Native Worm Lysis Protocol

Note:
Attached is the protocol for worm extract preparation that I wrote up a couple of years ago but Tali and others edited along the way. It says it's for "native" lysis extract but you'll see on the protocol the native extract parts are high-lighted in red. Also, this protocol seems to have been optimized for aggregate-extract but I think it's the generic one used by everyone here (with the exception of the filter step at #8--I think only Tali does that)...--Sue

Materials:

Lysis Buffer:		
1X:	10ML of 2X:	
50mM Tris pH 7.4	1 M Tris pH7.4	1 ml
5mM $MgCl_2$	1M $MgCl_2$	100 ul
2% Triton-X 100 [use 0.5% for native samples]	10% Triton X-100 (2.5%)	4 ml (1ml)
0.2mM PMSF (Sigma P-7626)	100mM PMSF (in EtOH)	40 ul
1ug/ml Leupeptin (Sigma L-0649)	1 mg/ml Leupeptin	20 ul
1ug/ml Pepstatin A (Sigma P-4265)	1 mg/ml Pepstatin A (in EtOH)	20 ul
Complete protease inhibitor cocktail	5x Complete Mini protease inhibitor cocktail tablets*	5
	dH_20	4.82 ml (7.82ml)
	*use at 5x concentration (i.e., for the Mini tablets resuspend tablet in 2ml instead of 10ml)	
Make aliquots and freeze 2XLysis Buffer for experiments (do not freeze/thaw more than 2-3 times)		

- Liquid Nitrogen or dry-ice/MeOH bath
- Protease Inhibitor: Roche Molecular Complete Mini, EDTA-free (11-836-170-001)
- Sand, white quartz: Sigma (S-9887)
- Millipore ultra-free-MC centrifugal filter unit 0.65µm (UFC30DV00 pk/100)
- Pestle (disposable blue plastic ones for microcentrifuge tubes): Kontes (from Fisher Scientific) K749520-00000 (pk/100)
- Gel loading tip (we use United Lab Plastics (cat# UP4091)

This protocol is designed to preserve small aggregates and large oligomers, while removing large aggregates visible in light microscope.

1. Wash worms in M9, last wash with chilled H2O, remove maximum liquid and flash freeze animals in liquid nitrogen (store at -80°C). Aim for a pellet of no more than 100ul.
2. Steps 2 and 3 need to be done quickly to avoid protein degradation. Grind the pellet with a chilled pestle for ~40 strokes up and down (keep on ice as much as possible), avoid making bubbles.
3. Estimate pellet volume and add equal volume of 2XLysis Buffer, mix by pipetting.
4. Use a pipetmen to measure total volume and correct the volume to your 1:1 estimation in the previous step. This step will bring the buffer concentration to 1X

and is useful to help in concentration (viscosity) consistency if preparing several samples.

5. Let sit for at least 15 min on ice.
6. Monitor efficiency of lysing by sampling a small aliquot of extract (1µl) under a microscope. If there are still intact adult worm bodies, pass through a fine needle a couple of times, taking care not to bubble, or ** optional, isn't good for finicky proteins - add sand (up to 50% of the volume) and vortex. Transfer extract to a new microfuge tube by using a gel-loading tip to avoid pipetting sand (long-thin tip).
7. Spin at 1000g (3000rpm in the large Eppendorf 5417C table-top) for 1 min to remove debris.
8. Transfer extract to a Millipore spin column (0.65µm*) and spin for 5-15 min at 1000-5000g in the cold room. 5000g is the maximum for these columns, 1000-2000g works fine. If the column gets clogged and lysate is not going through, either rotate the column 180 degrees, or transfer remaining lysate from the top of the column to a new one, and spin again.
9. Measure protein concentration, aliquot, freeze in -80C.

* Millipore has filters with different size pores, including much larger ones, but be careful to choose only Low Binding Membrane (Durapore).

Modification for including most of the large visible aggregates:

Steps 1-6 are the same, except do not use sand in step 6.
7. Spin at 30g (500 rpm in the large Eppendorf 5417C table-top) for 1 min, gently and slowly pipet the supernatant leaving behind the layer close to the pellet (the pellet is very fluffy and easy to disturb) and at the top of the tube (which has lipids and low density debris). Examine 1ul on a slide under the microscope, the aggregates will appear as bright discrete entities floating in the lysate, but there should not be other debris.
8. For protein concentration determination this lysate can be use directly if dilution factor is large enough, or spin a small aliquot at top speed.

Native PAGE + Western

Make 5%-7.5% 1.5mm (.75mm can be used if loading low amount of total protein) gels following resolving gel recipe minus the SDS, pour a continuous gel without the stacking gel. If need stacking gel, use the same pH for the Tris buffer! The running buffer is the same as the usual running buffer minus the SDS. Assemble the gel running apparatus, fill with running buffer and place in the cold room to cool down (optional).

Sample buffer (loading buffer) is 50mM Tris, 50% glycerol, some Bromophenol Blue (or other pigment) for color. This can be used as 5X – 10X.

Samples: to see YFP as either soluble protein or in aggregates/oligomers, load 25-30ug total cellular protein. For Western load less, depending on the sensitivity of your antibody. For MW markers, use FPLC standards, 3-5ug per protein band for Coomassie staining (these do not really allow MW determination, unless you protein has the same pKI and the same shape) but are useful as reference points between gels), BSA works well too, gives a monomer and a dimer bands.

Run gel slowly (60-100 V for short gel) to avoid smearing, preferably in the cold room. If the proteins in the gel are fluorescent, the run can be monitored by removing glass sandwich from the gel running apparatus and examining the position of the protein bands under the fluorescent microscope; the glass sandwich can then be returned to its position and the run continued. The large visible aggregates will stay in the well, the oligomers will enter the gel but stay close to the top, GFP alone will run close to the dye front in 5% gel. GFP and it's derivatives are very stable and remain fluorescent even in the standard SDS gel (if not boiled prior to loading).

After the run, the gel can be either directly scanned for fluorescent bands, or transferred for Western.

Direct scan: any scanner with blue laser will work, we use Storm Phosphorimager. Place gel directly onto the glass plate of the imager and choose blue laser to scan.

Western Blot: this may allow detection of species that do not have the folded and therefore fluorescent GFP, and is generally more sensitive that direct scan (depending on the antibody), but is not as quantitative. For GFP and derivatives, we use IR-labelled anti-GFP from XXX at 1:15000 or 1:20000 dilution.
Soak the native gel in a small volume of SDS running buffer (can use the same running buffers that the gel was run in with the addition of SDS to 0.1%) in 75-80 degrees Celsius oven (in the shaker room) for 20-30min, make sure the gel is completely covered. If needed, reducing agents can be added to the soaking solution (for example for SOD-1). Rinse with water, proceed with transfer as would with the normal SDS gel.

7/10/07