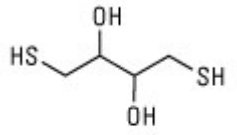
Protein extraction and quantification by Teresa Fan, University of Kentucky

Note: This procedure follows [Fan_Extract_Polar_Lipid_Prot_SOP] 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.
2. **DTT** (Dithiothreitol) is stored at -30°C as it is labile. When a 1 M solution is made up from the powder it is stored as 250uL 1M aliquots in -30°C freezer. When ready for use, dilute to 1mM using dH_20 (i.e. 0.1M= 1mM; 1:100 dilution).
3. **Working Buffer**. A 1:100 dilution of DTT: Blank buffer. Example calculation: 11uL DTT: 1.1mL (1100uL) Blank buffer= 1111 uL Working buffer (BB+DTT). Each sample will require 300 uL working buffer in 3 steps:
    a. 150uL WB→ added to tissue sample
    b. 50uL WB→ to rinse grinding pestle
    c. 100 uL WB→ added to supernatant of samples after centrifuge ½ (1 of 2)

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

5. Mechanically dissociate the tissue by adding WB Aliquot A (150uL) to tissue sample and using the grinding pestle to break up the tissue.
    a. Hold tube at top to avoid body heat to sample.
    b. 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.
    c. Grind until tissue/WB appears homogenous and translucent to light. Do not dump out material if you hold tube up. Keep tube vertically oriented.
6. Vortex dissociated tissue sample for about 3 seconds

7. Use WB Aliquot B (50uL) 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.

8. 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**.
9. Remove samples from centrifuge and place on ice. Remove the supernatant from the pelleted protein using fine tip gel loading pipette tips (e.g. USA Scientific, 1022-000). Leave pipette tip in microcentrifuge tubes with supernatant to use again.
10. Add WB Aliquot C (100uL) to protein pellet in microcentrifuge tubes on ice.
    a. Using labeled matched pestles from step 7, repeat step 5, 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 9 and pool with supernatant collected in step 9.
    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

11. Vortex supernatant samples for 3 seconds.
12. 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 8. 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.

PREPARATION OF BSA REAGENTS AND STANDARDS

13. 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
14. 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.
15. 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

16. Determine whether samples require dilution by comparing to Standard I (highest concentration). All samples can be run in this step, or you may identify the most concentrated samples based on their color density and appearance.
    a. Load highest concentration standard (3uL), and samples (1uL) into 386 well plate. Wet tip, squeeze out excess, touch bottom of plate, go to clean side of well before removing tip
    b. Add 1.33 uL of compatibility reagent. Touch tip to mid side wall, toward bottom, do not touch the sample with the tip.
    c. Add 2 ul of H20 to make sample volume only to sample, not to standard. Place on opposite side of compatibility reagent and tap the plate down
    d. Add 87 uL Working Solution (86.6 uL—but using automated pipette** See Appendix B for instructions). Anchor pipette tip to side of well and hover tip over the middle as WS goes into well.
    e. Shake plate side to side 5-10x. Place on paper so it doesn't make terrible noise. Do not move plate up and down or bounce on the bench top.
17. Incubate plate at 37°C for 5-15 min. within the spectrometer (See Appendix A for details).

18. Run the plate using the 384 well plate procedure. See Appendix A for instructions. If any of the samples read higher than the highest standard, these need to be diluted.
19. Dilute those samples requiring dilution with Working Buffer. After dilution, spin tube briefly before using.
20. 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).
21. Load samples: Pipette your samples in duplicate. 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

22. 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

23. 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

24. 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

25. Shake plate side to side 5-10x. Place on paper so it doesn't make terrible noise. Do not bounce the plate.
26. Incubate plate at 37°C for 15 min.
27. Run the plate. See Appendix A for instructions.
28. 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.

Appendix A: Spectrometer instructions

1. Turn on (will be turned off daily); Plate reader should be turned on first, then computer. Plate reader on button is on the front

2. Open Gen 5 2.04 program

3. Select New

4. Select protocol-procedure → 384 well plate

a. Set temp: 37 ®C

b. Read: Absorbance: Endpt Kinetic; monochromators

c. Wavelength: 562nm; deselect full plate, then select appropriate wells

d. Read speed: normal

e. Shake: 1 min

f. Linear frequency: Double orbit (i.e. figure of 8)

g. Dispense: will take care of later

h. Start kinetic: run 30 min; interval- 5 min

Drag Read A 562nm under start kinetic

Validate, save in a file if needed

Select and highlight pertinent wells

Double click on procedure, Yes to use lid

Read now is in tool bar

*****Must start running a program for plate reader to start warming to incubating temperature.

**** The tray that holds the well plate must be out of the machine when a program is either validated or begun. The first line in the program is to shut the door, so if it is not open the program will fail.