Total protein isolation from Arabidopsis leaves (modified protocol 1_Amr)

1. **Grind Arabidopsis tissue in liquid nitrogen to obtain a fine a powder.**
2. **Prepare an extraction buffer (0.175 M tris-HCL pH 8.8, 50 g/L SDS, 15% glycerol, 0.07% 2-mercapto-ethanol (Pavokovic et al., 2012), 0.4% protease inhbitor cocktail (Sigma) (Wang et al., 2013). Tissue powder (0.3 g) is dissolved in 2 ml ice-cold extraction buffer (Pavokovic et al., 2012)**
3. **Cell debris is removed by centrifugation at 500 g at 4 C for 15 min (Pavokovic et al., 2012)**
4. **The supernatant is transferred to a new tube, and proteins are precipitated by 4 volumes of ice-cold acetone containing TCA (10%), and 2-mercaptoethanol (0.07%). (Pavokovic et al., 2012)**
5. **Samples are stored in -20 C for at least 1 h.**
6. **Pellets are obtained by centrifugation at 15000 g at 4C for 45 min**
7. **Pellets are washed three times with an ice-cold solution of (acetone with 0.07 % (V/V) 2-mercaptoethanol).**
8. **Pellets are centrifuged at 15000 g for 15 min at 4 C between washes**
9. **Pellets to be dried under air at room temperature**
10. **Dissolve pellets in reducing buffer (100 mM NH_4HCO_3 pH 8.1, 8 M urea) (Wang et al., 2013)**
11. **The protein concentration is determined by Bradford method**
12. **The mixture was reduced 20 mM DTT at 37 C for 2 h and then alkylated by 40mM iodoacetamide in darkness for 45 min. (Wang et al., 2013)**
13. **100 mM NH_4HCO_3 buffer is added to adjust the urea final concentration to 1M. (Wang et al., 2013)**
14. **Trypsin is then added with an enzyme-to-protein ratio of 1/25, the digestion is performed at 37 C overnight and the resulting mixture is stored at -20 C. (Wang et al., 2013)**

Total protein isolation from Arabidopsis leaves (modified protocol 2_Amr)

This method is the same as August 2014, and only varied based on the solubilization buffers for the pellets. Because a very recent article indicated that Arabidopsis proteome improved based on the amount and PTMs when using different buffers to solubilize the pellet (Marondedze et al., 2015)

1. **Grind Arabidopsis seedlings from Wt and mutant in liquid nitrogen to obtain a fine a powder.**
2. **Prepare 20 ml of acetone+ 14 µl 2-mercaptoethanol (0.07%). Take 4.5 ml from the mixture and add 0.5 ml TCA (10%), to prepare the buffer for protein pptn (steps 3 and 4). The rest will be used in steps 5 and 10.**
3. **Transfer carefully the ground powder in 2 ml Eppendorf tube. Cover with (0.9 ml of cold acetone with 10% (W/V) TCA and 0.07% (V/V) 2-mercaptoethanol). Vortex and add the next portion of 0.9 ml (acetone with TCA and mercaptoethanol), place at -20 ^{o}C for 1 h.**
4. **Centrifuge at 10000 x g for 10 min at 4 ^{o}C.**
5. **Discard the supernatant and resuspend the pellet in 1.8 ml of (cold acetone with 0.07 % (V/V) 2-mercaptoethanol). Place the tube at -20 ^{o}C for 1 h.**
6. **Centrifuge at 10000 x g for 15 min at 4 ^{o}C**
7. **Discard supernatant. Wash pellet twice. The last step can be incubated -20 ^{o}C overnight.**
8. **Air-dry the pellet at room temperature.**
9. **Pellets were kept in -20, and transferred to Trondheim**
10. **Solubilize pellets (Marondedze et al., 2015)**
    - A. **in equal volume of 7 M urea, 2 M thiourea**
    - B. **in equal volume of 7 M urea, 2 M thiourea+ 4% NDSB (non-detergent sulfobetaine 201)**
    - C. **Two step isolation method**
        - i. **Solubilize in 7 M urea, 2 M thiourea, centrifuge at 20000 g for 15 min**
        - ii. **Take supernatant (fraction 1)**
        - iii. **The residual pellet is solubilized in 7 M urea, 2 M thiourea+4% NDSB (fraction 2)**
11. **1 mg of protein is reduced with 5 mM DTT for 2 h at 37 C, and then alkylated with 14 mM iodoacetamide for 30 min in the dark**
12. **Unreacted iodoacetamide is quenched with an extra 5 mM DTT for 15 min.**
13. **Proteins are diluted to 2 M urea with 50 mM triethylammonium biocarbonate buffer (NH_4HCO_3 can be used??), and incubated with trypsin at ratio 1:50 overnight at 37 C.**

Total protein isolation from Arabidopsis leaves (August 2014)

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6. **Centrifuge at 10000 x g for 15 min at 4 ^{o}C**
7. **Discard supernatant. Wash pellet twice. The last step can be incubated -20 ^{o}C overnight.**
8. **Air-dry the pellet at room temperature.**
9. **Pellets were kept in -20, and transferred to Trondheim**

In Trondheim (August 2014),

1- **Dissolve pellets in 100 ul 50 mM Ammonium bicarbonate (AmBic), 20 mM DTT (if it is not dissolving increase the volume)**
2- **Heat at 50 C for 1 h, or at 75 C for 20 min**
3- **Cool at RT**
4- **Add 20 ul 50mM AmBic, 1M iodoacetamide (pr. 100 ul)**
5- **Incubate 20 min, RT, dark**
6- **Add 50ul 50 mM AmBic**
7- **Add 2 ug trypsin (approx 1:20-50 dilution), we have put 4 ug for total proteins, and 1 for peroxisomes**
8- **Incubate at 37 C O/N**
9- **Centrifuge at top speed for 5 min, and transfer the supernatant to other tubes and avoid any type of precipitate; this helped to avoid the seed coats, and undigested proteins**
10- **Measuring the concentration of peptides by loading 2 ul on cards to be analyzed by DIRECT DETECT device (using the NIST-BSA-AM2 method, but AM1 for PBS buffer)**
11- **Transferring 20 ug for total protein extractions, and 400 ug for phospho-peptides enrichment to separate tubes for drying using the vacuum. The rest was kept at -20 at mass spec. room.**
12- **The dried pellet for 4 samples (WT-1, m1-1, m2-1, perox15.12) were dissolved in 50 ul of 0.1% formic acid, and transferred to vials for ELITE LC-MS/MS detection using column C18 and using high complex 270 min setup.**
13- **Phospho-peptide enrichments using** MagReSyn® Ti-IMAC (http://www.resynbio.com/ti_imac.htm) **for 4 samples as step 12, by following the instructions of the kit manual**

14- **Measuring the concentration on the peptide level by a chip-based method (direct detect), and drying the phosphopeptides by vaccum again.**
15- **Running the MS/MS ELITE**

- **Protease inhibitor cocktail, Phosphatase inhibitor 2 and 3 from Sigma will be added to step one**
- **DTT to break the di-sulfide bonds, and the iodoacetamide to alkylate the free S, not to make bonds again**
- **Trypsin (produc#1862748, Pierce TM trypsin protease, MS grade, thermo) was dissolved in 50 uM AmBic, and distributed in the tubes**
- **If some remaining Trypsin needs to be stored, Acetic acid should be mixed with it in order to lower the pH, to prevent self-digestion**
- **To search about MS/MS,, find the Planetorbitrap**
- **Bioinformatics tools will be Proteome Discoverer for extracting data from raw files (licensed, thermo),**

Marondedze C, Wong A, Thomas L (2015) Exploring the Arabidopsis Proteome: Influence of Protein Solubilization Buffers on Proteome Coverage. International journal of molcular sciences

Pavokovic D, Kriznik B, Krsnik-Rasol M (2012) Evaluation of protein extraction methods for proteomic analysis of non-model reclalcitrant plant tissues. Croatica Chemica ACTA **85:** 177-183

Wang X, Bian Y, Cheng K, Gu LF, Ye M, Zou H, Sun SS, He JX (2013) A large-scale protein phosphorylation analysis reveals novel phosphorylation motifs and phosphoregulatory networks in Arabidopsis. J Proteomics **78:** 486-498