C. Elegans methods
Floatation, hypochlorite treatment; liquid culture, RNA prep

adapted from protocols found at http://info.med.yale.edu/mbb/koelle/protocols/protocol_liquid_culture.html and information about neutralization of hypochlorite from Arza Ron

Reagents for flotation and hypochlorite treatment:
0.1M NaCl Ice cold~500ml-1L. Make, autoclave and store @ 4C.
60% sucrose Ice cold~5-10ml. Make. Filter sterilize and store @ 4C.
S-basal ~250ml
Na-hypochlorite (NaOCl, Fisher SS290-1)
NaOH
Na-thiosulfate 0.054M, 25ml
HCl

A. **Flotation of worms** (to clean worms for RNA, protein, or egg prep)

1. Collect Worms from 5-10 of densely populated but not starved 10cm plates in S-basal by washing plates (2x5ml) or pelleting from ~500ml-liquid culture. Pool to 50ml tube.

2. Pellet 3000rpm.

3. For washed agar plates: Add ~8vol (4ml) O.1M NaCl.

4. Transfer to 15ml tube. Vortex briefly.
   Broken agar chunks will stick to sides of tube.
   Worms will stay in liquid.

5. Transfer liquid to new tube.

6. Wash the agar in the first tube with 8 vol (4ml) 0.1M NaCl & vortex briefly.

7. Pool 2nd wash with first. & pellet worms 3000rpm.

8. Wash 1X with 20vol (10ml) cold (4C) 0.1M NaCl.

9. Pellet 3000 rpm.

10. Resuspend worms in 10 vol (5ml) cold 0.1M NaCl.

11. Add equal vol (5ml) ice cold 60% sucrose and mix quickly.

12. Layer 1/5 total vol (2ml) ice cold 0.1M NaCl on top of sucrose.

13. Spin 3000rpm 5min.

14. Live worms will be at interface of 0.1M NaCl and sucrose.

15. Transfer ~2.5 ml upper phase to new 15ml tube containing 10ml S-Basal & mix.

16. If all worms not collected, add 1-3ml 0.1M NaCl to top of remaining sucrose to assist in collecting remaining worms.

17. Add 2nd collection of worms to first and pellet at 3000rpm.

18. For RNA Prep. Lyse worm pellet at this point in GTC.

19. For othr purposes wash worm pellet with 10ml S-basal and pellet again. Use for western, hypochlorite treatment etc.

B. **Treatment with hypochlorite** (for timed hatching)

1. Prepare 500ul-1ml floated, washed worms.
   (Use clean worms to prevent hatched worms from eating junk.)

2. Add 25ml ICE COLD bleach mix:
   10ml Na-Hypochlorite 4% stock solution, (0.215M final)
   2.5ml 10N NaOH (1M final)
   12.5ml H_2O

3. Bleach ~5 min on end-over-end rotator at room temp until worms break up releasing eggs.

4. Neutralize with 25ml COLD (4C) 0.054M Na-thiosulfate.
   (It takes 0.1 M Na-thiosulfate to neutralize an equal vol 0.4M Na hypochlorite)

5. Mix quickly and then add. 2.25ml conc. HCl (to neutralize NaOH) and mix quickly. (Don't premix HCl and Na-thiosulfate. It will precipitate)

6. Pellet at 3000rpm.

7. Wash 3x 50 ml S-basal pelleting @ 3000rpm between washes.

8. Resuspend eggs in S-basal or m9 and plate on plates or start liquid culture.

Liquid culture of worms

By Michael Koelle and Tory Herman, adapted from Mir Hengartner

Media

1) superbroth (5 X 2 1/2 liters autoclaved in 6 liter flasks)

5 X 30 g Bactotryptone
5 X 60 g yeast extract
5 X 20 mL 50% glycerol stock solution
5 X 2.25 liters DDW
•250 mL of sterile 0.17M KH2PO4, 0.72M K2HPO4 **added after autoclaving**

To prepare 0.17M KH2PO4, 0.72M K2HPO4 :

46.2g KH2PO4
250.8g K2HPO4 per 2 liters; make 250 mL aliquots and autoclave.

2) S- medium (4 X 500mls autoclaved in 2 liter flask)

Make the following stock solutions:

1M potassium phosphate, pH 6.0; 1L
136.1 g KH2PO4
800 mL H2O

•Adjust to pH 6.0 with solid KOH (approx. 15g).
•Bring up to 1L.

5 mL 100X trace metals solution
0.346 g FeSO4.7H20
0.930 g Na2EDTA
0.098 g MnCL2.4H20
0.144 g ZnS04.7H20
0.012 g CuSO4.5H20 per 500 mL
Autoclave. Keep in dark (wrap in aluminum foil).

1M KCitrate, pH 6.0, 100ml
21.01 g citric acid, monohydrate
80 mL dH2O

adjust to pH 6.0 with solid KOH (approx. 17 g) before bringing up to volume. Autoclave to sterilize.

5 mg/mL cholesterol in 95% EtOH

Warm to 37°C O/N to dissolve. When this is added to an aqueous solution some cholesterol will precipitate; don't worry about this.

S-medium:

0.5 L	2 L	
2.9g	11.6 g	NaCl
25 mL	100 mL	1M potassium phosphate, pH 6.0
475 mL	1.9 L	ddH2O

Autoclave and cool then sterilely supplement EACH 500 mls with:

0.5 mL 2 mL 5 mg/mL cholesterol in 95% EtOH
1.5 mL 1M MgSO4
3 mL 0.5M CaCl2
5 mL 100X trace metals solution
5 mL 1M K-Citrate, pH 6.0
5 mL Gibco 100X Pen/Strp/Neo (optional, buy from Gibco, keep in freezer)
5 mL 100X Nystatin or fungizone(optional, buy from Gibco, keep in freezer).

3) worm plates- approximately 5 large plates of N2 (more for sick mutants) are needed to start each liter of liquid culture. Grow until the plates almost starve, or if you want to stage the worms, grow until the plates starve (they'll be mostly L1s).

GROWING THE BACTERIA (WORM FOOD)

1) Prepare 5 x 2.5 L of Superbroth in 5 x 6 L flasks (see above for recipe)
2) Innoculate ~5 mL of HB101 in Superbroth into each of the 5 6-liter flasks; grow O/N @ 37° C with shaking.

Warning: Be sure to have enough worm food! It is very bad to run out of food in the middle of a growth.

3) Spin down the bacteria in 400ml- jars (15 min / 4000 rpm / 4°C) using multiple spins per jar. (Pour off supernatant between spins.) Resuspend the pellet in a bit (3 mL per bottle works fine) of S Medium. A convenient

method is to put the 400ml jar back on a platform shaker for ~15 minutes. Transfer to a 50 mL polypropylene tube and store in fridge for up to 2 weeks, or store @ -20 or -80 °C for unlimited time. **Final vol =10ml/liter of culture**

GROWING THE WORMS

- Start with 5-8 almost starved large plates of N2 (more of mutant) containing a mixture of adults and young larvae.
- Wash the worms off the plates 2X with S medium.
- Split the worms into two 2 liter flasks containing 500 ml each S-medium.
- Add 12.5 mls of bacteria to each flask.
- Shake @ 20° C on a platform shaker at ~240 RPM.
- Add 25 mls more of bacteria to each flask after two days
- harvest the culture 4-4.5 days after it was started.
- Check worms every day:
    - Pipet 200 µl aliquot on an unseeded worm plate
    - check for growth and food
    - oval browinish debris and/or waste pellets will accumulate.
    - If dauers are forming you need more food.
- Add 25 mls more of bacteria to each flask after two days
- harvest the culture 4-4.5 days after it was started.
- If there aren't enough worms, it is possible to wait 1-3 extra days to harvest. Some mutants grow more slowly than N2 and will definitely require these extra days of growth.

Judging when the culture is ready to harvest takes a bit of experience. In the very best cultures, the bacteria are beginning to clear at the time of harvest (culture turns from milky to a clearer yellowish greenish brownish, and bacteria are less evident under the microscope), the worms are extremely dense, all stages of worms are present, and no dauers visible. If the worms get too dense all the worms will go dauer even if the food hasn't run out due to the accumulation of dauer pheromone. Of couirse, if the food runs out, the worms will also go dauer. Looking at 200 µl of liquid culture dumped onto an unseeded plate, when the culture is ready to harvest you should see about as many worms as you would find on a plate of worms (grown normally, on a seeded plate) that is about 0.5-1 days before starving.

Purify worms by flotation on sucrose (see first page) after harvest for all uses

C. elegans RNA prep

HH adapted from protocol found at:
http://info.med.yale.edu/mbb/koelle/protocols/protocol_worm_RNA_prep.html

by Michael Koelle and Tory Herman, adapted from Sambrook et al., "Molecular Cloning" 4/6/94

C. elegans RNA preps that have been widely used previously involved fairly slow lysis of the worms, potentially leading to degradation of the RNA. In addition, they failed to separate RNA from genomic DNA, leading to difficulties in determining the yield and purity of the RNA. Also, the contaminating AT-rich genomic DNA may interfere with poly A selection of the RNA. This protocol is based on standard methods that have been used for many years to isolate RNA from mammalian tissues and *Drosophila*; it involves very rapid lysis of the worms and subsequent purification of the RNA to homogeneity.

Precautions against RNase contamination (see Current Protocols online)

Solutions:

Caution: Wear gloves and avoid getting DEPC on your skin.

RNase free water (make several 100 ml bottles)
To ddH20, add DEPC to 0.1%

Shake to get the DEPC droplets into solution
Leave at Room Temp 15 min Autoclave 20 minutes to destroy the DEPC

1.0 M Tris pH7.5
Make using clean technique and DEPC treated water
autoclave
Tris solutions cannot be DEPC treated.

TE (10 mM Tris pH7.5, 1 mM EDTA)
Make using clean technique and DEPC treated water
autoclave
Tris solutions cannot be DEPC treated.

TE + 0.1% SDS
Make using clean technique and DEPC treated water.
Autoclave. Tris solutions cannot be DEPC treated.

20% sarcosyl (sodium laurel sarcosinate)
Make using clean technique, add DEPC to 0.1%
Shake to get the DEPC droplets into solution
Leave at Room Temp 15 min Autoclave 20 minutes to destroy the DEPC

Homogenization buffer
4.0 M guanidinium isothiocyanate
0.1 M Tris pH 7.5 (use the clean 1.0 M Tris solution described above)

- sterile filter to remove particulate matter
- just before use, add ß-mercaptoethanol to 1%

Cesium cushion solution
5.7 M CsCl, 0.01 M EDTA pH 8
96 g CsCl with
2 ml 0.5 M EDTA pH 8
H20 Vol. to exactly 100 ml,

- mark the level of the meniscus
- Shake to get the DEPC droplets into solution
- Leave at Room Temp 15 min Autoclave 20 minutes to destroy the DEPC
- After autoclaving, some volume may have been lost due to evaporation.
- Add DEPC treated water to bring the meniscus up to the mark, and mix.

3M sodium acetate pH 5.2
Make using clean technique, add DEPC to 0.1%
Shake to get the DEPC droplets into solution
Leave at Room Temp 15 min Autoclave 20 minutes to destroy the DEPC

RNase free 100 % EtOH
Use a fresh bottle, then label RNase free and keep clean

RNase free 70% EtOH
Prepare using the clean 100% EtOH and DEPC treated water

RNA prep method:

1. Start with a pellet of sucrose cushion floated/purified worms grown in liquid culture. It is best to have 1-1.5 mls of worm suspension (~90% worms in 0.1 M NaCl) in a 15 ml disposable snap cap tube. I

2. Using a motorized tissue homogenizer, add 5 volumes of homogenization buffer with fresh BME, and homogenize for 2 minutes.

3. Add sarcosyl to 0.5 % (using the RNase free 20% solution) and mix.

4. (optional) Pour the mixture into a 28 ml polycarbonate Oak Ridge tube. Spin in the 70Ti ultracentrifuge rotor for 20 minutes at 30K RPM at 20° to pellet debris. Pour off the supernatant into a clean 15 ml disposable tube. It should be brownish yellow.

5. Prepare one cesium gradient for each 8 mls of homogenate. Use RNase free polyallomar tubes for SW41 rotor.

6. Measure out 4 mls of the cesium solution (use a disposable plastic pipette) into each tube.

7. Layer the sample over the cesium pad by slowly drizzling it down the side of the tube. With a felt tip pen make a mark at the interface between the cesium pad and the homogenate.

8. Spin at 20° C for 18-24 hours at 27K RPM.

9. After the spin, carefully remove the tubes from the buckets into a rack, taking care not the disturb the gradient. The brownish proteins should still be above the felt tip pen mark; you may see a whitish doublet band within this brown material. Further down in the clear part of the cesium gradient there should be another white band; this is the DNA. The RNA is a crystal clear gelatinous pellet LOOSELY attached to the bottom of the tube; it will remain invisible until almost all the liquid is removed. The pellet looks about like a wispy flattened chunk of low melt agarose, with a volume of ~30 µl.

10. Removing the liquid requires great care, both to avoid contaminating the RNA with material from higher up in the gradient, and to avoid losing the difficult-to-see RNA pellet.

11. Carefully suck off the supernatant down to the felt tip pen mark. Do this by holding the pipette tip at the very surface of the liquid, so that both liquid and air are sucked up.

12. Rest tube 1min to let drops fall, and suck off more liquid, in the same manner, down to just below the whitish DNA band.

13. Rest tube 1 min to let drops fall and suck most of the rest of the liquid, leaving ~0.5 mls, so that liquid just fills the curved bottom part of the tube. The remaining liquid will have to be removed extremely carefully to avoid losing the RNA pellet.

14. Hold a new razor blade in a bunsen burner (using forceps or a hemostat) until it is red hot, and use the blade to cut (really melt) through the tube just above the level of the remaining liquid.

15. Using an RNase free 200 µl pipette tip, VERY CAREFULLY remove the remaining liquid. The clear gelatinous RNA may be floating or in several pieces at this point; sometimes it helps to tilt the tube bottom around to try to separate the liquid from the pellet so that the liquid can be safely sucked off.

16. Sometimes it is truly impossible to remove the last ~30 µl of liquid without sucking up chunks of RNA. In this case, transfer the remaining liquid with chuncks to an RNAse free 1.5ml tube and spin 14,000rpm for 1 min. Remove all liquid above soft grey/ clear pellet. Keep the trimmed centrifuge tube and

17. Add 500ul GTC lysis buffer with 0.5% sarcosyl and 1% BME to each tube, and suspend the RNA by pipetting up and down.

18. Transfer to the 1.5ml microfuge tube containing the RNA chuncks or a new tube.

19. Rotate 5 min at RT on an end –over – end rotator to dissolve.

20. Phenol extract with 500ul 4C tris saturated phenol. Spin max 10min 4C.

21. Transfer upper phase to a new tube and extract with 500ul 50%phenol50%chloroform. Spin max 5 min 4C.

22. Transfer upper phase to a new tube and add 1ml 100% EtOH. Chill 15 min on ice then spin max 15min 4C.

23. Discard the supernatant, wash the pellet with 70% ethanol, dry, and dissolve in a small volume of water, (perhaps ~80 µl, depending on how big the pellet looks).

24. Measure the concentration and purity of the RNA by making an appropriate dilution (~1000 fold) in water and taking the OD260 and OD280. RNA prepared by this method should be comletely pure, with an OD260/OD280 of 2.0. For calculating the RNA concentration, a solution of OD260=1.0 is 40 µg/ml.

25. Store the RNA at -80°. Diluting all samples to a standardized concentration (say 5 mg/ml) and aliquoting it is a good idea.

26. Yield: 0.8 to 1.1 mg of pure total RNA per ml of packed worms. These preps will be contaminated to some extent with RNA from *E. coli* that were in the starting material. The proportion of *E. coli* RNA in the prep can be assessed by running the RNA out on a gel, blotting, and staining the RNA on the blot with methylene blue; the *C. elegans* rRNAs, which run at 3.5 and 1.7 kb, can be resolved from the *E. coli* rRNAs, which run at 3.0 and 1.5 kb (see our Northern blot protocol for details on these methods). We found that if the worms were prepared using our liquid culture protocol, the amount of *E. coli* RNA in the final prep varied from undetectable to about 40% of the RNA.