample.
- ☐ Optional for RNA preparation: Place clean metal spatulas in 70% EtOH to sanitize. Wipe spatulas dry with a Kim-Wipe and place in benchtop Liquid Nitrogen dewar containing ~ 5-10 cm of LN2.
- ☐ Make fresh urea lysis buffer- (see below).

B. Pulverize tissue:

- ☐ 1. Remove tumor samples from to -80°C freezer or LN2 tank and place tubes with tumor on dry ice (sets of 8 to 16 samples processed at a time).
- ☐ 2. Weigh each tumor sample tube and record mass
- ☐ 3. Place tumor sample in pre-cooled Covaris tissue bag, connect to glass tube (loosen slightly) and return to dry ice. Repeat for all samples
- ☐ 4. Cool tissue sample in Covaris tissue bag by brief emersion in LN2
- ☐ 5. Turn on Covaris Tissue Impactor, set to Impact Level to 4
- ☐ 6. Place bag assembly into Tissue Impactor and pulverize sample, rotate the tube 180° and pulverize a second time
- ☐ 7. Briefly refreeze sample in LN2 and invert assembly to transfer pulverized tissue into glass tube
- ☐ 8. Remove and discard tissue bag
- ☐ 9. Optional step if RNA will be generated:
    - ☐ Using a metal spatula cooled in LN2, remove a small aliquot of pulverized tissue and place in a pre-cooled 1.7 mL microfuge tube for RNA analysis.
    - ☐ Keep tube on dry ice before transferring to -80°C freezer
    - ☐ Dirty spatulas are soaked in 10% bleach solution for at least 15 minutes before washing.
    - ☐ Consult your RNA Seq facility for details on RNA extraction instructions
- ☐ 10. Place glass tube containing pulverized sample on dry ice and repeat procedure for remaining samples

C. Generate protein lysates

- ☐ 1. Transfer glass tubes containing pulverized sample to wet ice
- ☐ 2. Add the appropriate volume of ice-cold urea buffer to each glass tube (buffer volume depends on tumor size and type).
- ☐ 3. Cap each tube and vortex each sample 15 seconds on maximum speed, return to ice bucket
- ☐ 4. Bump down lysates in bench-top centrifuge (3,000 xg, 1 second, 4 °C)
- ☐ 5. Optional: we often take a photograph of the samples at this point to document levels of blood contamination (see figure 3 below).
- ☐ 6. Remove metal ejector shaft from a L1000 pipetter.
- ☐ 7. With a wide-bore 1,000 μL pipet tip, transfer lysate and tissue chunks to pre-labeled 1.7 mL screw-top microfuge tubes.
- ☐ 8. Sonicate samples 3x in cup horn probe (filled with ice water) at 50% power for 30 seconds, incubate on ice for at least 10 s between each sonication step

9. Clear lysate by centrifugation: 20,000 xg, 10 minutes, 4 ºC
10. On ice, transfer lysate to pre-labeled Cryo-vial avoiding the pellet
11. Transfer 20 uL of each lysate to a 1.7 mL microfuge tube for quantitation by BCA and for SDS PAGE analysis
12. Weigh each empty tumor sample tube, record mass and calculate tumor mass.

Summary of Samples

Sample	Tube	Storage	Usage
Lysate	Cryovial	Vapor phase of LN2 tank	Proteomic analysis by Mass Spec
20 uL Lysate aliquot	1.7 mL microfuge tube	-80 oC freezer	For BCA and SDS PAGE
Tissue Aliquot for RNA	1.7 mL microfuge tube	-80 oC freezer	For RNA extraction
Tissue pellet	1.7 mL screw-top tube	-80 oC freezer	Hold for backup extraction
Empty sample tube	1.7 mL screw-top tube	Room temp then discard	Confirm sample IDs

Solutions:

Lysis Buffer. **Must be made fresh daily**:

- 4 Parts 7.5 M Urea (see below)
- 1 Part 5x Lysis Buffer Stock Solution (see below).
- Add 1% Sigma phosphatase cocktail 2
- Add 1% Sigma phosphatase cocktail 3
- Add 1% Sigma Protease Inhibitor
- Mix well, cool on ice

5x Lysis Buffer Stock Solution. May be made in advance and stored at 4 ºC or room temp.

- 12.5 mL 1M Tris (pH8.0)
- 1.0 mL 0.5 M EDTA
- 1.0 mL 0.5 M EGTA
- HPLC water to 100 mL
- Sterilize with 0.22 um filter.

7.5 M Urea**. Make fresh daily**.

- Add 13.0 mL HPLC water to a 50 mL Falcon tube.
- Add 9.0 g Urea to the 50 mL Falcon tube.
- Mix until Urea is in solution, final volume should be 20 mL.

Final Urea buffer: 6M Urea, 25 mM Tris (pH8.0), 1 mM EDTA, 1 mM EGTA

Reagents:

- Urea (Sigma, U0631)
- 1 M Tris (pH8.0) (Sigma, T2194)
- EDTA (Sigma, E7889)
- EGTA (Sigma, E0396)
- TCEP (Sigma, C4706)
- HPLC water (Fisher, W6-4)
- Protease Inhibitor (Sigma, P8340). Aliquot into amber microfuge tubes, store at -20 ºC
- Phosphatase Cocktail 2 (Sigma, P5726) Aliquot into amber microfuge tubes, store at 4 ºC
- Phosphatase Cocktail 3 (Sigma, P0044) Aliquot into amber microfuge tubes, store at 4 ºC

Supplies and equipment:

- Refrigerated Benchtop centrifuge (Eppendorf, 5810R)

- Refrigerated Microcentrifuge (Eppendorf, 5417R)
- Covaris cryoPREP CP02 Impactors
- Covaris tissue bag (TT1, 520001)
- Kimble culture tubes, 16x100 mm (45066A-16100)
- Fisherbrand Spatula, 7 inch (Fisher, 14374)
- Nalgene Autoclavable Polypropylene tray, 32 x 26 x 7 cm (Fisher, 6902-3000 or similar)
- Nalgene HDPE tray, 54 x 43 x 13cm (Fisher, 13-359-26 or similar)
- Ice buckets (Fisher, 07-210-123 or similar)
- 1.7 mL Screw Cap Microcentrifuge Tubes (VWR, 16466-046)
- 1.7 mL Graduated Microcentrifuge Tubes (VWR, 490004-436)
- Tough-Tag labels (Diversified Biotech, TTLW-2016)
- 50mL Conical Centrifuge Tubes (Fisher, 14-432-22)
- Benchtop Liquid Nitrogen Container (Fisher, 11-670-4B or similar)
- Dry ice (Small pellets preferred)
- Liquid Nitrogen (LN2)

Figure 1. Assembled tissue tubes and tissue bags pre-cooling on dry ice.

Figure 2. Sample tubes pre-cooling on dry ice.

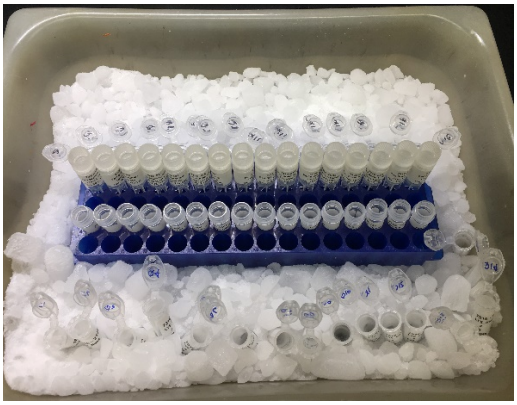

Figure 3. An example of an optional image of tumor lysates after extraction in Urea Buffer and before sonication.