Cryopreservation and DNA Preps of Caenorhabditis Nematodes
March 2020

This protocol will follow the **Collections protocol for wild collections** and/or the **Andersen Lab Strain Intake protocol for receiving collections from other labs**. You will track your progress of cryopreservation and DNA pellets/preps on the INSERT MASTER SHEET.

*After completing Andersen Lab Strain Intake protocol <u>for receiving collections from other labs</u>, begin at "Preparing to Freeze."

<u>Starting the Wild Collections Intake Process:</u>

1. <u>Preparing the strains:</u>
   The labels used during this process will be printed by the person in charge of the wild collections. See the collections_protocol (Labels for the Wild Collections Intake Process) for information about making labels.
   a. <u>Day one:</u> Chunk strains. Use 1 initial chunk plate clear label.
   b. <u>Day two:</u> Bleach strains. Bleach 5-15 gravid animals:
      1. Use 1 clear label prepared for bleach and write "bl".
      2. Place 16 µl of bleach on a labeled 6 cm plate, away from the bacterial lawn.
      3. Pick 5-15 gravid animals into the bleach. You have to pick quickly before the bleach absorbs into the plate.
      4. Leave the bleach plate lid-side up at room temperature for 24 hours.
   c. <u>Day one-post bleach:</u> Pick 15-20 L1s from the bacterial lawn of the bleach plate to a new, labeled 6 cm plate. Parafilm both the bleach plate and the clean plate. Discard the initial chunk plate. Let the L1s plate starve.
   d. <u>Day 7-8 post L1 pick:</u> Once the strain is starved, chunk the strain to four 10 cm plates: 1 labelled freezing plate and three DNA prep plates. Parafilm all four plates. Put the chunked plates at 20ºC. Let the four 10 cm plates starve.
   e. <u>Storage:</u> Store the parafilmed bleach and clean plates at 15ºC.

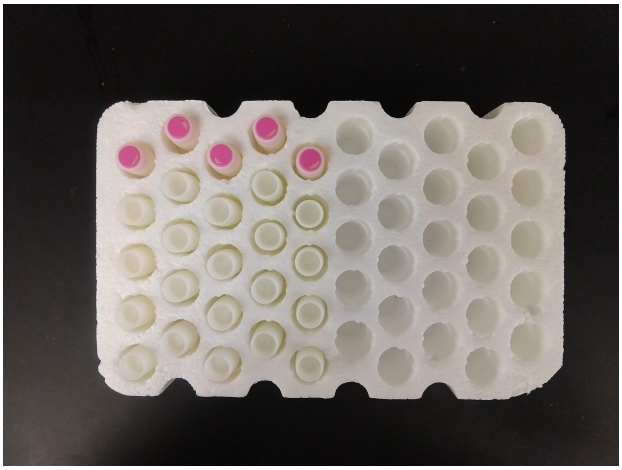
<u>Preparing to Freeze:</u>

1. Using the starved 10 cm plates, three plates will be used for DNA pellets, **one for cryopreserving.**
   - *Check to make sure your labels were made; all labels are made when you receive a box to process.*
   - The strain is ready to freeze when there are no bacteria on the plate and there are few or no embryos remaining on the plate. Most of the animals should be L1, L2 or dauer. Strain health, where the box is stored, and initial chunk size will influence how long it takes for plates to be ready.
2. Prepare the number of cryotubes you need (Fisher 12-565-163N) with cryo-safe labels (Fisher 15930A). For each strain, label **five** cryogenic tubes. The first tube

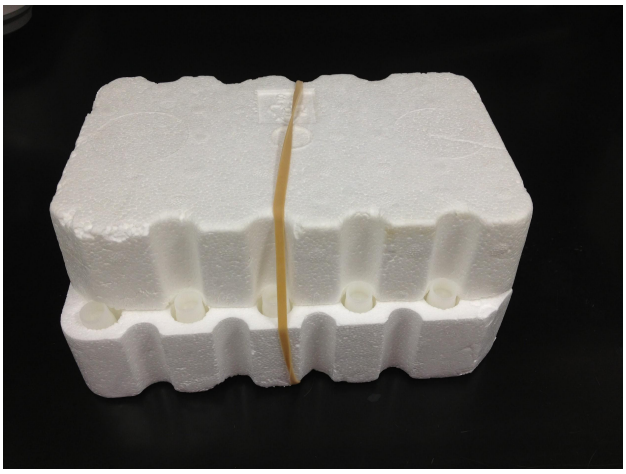
of the strain (of the five tubes per strain) will be marked with a pink cap insert; the remaining four tubes are not marked with any color.
    - *Freezing many strains at a time can be a challenge; it is best to be as organized as possible and prep everything together. When you can, label all of your tubes ahead of time and add freezing solution to the last two tubes.*
    - The fourth and fifth tubes need “normal” freezing solution in them. Use sterile technique to aliquot 0.75 mL of “normal” freezing solution into each of those tubes with a sterile plastic pipette.

Freezing:

1. Set the water bath on the freezing bench to 50˚C.
2. Microwave soft agar until fully melted (no chunks!). Confirm that agar is not contaminated —sometimes fuzz balls of mold are not visible until agar has melted. If contaminated, throw away.
    - Make sure the cap is loosened before microwaving.
    - Be careful when microwaving: the agar at the bottom can melt and boil before the rest of the agar. This can lead to explosions in the microwave! I heat for 10-15 seconds, swirl the bottle, heat again, etc.
3. Incubate the soft agar in the 50˚C water bath for an hour. Swirl periodically. Be sure agar has equilibrated to 50˚C in the water bath. Agar too hot will kill the worms.
4. Place cryotubes in a blue rack in alphanumeric order, making sure the order of cryotubes matches your freezing plates, and put on ice.
5. Keep tubes on ice for five minutes before starting the freezing process.
6. Using a sterile, glass pipette, transfer 4.5 mL of M9 onto your starved 10 cm plate. Swirl the plate with M9 to reach the edges of your plate. The goal is to wash animals off the 10 cm plate and into your cryotubes.
7. Add 0.75 mL of worm/M9 mixture to each of the five tubes. Lightly cap the first three tubes. Recap the last two tubes (four and five) tightly and invert to mix.
8. Chill tubes on ice 5 min.
9. Add 0.75 mL soft agar (50˚C) to the first three tubes that do not contain “normal” freezing solution. Recap tightly. Invert to mix. Chill on ice 5 min.
10. Transfer tubes to Styrofoam holders, making sure to keep the strains in alphanumeric order (the transfer is from a horizontal position to a vertical position).



11. You will use two Styrofoam holders and one rubber band to secure them together. *The bottom row of tubes will be exposed.*



12. Label the top of the Styrofoam holder with the date, your initials, and box number (if you freeze more than ten strains). Place at -80˚C.
13. Record the strains you froze in the Freezer Log **in the same order that you froze them and placed them in the freezer box**. Record the names of the strains by looking at the plates you used, which should still be in the order that you froze. Robyn moves strains to permanent storage location (-80˚C or liquid nitrogen).
14. Record your work in the Intake Binder.

<u>Thawing cryopreserved strains:</u>

1. Maintain a metal tube-holder block at -80˚C (same ones used in heat blocks; must fit cryotubes).
2. Transfer cryotubes from their storage location to the metal block. Bring the block to your bench. Working one strain at a time, remove a cryotube and warm it briefly in your hands. Flame a metal spatula as you would for chunking. Scoop out a plug of frozen agar and transfer it to a 6 cm plate.

3. Viability declines with each thaw. On the third or fourth thaw, thaw the entire tube and distribute across two or three 6 cm plates. This strain will have to be refrozen after minimal passaging.

1X M9 (1L)

- 3 g KH_2PO_4
- 6 g Na_2HPO_4 anhydrous
- 5 g NaCl
- H2O up to 1 L
- 1000 µL 1M $MgSO_4$

1. Mix KH_2PO_4, Na_2HPO_4, NaCl in less than 1 L of water, once dissolved pour into a graduated cylinder and add remaining water up to 1 L, cover and invert to mix thoroughly.
2. Filter sterilize with 0.45µM vacuum filters into 500mL aliquots.
3. Autoclave on liquid cycle 40 minutes.
4. Once bottles are cool, add 500 µL 1M $MgSO_4$. MgSO4 sometimes precipitates out of solution if it is too hot. After the solution cools, swirl to dissolve.

Soft Agar Freezing Buffer

1. Place a 1 L glass beaker on a scale and add:
    - 300 g glycerol
    - 4 g Difco Agar
    - You must use a glass beaker to heat the solution enough to dissolve the agar.
2. Bring up ~0.5 L with M9. Add stir bar and heat/stir to dissolve agar. There should be no visible granules.
    - This may take a long time.
    - You will need to heat it close to 200℃.
    - Add M9 to just shy of 1 L, stirring to mix.
3. Transfer to a glass graduated cylinder and bring up 1 L.
4. Transfer to glass bottles in 200 or 100 volumes. Label your bottles. Autoclave 20 min.
5. Store at room temperature.

Freezing Solution

1. Mix the following:
    - Potassium phosphate buffer, 1 M pH 6 (100 mL)
    - NaCl, 5 M (40 mL)
    - Glycerol, 100% (600 mL)
    - dH2O (up to 2 L)
2. Divide into 250 ml aliquots.
3. Autoclave on liquid cycle for 30 minutes.
4. Add MgSO4 to a final concentration of 0.3 mM (e.g. for 200 ml of freezing solution, add 60 µl of 1 M MgSO4).

Nematode DNA Isolation using Qiagen DNAEasy Kit (cat #69506)

1. *This step will take place after the Andersen Lab Strain Intake protocol, step 7, day 7-8. Plates should have been chunked from L1 stage 6 cm plates to four 10 cm plates, parafilmed, and starved, before beginning DNA pellets.* **Three plates will be used for DNA pellets**, one for cryopreserving.
2. Prepare one 15 mL conical and one 1.5 mL microcentrifuge tube PER STRAIN. Write (or print labels using the Dymo printer) the strain name on the individual 15 mL conical caps and on the side of the conicals. Place in a conical rack. Label your 1.5 ml tubes with your dot labels (printed previously).
3. There are four dot labels per strain. One dot label will be placed on the 1.5 mL tube for each strain. The remaining three dot labels should be stored until the actual DNA prep is carried out..
4. Wash the nematodes off the three 10 cm plates:
    a. Using a sterile, glass pipette take up 12 mL of M9. Squirt 6 mL of the M9 on one plate, swirling the plate and transferring the liquid to the second and then third plate.
5. Pour the M9/worm mixture into your 15 mL conical.
6. Squirt the remaining 6 mL of M9 onto the first plate and repeat the process until the M9/nematode mixture is in the 15 mL conical.
7. NOTE: If the plates are too starved (i.e. - the worms have started to burrow), you can increase your yield by washing the worms off into the conical, allow the plate to sit for 5-10 minutes, wash the plate again into a second 15 ml conical. Waiting after some liquid is on the plate might help flood the burrowed worms back to the surface.
8. Allow the worms to settle in the conical for one hour.
9. Aspirate off the M9. Be careful not to aspirate off any worms. *If the aspirator flask is full, empty out appropriately.*
10. Add 5 ml of M9 to the 15 ml conical and allow the worms to settle again; one hour.
11. Aspirate off the M9.
12. Transfer the worms in the remaining liquid with a bulb/Pasteur pipette to the labeled 1.5 ml microcentrifuge tube. Allow the pipette to remain in the 1.5 ml tube while you add a little (less than 1 ml) of M9 to the conical tube to wash out the tube. Use the Pasteur pipette to transfer any remaining worms to the microcentrifuge tube.
13. Allow the worms to settle; 25 minutes. Then, use a p200 to take off the remaining ~100 µl.
14. Aspirate off the majority of the M9 and use a p200 pipette to manually remove the majority of the remaining liquid. Be careful to not remove any of the worm pellet.
15. Store the worm pellets at -80°C in a box labelled “wild DNA preps”until you are ready to isolate DNA.

DNA Prep

16. Turn on a heat block to 56°C.
17. Make a master mix of lysis solution: 180 µl of Buffer ATL and 20 µl of Proteinase K (provided with the kit) per samples you have plus one.
    a. As with all master mixes, prepare for at least one extra sample to compensate for pipetting errors. (*e.g.* if you are prepping 24 samples, make enough master mix for 25).
    b. If buffer ATL seems to have particulates, heat at 37C until particulates go back into solution.
18. Incubate at 56°C with vortexing for 18-22 hrs.
19. Add 4 µl of RNAseA (100 mg/ml) (Fisher, cat#BP2539100). Incubate at room temperature for two minutes.
20. Add 200 µl buffer AL. Incubate at 56°C with vortexing (or vortex a few times during incubation) for 10 minutes.
21. Add 200 µl EtOH and vortex to mix. Transfer contents to a labeled spin column in a collection tube.
22. Spin at 10,000 rpm for one minute. NOTE: if all the contents did not go through the column, repeat the spin.
23. Remove the spin column and transfer to a new collection tube. Add 500 µl Buffer AW1. **If you will be using a new bottle, be sure to add EtOH.*
24. Spin at 10,000 rpm for one minute.
25. Remove the spin column and transfer to a new collection tube. Add 500 µl Buffer AW2 **If you will be using a new bottle, be sure to add EtOHh.*.
26. Spin at maximum speed (13,000-14,000 rpm) for three minutes.
27. Remove spin column and transfer to a clean, labeled 1.5 ml microcentrifuge tube. Add 200 µl Buffer AE.
28. Incubate at room temperature for one minute.
29. Spin at 10,000 rpm for one minute.
30. Remove spin column and transfer to a new clean, labeled 1.5 ml microcentrifuge tube. Add 200 µl Buffer AE.
31. Incubate at room temperature for one minute.
32. Spin at 10,000 rpm for one minute.
33. Combine eluates from steps #29 and 32,
34. Determine DNA concentration (ng/µl) using Qubit Broad Range (cat# Q32850; follow Qubit-provided protocol).
    a. Label enough Assay Tubes for your samples, standard 1 and standard 2.
    b. Prepare the Qubit Working Solution by diluting the Qubit BR reagent 1:200 Qubit BR buffer. Prepare 200 µl of Working Solution for each standard and sample, plus one extra.
    c. For each standard, add 190 µl of of Working Solution to the appropriate tubes.
    d. For each sample, add 198 µl of Working Solution to the appropriate tubes.
    e. Add 10 µl of each standard to the appropriate tube.
    f. Add 2 µl of each sample to the appropriate tube.
    g. Briefly vortex all tubes.

    h. Incubate the tubes for 2 minutes at room temperature.
    i. Insert tubes in the Qubit Fluorometer to take readings.
35. If the concentration is very low, you can concentrate the sample using a speed-vac.
36. Store extracted DNA in the cold room.
37. Record DNA concentrations in the lab notebook and the Andersen Lab Strain Intake Sheet.