Total Protein Extraction for IEF and 2D SDS-PAGE

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November 2006

This procedure is a modification of Hurkman & Tanaka, 1986, *Plant Physiology* **81**:802-806, designed to obtain protein from protein-poor tissues from plants.

1. Grind 1 g of fresh tissue to a powder with liquid nitrogen in a mortar and pestle.
2. Add 2.5 mL of Tris pH8.8 buffered phenol and 2.5 mL of extraction media (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose) and continue grinding for an additional 30 sec in a fume hood. Alternatively, transfer to a 15 or 50 mL Falcon tube and homogenize in polytron homogenizer for one minute.

Note: If extracting from liquid/buffer, add 4 volumes of the Tris-EDTA/sucrose buffer and mix thoroughly. Then add a volume (equal to the volume you now have) of the Tris-phenol and proceed with the extraction.

3. Transfer to Falcon tube.
4. Agitate for 30 min at 4° C.
5. Centrifuge 10 min at 5000 g, 4° C.
6. Remove phenol phase (if sucrose was added to Tris-EDTA buffer, phenol layer should be top phase; layers are reversed if no sucrose was used) and place into fresh Falcon tube.

Note: Do not remove the interphase "junk" between the phenol and aqueous layer. Leave for the next extraction.

7. Add an equal volume of fresh Tris-phenol to the aqueous phase (2^{nd} extraction). Repeat steps 3-5, then add the phenol layer to the previous phenol layer from the 1^{st} extraction.
8. Back-extract the combined phenol phases with an equal volume of fresh Tris-EDTA/sucrose extraction media. Repeat steps 3-6.
9. Precipitate the phenol extracted proteins by adding 5 - 10 volumes of 0.1 M ammonium acetate in 100% methanol (pre-chilled to -80° C) to the phenol phase.
10. Vortex and incubate at -80° C for at least 2 hr (overnight may be needed, but is not necessary).
11. Collect the precipitate by centrifugation (30 min, 4000xg, 4° C).
12. Wash the pellet 2x's with ice-cold 0.1 M ammonium acetate in methanol containing 10 mM DTT, then 2x's with ice-cold 80% acetone containing 10 mM DTT. (Optional: final wash 1x with cold 70% ethanol. Instead of 70% EtOH, substitution of a final wash in 80 % acetone/10mM DTT can improve protein solubility in IEF buffer).

NOTE: Completely resuspend the pellet each time with vortexing and if necessary, sonication (this usually takes longer with the first wash). Place the resuspended sample at -20° C for at least 20 min in between each wash. You can store the last resuspended pellet in 80 % acetone at -80° C until ready for IEF.

CAUTION: freeze-thawing samples in IEF buffer causes problems. We recommend making aliquots at the last wash stage i.e. leave precipitated protein in 80% acetone/DTT, vortex and aliquot immediately, then store at -80ºC.

13. Allow the final pellet (after removing the wash solution) to air dry on the benchtop.

CAUTION: Do not overdry the protein pellet. A completely dry pellet is impossible to resuspend in IEF buffer. For an average size pellet, usually 10 – 15 minutes of air drying is needed.

14. Resuspend final pellet in 0.2-0.5 mL of IEF extraction solution (8 M urea, 2 M thiourea, 4 % CHAPS, 2 % Triton X-100, 100 mM DTT and 1 % pH 3-10 ampholytes) by pipetting and vortexing at 25° C. Incubate sample for 1 h at room temperature with agitation.

CAUTION: Do not heat sample under any circumstances as this will lead to carbamylation of proteins.

15. Place sample on ice for storage until quantification and rehydration solution can be prepared.
16. If protein quantitation is necessary, precipitate protein sample with TCA or acetone prior to performing Bradford or Lowry assay as detergents and reducing agents interfere with these assays. However, we recommend using the new protein quantitation kits (e.g. EZQ from Invitrogen) that tolerate detergents and reducing agents.

Notes:

- Preparation of samples must be performed with labware 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.