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Assay to Access Anthelmintic Activity of Small Molecule Drugs Using *Caenohabidtis elegans* as a Model

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[Abstract] This protocol proposes to use the nematode *Caenorhabditis elegans* as a model to screen and study the anthelmintic activity of natural and synthetic compounds and to observe their effects on the morphology and the ultrastructure of the helminths. Furthermore, *C. elegans* can be used to investigate the anthelmintic activity in embryonated eggs, larval stages and in the adults' survival. As most current anthelmintics are not effective against all nematode life stages, this protocol can contribute to the identification of new alternatives to helminthic infections (Sant'Anna *et al*., 2016).
Keywords: *C. elegans*, Nematodes, Anthelmintic drugs, Chemotherapy

[Background] *Caenorhabditis elegans* is a model organism for parasite nematode research and an excellent system for the screening of compounds with potential anthelmintic activity, because it is inexpensive, readily available, and easy to work with. In addition, the use of *C. elegans* in assays to investigate nematode behavior, locomotion, reproduction and death is uncomplicated and reliable (Simpkin and Coles, 1981). The protocols for screening new compounds on *C. elegans* were first carried out in axenic liquid medium in deep well microscope slides (Tomlinson *et al.*, 1985) or using the drugs added to melted modified NGM agar (Driscoll *et al.*, 1989). These methods are not effective in drug screening as axenic cultures, containing low food supply, trigger the intra-uterine birth causing maternal death (*endotokia matricida*) (Lenaerts *et al.*, 2008) and drugs added to melted agar can modify drug stability due to the high temperatures. In this protocol, we used 96-well plates with liquid medium supplied with *Escherichia coli* to evaluate each stage (eggs, L1-L2 larvae, L3-L4 larvae and adults) independently.

Materials and Reagents

1. Transfer pipette
2. 15 ml Falcon tubes (Corning, Falcon®, catalog number: 352095)
3. 96-well plate, flat bottom, polystyrene, 0.32 cm^2, sterile. TPP tissue culture plates (Sigma-Aldrich, catalog number: Z707910)

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4. Tissue culture dishes of polystyrene TPP- diam. 60 x 15 mm, surface area size 22.1 cm^2 with NGM (Sigma-Aldrich, catalog number: Z707678)
5. *C. elegans* N2 strain
6. *Escherichia coli* OP50 strain
7. Drugs to screen
8. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 795429)
9. Hypochlorite (NaClO) (Sigma-Aldrich, catalog number: 13440)
10. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
11. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S5136)
12. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
13. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), BioUltra ≥ 99.5% (KT) (Sigma-Aldrich, catalog number: 63138)
14. Potassium phosphate dibasic (K_2HPO_4), ACS reagent, ≥ 98% (Sigma-Aldrich, catalog number: P3786)
15. Cholesterol (Sigma-Aldrich, catalog number: C3045)
16. Ethanol (p.a., without additive, ≥ 99.8%) (Sigma-Aldrich, catalog number: 24102)
    Note: This product has been discontinued.
17. Citric acid monohydrate (ACS reagent, ≥ 99.0%) (Sigma-Aldrich, catalog number: C1909)
18. Tri-potassium citrate monohydrate (Sigma-Aldrich, catalog number: 6100-05-6)
19. Disodium EDTA (98.5-101.5%, BioUltra) (Sigma-Aldrich, catalog number: E1644)
20. Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Sigma-Aldrich, catalog number: 215422)
21. Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (Sigma-Aldrich, catalog number: 203734)
22. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (BioReagent, suitable for cell culture) (Sigma-Aldrich, catalog number: 7446-20-0)
23. Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) (BioReagent, suitable for cell culture, ≥ 98%) (Sigma-Aldrich, catalog number: C8027)
24. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C1016)
25. Magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, catalog number: M7506)
26. Lysing solution (see Recipes)
27. M9 buffer (1 L) (see Recipes)
28. S medium (1 L) (see Recipes)

Equipment

1. Clinical centrifuge (Thermo Fisher Scientific, catalog number: 22-029-416)
2. Inverted microscope (ZEISS, model: Axio Vert.A1)
3. Biochemical oxygen demand (BOD) incubator (Thermo Fisher Scientific, Fisher Scientific™, catalog number: 37-20)

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4. Micropipet, 100-1,000 µl volume (Nichipet Eco pipette, catalog number: Z710199)
5. Autoclave

Procedure

A. Culture synchronization (adapted from Stiernagle, 2006)

1. Begin the procedure with *C. elegans* plates containing nearly 500 gravid hermaphrodites. Add 5 ml of M9 buffer to the plate and gently stir the liquid with a pipette to dislodge the worms from the agar.
2. Using a pipette, transfer the worms to a 15 ml sterile Falcon tube, centrifuge at 800 *x g* for 1 min. The worm pellet must be suspended in 3.5 ml total volume.
3. Add 1.5 ml of lysing solution to the tube. Shake the tube gently for 5 min, manually, looking under a microscope to check if worms have disintegrated or not. Most of the worm bodies have dissolved, centrifuge at 800 *x g* for 1 min.
4. Remove most of lysing solution without disturbing the egg pellet.
5. Then, suspend the pellet in 5 ml of M9 buffer, centrifuge 1,000 *x g* for 5 min, remove the supernatant, add fresh M9 and repeat these steps 2 more times.
6. The eggs obtained can be conducted in three different procedures in parallel.
   I, The eggs can be incubated with the drugs directly for 15 h at 20 °C.
   II, The eggs can be incubated at 20 °C for 15 h to produce larvae, which can be incubated at maximum for 24 h with the drugs, to avoid development in advanced stages.
   III, The eggs can be incubated at 20 °C for 3 days to produce adults. Adults can be incubated, at maximum, for 3 days with the drugs to avoid new adult generations. Figure 1 summarizes the culture synchronization and the use of the three different life stages of *C. elegans* – embryonic eggs, larvae and adults (steps B, C and D) to evaluate anthelmintic activity of drugs.

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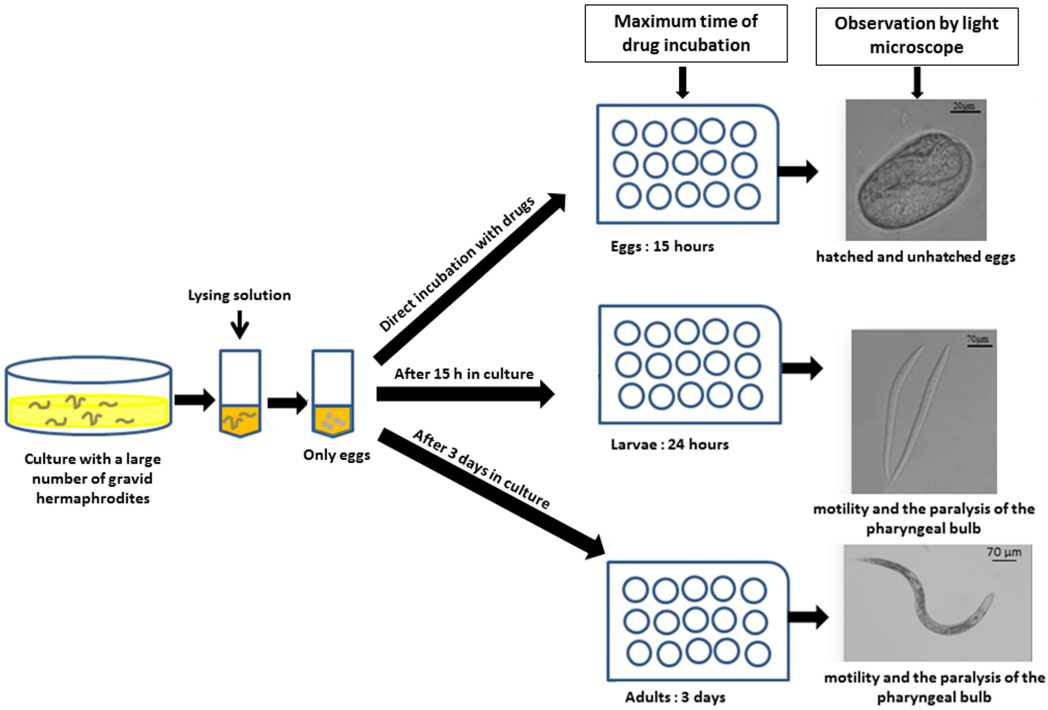

Figure 1. Scheme summarizing the culture synchronization procedure and the use of, embryonic eggs, larvae and the adults (steps B, C and D) of *Caenorhabditis elegans* to evaluate anthelmintic activity of drugs

B. Egg hatch assay

1. After the synchronization procedure, transfer the eggs to a 96-well plate with a pipette.
2. Add approximately 30 eggs per well in 200 µl of S medium.
3. Count and identify the embryonic stages inside the eggs with an inverted microscope to observe the larval development.

   Note: The presence of the gastrula form is important to determine the initial pattern before treatment with the drugs and to verify if the lysing solution affected or not the eggs. The gastrula form can be identified according to Figure 2B.
4. Add the different concentrations of drugs and incubate for 15 h at 20 °C in a BOD incubator.
5. At the end of the incubation, the percentage of hatched and unhatched eggs and the L1 larvae will be determined for each of the different drug concentrations by light microscopy. Triplicates of six independent experiments should be performed.

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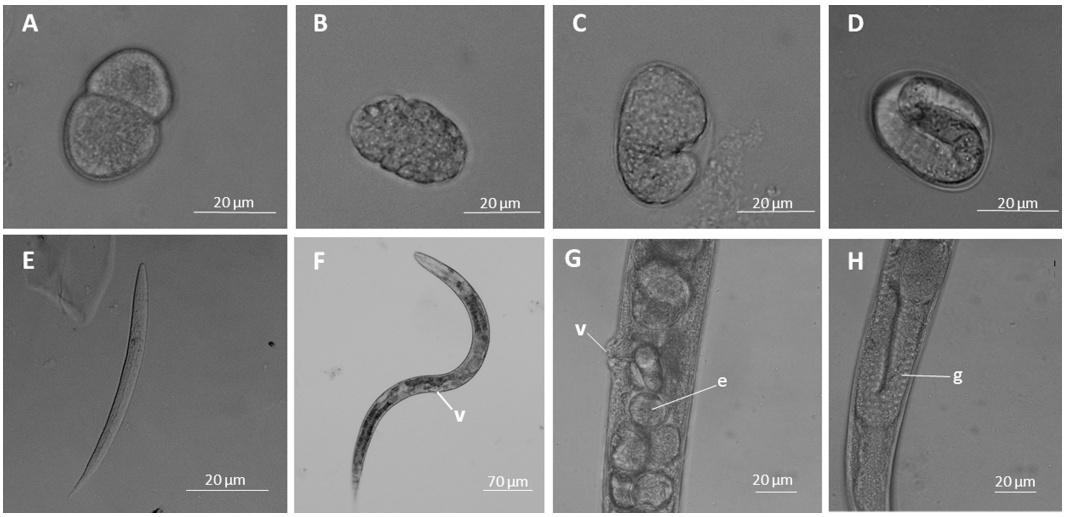

Figure 2. Phase contrast light microscopy images showing different *C. elegans* stages. A. Embryo in the first cleavage; B. Gastrula form; C. Embryo with comma form; D. Egg with a larva inside (the 3-fold stage indicates the complete larval development); E. L1 stage; F. Hermaphrodite, the vulva (v) can be observed; G. Vulva (v) and numerous eggs (e) in higher magnification; H. The hermaphrodite gonad (g) can be observed.

C. Larval development assay

1. After the synchronization procedure, put the eggs in microtubes containing S medium and *E. coli* under gentle agitation, for 15 h at 20 °C at the BOD incubator.
2. After this time, collect the larvae at the first stages (L1/L2) (Figure 2E). Alternatively, the eggs can hatch in the absence of food as development will be arrested and larvae will stay as L1.
3. Adjust the concentration of larvae to 20 larvae/50 µl in S medium.
4. Incubate for 24 h at 20 °C in a 96- well plates containing S medium supplemented with *E. coli* and the different concentrations of the drugs to be analyzed. Triplicates of six independent experiments should be performed.

D. Assays using adults

1. After the synchronization procedure, transfer the eggs to NGM (Nematode growth medium) plates seeded with *E. coli* with a pipette. The eggs were incubated at 20 °C for three days in the BOD incubator to obtain most of the adult nematodes at the same age.
2. Collect the adult worms with a transfer pipette by washing the NGM plates with 5 ml of M9 to dislodge the worms and centrifuge at 800 *x g* for 5 min in a Falcon tube. Remove the supernatant and wash the pellet in the same buffer (three times).
3. Place thirty nematodes per well in a 96-well plate containing S medium 200 µl supplemented with *E. coli.* The bacteria can be autoclaved to avoid excessive growth during the period of incubation. We suggest 2 x 10^3 bacteria/ml approximately.

4. Add the different concentrations of the drugs to be screened in the study. The drugs must be omitted in the wells used as negative controls.
5. Incubate at 20 °C for 3 days in the BOD incubator. Three days should be the maximum period of incubation to avoid the presence of new generations of adults.
6. After this period, the survival was evaluated by counting the live and dead worms by light microscopy considering the motility and the paralysis of the pharyngeal bulb and the total loss of motility with the occurrence of straight bodies as shown in Video 1. Use an inverted microscope to make these observations. Triplicates of six independent experiments should be performed.

Video 1. Movie illustrating the motility and the paralysis of the pharyngeal bulb by phase contrast light microscopy with the occurrence of straight bodies of dead worms

Data analysis

1. To evaluate the survival of adults and larvae and their motility, living worms must be counted by optical microscopy excluding the larvae stages, which hatched during the assay. The size and the presence of reproductive organs are used as criteria to follow the original adult population (Figures 2F-2H).
2. In the egg hatch assay, the percentage of hatched and unhatched eggs and survival of L1 larvae should be determined for each different drug concentration (as in Sant'Anna *et al.*, 2016).
3. Kaplan Meyer tests can be used to analyze the survival of individuals along the treatment and the survival curves should be compared by the log rank test (Kaplan and Meier, 1958).

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Notes

The absence or low concentration of cholesterol affects the embryos survival, because it is essential for the development of the oocytes. The S medium should contain adequate concentrations of cholesterol (Greenstein, 2005).

Recipes

1. Lysing solution
   5 N NaOH
   1% hypochlorite
   Prepared fresh for each batch
2. M9 buffer (1 L)
   3 g KH_2PO_4
   6 g Na_2HPO_4
   5 g NaCl
   0.25 g $MgSO_4 \cdot 7H_2O$
   Autoclave and stock at 4 °C
3. S medium (1 L)
   a. S basal
      5.85 g NaCl
      1 g K_2HPO_4
      6 g KH_2PO_4
      1 ml cholesterol (5 mg/ml in ethanol)
   b. 10 ml 1 M potassium citrate, pH 6
      20 g citric acid monohydrate
      293.5 g tri-potassium citrate monohydrate
      Add H_2O to 1 L
      Sterilize by autoclaving
   c. 10 ml trace metals solution
      1.86 g disodium EDTA
      0.69 g $FeSO_4 \cdot 7H_2O$
      0.2 g $MnCl_2 \cdot 4H_2O$
      0.29 g $ZnSO_4 \cdot 7H_2O$
      0.025 g $CuSO_4 \cdot 5H_2O$
      Add H_2O to 1 L
      Sterilize by autoclaving and keep protected from light
   d. 3 ml 1 M $CaCl_2$
   e. 3 ml 1 M $MgSO_4$

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Note: Manipulate the components under sterile conditions; do not autoclave the complete medium. All the recipes were adapted from Stiernagle (2006).

Acknowledgments

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