Video Article

An Introduction to Worm Lab: from Culturing Worms to Mutagenesis

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Abstract

This protocol describes procedures to maintain nematodes in the laboratory and how to mutagenize them using two alternative methods: ethyl methane sulfonate (EMS) and 4, 5', 8-trimethylpsoralen combined with ultraviolet light (TMP/UV). Nematodes are powerful biological systems for genetics studies because of their simple body plan and mating system, which is composed of self-fertilizing hermaphrodites and males that can generate hundreds of progeny per animal. Nematodes are maintained in agar plates containing a lawn of bacteria and can be easily transferred from one plate to another using a pick. EMS is an alkylating agent commonly used to induce point mutations and small deletions, while TMP/UV mainly induces deletions. Depending on the species of nematode being used, concentrations of EMS and TMP will have to be optimized. To isolate recessive mutations of the nematode *Pristionchus pacificus*, animals of the F2 generation were visually screened for phenotypes. To illustrate these methods, we mutagenized worms and looked for Uncoordinated (Unc), Dumpy (Dpy) and Transformer (Tra) mutants.

Video Link

The video component of this article can be found at http://www.jove.com/video/2293/

Protocol

Part 1: Preparing nematode growth media (NGM) Petri plates

Experimental nematodes such as *C. elegans* and *P. pacificus* are typically cultured in the laboratory on 6 cm Petri plates containing Nematode Growth Medium (NGM) [1]. Worms are kept at 20°C, but can be incubated at a range of temperatures (between 4°C - 25°C), depending on the nematode species and experimental design. To prepare plates with NGM, standard sterile techniques have to be used to prevent fungal and bacterial contamination. Following is the protocol to prepare NGM plates:

1. Weigh 15 g agar, 2.4 g NaCl, 2 g Tryptone, and 2.72 g KH_2PO_4 in 2 L conical flask, make volume up to 1 L with water, autoclave and allow it to cool to 60° C in a water bath.
2. Add 0.8 ml each of 1 M $CaCl_2$, cholesterol (5 mg/ml in ethanol), and 1 M $MgSO_4$. To prevent fungal and bacterial contamination, 1 ml streptomycin (100 mg/ml) and 1ml nystatin (10 mg/ml) can be added to each liter of medium.
3. Using a Unispense machine and maintaining sterile conditions, pour the media into the desired plates. The plates should be filled to about 2/3 rd full. A standard 60 mm diameter plate requires about 10 ml of the media. Do not move the plates until the agar is completely gelified; otherwise there will be an uneven surface of agar, making it difficult to focus worms on the stereomicroscope.
4. Once dried, the plates can be seeded immediately or stored at 4° C until seeding with bacteria.

Part 2: Preparing OP50 bacteria and seeding NGM Petri plates

P. *pacificus* is cultured in the laboratory by feeding on the *E.coli* OP50 strain. OP50 has a limited growth on NGM plates, thereby forming a thin lawn that facilitates the visualization of the worms[1]. To grow a culture of OP50, use LB-broth:

1. Add 5 g bactotryptone, 2.5 g yeast extract and 2.5 g NaCl to a 500 ml screw cap bottle, make up the volume to 500 ml with distilled water and autoclave.
2. Inoculate the broth with a single colony of OP50 from the culture plate under sterile conditions and let it grow overnight at 37° C.
3. LB-OP50 is ready to be used and can be stored at 4° C for several months if sterility is maintained. Whenever plates need to be seeded, pour a small amount of the broth into a small sterile beaker and this will help prevent contamination of the stock culture.
4. To seed plates, dispense 100-120 µl of the OP50 onto the 60 mm diameter NGM Petri plate and swirl to spread it.
5. Incubate these seeded plates overnight at room temperature for the lawn to grow. These seeded plates can now be stored at 4° C for up to 3 weeks. Storing them in an inverted position helps prevent rapid drying.

Part 3: Making a pick

In this section we describe how to make a pick for worms. The pick is used to transfer worms from one location to another. The pick is made of 30 gauge 90% platinum 10% iridium wire, though some researchers may prefer slightly different metal compositions:

1. Cut about 3-4 cm of wire, place 0.5 cm of it inside the tip of a Pasteur pipette (whose tip was broken to a smaller length), and then seal it into the glass over a Bunsen burner. The length of the wire protruding from the glass is about 3-3.5 cm, but can vary according to individual preferences.
2. Flatten the end of the wire that is protruding using a hammer or pliers. Then bend the flattened portion upwards to form a scoop.
3. Smoothen the sharp edges of the pick with a sand paper to prevent damaging the worm or the agar.

Part 4: Picking worms

1. To pick worms, sterilize the platinum wire on a flame and drag the flattened tip of the pick along the bacterial lawn to coat the tip with thick sticky bacteria, taking care not to puncture or damage the agar surface.
2. Using the stereomicroscope, very lightly brush the sticky tip against the worm/worms to be picked till the worm sticks on to the bacteria on the pick.
3. Once the worm is on the tip of the pick, transfer it immediately to the new plate by touching or sliding the tip of the pick against the bacterial lawn of the new plate, and the worm should crawl off. Care must be taken not to damage the surface of agar on the plate else the worms crawl into the holes making it difficult to retrieve them. If the worm stays on the pick for too long, it might desiccate.

Part 5: EMS mutagenesis

Ethyl methane sulfonate (EMS) induces a broad spectrum of mutations in the in the genome 2. Mutants can be identified by defects like egg laying, muscle defects, sex determination, dauer formation, behavior, and gonad formation. The protocol for EMS mutagenesis is similar to that described for *C. elegans* [1].

1. Prepare 500 ml (or more) of 1N NaOH.
2. Take 4-5, 6 cm diameter Petri plates full of worms, containing a few hundred worms at the third larval juvenile (J3) stage and wash the worms off using 2-3 ml of sterile M9 per plate.
3. Collect all the worms in a sterile, disposable 15 ml centrifuge tube. Centrifuge at 1500 x g for 5-7 minutes or until the worms form a pellet.
4. Aspirate the liquid and use 2 ml of M9 to re-suspend the worms. Add 20 µl EMS to a tube containing 2ml of M9 and shake to dissolve it. Then add this EMS solution to the 2ml of worm suspension and swirl gently. The final concentration of EMS will be 47 mM. Caution: EMS is a very strong mutagen and should be handled in a fume hood. All materials (gloves, pipettes, tips) that come in contact with this mutagen should be treated with 1N NaOH to inactive the EMS. Wear double gloves during the entire EMS mutagenesis procedure. Dispose the hazardous waste according to your institution's guidelines.
5. Place the centrifuge tube and cover the cap with parafilm; place it horizontally in the fume hood and let the worms incubate for 3.5 hours. The tube can also be placed on a low speed rocker.
6. Turn the tube and cover the cap with parafilm; place in vertical position and incubate until the worms sink.
7. Aspirate the supernatant and discard it into a beaker/flask containing 1N NaOH. This will inactivate the EMS.
8. Add 5 ml M9 to the worms, invert the tube 25-30 times to wash the worms, centrifuge at 1500 x g for 5-7 mins or until the worms form a pellet. Aspirate the supernatant and discard into the 1N NaOH beaker.
9. Repeat the washing step at least 3 times.
10. After the final wash, re-suspend the worm pellet in minimal amount (~500 µl) of M9 and pipette them out onto NGM plates containing a lawn of OP50 bacteria. At this point you no longer need to work in the chemical hood.
11. Let the liquid dry for a few minutes and then pick and transfer healthy looking J4 larvae to fresh seeded plates. Discard the original plate according to your institution's hazardous waste guidelines.
12. Let the mutagenized worms self fertilize. Using a stereomicroscope, screen the F2 generation phenotypes for recessive mutants of interest.

Part 6: Psoralen mutagenesis

In this section we describe the procedure for mutagenesis using TMP in conjunction with long wavelength ultraviolet (UV). TMP/UV method of mutagenesis is widely used to induce deletion mutations within a genome [3].

1. Collect worms from 4-5, 6 cm diameter Petri plates into a 15 ml centrifuge tube as described in the previous procedure.
2. Add 10 ml M9, invert 20 times to wash the worms, and centrifuge at 1500 x g for 5 minutes. Discard the supernatant and repeat the washing for a total of 3 times. Aspirate the liquid and re-suspend the worms in about 10 times their volume of M9 containing 30 µg/ml of TMP. Pipette 20 µl of TMP (3 mg/ml) into 2 ml of M9. Like EMS, TMP is a potent carcinogen and must be handled taking proper safety measures. To prevent from potential risk of the UV light proper eye wears should be used. The disposables must be discarded in proper biohazard bags.
3. Incubate the tube in the dark for 15 minutes at room temperature on a low speed rocker (40 UPM). Set the covered tube vertically for 10 minutes to let all the worms sink.
4. Remove the worms from the bottom of the tube with a Pasteur pipette and transfer them onto an unseeded NGM plate. Discard the supernatant following your institution's hazardous chemical guidelines.
5. Keep the plate in dark until the excess TMP is absorbed into the plate.
6. Using a UV intensity meter, set the distance between the long wave UV source and the plate such that the intensity on the plate is 500 µW/cm^2 [4]. Remove the aluminum cover and lid and cover and expose the plate containing the TMP treated worms to the long wave UV radiation for 50 seconds.
7. Cover and leave the worms in the dark for 5 hours at room temperature.
8. Pick and transfer healthy looking J4 larvae to fresh seeded plates. Discard the original plate following your institution's hazardous chemical guidelines.
9. Using a stereomicroscope, screen the F2 generation phenotypes for the mutation of interest.

Part 7: Representative Results

Some of the *P. pacificus* mutant phenotypes resulting from the mutagenesis (either by EMS or TMP/UV) are shown in Figure 1. Each mutant has a characteristic phenotype that is easily distinguishable from the wild type animal. From the worms that were treated with the mutagen, 30 J3/J4

larvae from the parental generation (P0) were placed in separate plates and allowed to lay eggs, which are the F1 generation. From the plates that showed healthy F1 progeny, a total of 1000 F1s were transferred on to individual plates and the F2 progeny was screened for phenotypes. Animals with Dumpy [5,6], Uncoordinated[6] and Transformer[7] phenotypes were found.

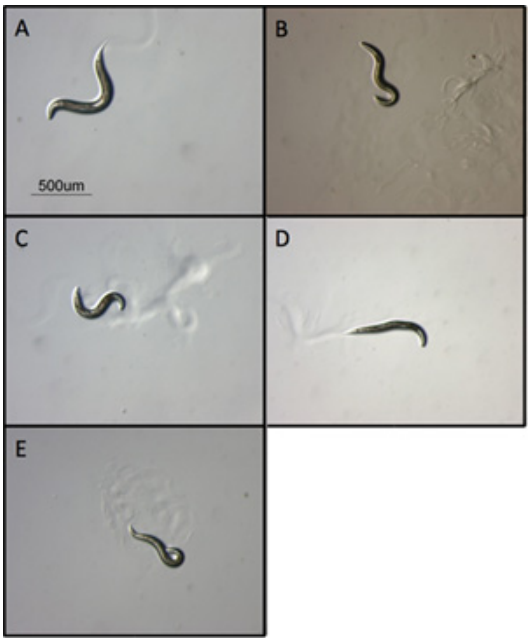

Figure 1. *Pristionchus pacificus* wild type and mutant animals. A. wt hermaphrodite B. wt male C. *dpy* mutant *D. unc* mutant *E. tra* mutant . Top two panels showing wild type hermaphrodite (left)and male (right) animals to compare with the different mutants in the remaining panels.

Discussion

Mutagenesis is a widely employed technique for genetic studies in various experimental model organisms. Mutagenesis experiments are often used to identify the function of a gene[2]. The mutagenesis procedures described here can be used not only for *P. pacificus*, but also for *C. elegans* and other related nematode species. Here we describe two methods, EMS and TMP/UV mutagenesis.

EMS is a chemical that induces point mutations or small deletions throughout the genome[3,8]. Psoralen mutagenesis (TMP/UV) mostly induces deletion mutations in the DNA due to possible breakage induced by the UV light. The deletion fragments are typically in the range of 0.10 to 15 kb in length [3].

We screened for phenotypes that are relatively common to find. In a screen using EMS, for each 400 plates with F2 animals, about one plate had a Dpy, Unc or Tra. Dpy can be recognized by its shorter size[7], Unc for its abnormal movement[6] and Tra for having a male phenotype and an XX karyotype[7]. TMP/UV mutagenesis has a lower frequency of generating mutants compared to EMS [3], but it generates bigger deletions.

For nematodes, the late fourth stage larva or young adult is the most effective developmental stage to carry out mutagenesis because they have the maximum number of germ line nuclei. It is therefore useful to start the experiment with a synchronized parental population with many fourth stage larvae. A simple way of making synchronized cultures is by making new cultures with eggs instead of adult worms. In the following generation, most worms will be about the same age. Recessive mutations can be detected by inspecting the progeny of F1 individuals, which will produce about twenty five percent of homozygous.

The power of genetic screens in nematodes can be further exploited by modifying the design of the screen[9]. The combination of hermaphroditic lifestyle and rapid generation time makes nematodes ideal for genetics studies.

Disclosures

No conflicts of interest declared.

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