Cleaning Worms

Mix equal volumes of 13% NaOCl (fresh), 2M NaOH, put 10 µl drop on small plate + 6 gravid hermaphrodites (which should dissolve), put them at 20C

Freezing worms

Wash 1 plate with 5 ml S-basal, put into blue cap 15 ml falcon, let worms settle down, remove S-basal to 1.5 ml, add 1.5 ml freezing solution - 3 aliquots of 1 ml into freezing tubes into freezing box (big styropor box with holes for tubes, there should be no air in it! Thaw 1 tube 24 hours later to check if worms are ok!

Production of dsRNA

PCR of gene of interest:

optimal are 1000 bp coding sequence (can be genomic or cDNA), 1 PCR primer with T3 and the other with T7 overhang + 20 bp corresponding to template sequence, conc 100pmol/µl(= 100 µM)

T3 and T7 sequences

AAT TAA CCC TCA CTA AAG G
TAA TAC GAC TCA CTA TAG G

1 µl of genomic worm DNA (1:50 diluted from Silke)
20 µl dNTPs 1 mM
1 µl of each primer
0.8 µl Taq
10 µl 10X buffer
66.2 µl H2O

if necessary MgCl2 concentration can be varied: 1.5 - 5.5 mM

in vitro transcription:

rNTP mix (Ambion Kit) 2 µl of each: rATP, rCTP, rUTP, rGTP
transcription mix : 8 µl DNA (PCR product, if very concentrated it can be diluted), 8 µl dNTP mix, 2 µl T7/T3 enzyme mix, 2 µl 10X buffer,

incubate 3-5 hours at 37C (incubator or heat block)

add 1 µl DNAse (do not put sample on ice)

incubate 15 min at 37C

add 10 µl of 10% SDS (normal solution, not DEPC water)

add 170 µl H2O

add 18 µl stop solution

mix well

add 1Volume (200µl) of Phenol (pH 8), vortex for 1 min, centrifuge, extract

add 1 V of chloroform, vortex for 1 min, centrifuge, extract

add 1 Volume of isopropanol - into liquid nitrogen, then for 20 min at -20C (not overnight!)

spin for 15 min at 4C, wash pellet with 70% Et OH (70%, DEPC)

dissolve in about 25 µl H2O-DEPC (it takes some time until it is dissolved - leave it on ice and flick tube every now and then)

keep 1 µl of each strand to analyze it on gel

anneal the two strands: 20 µl of each strand + 20 µl 3X injection buffer, 10 min 68C, then 30 min 37C (in PCR machine)

1 µl onto gel, should be shifted (higher) compared to single strands (Gel: TBE buffer prepared with DEPC water)

measure amount of RNA (260nm/280nm; factor = 40) dilute about 1:200; usually concentrations of 1 mg/ml are obtained

aliquot RNA and freeze in liquid nitrogen

RNAi to γ-tubulin (tbg-1)

1. PCR from cDNA

   Primer sequences

   CTC AAG CCT TCT GGA AAT CG + GCA TTG CAT CGT CCA TCA TA or
   TCC TCG ATA ATG CTG CAC TG + CTT TTG TAC GAC CGC CTT GT
2. Inject L4s and incubate 36 hours at least at 20C

RNAi to CeGrip-1

1. Generate RNA form cDNA to knock out CeGrip-1 (genomic sequence very large, big introns!)
2. Use C67 cDNA (Maxi Prep made by Sonja), dilute 1:10 for PCR (~170 ng), primers SPC98 left and right (100 pmol/µl)

   Primer Sequences: AGA ATG AGC AGA GCG GAG AG + GCG AGC TCT TTT TCG ATT TG
3. Inject L4s and incubate 36 hours at least at 20C

Double RNAi experiments

Zyg-9 : γ RNA = 1:3 (measure OD to determine concentration) zyg-9 RNA is much more efficient!

Dynein : γ = 1:3

PCR from wormprep (e.g. for sequencing tbg-1 in t1465)

1. Lysis of worms

   Buffer:

   100 µl 10X PCR buffer
   10 µ MgCl2 (100 mM: 1 mM final)
   10 µl Proteinase K (6 mg/ml stock)
   100 µl 4.5% Tween-20
   H2O final 1 ml
2. 10 µl of buffer into PCR tube
3. add 50 worms (t1465 uncs, be sure that no embryos are on them, which could be heterozygous, pick L4s, so that they can't be fertilized with wildtype sperm
4. 10 min into liquid nitrogen
5. 60 min 60C + 15 min 95 C (PCR machine)
6. 10 µl of this DNA solution for PCR reaction + 8.52 µl PCR buffer + 20 µl dNTPs (1mM) + 3 µl of each primer (100 µM) + 1 µl Taq + H2O up to 100 µl.

Cosmids

Handling cosmid stabs

In order to have the best chance of retaining an intact cosmid stock, it is a good idea to make an instant glycerol stock. This is done by taking an inocculum straight from the stab into a small volume (o.1 ml) of medium + 30 % glycerol. Vortex and freeze immediately at -80C.

Also immediately, colony purify from each stab. Streak out the stab to generate well isolated colonies and pick smallish but not pin prick colonies for a small volume (4 ml) liquid culture. Avoid large blobby

colonies at all cost. Grow several such overnight cultures, and take an aliquot of each for glycerol stocks. Ideally miniprep cosmid DNA from the remaining culture and assess the integrity of the cosmid DNA on 0.4% agarose gels.

Cosmid prep

Do not use miniprep spin kit, because it works only < 8 kb and cosmids are usually larger!!

1. 9 ml culture into 50 ml Falcon, spin at 5000 rpm for 5 min
2. add 900 µl cold P1, resuspend thoroughly, transfer into centrifuge tubes for SA-600)
3. add 900 µl P2 5 min RT
4. add 900 µl cold P3 (on ice)
5. spin at 14000 rpm 10 min RT (SA-600)
6. SN into new centrifuge tubes + 1.9 ml isopropanol
7. Spin 30 min at 14000 RT in SA-600
8. Discard SN, resuspend DNA pellet in 100 µl H2O + 1 ml QBT
9. Put DNA onto equilibrated tip 20
10. Wash 4x
11. Elute with QF 0.8 ml into 0.56 ml isopropanol in eppi
12. Spin 30 min at 13000 rpm
13. Dry pellet in speedvac, wash with EtOH, resuspend in 30 µl H2O

Rescue Experiment

1. Inject cosmid F58A4 (300µg/ml) at a concentration of 15 ng/ul together with a GFP_Marker (for muscle form Sophie) at 20 ng/ul and Bluescript (uncut, purified from Sophie) at 100ng/ul
2. Inject ~ 30 heterozygous t1465, put 3 worms on 1 small plat
3. *3 days later:*

   screen worms at GFP dissection scope - I had 62 green uncs (F1), which I put each on an extra plate (if progeny of these survives, then the rescue worked)I picked also 8 GFP worms in case that there is no rescue in F1, then the progeny of these GFP worms can be checked for rescue in F2.

4. *2 days later:*

4/62 worms had progeny (F2)

39

4 L4s
7 ~L1s
38 embryos

41

28 L1s
43 embryos

51

6 L4s
13 L1-L3s
93 embryos

60

44 L4s
2 L1s
99 embryos

5. Put F1s onto new plates, count again 1 day later and repeat procedure one more day to get all the progeny of the F1 animal, then determine how many living adult F2s you get from all the embryos of the F1 animal:

39: 14/94

41: 32/67

51: 24/152

60: 52/121

in total 122/434

6. I need to do a negative control: take 5 t1465
7. After 2 days start counting progeny and put adult on new plate. In total 0/247 embryos hatched.

Nocodazole experiment

Nocodazole from stock 5 mg/ml (prepared by Stephan) - dilute 1:500 (10 μg/ml)

Dissect worms on slides coated with 1 mg/ml polylysin (martin method doesn't work!) put coverslip on top, apply gentle pressure to crack egg shell (e.g. pipette tip) incubate in a humid chamber (weigh boeat with wet klenex) for 7 min, plunge into liquid nitrogen.

Nocodazole experiment (Pierre's method)

6 worms into 2.5 ul nocodazole (stock solution 100 mM - dilute 3 ul in 100 ul))
dissect them, put coverslip on top and tip on it with forceps or yellow tip (softly)
put petri lid on top and leave it for 7 min, then plunge the slide into liquid nitrogen.

Regrowth experiment

20 worms into 4μ ???????? on polylysine slide, put coverslip on top, apply gentle pressure (to squeeze out the embryos). Place slides on an aluminium block in an ice bath under a humidifying cover for 5 min, then transfer it to a alu-block at RT (waterbath with thermometer, 22-25C) for 15, 30, and 90 s under a humid chamber), plunge into liquid nitrogen.