
Pristionchus pacificus genetic protocols*

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1. Introduction

The diplogastrid nematode *Pristionchus pacificus* has been recently established as a new genetic model system for evolutionary studies. Forward and reverse genetics tools have been developed to allow detailed comparisons to the rhabditid nematode *C. elegans*. *P. pacificus* has distinctive body morphology and diverges in development both at the cellular and molecular level when compared to *C. elegans* (see [Evolution of development in nematodes related to *C. elegans*](#)). Despite these differences, *P. pacificus* shares many traits with *C. elegans* that are of advantage for genetic analyses: it is hermaphroditic, has a small genome size (160 Mb), small number of chromosomes (haploid set with 5 autosomes and 1 sex chromosome), short life-cycle (4 days at 20°C), and produces large brood sizes (150–200 eggs). Similarly to *C. elegans*, *P. pacificus* has 4 larval stages (J1-J4). However, the first larval stage molts within the eggshell (Sudhaus et al., 2003).

2. Protocols

2.1. Part A: Freezing worms

P. pacificus can be frozen in liquid nitrogen, although with lower efficiency than *C. elegans*. It was observed that the addition of calcium to the M9 dramatically increased *P. pacificus* freezing efficiency. As for *C. elegans*, *P. pacificus* early hatched larvae are most likely to survive freezing and thawing. Some modifications were added to the common *C. elegans* freezing protocol to increase efficiency (Protocol 1).

*Edited by Ralf J. Sommer. WormMethods editor, Victor Ambros. Last revised June 29, 2006. Published July 17, 2006. This chapter should be cited as: Pires da Silva, A. *Pristionchus pacificus* genetic protocols (July 17, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.114.1, <http://www.wormbook.org>.

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Protocol 1. Freezing *P. pacificus*

Equipment and Reagents

- Plates with Nematode Growth Medium (NGM)[15 g agar, 2.4 g NaCl, 2 g Tryptone, 2.72 g KH_2PO_4 , H_2O to 1 liter. Autoclave, let the solution cool to 60°C. Add 0.8 ml 1 M CaCl_2 , 0.8 ml cholesterol (stock solution: 2.5 g in 500 ml ethanol), 0.8 ml 1 M MgSO_4]. Add 250 μl of *E. coli* OP50 to each 6 cm diameter plate.
 - M9 Buffer [Protocol 6 (see [Maintenance of *C. elegans*](#))] containing 1 mM CaCl_2 .
 - Soft Agar Freezing Solution [0.58 g NaCl, 0.68 g KH_2PO_4 , 24 ml glycerol, 0.56 ml, 0.4 g agar, H_2O to 100 ml (autoclave). Add 3 ml of 0.1 M MgSO_4]
 - 1.8 ml cryotube vials
1. Grow worms on about 10 NGM plates until one day beyond the time the plates are just cleared of bacteria. Heat-shock worms on the plates at 30°C for 2–3 hours.
 2. Wash each plate with 2 ml M9, and transfer to a sterile 15 ml tube. Centrifuge worms at 1500 x g for 5 minutes, and combine all worms in one tube in 3 ml of M9. Add an equal volume of Freezing Solution. Mix well.
 3. Aliquot 1 ml of the mixture into 1.8 ml cryovials labeled with the strain name and date.
 4. Put cryovials into a Styrofoam tube tray and cover with another (an alternative is to take a Nalgene Cryo 1°C Freezing Container filled with 100% isopropyl alcohol). Fasten the racks with a rubber band, enclosing the samples inside the box. This ensures a slow constant rate of cooling.
 5. Place the box at –80°C for at least one day.
 6. On the following day, once completely frozen, move the vials to the permanent location in a liquid nitrogen tank.

2.2. Part B: Mutagenesis

Ethyl methanesulfonate (EMS) has been widely used in *P. pacificus* to isolate a wide variety of mutants with defects in the egg-laying system, muscle, sex determination, dauer formation, behaviour, and gonad formation (Protocol 2). The protocol for EMS mutagenesis used is the same as for *C. elegans* (Brenner, 1974). For mutagenizing *P. pacificus* with psoralen, however, some modifications were introduced (Protocol 3).

Protocol 2. EMS mutagenesis

Caution: EMS is a very strong mutagen and should be handled in a fume hood. All materials (gloves, pipettes, tips) that have been in contact with this mutagen should be treated with 1N NaOH to inactivate the EMS.

Equipment and Reagents

- M9 Buffer [Protocol 6 (see [Maintenance of *C. elegans*](#))]
 - EMS (ethyl methanesulfonate; Sigma, cat no. M-0880)
 - Well fed, young adult *P. pacificus* worms
 - NGM plates seeded with *E. coli* OP50 (to prepare NGM plates, see [Protocol 1](#)).
1. Wash the worms from 4–5 6 cm diameter plates using 2 ml of M9 for each plate. Collect in a 15 ml tube.

2. Centrifuge for 5 minutes at 1500 x g and resuspend the worms in 2 ml M9.
3. Add 20 µl of EMS into another tube containing 2 ml of M9. Swirl the tube until the EMS has dissolved.
4. Add the EMS solution to the worms (this concentration of EMS corresponds to 47 mM). Incubate the worms at 20°C for 3.5 hours in horizontal position, on a rocker at low speed (40UPM).
5. Incubate for 30 min in a vertical position to let worms sink.
6. Remove as much supernatant as possible. Discard the supernatant to a container with 1 N NaOH to inactivate the EMS.
7. Wash the worms 3 times with 5 ml of M9, and centrifuge each time at 1500 x g for 5 min.
8. Resuspend the worms in 500 µl of M9 and dispense them onto 2 NGM plates with OP50.
9. After the solution has soaked in the plate, pick moving J4 animals to new plates.
10. Depending on the mutation of interest (dominant, recessive, maternal effect), pick animals of the relevant generation onto fresh plates, and screen for desired mutant phenotypes.

Protocol 3. Psoralen mutagenesis (modified from Yandell et al., 1994)

Equipment and Reagents

- M9 Buffer [Protocol 6 (see *Maintenance of C. elegans*)]
 - 3 mg/ml TMP solution in DMF (Trioxsalen; Sigma, cat no. T6137; 4,5',8-Trimethylpsoralen)
 - Well fed, young adult *P. pacificus* worms
 - Unseeded and seeded NGM agar plates (to prepare NGM plates, see Protocol 1).
 - Long-wave ultraviolet (UV) source (365 nm; Model UVL-21 Blak-Ray lamp, UVP, Fisher Scientific cat no. 11-984-40)
 - UV intensity meter (Model J-221, UVP)
 - Rocker
1. Wash the worms from 4-5 6 cm diameter plates using 2 ml of M9 for each plate. Collect in a 15 ml tube.
 2. Using M9 buffer, wash worms off NGM plates, and combine in a 15 ml tube.
 3. Wash 2 times with M9, each time pelleting worms at 1500 x g for 5 minutes.
 4. Remove as much liquid as possible and resuspend in 30 µg/ml of TMP
 - e.g.: 20 µl of TMP (3 mg/ml) in 2 ml of M9
 - Note: the worms should be resuspended in about 10 times their volume
 5. Wrap the tube in aluminum foil and incubate the worms for 15 minutes at room temperature on a rocker at low speed (40 UPM). Afterwards, incubate for 5 min at a vertical position to let worms sink.
 6. Remove worms with a Pasteur pipette and place them in an unseeded worm agar plate at 20–25°C. Wrap the

plate in aluminum foil and let excess TMP to soak into the plate.

7. Calibrate the distance between the long-wave UV source and the plate using the UV intensity meter. Irradiate the worms for 50 seconds at $500\mu\text{W}/\text{cm}^2$ (the distance between the plate and the UV lamp is about 10 cm).
8. Keep the worms in the dark for 5 hours.
9. Transfer worms to seeded plates.

2.3. Part C: Construction of deletion libraries to generate *P. pacificus* gene knock-outs

A powerful method to isolate *C. elegans* mutants in a gene of interest for which sequence information is available is by chemical mutagenesis followed by polymerase chain reaction (PCR). The generation of knockouts in a high throughput manner by using deletions in a library of worms has proven very successful for *C. elegans*. The protocol adopted for *P. pacificus* does not include the freezing of the library, since the efficiency of recovery following freezing of *P. pacificus* is too low (Protocol 4). The library comprises 12 96-well microtiter plates and includes the arrayed progeny of nearly 5×10^5 F1 animals representing 10^6 mutagenized genomes. Worms are grown in plates until the food is exhausted, generating, on the average, 100 F2 progeny per F1 animal.

Protocol 4. Deletion library

Equipment and Reagents

- M9 Buffer [Protocol 6 (see Maintenance of *C. elegans*)]
- Basic hypochlorite solution: 30 ml 0.1M NaCl, 5 ml NaOH 5M and 10 ml 6% Sodium hypochlorite.
- Wild type worms
- 130 NGM plates (10 cm diameter) seeded with 1 ml of OP50 (to prepare NGM plates, see Protocol 1).
- 1200 NGM plates (6 cm diameter) seeded with 100 μl of OP50.
- 12-well NGM plates seeded with 50 μl of OP50.
- 20 ml 10 X lysis buffer [10 ml 1 M KCl, 2 ml 1M Tris pH 8.2, 0.5 ml 1M MgCl_2 , 0.9 ml NP-40, 0.9 ml Tween-20, fill to 20 ml with water]
- Proteinase K (ptnK) 10 mg/ml
- MicroAmp Clear Adhesive films (AB Applied Biosystems, Part 4306311)
- Oven 65°C
- Refrigerated incubator at 12°C
- 1.2 ml 96-well plates
- 200 μl 96-well plates
- 10 X yellow loading dye (6 g Ficoll, 30 mg Tartrazine, to 30 ml with water)

2.3.1. Designing primers for the gene of interest

Target a region of around 1.5 kbp (1.0-2.5 kbp), with nested oligonucleotides of around 20 bp (annealing temperature of 60°C). The larger the region targeted, the more difficult it is to detect small mutations in agarose gels. The smaller the region, the more difficult it is to find a deletion that happens to have occurred between the primers.

2.3.2. Growth synchronization

1. Transfer freshly starved larvae from 10 6 cm diameter plates to 15 10 cm diameter plates seeded with 1 ml of OP50 per plate. Incubate at 25°C (or 20°C) until plates are full of eggs.
2. Transfer worms and eggs to a 50 ml Falcon tube with M9 and centrifuge for 5 minutes at 1300 X g. Discard the supernatant. Add 30 ml of basic hypochlorite solution and incubate at room temperature for about 4 minutes. Collect the eggs by centrifugation (2000 x g, 5 min).
3. Wash the eggs 2 times with 30 ml of water and 1 time with 30 ml of M9 buffer. Leave the worms to hatch overnight in the buffer or in a 10 cm unseeded NGM plate.
4. The next day, pipet 10 µl of the suspension onto each of three plates and count to determine titer. The total number of worms in 50 ml should be around 100 000 worms (20 worms/10µl). Distribute about 2000 worms per plate onto 50 seeded 10 cm NGM plates.

2.3.3. Mutagenesis

5. Culture synchronized culture of worms for about 52 hours at 20°C. Follow [protocol 3](#) for TMP/UV mutagenesis. After mutagenesis, plate the worms on 50 fresh seeded 10 cm NGM plates, with 2000 worms/plate.

2.3.4. Plating the library

6. After a couple of days, when P0s have laid the F1 eggs, bleach the 50 10 cm plates (as in part B, 2) and leave the eggs in 50 ml of M9 buffer overnight.
7. The following day, count the number of L1 worms. Distribute about 400 F1s/small plate (total 1200 6 cm plates).
8. Wait one generation, until the F2 eggs have hatched. Wash plates by adding 800 µl of water and transferring 150 µl of the L1 suspension into a 1.2 ml 96-well plate (to avoid mistakes, leave the yellow tip inside the well). This step will take about half an hour/plate; in total it will result in 12 x 96-well plates. Leave the washed plates at RT until they dry. Then transfer them to 12°C.
9. Add 150 µl of lysis buffer + proteinase K (120 µg/ml) to each well of the 1.2 ml 96-well plates, seal them with MicroAmp Clear Adhesive films and incubate them first at -80°C for 2 hours and then at 65°C overnight with shaking.
10. The following morning, centrifuge the 96-well plates for 10 min at 4000 rpm.
11. Transfer 150 µl of the suspension into a 200 µl 96-well plate and inactivate the proteinase K at 95°C for 10 minutes. The remaining 150 µl should be stored at -80°C.
12. Pool the rows and columns from each of the 12 plates by combining 75 µl of each of the wells into a single 1.2 ml 96-well plate. For example, pool wells A1 of all 12 plates into the position A1 of the 1.2 ml 96-well plate. Repeat this procedure for each well, making a total of 96 pools. This is the master plate with pooled DNA.

2.3.5. Screening the library by PCR

13. Take 5µl as template for a 20 µl PCR reaction. It is necessary to perform nested PCR. For the 2° round, use 1 µl of the 1° round.

a. Example for PCR mix:

1st PCR round: [final Volume 10 μ l]

| | |
|--|-------------|
| H ₂ O | 1.7 μ l |
| dNTP (10mM each of dATP, dCTP, dGTP, dTTP) | 0.2 μ l |
| forward primer (10 μ M) | 0.5 μ l |
| reverse primer (10 μ M) | 0.5 μ l |
| Buffer 5X | 2.0 μ l |
| Taq (5 U/ μ l) | 0.1 μ l |
| DNA (pooled Library) | 5.0 μ l |

94° C for 2 min.

94° C for 30 sec.

58° C for 20 sec. 35 cycles

72° C for 3 min.

72° C for 6 min.

→ add 20 μ l H₂O to 1° round.

2nd PCR round: [final Volume 10 μ l]

| | |
|---|-------------|
| H ₂ O | 3.7 μ l |
| dNTP (10 mM each of dATP, dCTP, dGTP, dTTP) | 0.2 μ l |
| forward primer (10 μ M) | 0.5 μ l |
| reverse primer (10 μ M) | 0.5 μ l |
| Buffer 5X | 2.0 μ l |
| Taq (5 U/ μ l) | 0.1 μ l |
| yellow Loading dye | 2.0 μ l |
| DNA (1:3 diluted) | 1.0 μ l |

Same cycling conditions as 1st round.

14. Select samples which show bands that are smaller than the wild-type for further tests. Determine the precise address of the candidate sample by repeating the PCR using 5 μ l of the corresponding well of each of the twelve 96-well plates (200 μ l 96-well plates). This will give the number of the plate (2-A5, for example).

2.3.6. Sib selection

15. Resuspend the original plate in 50 ml water or M9 (50 ml Falcon tube). Count the number of worms/ μ l, by pipetting 3 times 10 μ l into a plate. Calculate the average of worm/ μ l.
16. Dispense 50 worms into the wells of 8, 12-well plates. The remaining worms (which were not dispensed) should be centrifuged and put back in the original plate, without bacteria. Keep this plate at 12°C.
17. Culture the worms at 20°C for 5 days. Harvest a portion of each well by washing with 300 μ l of water. Transfer 75 μ l of the suspension into a 1.2 ml 96-well plate, plus 75 μ l lysis buffer with ptnK. Incubate overnight at 65°C with shaking. The agar plates with worms should be kept at 12°C.
18. Transfer 100 μ l to a 200 μ l 96-well plate.
19. Inactivate the ptnK by incubating the 96-well plate at 95°C for 15 minutes.
20. Screen the plate by PCR (5 μ l as template), identify the plate and repeat procedure by dispensing 96 x 15 worms/plate.
21. Repeat procedure above with 5 worms/plate and later 1 worm/plate.

2.4. Part D: RNAi and morpholino by injection

The possibility of rapidly knocking-down genes by RNA interference (RNAi), together with the availability of the *C. elegans* genome sequence, stimulated the study of gene function in a global scale (see [Reverse genetics](#)). Currently there are three different ways of delivering double-stranded RNA (dsRNA) into *C. elegans*: injection, soaking and feeding. Preliminary experiments with *P. pacificus* indicate that RNAi works when injecting dsRNA into the gonad (personal observation). The conditions used for dsRNA preparation and injection were basically the same as described for *C. elegans* (see [Reverse genetics](#)). The injection of a 1081 bp *Ppa-tra-1* dsRNA, for instance, resulted in 20% (n=346) progeny with the transformer phenotype. For genes involved in vulva development, however, the efficiency of the RNAi seems to be much lower (Zheng and Sommer, personal communication). Similar observations have been made with vulva genes in *C. elegans* (Chen et al., 2004). Another knockdown technology, using morpholino oligonucleotides, has proven robust for *P. pacificus* (Protocol 5) (Pires-daSilva et al., 2004; Zheng et al., 2005). Morpholino-oligonucleotides, widely used for knocking down genes in other model systems (e.g., sea urchin, zebrafish), are modified oligonucleotides that efficiently block the translation or splicing of specific mRNAs. The properties of morpholino oligonucleotides, which combine nuclease-resistance, water-solubility and high specificity, are of major advantage. However, the injection of morpholinos causes a high rate of lethality in the *P. pacificus* F1 progeny (about 50%). When selecting oligo sequences, it is recommended to design at least two non-overlapping oligos. In this way, the consistency of the resulting phenotype can be tested. A good target sequence has minimal mRNA secondary structure and includes the translational start codon and/or the 5'UTR region.

Protocol 5. Injection of morpholino oligonucleotides

Equipment and Reagents

- Agarose
 - Glass coverslips (22 x 50)
 - Tips for injection Microloader (Eppendorf 5242956.003)
 - Light mineral oil (Sigma M-3516)
 - Glass thin filaments 1.0 mm (World Precision Instruments)
 - Transjector (Eppendorf 5246)
 - Micropipette puller
 - Inverted microscope (Zeiss, Axiovert 135)
 - Morpholino oligonucleotides (Gene Tools)
1. Prepare 5 ml of 2% agarose in water, and melt it in the microwave.
 2. Drop the melted agarose on to a coverslip. Put another coverslip on top. Repeat this procedure for 10 more pairs of coverslips.
 3. Separate the two coverslips and leave the agarose pad in an 80°C drying oven for one hour.
 4. Dissolve the oligonucleotide to 100 µM in water.
 5. Pull a glass thin filament with the needle puller (the tip of the needle should taper quickly and smoothly).
 6. Load about 500 µl of the morpholino into the needle using Microloader tips. To discard bubbles accumulating in the tip of the needle, hold the loaded needle in vertical position for a few minutes.
 7. Attach the needle to the transjector. To break the tip of the needle, rub the end of the tip against a thin glass bar immersed in light mineral oil. Monitor the flow of liquid out of the needle under the 40 X objective.

8. Add a drop of the light mineral oil to the agarose pad. Pick a few worms and place them into the oil. Gently force the worms to the agarose pad with the wormpick, so that the entire body of the worm sticks to the pad.
9. After aligning the needle with the worms, move the gliding stage against the tip of the needle. In this way the needle will penetrate the cuticle of the worm and reach the gonad. Apply pressure to expel the morpholino solution into the gonad.
10. Recover the worms from the agarose pad by adding a drop of M9 buffer onto the surface of the oil. Transfer worms to a freshly seeded plate with a mouth pipette.

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