Expansion of Worms for Microarray, IP, ChIP and Similar Experiments

Peichuan Zhang*

Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA
*For correspondence: peichuan.zhang@ucsf.edu

[Abstract] This protocol describes the basic work-flow of expanding nematode culture under lab conditions, which serves for the subsequent preparation of RNA (microarray), protein (IP), and DNA/protein (ChIP).

Materials and Reagents

A. Plates and culture materials:
   1. High growth (HG) plates
   2. Normal growth (NG) plates
   3. OP50 or RNAi bacteria culture
   4. LB medium (autoclaved)

      Note: Prepare enough plates (with extra ones to replace contaminated plates).

B. Other materials:
   1. Antibiotics (carbenicillin, tetracycline)
   2. IPTG
   3. Cholesterol
   4. $CaCl_2$
   5. $MgSO_4$
   6. KPO_4
   7. KOH
   8. Sodium hypochlorite
   9. M9 buffer
   10. NP40 buffer
   11. O/N culture
   12. Triton X-100 (Sigma-Aldrich, catalog number: 9002-93-1)
   13. Sodium hypochlorite (Thermo-Fisher Scientific, catalog number: SS290-1)
   14. 2x bleach solution (see Recipes)

Equipment

1. Beckman centrifuge and rotor (Beckman Coulter)
2. Low-temperature incubator (Thermo-Fisher Scientific)
3. 15-ml conical tubes

Procedure

A. Before Day 1

1. Prepare HG plates (10 cm in diameter) and NG plates (15 cm). Prepare enough medium as described in recipe. Autoclave, cool down to 60 °C, and then add all the supplements (cholesterol, $CaCl_2$, $MgSO_4$, KPO_4, antibiotics, etc). Pour ~15 ml/ 10 cm-plate, ~100 ml/ 15 cm-plate.

   Tip 1. Certain RNAi bacteria do not appear to grow well in the presence of both carbenicillin and tetracycline. In this case, omit tetracycline from the culture and use.

B. Day 1

1. Inoculate a single colony of OP50 in 5 ml LB or RNAi bacteria in 5 ml LB (supplemented with 100 µg/ml carbenicillin & 12.5 µg ml^{-1} tetracycline) O/N (~16 h), 37 °C, 300 rpm.

   Tip 2. Certain RNAi bacteria do not appear to grow well in the presence of both carbenicillin and tetracycline. In this case, omit tetracycline from the culture and use carbenicillin only to keep the selection pressure. The Tn10 transposon confers tetracycline resistance, which simultaneously knocks out the rnc14 gene that encodes an RNase to enhance the production of dsRNA.

   Tip 3. Prepare a mini-prep of plasmid for the RNAi bacteria and confirm the insert for your gene of interest by PCR (T7 primers) & DNA sequencing (M13 forward primer). The RNAi clones from Julie Ahringer's lab are based on low copy-number plasmid, so don't be surprised if the concentration is below detection by spectrophotometry.

2. Inoculate the O/N bacteria culture in 1 L of LB or LB with antibiotics. Grow O/N (~16 h), 37 °C, 300 rpm.

   Cool the O/N culture on ice (it can be stored in the cold room for a few days). Transfer the culture into centrifuge bottles, spin at 7,000 rpm, 10 min, 4 °C.

   While spinning, make - Make 60 ml NP40 buffer before beginning (30 ml 2x buffer + 6 ml 10% NP40 + 24 ml cold ddH_2O - chill on ice!).

   Washing sample in each bottle w/ 250 ml cold H_2O. Spin 7,000 rpm 10 min.

   Tip 4. Be extremely careful to avoid any possible contamination to the OP50 culture. Good practice of antiseptic operations is always a must to start your own experiments. Seed a few OP50/NG plates to check contamination. OP50, a uracil auxotroph, should

form only a thin layer on NG plate. It is considered to be non-pathogenic to worms. Any weird change of color/smell of your bacteria culture may indicate potential contamination by other types of bacteria.

3. Seed OP50 (0.5 ml) on 10-cm HG plates (diameter = 8.5 cm, surface area = 57 cm^2). Seed RNA bacteria (1.0 ml) on 15-cm NG /carbenicilin (100 μg/ml)/ IPTG (0.5 mM to 1.0 mM) plates (diameter = 14.0 cm, surface area = 154 cm^2).

   Incubate the plates at 37 °C, O/N. Cool down to room temperature before use. Alternatively, put on bench for a few days (adding too much bacteria is not a good idea, since it takes time to dry on the plates).

   Store the plates (seeded with bacteria) in cold room, if not used immediately. Most RNAi bacteria plates can be used within 2 weeks to 1 month (and particularly, for certain potent RNAi clones).

   Tip 5: Typically, 6 to 8 HG plates should provide sufficient numbers of gravid adult animals for hypochlorite synchronization (bleaching) and production of eggs (up to several hundred thousand) for 40 to 50 NG/ carb/ IPTG plates.

   However, different strains could have different development/growth conditions (*e.g.,* mitochondria mutant animals such as *clk-1* or *isp-1* display slow growth and have less progeny) and optimal conditions should be determined before you start any large-scale experiments.

C. Day 2

1. Place two agarose plugs of well-fed animals (*e.g.,* wild-type N2) onto each HG plate.

   Prepare another set on the 2nd day, 3rd day, so on and so forth, to prepare enough biological replicates.

D. Day 4

1. When most worms are at d1-d2 stages (it takes ~ 4 to 5 days from starved population), start collection.
2. Wash worms off from HG plates with M9 containing 0.2% Triton X-100 (0.2 ml per 100 ml). Use glass pipet and 15-ml conical tubes.
3. Need to wash the plates a few times in order to collect as many gravid animals as possible.
4. Spin in a bench-top IEC clinical centrifuge at speed 5 (~700 *x g*), 30 sec.
5. Remove the supernatant carefully and wash worms w/ M9 buffer, once. Centrifuge again, remove supernatant and leave ~7 ml liquid in the 15-ml conical tube.
6. Add equal volume of 2x bleach. Agitate/shake the tube slowly yet firmly for ~ 2 to 3 min.

Tip 6: Optimal incubation time with bleach solution requires a pilot test. The best time to stop is when you see broken carcasses are about to disappear. Remember that you need to collect the eggs by centrifugation for another 30 sec, and during this short period of time, bleach will continue to exert more effects to break the body, and also, causes damage to the eggs. Definitely, avoid over-bleaching!

7. Start centrifugation for 30 sec to spin down eggs when can still be seen in the tubes. Remove supernatant, and wash immediate*ly* with plenty of M9 buffer, at least twice.
8. Add 10 ml M9 buffer and rock the tube on a rotator at desired temperature (typically, 20 °C), O/N.

   Please refer to *Caenorhabditis elegans/Phenotypic assay/Synchronization of worm* for further description of hypochlorite synchronization.

E. Day 5 and beyond

1. Take a few microliter of M9 and count the numbers of arrested L1 (swimming). Presence of many dead eggs would suggest over-bleaching, which may or may not affect your experiments.
2. Seed ~6,000 arrest L1 per 15-cm NG/ Carb/ IPTG RNAi plate (or ~2,000 - 2,500 per 10-cm plate). At least 4,000 animals are required to prepare ≥5 µg of total RNA. You need to concentrate arrested L1 if the density is too low. Otherwise, adding too much M9 buffer on a plate will cause hypoxia.

   Tip 7: Do not add too many animals on a plate. Nematodes love to burrow, and this is one reason why you should not use old plates. One way to solve this problem is to increase the concentration of agar from 2.0% to 3.0%.

F. Whenever necessary, feed animals with concentrated bacteria that you have prepared before. Start with less arrested L1 (*e.g.,* 3,000 - 5,000) to avoid starvation.

   Tip 7: It takes wild-type animals ~50 - 54 h to come out of arrested L1 and develop to young adult (YA) (w/o eggs). Majority (>90%) of animals should be synchronized as young adults at this point. The time is ~68 - 72 h for *daf-2* mutant animals, such as *daf-2(e1370)*, while these animals could never be perfectly synchronized (you will see a mixed population of L3, L4 and YA).

G. Collect animals at appropriate time points according to your own experiments.

   Tip 8: Compact pellet of ~4,000 YA/d1 adult is ~25 µl in volume. Typically, less than 0.5 ml of worm pellets can be collected from a single 15-cm plate, which can provide enough RNA for at least 200 RT-PCR reactions. We can collect up to several hundred mg of proteins from 10,000 adults.

Recipes

1. 2x Bleach solution (prepare fresh solution every time before use)
   60 ml sodium hypochlorite
   30 ml 5 N KOH
   10 ml ddH_2O

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References

1. The Ahringer RNAi library

