

Liquid Worm culture

Na 22 culture in 2X YT 10 ul/500 ml - 6 flasks, spin down in Beckmann bottles JLA 8.1 rotor, 4500 rpm, 10' brake slow

Resuspend pellet of 1 l in 500 ml S-complete, add worms (3 plates) wash each with 3 ml M9, then use 3 ml sequentially on all 3 plates, let worms settle down, remove SN, add ~ 3 ml M9, shake and put into medium, start shaking at 20C 200 rpm until most of them are gravid adults. The worms should be cleaned prior to putting them into liquid culture: make a bleach spot on small plates by pipetting 5 µl of 2M NaOH and 5 µl of 10% bleach. Add 100 adult hermaphrodites in this spot, keep plates at 24 C for three days, then seed large plates with 15 adult hermaphrodites, let them grow until food is completely consumed, then start liquid culture.

Harvest

1. Settle worms in 1 l sterile cylinders in the cold room overnight,
2. aspirate the medium off as much as possible,
3. transfer the worms and residual fluid in 50 ml conicals, bring up to 50 ml with cold M9, spin at 120g for 2 min(800rpm Heraeus Megafuge), wash 2 times with cold M9
4. resuspend washed worms in 25 ml of ice cold M9, add 25 ml of ice cold 60% sucrose, mix by inversion
5. centrifuge worms immediately after adding sucrose at 120 g for 5 min, collect the worms floating on top using a plastic pipette and collect them in 2x50ml conicals
6. bring the worms in each conical to 50 ml with M9, spin, wash 2 more times, freeze by dropping worms into conicals filled with liquid nitrogen
7. For preparation of extract: thaw pellets in RT water!!! Add extract buffer (H100): (50mM Hepes pH 7.5, 100 mM KCl, 1 mM MgCl₂ (commercial solution!), 1 mM EGTA, 10 % glycerol) + protease inhibitors (LPC 1:1000, E-64 1:1000. PMSF 1:100 and DTT 1 mM). See protocol for egg plate culture.

Egg Plates

NGM plates (diameter 20 cm), 2% agar

Day -2

start 2x 100 ml culture of wt E.coli (e.g. feeding strain, take out of freezer with inoculation needle), shake 12 h, start 4 x 500 ml cultures in 2 l flasks overnight.

Day -1

1. spin down the bacteria 5' 3600 rpm (JA 8.1, 1 l bottles, clean them before with 70% EtOH and optional put the open in hood with UV on)
2. Resuspend the pellet (in the hood) by pipetting up and down with ~ 10 ml H₂O, put this in a sterile peaker,
3. crack 2 eggs in a peaker "mix" by using a 10 ml syringe + needle 1.2 mm (remove "embryo which clogs the needle), mix with bacteria, distribute onto ~12 plates (~8 ml on each) with the syringe, let the plates dry (open in hood, ~1 hour). Incubate overnight at 37C.

Day 0

wash N2s off 2 small plates (adults) with 2x 2 ml H₂O, distribute them on 4 egg plates, incubate at 25C until they are dauers (~8 days). Store the rest of the plates in a plastic box in the cold room.

Harvest Dauers

These steps can be done on the bench since the plates will be incubated only for 36-48 hours

1. Prepare 1% SDS - filter and 60% sucrose - filter
2. Wash worms off the plate with cold water, put 10-15 ml, let it soak a little bit, but not too long! Because then all the water goes into the agar...
3. Spin worms for 2-3 min at 800rpm in Heraeus (without brake), repeat 1-2 times this wash
4. Fill up 50 ml conical with 1% SDS, leave on shaker for 15 min
5. Wash 2x with cold water, pool the worms in 1-2 falcons
6. Add equal volume of ice cold sucrose to worms, mix by inversion, spin immediately at 300rpm 5 min and then at 800rpm 5 min without brake, transfer upper layer of worms to new tube
7. Wash with a lot of cold M9 2x (check under microscope if there are worms and not too much agar!)

My first harvest was 10 ml (10,5g), diluted 2 μ l in 500 and counted worms, $\sim 30 = 750$ worm/10 μ l = 75000/ml. Alex uses 300.000/plate - I made a mistake and used only 150000 which was fine, too. It should just not be too many, so that they have enough food.

1. Incubate 36-48 hours
 2. wash worms off the plates
 3. wash them 2x with H₂O
 4. flotata with sucrose (see above)
 5. wash them with H 100 (50 mM Hepes pH 7.5 (KOH), 100 mM KCl, 1 mM MgCl₂, 1 mM EGTA).
- I obtained 2 pellets with ~ 6.5 g = 7.5 ml, which I resuspended in an equal volume of H100 (7.5 ml) + Protease Inhibitor Tablet (Roche) (1 tablet/5 ml, because it's 2x:7.5 + 7.5), freeze worms in liquid nitrogen (precooled Falcon with liquid nitrogen inside).

Immunoprecipitations

Preparation of antibody beads:

Random IgG (Jackson Immunoresearch) + antibodies of interest

1. Equilibrate some affiprep beads (BioRad) into PBST (PBS + 0.1 % Tween): I had 4 antibodies + 1 control (Randon IgG), so I needed ~ 500 μ l of beads - I took some out into a 15 ml Falcon, spun them down and checked on the tube if I have 500 - washed them 3x with 10 ml PBST (spin at 500 rpm in Heraeus for 1 min).
2. Resuspend the beads in 2.5 ml total, put 500 μ l each in 5 eppis (should be 100 μ l resin) and added 55 μ g of each antibody.
3. Rotate the beads for 30 min on a wheel, wash them 3 times with PBST (1 ml), resuspend in 550 μ l and take out 25 μ l and add 25 μ l 2x sample buffer
4. Wash the beads 3x with 1 ml of 0.2 M sodium borate, pH 9.0. Make this from a stock of 1 M NaBorate pH 9.0. Make this stock form Boric acid - pH with NaOH. After the final wash add 900 μ l of 0.2 M NaBorate to 100 μ l of resin to bring to 1 ml.
5. Add 100 μ l of 220 mM dimethylpimelimidate (Sigma) to each tube (20 mM final) and rotate the tubes gently for 0.5 - 1 hour at RT.
6. To make DMP: let the bottle (which should be stored with silica at -20C) stay at RT for 20-30 min before opening. Weigh out the DMP and leave dry until just before use. Resuspend 32 mg in 561 μ l.
7. Wash the beads 2x into 0.2 M ethanolamine pH 8.5 to inactivate the residual crosslinker and rotate at RT for 1 hour. Resuspend the beads to 525 μ l with buffer and take a sample of 25 μ l + 25 μ l 2x sample buffer.

The beads can be stored for some time at 4C in Ethanolamine.

The samples can be run on a gel and stained with Coomassie (2.5 g Coomassie Brilliant Blue, 900 ml MeOH:H₂O = 1:1, 100 ml acetic acid (100%))

The sample buffer should contain beta-Mercaptoethanol as reducing agent (so that light and heavy chain of antibody are separate). B-ME should not destroy covalent bonds (produced by the cross-linker), so after the DMP treatment there should be less antibody in the sample since it should not come off the beads! Because of SDS - antibody-bead bond is destroyed, so before cross-linking the antibodies come off.

Preparation of extract

1. Thaw pellets in RT waterbath (I had frozen them in extract buffer, Karen usually adds the buffer when she thaws the worms). To ~ 3.5 ml pellet, I added DTT and NP-40 to 0.5 mM and 0.05%, respectively.
2. Sonicate 3x 15s (30% with 45s pause in between) - check under microscope if embryos open, if not 3x 15s (40%, 45s pause)
3. Take sample of 20 μ l from this crude extract + 80 μ l 1x sample buffer with beta-ME and DTT (diluted from 4x sample buffer, which contains beta-ME, and I added 1M DTT to 50mM final + Bromphenolblue, then I diluted it 1:4)
4. Spin extract in TLA 100.3 (table ultracentrifuge) for 10 min 20 K, take 20 μ l of **Low Speed** Supernatant and add 80 μ l of 1x Sample Buffer
5. Spin LSS in TLA100.3 for 10 min at 50 K, take 20 μ l of HighSS sample + 80 μ l SB
6. Keep 2x1 μ l of each - crude, LSS and HSS and dilute in 800 μ l H₂O) for Bradford.

Immunoprecipitations

1. I had ~ 100 μ l of beads in 500 μ l ethanolamine - I mixed them, took out 250 μ l, which should correspond to 50 μ l of beads, which I pre-eluted 3x with 1 ml 100 mM Glycine pH 2.5
2. Wash the beads 2x into extract buffer (1 ml) to neutralize - use pH paper to see if it occurred
3. Mix 50 μ l of beads with about 900 μ l of extract for 1 hour at 4C (wheel)
4. Rinse the beads 3x with 1 ml of extract buffer containing 0.05% NP-40 and 0.5 mM DTT + protease inhibitors:
 - LPC 1:1000, Chemicon, 10 mg/ml
 - E-64 10 mM in DMSO, Biomol, 1:2000
 - PMSF 100 mM in EtOH, Sigma??? 1:100
5. Wash the beads 2x 5 min with extract buffer
6. Wash them 1x with buffer without NP-40
7. Elute beads by heating at 50C for 10 min (heat block with shaker) with 50 μ l of 2x sample buffer without DTT: 4 ml SDS 10 %, 1,25 ml 1M Tris pH .8, 2,5 ml glycerol 40%, 2,25 ml H₂O
8. Spin to pellet the beads, transfer supernatant to new tube and add DTT to 100mM (5 μ l of 1M DTT for 50 μ l), boil the beads 995C) in 2x sample buffer with DTT and beta-ME (1:2 diluted 4x)
9. Bradford to determine protein concentration of HSS and LSS (1 μ l in 800 μ l). Dilute 1 mg/ml BSA: 0, 2.5, 5, 7.5, 10 μ l in 800 μ l H₂O (0 - 10 μ g/ml), add 200 μ l of Biorad Reagens to all tubes.
10. measure absorbance at 595 nm (I had ~20 μ g/ml in extract!)

Silver Stain

1. Incubate 2 - 12 hours RT in 5 gel volumes EtOH:glacial acetic acid:water = 30:10:60 to fix proteins
2. Discard fixing solution and add at least 5 gel volumes 30% EtOH 2x 30 min RT
3. 10 gel volumes of deionized water 10 min RT
4. 5 gel volumes of 0.1% AgNO₃ (freshly diluted from 20%stock, tightly closed brown bottle, RT), 30 min RT
5. discard, wash both sides of gel 20" each under a stream of deionized water
6. 5 gel volumes 2.5% Na₂CO₃, 0.02% formaldehyde (usually 37% in H₂O)
7. shaking at RT until bands appear within a few min
8. quench reaction by washing gel with 1% acetic acid, then with deionized water.

Western Blot of *C. elegans* worm/embryo extracts

Preparation of worm extracts

For a 50 worms sample make a mark on an eppendorf tube at 25 μ l. Put about 100-200 μ l of M9 into the tube. Pick 50 worms with a forceps and put them into the M9. Wash the worms 3 times with M9 (let the worms either settle by gravitation or spin them down gently at 200g for 1 min between the washes).

Pipet off the M9 leaving worms in 25 μ l of M9. Snap freeze in liquid nitrogen. Thaw and add 25 μ l of 2X sample buffer. Sonicate for 2 min in waterbath sonicator (Sonorex Super RK 102H), put immediately at 95C for 5 min to inhibit proteases.

Load 15 μ l (for Coomassie) or 10 μ l for western blot.

Preparation of embryo extracts

1. For a 240 worms sample, make a mark at 50 μ l on an eppendorf tube. Put 1 ml of M9 + 240 worms into it. Wash worms 3 times with M9.
2. Pipet off M9 leaving worms in 25 μ l of M9.
3. Add 50 μ l of freshly made 2X bleach solution (150 ml H₂O + 500 ml 2M NaOH + 350 ml bleach). Vortex for 3 min. Briefly spin down, then transfer to a small eppendorf tube (with a mark at 10 μ l) containing 300 μ l of embryo isolation solution (M9 + 10% glycerol + 0.1 % triton X-100).
4. Centrifuge at 1000g for 1 min to recover embryos.
5. Using a 200 μ l pipet tip, remove all of the buffer except 10 μ l. Add 400 μ l of embryo isolation buffer and repeat spin 2x.
6. Pipet off buffer, leaving embryos in 10 μ l isolation solution. Add 10 μ l of 2X sample buffer and sonicate for 10 min at 80C in a waterbath sonicator, put sample at 95C, load 15 μ l for western blot.

7. Run gel (large plates, spacer 0,75 mm, comb with small teeth!!) at about 20 mA, blot overnight at 4C at 100mA
8. Transfer the nitrocellulose membrane after blotting into 5% milk in PBT for 1 hour
9. Then incubate with anti γ -tubulin antibody (1 μ g/ml) for 45 min
10. Wash several times with PBT
11. Incubate with anti rabbit horseradish peroxidase-conjugated secondary antibody (1:3000, Bio Rad) for 30 min
12. Wash several times with PBT
13. Incubate with ECL reagent (Amersham) for 1 min, expose on X-Ray film, develop film
14. Take nitrocellulose out of X-Ray cassette and wash it with PBT, leave it in PBT for about an hour to wash off all the ECL
15. Incubate with dm1 ??????????????ma) for 1 hour
16. Wash with PBT
17. Incubate with anti mouse alkaline phosphatase labelled secondary antibody (1:2000, Jackson ImmunoResearch Laboratories)
18. Wash thoroughly
19. Incubate in Western Blue Stabilized Alkaline Phosphatase Substrate (Promega) for about 20 min - band becomes pink, reaction has to be stopped (remove substrate, add water) as soon as band is clearly visible and before background gets too strong!!!

Stripping of blots

1. Put blot in closed box in 55C waterbath in following solution:
 - 10 ml SDS (10%)
 - 3.13 ml 1M Tris pH6.8
 - 0.35 ml b-Mercaptoethanol
 - add H2O to 50 ml
2. incubate for 40' (depending on signal 30-40')
3. wash 2X 10' with PBT
4. block with milk, reprobe

Antibody purification

Coupling to Sulfolink resin

1. Pack homemade columns (???) containing 2 ml each of Sulfolink resin
2. dissolve 5 mg of each peptide in 100 μ l DMSO. Just before coupling, add this to 4 ml of 50 mM Tris pH 8.5, 10 mM EDTA
3. run about 6 column volumes of 50 mM Tris pH 8.5, 10 mM EDTA over the column. (all flow rates \sim 1.5 ml/min)
4. recirculate the peptide over a column containing 2 ml Sulfolink resin for 45 min
5. recirculate 4 ml of 50 mM cysteins in 50 mM Tris pH 8.5, 10 mM EDTA over the columns for 30 min.
6. wash with 10 mM Tris pH 8 for 5 min
7. wash with 0.1 M glycine pH 2.0 for 5 min
8. wash with 10 mM Tris pH 8 for 5 min
9. wash with 0.1 M triethylamine pH 11.5 for 5 min (prepare fresh!)
10. repeat steps 6-9
11. wash with PBS for 15 min

Serum recirculation

1. Thaw serum at 37C until a small clump of ice remains, add an equal volume of sterile PBS and NaN3 to a final concentration of 0.1% and filter using a SteriCup(0.2 μ m)
2. Replace lid on SteriCup with a special two-holed lid and attach the HiTrap column to the lid and insert the capillary tube through the other hole.
3. Recirculate overnight.

Wash and elution

1. Remove HiTrap column from antiserum bottle
2. Wash with wash buffer for 1 hour
3. Wash with PBS for 20 min
4. Equilibrate column with 2 M Tris pH 8 (test if 100 ul of this buffer can neutralize 900 ul glycine pH 2.6 and triethylamine pH 11.5 e.g. for CeGrip 200 ul were needed + 800 ul base) Prepare 10 tubes with 100 ul Tris for acidic elution (pipette 900 ul glycine onto column and collect eluate or pump slowly over column, don't put let more than 1 ml into tube), if 200 ul of Tris are required for neutralization, prepare tubes with 200!, elute with base solution as described above. Measure OD of each fraction (280 nm; reference 100/200 Tris + 900/800 glycine/triethylamine): take out 100 ul for measurement - put it back into the tube!
5. Pool fractions which contain antibody (~ everything above 0.1) Dialyze overnight against PBS, repeat a few times, change PBS as often as possible. Use big container and stick one end of the dialyze tubes to the rim of it - they should be completely covered with PBS to prevent drying out. Stir continuously.
6. The columns should be stored in 55 % glycerol in PBS + 0.1% azide (dilute 1:4, let it run over column, store it at 4C; for longer storage undiluted buffer should be used and columns kept at -20C)
7. After dialysis: in the last step use 55% glycerol in PBS (11?), spin antibody in TLA 100.??? 10' 15K Measure OD280: expected ~ 100 ug/ml OD 1.35 = 1 mg/ml (dilute ~ 1:10)

Wash Buffer

PBS
0.5 M NaCl
0.1% TritonX100

Storage Buffer

1X PBS (dilute from 10X)
55% glycerol

All solutions need to be sterile filtered!