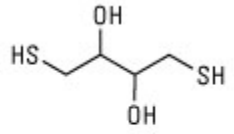
Protein extraction and quantification by Teresa Fan, University of Kentucky

Note: This procedure follows [Fan_Extract_Polar_Lipid_Prot] Step 11 (middle part, in 1.5 ml tube).

BUFFER PREPARATION AND STORAGE

1. **Blank Buffer** is 62.5mM Tris with 2%SDS (pH=6.8). Blank buffer can be stored at 4°C up to 6 months.
2. **DTT** working concentration is 0.1M in dH_2O. (Dithiothreitol) is stored at -30°C as it is labile. DTT Master stocks of 1 M solution can be store in 250uL aliquots at -30°C freezer for 12 months. When ready for use, dilute to 0.1M using dH_20.
3. **Working Buffer**.1mM DTT in Blank Buffer (1:100 dilution of 0.1M DTT.)
   Example calculation:
   For 5 samples: Each (2 mg < sample > 4 mg) sample will require 300 uL working buffer in 3 steps:
   a. 150 uL WB→ 1st grinding extraction
   b. 50 uL WB→ to rinse grinding pestle
   c. 100 uL WB→ 2nd grinding extraction pooled with 1st extraction.
   Calculate for 6 to ensure enough working buffer for extraction and for protein analysis blank. (6 samples x 300 ul = 1800 ul. The required DTT from a 0.1M stock is 1/100 of volume. (1800 ul / 100 = 18 uL) Therefore add 18 ul DTT (0.1M) to 1782 uL Blank buffer to give 1800 ul Working buffer (BB+DTT).

Note: Once DDT is added to blank buffer, it must be used that day because DDT is a labile factor. This is a disulfide reducing agent. Blank buffer will not be suitable for use if DTT remains in it for > 1 day.



Dithiothreitol (DTT)
MW 154.25

4. **Tissue pellet** is stored at -80°C until ready for use. Limit amount of time this material is out of cold unit/on ice.

TISSUE DISSOCIATION AND PREPARATION

1. Determine the amount of buffer to use based on the weight of the protein containing residue:
   a. If residue weight < 2 mg, use 200 ul of Working Buffer
   b. If 2 mg < residue weight > 4 mg, use 300 ul of Working Buffer
   c. If residue weight > 4 mg, use 500 ul of Working Buffer

2. Mechanically dissociate the tissue by adding WB Aliquot A (half of total volume from step 1) to tissue sample and use a combination of the following steps:
    a. Use the grinding pestle to break up the tissue. Press tip of pestle to the bottom of the tube and use clockwise and counterclockwise twisting motion to grind tissue. Bilateral counterforces are helpful to increase grinding work, decrease grinding time, and reduce fatigue. Do not use up and down motions frequently or you will create bubbles and lose tissue.
    a. For "gooey" samples (usually cell cultures), when you can see long strands of mucusy material sticking to the pestle, use an insulin syringe to shear the long strands. This is complete when you can see the sample come drop-by-drop from the needle of the syringe. Keep syringe for rinsing in step 7.
    b. For "fluffy" samples (usually tissues), when you cannot get the residue to break up and solubilize in the buffer, use small stainless steel scissors to break it up. This is complete when you no longer see bits of solid material in the solution.
    b. Grind until tissue/WB appears homogenous and translucent to light. Do not dump out material if you hold tube up. Keep tube vertically oriented.

Note: Hold tube at top to avoid body heat to sample.

3. Vortex dissociated tissue sample for about 3 seconds
4. Use WB Aliquot B (half of remaining volume) to rinse the pestle.
    a. Dribble rinse on the bottom third of the shaft of the pestle and allow it to run down pestle into the sample
    b. Scrape pestle on side of tube repeatedly (including the ridge of the pestle) to ensure all tissue sample is returned to tube.
    c. Place dissociated sample back on ice. Label pestle according to sample number and place it in ice as well, not allowing it to touch anything. You will use it again.

Note: samples should be kept on ice unless you are directly handling them.

5. Centrifuge to extract the protein at 4° C, 14,000rpm for 20min.
    a. While samples are under centrifugation, label and weigh tubes intended for supernatant. Make sure that the tube you select will make good contact with the heat block. Weigh the tubes on the 4 place balance, setting the tare to zero before each and recording as "Protein ext tare (g)" in your spreadsheet.
    b. Also a good time to turn heating source on to 90-95**°C**.
6. Remove samples from centrifuge and place on ice. Transfer the supernatant from the pelleted protein to the weighted tube using fine tip gel loading pipette tips (e.g. USA Scientific, 1022-000). Leave pipette tip in microcentrifuge tube with supernatant to use again.
7. Add WB Aliquot C (remaining 1/4 of total volume) to protein pellet in microcentrifuge tube on ice. If you used a syringe, remove the barrel from the

syringe and pipet the buffer through the syringe, replacing the barrel and pushing the buffer through to rinse. Dispose of syringe.

a. Using labeled matched pestles from step 4, repeat step 2a, grinding the protein pellet again to extract all remaining protein.
b. Centrifuge at 4° C, 14,000rpm for 20min
c. Remove the supernatant from the pelleted protein on ice using pipet tip reserved in step 6 and pool with supernatant collected in step 6.
d. Weigh tubes of supernatent and record weights as "g Tare+Ext" in your spreadsheet. Make sure to blot dry tubes prior to weighing them. Determine the difference in the weights as "g protein ext" in the spreadsheet.

Note: Pestles can be placed in cleaning solution and save after this step. Keep tissue pellets for future use. Store in -80°C.

DENATURE PROTEINS

Note: Samples should be kept on ice unless you are directly handling them

8. Vortex supernatant samples for 3 seconds.
9. Heat samples for 10 minutes in dry bath incubator. Do not go longer than that because DTT will degrade. Heat source should be ready by now if you started it in Step 5. Use boiling clamps to prevent condensation from getting into tubes. (Parafilm and lab tape are acceptable if you can't find clamps. Or use screw cap tubes). After 10 minutes of heating promptly move samples back to ice. Spin samples in the flexfuge to bring all liquid to the bottom of the tube.

PREPARATION OF BSA REAGENTS AND STANDARDS

1. Prepare BSA standard curve from ampules containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide stored in 4°C.
    a. To open ampule, tap very lightly to ensure all liquid is at base of ampule. Always use towel to protect your hand when breaking vial.
    b. Create 7 standards using serial dilution in nanopure water. Example serial dilution follows:
        I. 2000 ug/mL= 0:500uL ; dH_20:stock solution (2.0 mg/ml)
        II. 1000 ug/mL= 250 uL:250 uL ; dH_20:stock solution (2.0 mg/ml)
        III. 500 ug/mL= 250 uL:250 uL ; dH_20: 1000 ug/mL (standard II)
        IV. 250 ug/mL= 250 uL:250 uL ; dH_20: 500 ug/mL (standard III)
        V. 0.125 ug/mL= 250 uL:250 uL ; dH_20: 250 ug/mL (standard IV)
        VI. 0.0625 ug/mL= 250 uL:250 uL ; dH_20: 0.125 ug/mL (standard V)
        VII. Blank= 1,000 uL dH_20 ; none
2. If using, prepare Compatibility Solution by puncturing foil over one of the small vials on the tray with clean pipette tip. Add 100 uL dH_20; Pipette this solution up

and down gently 15-20x to ensure that it is dissolved. Transfer this to a microfuge tube on ice.

3. Prepare the BSA Working solution by combining 50:1 (Reagent A:Reagent B) in an appropriate vial on ice. Example calculation for mixing ratio: 3ml A + 60 uL B.

Note: BCA™ Reagent A=sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide; BCA™ Reagent B= 25 ml, containing 4% cupric sulfate. Purpose of these reagents: BCA, or bicinchoninic acid, is used for the colorimetric detection and measurement of protein. The purple color change you will notice is due to the chelation of 2 BCA with 1 Cu+. In a basic environment (created by these reagents), protein reduces Cu++ to Cu+. The Cu+ with the BCA makes a water soluble complex that absorbs at 562 nm and works with protein concentrations over a large range.

SAMPLE/REAGENT LOADING AND ANALYSIS

1. Dilute those samples requiring dilution with Working Buffer. After dilution, spin tube briefly before using.
2. Load standards to a clean 386 will plate. Pipette two rows of standards. You will use the average of two independent readings for quantification! Add 3 uL of each standard. Begin with the blank and pipette each standard twice, working from least to most concentrated. (Ex. Row A1-7; Row B 1-7 would be your respective standards).
3. Load samples: Pipette your samples in duplicate, also into adjacent rows. You will use the average of two independent readings for quantification! Add 1 uL of dilute sample (if it was diluted, otherwise, just add the sample).

Pipetting Tip: Wet tip, squeeze out excess, touch bottom of plate, go to clean side of well before removing tip

4. Add 2 ul of H20 to make sample volume. Add to samples only, not to standards.

Pipetting Tip: Place on opposite side of compatibility reagent and tap the plate down

5. If using, add 1.33 uL of compatibility reagent

Pipetting Tip: Touch tip to mid side wall, toward bottom, do not touch the sample with the tip.

6. Add 86.6 uL Working Solution

Pipetting Tip: Anchor pipette tip to side of well and hover tip over the middle as WS goes into well

7. Shake plate side to side 5-10x. Place on paper so it doesn't make terrible noise. Do not bounce the plate.
8. Incubate plate at 37°C for 30 min.
9. Run the plate.

10. Save the resulting datafile and move to your computer for processing.
    a. Compute the average reading from the two analytical replicates of all standards and samples.
    b. Subtract the average reading of the blank (0 mg/ml BSA) from all standards and sample readings.
    c. Use a linear regression of the blank subtracted average readings for standards against the known standard concentrations to derive a slope and y-intercept for interpolation of sample concentrations.
    d. Apply the regression equation to the blank subtracted average readings for samples to compute unadjusted protein concentrations. Multiply by the dilution factor (at least 3, since standards were pipetted at 3 ul and samples only at 1 ul – but if additional sample dilutions were done, multiply up by that amount as well.)
    e. Copy and paste the resulting protein concentrations to your spreadsheet (mg/ml protein).
    f. Compute (mg protein) by multiplying protein concentration by (g protein extract), which is the volume in ml of the protein extract.