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Extraction, Clean-Up, and Assay of Protein

Extraction:

Phenol Extraction Followed by Methanolic Ammonium Acetate Precipitation

Variation on Hurkman and Tanaka, 1986, Plant Physiology 81:802-806

1. Grind 2 g (0.3g for small scale extractions) of fresh tissue to a powder with liquid nitrogen with a mortar and pestle. Keep the tissue frozen at all times. Transfer the tissue to 50 ml conical tubes (2 ml Eppendorf tubes) and drop into liquid nitrogen if not ready to begin step 2 immediately.

2. Add 5 ml (600 µl) of chilled Tris pH 8.8 buffered phenol and 5 ml (600 µl) of chilled extraction buffer and vortex immediately until all tissue is wet and immediately place sample on ice. Use phenol and extraction buffer only IN THE HOOD.

CAUTION: Wear two layers of nitrile gloves when using phenol. Change the outer layer frequently to avoid workspace/instrument contamination. If phenol droplets get on the gloves, change gloves as soon as possible.

3. Vortex on high for 30 min at 4 °C (or periodically chilling on ice). If you have many samples, rotate them between vortexing and chilling in the ice bucket every 60 sec. Adjust the volume of the samples with extraction buffer so that they may be balanced in the centrifuge.

4. Centrifuge 10 min at 5000 g, 4 °C.

5. Remove phenol phase (should be top phase) and transfer to a new 50 ml conical tube (2 ml tube). [If you do not plan to do step 9 then transfer to ultracentrifuge tube.] Use a 1000 µl tip with a 10 µl tip on the end to avoid transferring other layers. Store on ice.

6. Back-extract aqueous phase: Add 4 ml extraction buffer (0 µl) and 4 ml (400 µl) phenol to aqueous phase and vortex until thoroughly mixed (1-2

minutes). Adjust volume with extraction buffer to balance tubes.

7. Centrifuge 10 min at 5000 g, 4 °C.

8. Remove phenol phase and combine with first extraction.

9. Recommended step: [If your extract is clean, you may transfer it directly to the ultra-centrifuge tube and skip step 9.] Centrifuge combined extract (phenol phases) for 15 min at 5000 g, 4 °C then remove the phenol layer (leaving behind the lower layer that previously contaminated the phenol layer) and transfer to a clean 40 ml silicone ultra-centrifuge tube (2 ml eppendorf for small scale extractions). Contaminating layer may be discarded, along with the aqueous phase into the non-chlorinated waste. Leave empty tubes in hood to air out for a few weeks before discarding in trash.

10. Precipitate phenol extracted proteins by adding 5 volumes of COLD 0.1 M ammonium acetate in 100% methanol (chilled at –20 °C) to phenol phase. Balance tubes.

11. Vortex until thoroughly mixed and incubate at –20 °C for at least 1 h or overnight.

12. Collect the precipitate by centrifugation (20 min, 20,000 g, 4 °C).

13. Remove the supernatant and dispose in the non-chlorinated waste. Wash pellet by adding 30 ml (1.5 ml) of COLD 0.1 M ammonium acetate in methanol (-20 °C). Suggestion: First add only ¼ of the buffer and use this to break up the pellet as much as possible, then wash the protein from this tip into the sample tube with the remaining buffer. Break up the pellet as much as possible by vortexing and sonication. Do not sonicate longer than 30 seconds at a time or longer than 1.5 minutes total time. If the sample starts to warm up, chill it before sonicating again. Balance the tubes.

14. Place the resuspended sample at –20 °C for at least 15 min, then centrifuge 20 min, 20,000 g, 4 °C.

15. Repeat the washes as instructed above 1X more with COLD 0.1 M ammonium acetate in methanol, 1X to 2X with COLD 80% acetone, and 1X with COLD 70% methanol. Dry the last pellet using the SpeedVac

(cold trap ON, 1-2 minutes, do not overdry) or after removal of last wash, centrifuge hard and remove the ~10 microliter of supernaten (repeat 2x) until removing all liquid (the latter is best).

Hint for large scale extractions: after last wash, transfer pellet from 40 ml tube to 2 ml tube by: cut off 1 ml tip with a scalpel to widen the opening. Add 0.5 ml cold 70% methanol with cut tip and use it to break up the pellet. Using cut tip, transfer as much of sample as possible to the new 2 ml tube. Wash the cut tip into the 40 ml tube with 0.5 ml 70% methanol and then use cut tip to transfer this solution and the remaining sample to the 2 ml tube. Wash the cut tip into the 2 ml tube with the 0.75 ml 70% methanol. Collect the protein by centrifugation and discard supernantant.

16. Resuspend dried pellet immediately in appropriate buffer (e.g. IEF sample buffer) by pipetting and vortexing at 25 °C. Incubate sample for at least 1 h at room temperature with agitation (on the shaking platform; avoid vortexing very long whereas foam easily forms). Do not heat sample under any circumstances as this will lead to carbamylation of proteins. For small-scale extractions, resuspend sample in 180 µl of sample buffer, 300 ul for large-scale extractions. Avoid adding more than this until after quantifying the proteins. If the sample does not completely resuspend, you may sonicate in intervals of 15 s for no longer than 45 sec total. If, after hours of shaking, it does not solublize, you may add more buffer.

Additional Notes:

- Preparation of samples must be performed with lab ware that has never been in contact with nonfat milk, BSA, or any other protein-blocking agent to prevent carryover contamination.

- Always use non-latex gloves when handling samples; keratin and latex proteins are potential sources of contamination.

- Never re-use any solutions, abundant proteins will partially leach out and contaminate subsequent samples.

Extraction buffer

(0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose)

For 200 ml:

1.5 M Tris pH 8.8	13.3 ml
500 mM EDTA	4 ml
β-ME	0.8 ml
sucrose	61.6 g

Do NOT add β-ME until just prior to use. You may store the buffer at RT without the βME and then add just before use (80 µl β-ME per 20 ml of buffer).

Sample Clean-Up (if necessary)

To remove agents known to interfere with iso-electric focusing (IEF), it may be necessary to clean up your sample.

If this step is necessary, follow the instructions for the Perfect-Focus clean up kit (Genotech Inc.).

The Perfect-Focus kit is kept on the second shelf above the bench 2 workspace.

Protein Assay

To determine how much protein is in your sample, follow the instructions for the CB-X Protein Assay (GBiosciences, Cat # 786-124) located on the following page. The assay kit is kept in the 4°C freezer.

Use the spectrophotometer in the Taylor lab and print out your results.

Compare the absorbance of your sample to that on the CB-X table to estimate the amount of protein.

**note: use the table for 1cm length cuvette.