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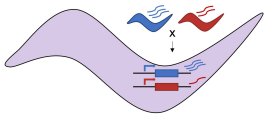
Generating multiple stage-matched C. elegans hybrids and parental strains simultaneously



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We use this protocol and it's working

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Disclaimer

This is an experimental protocol; no outcomes are guaranteed.

Abstract

Here we provide a protocol for generating RNA from synchronized C. elegans F1s from crosses of multiple wild strains to a common reference strain (for example, for allele specific expression analysis). This protocol, which comprises 9 sequential days (6 active days) followed by RNA extraction, is optimized for synchronization of parental and offspring worms, retention of embryos, and timing. Others could easily adapt this protocol for crossing designs other than the specified multiple wild strains x common reference. This protocol accommodates 8 concurrent crosses and one extra parent with 3-4 active worm pickers on Days 3 and 6, and should accommodate increased or decreased numbers of crosses with commensurate personnel adjustments.

Materials

REAGENTS AND CONSUMABLES

- M9 buffer. Recipe:
  - 3g KH2PO4
  - 6g Na2HPO4
  - 5g NaCl
  - H20 to 1 liter. Sterilize by autoclaving
  - Add 1 mL 1M MgSO4 (filter sterilized)
- Bleaching solution (2:1 commercial bleach : NaOH 5M)
- Tween-20 (#BP337-100)
- TRIzol (Invitrogen #15596026)
- Chloroform (Fisher #C298-500)
- Pure ethanol; fresh 70% ethanol day of RNA extraction
- liquid nitrogen
-1.5mL mini centrifuge tubes
-15mL conical tubes

PLATES

Recipe: Nematode growth medium (NGM)/Agar:

- 1.25% Agarose
- 0.75% Agar
- 2.70% NaCl
- 2.25% Peptone
- Sterilize the above by autoclaving. Then, for every liter of agar add:
- 1 mL 1M MgSO4
- 1 mL 1M CaCl2
- 1 mL Cholesterol (5mg/mL)
- 25 mL KPO4 1M pH 6

Food for plates: *E. coli* OP50.

- Inoculate 2-3 OP50 colonies and grow overnight (16-20 hours) in a shaker (37C, 300 rpm). Use this to seed plates; plates can be kept at 4C prior to use.

Plate types to make for this experiment:

1. Growing plates
2. Mating plates
3. Un-syncing plates

EQUIPMENT

- Rocker
- 37°C heat block
- Room temp mini centrifuge
- 4°C mini centrifuge
- Big centrifuge (15mL conical tubes)

- Vortex
- Shaker for 1450rpm (e.g., vortexer with mini tube holding head)

Worm maintenance prior to start of experiment

1 In general, follow standard worm culture procedures (Stiernagle 2006). Grow worms at 18°C or cooler if any strains have known or suspected mortal germline phenotypes (Frezal et al. 2018)

2 Grow healthy cultures of each strain that will be a parental strain. For the reference strain that will be crossed to all wild strains, make sure to have many plates growing (e.g., 15 growing plates).

3 For all strains that will be crossed to the common reference strain, start and propagate male-heavy cultures (to have enough males to mate)

3.1 Set up mating plates with 20-30 hermaphrodites and as many males as you can find (as few as 3 and up to 45) from the wild strain

3.2 Repeat setting up mating plates until you can visibly see a lot of males; then, these male cultures can be propagated as normal (e.g., through chunking)

4 For all strains, keep the original hermaphrodite-heavy culture going

Cross set up

4d

5 ***Day 1.*** Bleach synchronize all parental strains and re-plate embryos.

4h

5.1 Inspect plates, only use plates that are not starved.

5.2 Wash 2-3 plates of the wild strains and 10-15 plates of the reference strain to a conical 15 mL tube using water.

5.3 Centrifuge tubes (2000 rpm for 2 minutes) and remove the supernatant careful of not disturbing the worm pellet.

5.4 Resuspend pellet with 7 mL of water and add 3 mL of a 2:1 Bleach:NaOH (5M) bleaching solution. Gently shake tubes constantly for 5-7 minutes. During this time, inspect tubes using a stereoscope. When carcasses of the worms are largely dissolved (often around 7 minutes), centrifuge tubes as described before.

5.5 Remove the bleaching solution from the tube and resuspend the pellet in 13 mL of water. Shake tubes vigorously and centrifuge as before.

5.6 Repeat previous washing step one more time. Remove washing solution (water).

5.7 Resuspend embryos in water and transfer to NGM/Agarose plates as necessary.

5.8 Make sure to have several growing plates per strain to distribute across temperatures.

6 Grow reference-strain worms on growing plates at multiple temperatures (e.g., 18°C, 19°C, 20°C, and room temperature) to ensure worms at the proper stage of development will be available throughout a wide window when generating mating plates. Grow wild worm cultures at a couple of these temperatures. 2d

7 ***Day 3*** (starting 48 hours after bleach syncing): set up mating plates. 1d

7.1 Add 60 L4 hermaprodites from the reference strain to each of 5 mating plates per wild strain.

7.2 Screen these plates to ensure no males are on them.

7.3 Add 40 L4 males from the wild strain to the 5 mating plates for that strain.

7.4 Add 80 L4 hermaphrodites from each of the wild strains to each of 3 mating plates for each strain. (The selfed offspring of these worms will be the matched 'parent' worms for the F1 crosses)

8 Leave worms to mate for 48 hours at 20°C 2d

Synchronizing experimental generation embryos 4h

9 ***Day 5*** after allowing 48 hours of mating: wash all worms, bacteria, and embryos from one cross (or parental selfing) into a single 15mL conical tube, being careful to collect all embryos.

9.1 Rinse plates twice with water, then gently swipe tip of glass pipet back and forth across the surface of the plate while expelling water, holding plate at an angle. Goal is to remove all the embryos but not damage them, and not dislodge any agar into the liquid.

9.2 Repeat several times, reusing the same water (collecting in bottom edge of plate; very gently but methodically swipe the pipet tip back and forth across the surface of the plate in a comprehensive pattern), until lawn is completely removed by eye.

9.3 Check under scope, re-focus on rinsing remaining areas as needed.

10 Add water to 14mL (so that all tubes have equal volume)

11 Spin 2000rpm x 2 min

12 Use vacuum to remove most of liquid, taking care not to disrupt loose pellet (remove as much bacteria as possible – but don't risk the pellet!).

13 Resuspend each tube to 3.5mL with fresh water

14 Add 1.5 mL bleaching solution (2:1 bleach:NaOH). Start timer.

15 Shake tubes every 30s, examine.

16 When adult carcasses fully dissolve (often around 7 minutes), add water to each tube up to 14mL.

17 Spin 2000 rpm x 2 min.

18 Vacuum and long glass pipet aspirate bleaching solution, getting as close to pellet as is safe.

19 Resuspend to 10mL with water and shake

20 Spin 2000 rpm x 2 min.

21 Vacuum aspirate only to the 1.5mL mark (avoid embryo loss).

22 Resuspend with water + 0.25% tween-20 to 15mL

23 Spin 2000 rpm x 2 min, being careful to position the tubes within the centrifuge so the tube sides with the interior striations are not on the inside where embryos would collect.

24 Vacuum aspirate as close to the pellet as possible.

25 Resuspend with water (to wash out detergent)

26 Spin 2000 rpm x 2 min

27 Vacuum aspirate as close to the pellet as possible

28 Resuspend embryo pellet to 10mL with M9 buffer.

29 Leave tubes gently rocking at room temperature. Embryos will develop into L1s and arrest.

Growing synchronized experimental generation L1s

3h

30 ***Day 6/7 – 30ish hours after leaving embryos in buffer.*** Add tween-20 to 0.25% to each tube

30.1 Add 25 µl Tween-20 directly to the synced worms in tubes

30.2 OR, combine 500 µl Tween-20 to 4mL of water and mix well, then add 200uL of this diluted solution to the synced worms in tubes

31 Spin 2000rpm x 2 min

32 Aspirate using vacuum, leaving under 0.5mL volume in tube. The pellet of L1 worms is loose, so take care not to disturb it.

33 Quick count worms to determine their density. For each tube:

33.1 Add 10µl of mixed worm mixture as a single droplet to an empty 6cM plate

33.2 Roughly count the number of L1s immediately after adding the droplet by eye under the scope. Record this number.

33.3 Calculate the volume of liquid containing 400 worms

34 Add 400 worms to 3 or more un-syncing plates (6cM large-lawn plates) per strain.

34.1 Spot directly onto the lawn

34.2 Split the 400 worms into multiple smaller spots on the lawn if possible to facilitate drying

34.3 Leave lids off the plates until droplets absorb, typically a few minutes

35 Leave closed plates at 20°C

Male removal

1d

36 ***Day 8.*** Remove males from at least 3 plates from each strain. F1s will be ~50% males so this is a challenge; parentals only have spontaneous males so just checking them a couple times should suffice.

Males start to be visible ~32 hours after un-syncing, however, they're very young. Males are much more evident 36+ hours after un-syncing.

Worm harvesting

37 ***Day 9.*** Prepare liquid nitrogen for flash-freezing worms after harvest.

38 Identify strains where most hermaphrodites on the plate are gravid and embryos have been laid on the plate, typically 55-60 hours after un-syncing at 20°C. Several strains may become mature young adults like this at once while others lag; all strains should reach this within a few hours on this day.

38.1 While looking at strains, remove any straggler males that made it through the initial screen.

38.2 Group strains that are developmentally similar and do the following harvesting in these similar groups.

39 Rinse all plates from one strain with M9 buffer into 15mL tubes

39.1 Re-use 1mL of buffer on several plates

39.2 Make sure each plate gets rinsed at least twice

39.3 Check to make sure all worms are removed, especially for strains with fewer worms (F1s). Do not scrape up the bacteria or rinse embryos, focus on adult worms.

40 Spin 2000rpm x 2 min

41 Remove buffer using vacuum down close to pellet

42 Resuspend in 13 mL M9 buffer.

43 Spin 2000rpm x 2 min

44 Remove buffer using vacuum down close to pellet

45 Add TRIzol to a total volume of ~1500 µl /1.5mL. (this is probably adding ~1200uL TRIzol, but try to have final volume of worms + trizol consistent across strains).

46 Transfer equivalent volume of worms in trizol to each of 3 1.5mL centrifuge tubes, ending with ~500µl per tube. Using smallest glass pipette works well.

47 Quick spin down tubes

48 Flash freeze tubes in liquid nitrogen

49 Store at -80°C until ready to proceed with RNA extraction.

RNA extraction (following He 2011)

50 Set up for RNA extraction. Full details on reagents and equipment are found in the appropriate sections; this step describes set up for this specific experiment.
Plan to extract RNA in batches of no more than 18 samples/batch, with one sample per strain in each batch (likely 1 or 3 batches)
Equipment:
- Heat block set to 37°C
- Procure dewer of liquid nitrogen for freeze-thawing
- Centrifuge at (or set to) room temperature
- Centrifuge at (or set to) 4°C

Reagents:
- TRIzol (fume hood!)
- Chloroform (fume hood!)
- Freshly prepared 70% ethanol
- Qiagen RNeasy buffer RW1
- Qiagen RNeasy buffer RPE (with ethanol added as directed by the kit)
- RNAse-free water

Sets of tubes to label:
- 1.5mL tubes for aqueous phase after TRIzol-chloroform spin
- RNeasy spin columns
- 1.5mL tubes for eluted RNA collection

Clean with RNAse away:

- Gloves
- Fume hood
- Outside of tip boxes, reagents to be used
- Freeze thaw area
- Centrifuge area

51 Break up worms

51.1 Vortex worms (previously stored at -80°C in TRIzol) for 2 minutes

51.2 Thaw worms at 37°C

51.3 Quick spin tubes

51.4 Freeze by submerging tube in liquid nitrogen

51.5 Thaw at 37°C

51.6 Repeat the previous 3 steps for a total of 6 freeze thaw cycles. Spin down.

52 TRIzol-chloroform extraction (do in fume hood)

52.1 Add 200µl fresh TRIzol to each tube

52.2 Incubate for 5 minutes at room temperature

52.3 Add 140µl chloroform to each tube

52.4 Shake at 1450 rpm for 1 minute (make sure thorough mixing has occurred)

52.5 Incubate for 2 min at room temperature

52.6 Spin at 12,000g for 15 minutes at 4°C

52.7 Remove top aqueous phase to new tubes; this is what will be used downstream

52.8 Add 1 volume of fresh 70% ethanol to the aqueous phase; mix and spin down

53 RNA elution using Qiagen columns

53.1 Transfer 650µl of each sample to a Qiagen RNeasy spin column (1 per sample)

53.2 Spin at max speed for 30 seconds; discard flow through

53.3 Add remaining sample to Qiagen RNEasy spin column with 700µl buffer RW1 (from the Qiagen kit)

53.4 Spin at max speed for 30 seconds; discard flow through

53.5 Add 500 uL buffer RPE (from Qiagen kit)

53.6 Spin at max speed for 30 seconds; discard flow through

53.7 Repeat buffer RPE wash (add, spin, discard – previous two steps)

53.8 Spin column at max speed for 2 minutes to remove any residual buffer

53.9 Transfer spin column to new tube (labeled, where RNA will be collected)

53.10 Add 50uL RNAse-free water to column

53.11 Incubate for 1 min at room temperature

53.12 Spin at max speed for one minute, retain eluant

53.13 Place samples on ice; quantify; store at -80°C

Protocol references

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