**How to isolate nondenatured protein extracts from Neurospora for western analysis.**

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Procedure:

Reagents

Extraction Buffer: 50mM HEPES (pH7.4), 137 mM NaCl, 10% Glycerol.
Add protease inhibitors fresh to the buffer just before the extraction.
Protease inhibitors final concentration:
Pepstatin A (1mg/ml), Leupeptin (1mg/ml), PMSF (1mM)

Stock kept in -20˚C:
Pepstatin A: 1mg/ml in methanol.
Leupeptin: 1mg/ml in $H_2O$.
PMSF: 100 mM in isopropanol.

For certain experiments with PPase inhibitors (made fresh):
25mM NaF, 10mM $Na_4P_2O_7.10H_2O$, 2mM $Na_3VO_4$, 1mM EDTA.

1. Grind tissue in liquid nitrogen with a mortar and pestle.

2. Suspend ground tissue in ice-cold extraction buffer containing protease inhibitors. Mix by gentle inversion. Let sit on ice for 5 min and then mix again.

3. Centrifuge cell homogenate at 12,000 rpm for 15 min at 4˚C.

4. Keep the supernatant, and measure the protein concentration.
   2 ml protein extract to 800 ml $H_2O$, then 200 ml Bio-Rad protein assay dye. Mix and measure $OD_{595}$ and calculate protein concentration.

5. Prepare protein sample for SDS-PAGE. For small gel (Bio-Rad Protean III gel) ~20-40mg per well.

| | Small gel (50mg) |
|---|---|
| $H_2O$ | (20-x) ml |
| 2X loading buffer | 20 ml |
| protein extract | x ml |

6. Boil the sample on heat block for 5min, chill on ice and load onto SDS-acrylamide gel.

Urea containing 2X loading buffer (the addition of Urea is optional):
100mM Tris pH6.8, 4% SDS, 2mM EDTA, 2% glycerol, 6M Urea.
To use: mix 900 ml buffer, 50 ml 2M DTT and 50ml 5% Bromophenol Blue.

References:

Garceau NY, Liu Y, Loros JJ, Dunlap JC. Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell. 1997, 89(3): 469-76.