Nematode Collection Protocol
By C. Buchanan and T. Crombie, March 1, 2020

Introduction

The Andersen Lab uses a variety of genetic and genomic tools to investigate the molecular, evolutionary, and quantitative genetics of *C. elegans* natural populations. This protocol describes the step-by-step process for collecting wild isolates and incorporating the strains into the Andersen Lab and CaeNDR.

Collections, in bags and on plates, are labeled with C-labels. Isolated nematodes are labeled with S-labels. The C-labels are used to identify unique collections, and the S-labels are used to identify unique nematode isolates. These two types of labels are used to make the connection between a particular collection (C-label) and the nematodes isolated from that collection (S-labels).

Two identical C-labels (one for collection bags and one for corresponding collection plate) and one S-label will be used for every strain. The labels are stored in bench 2, cabinet D7. To find the appropriate starting label, find the last sheet of labels printed and continue in succession. The labels can be printed from the Dropbox/AndersenLab/Collections folder (S-labels 1-99999.pdf and C-labels 5000-99999.pdf). If you need more labels, go to Barcode Generation below.

Barcode Generation

Barcode generation creates C-labels and S-labels. They are generated using a script called generate_labels.py in the GitHub Hawaii2017 repo (LINK). The script generates labels for use with Avery Durable ID Labels with TrueBlock® Technology, 61533. These labels can be printed from most laser printers. The script can be run after the requirements are installed. Be sure you have the latest version of pip prior to installation as well. Navigate to the repo directory and run:

```
pip install --upgrade pip
pip install -r requirements.txt
```

Then you can run the script from within the repo directory by running:

```
python scripts/generate_labels.py
```

The number of labels, label prefixes, and type of label can be modified by editing LABEL_USE and LABEL_SETS in the following lines of the script:

```
# Configuration
LABEL_USE = "a_61533" # Type of label
LABEL_SETS = {'C': 120, 'S': 240} # Label prefixes and counts
```

The script will output a set of labels and an associated count as defined in the python dictionary LABEL_SETS. The script will output two PDF files:

- C-labels.pdf (120 labels)
- S-labels.pdf (240 labels)

The PDF files are saved in the same directory as the script.

Notes Prior to Collection

We have found that collections work more efficiently with two people per team. One person performs data entry to the Fulcrum app, carries the ambient humidity/temperature detector, takes the picture of the sample, and distributes the empty labeled sampling bag. The second person identifies the substrate to sample, records the substrate temperature, and collects the sample. The first person should scan a bag to be used in the next collection before the next collection is identified. This step speeds up sampling significantly.

Plan hikes and alternative hikes, parking sites, time, weather, etc.

1. Download Google maps for the entire collection area (once per trip). It is free and can be accessed without WiFi or data coverage.

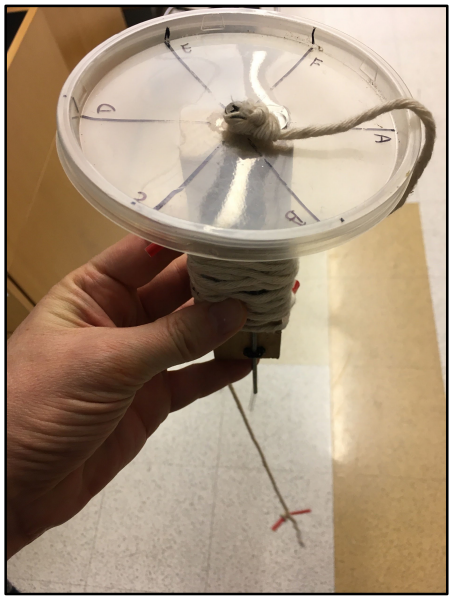
2. Determine which method of shipping you will use. FedEx is easiest for the lab. If UPS is the only shipper available, labels will need to be made by the manager for Life Sciences shipping, Robert Gill (r-gill@northwestern.edu). Unless you are going to a remote location, boxes can be purchased from the shipping company.

Materials for Collecting

This equipment is used in conjunction with the data collection app, Fulcrum, to collect wild isolates in nature. Fulcrum records data for the parameters the lab uses to investigate nematode collection sites.

1. Fanny pack
2. Collection coolers and cooler packs to keep samples cool when ambient temperature is above 25°C
3. Preciva Digital temperature/humidity meter (product number: HT154001) to record air temperature and humidity
4. Infrared thermometer temperature gun (ASIN number: B00837ZGRY) to determine the temperature of the sample
5. Bundles of bags with C-label barcodes, each bundle should contain 25 barcoded bags (located at Erik's bench, D7)

Figure 1



6. Grid sect tool (optional; **Figure 1**) to sample at fixed positions within a ~30 m^2 circular sampling area. The grid sect tool is pushed into the ground at the central sampling point. Additional sampling points are located 1, 2, and 3 meters away from the center in six directions, each direction 60° apart from each other.
7. Charged phone in airplane mode. The Fulcrum app GPS positions are inaccurate compared to the GPS positions extracted from pictures. This setting ensures that you use less power and get more precise GPS measurements.
8. Extra batteries for sampling equipment
9. External battery charger and charging cable for iPhone or Android
10. Access to Fulcrum app (LINK) on your phone or tablet. **Erik has to provide the lab member with the initial Fulcrum access; the wild collections czar (Claire) can add the lab member to a project. *Project format is always YearMonthLocation*
11. Backpack for your samples, food, and water for the day
12. First aid kit
13. Paper towels for absorbing excess moisture in a bagged sample.
14. FedEx shipping labels. **These labels and the boxes (below) do not need to be with you in the field, but prepared and taken with you on your collection trip.*
15. Broken down boxes for your samples *if you are working in a remote area*. Boxes can be purchased at the field sites, but it will depend on what resources are available. It is more convenient to purchase boxes if you are at a location where Fedex, UPS, or DHL are available.

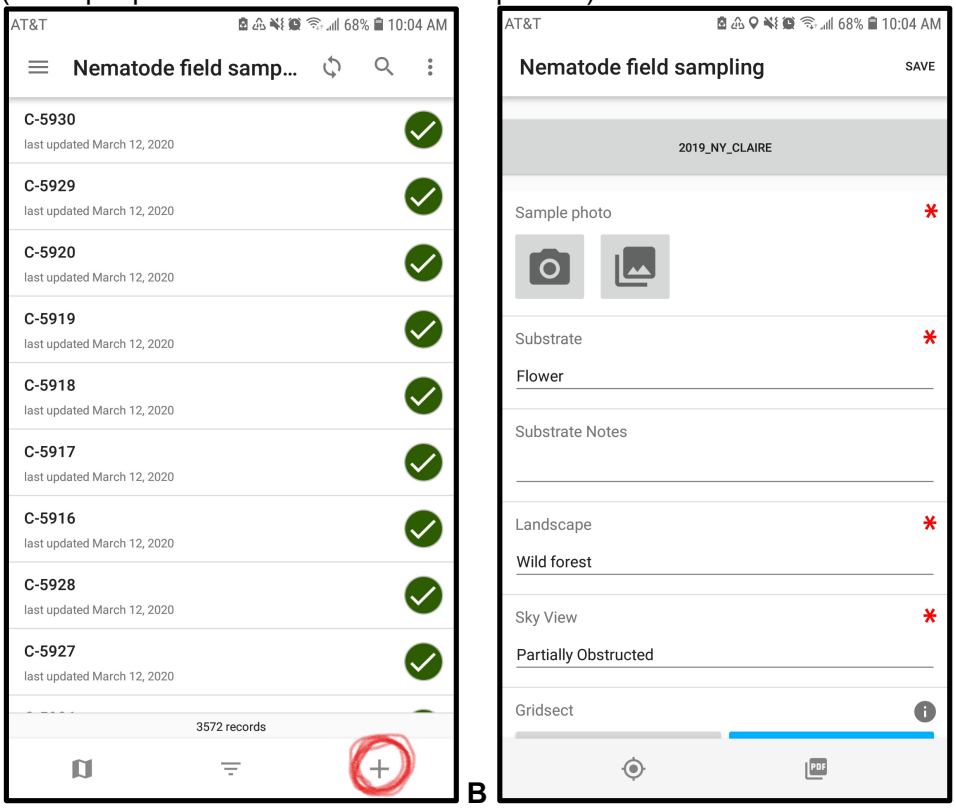
Field collection

1. Locate collection material (leaves, rotting fruit, tubers, flowers, dirt, nuts, berries, etc.). Samples should be actively rotting to provide ample bacteria for propagation of nematodes. For fruits, collapsed almost grainy looking fruits are best. Rotting, wet samples are not good substrates for nematodes. Rotting leaf litter should be rotted and dark but (again) not wet. (see **Figure 2** for examples)

Figure 2

2. Open the Fulcrum app. Go to *Nematode field sampling* from the drop-down menu.

Figure 3 (Example photos are from an Android phone.)



3. Press + to start a new record in your project (red circle in **Figure 3A**). Take a photo of the substrate. Click OK to capture the image.
4. Make sure you are in the correct project by checking the gray box at the top. If it is not the correct project, click on the box to select the correct one (**Figure 3B**).
5. Choose the 'C-label' option and choose “Scan” when the prompt appears. Scan the barcode on the collection bag.

6. Choose substrate from the drop-down menu. A variety of substrates are pre-entered. Pick the substrate that best represents your sample. You can add notes in the line right below 'Substrate,' in 'Substrate Notes.' If you do not see your sample, please select Other and then enter it.
7. Collect about a tablespoon of substrate without sticks or other hard pieces. The substrate should fit easily on a 10 cm plate.
8. Choose a landscape from the drop-down menu. Pick the landscape that best represents where you are collecting.
9. Choose a sky view. When choosing skyview, look up at the location where you picked up your sample. Describe how clearly you can see the sky (*e.g.* I can see the sky without any trees impeding my view = full).
10. Invert the bag or use it as a "glove" to collect the substrate. Seal the bag. Put a paper towel in the bag if the sample is particularly moist.
11. Save the record in Fulcrum.
12. Put collection bags into investigator backpacks on hikes and then in collection coolers (with cool packs) in investigator car trunks after hikes.

Tips for the Digital Temperature/humidity Meter and Infrared Thermometer Temperature Gun

- Make sure neither device is on 'hold.' Both devices have visual indicators, but it is easiest to make sure the measured parameter changes during the measurement time.
- Make sure you are no more than 14 inches from the substrate while recording the substrate temperature.
- Make sure that the probe tip of the ambient temperature meter does not get wet with rain or dew. To toggle between ºC and ºF, turn off, and then on and hold the max button for five seconds. It will change when the button is released. Try to keep the ambient tool outside of a bag and away from your body.

Gridsect Collections

1. Identify an area to perform a gridsect collection.
2. Sample the center of the area with the 'Gridsect' option set to 'Yes'.
3. For the center point, set the 'Grid sect direction' to A and the 'Gridsect Radius' to 0.
4. Put the gridsect sampling tool into the ground at the point where A-0 was collected. Point the 'A' direction north.
5. Extend the string along the A direction and sample every meter up to three meters (marked by red straws on the gridsect tool). Make sure to modify the gridsect direction and radius fields in Fulcrum as you sample.
6. Continue sampling through the F direction.

After Collection of Wild Isolates

1. Clean all materials, take out batteries, re-freeze freezer packs.
2. After each day, make notes about the trails and conditions you hiked. Include how many bags you used and anything relevant.
3. **Make sure to sync your Fulcrum data!** Uploads can take several minutes. You might want to wait until you have WiFi Data access. Picture files are large.
4. Ship your samples to the Andersen Lab using the steps below.

Shipping to Andersen Lab

1. Shipping labels should be printed at the lab prior to your trip. The collector will bring these labels with his/her collection materials.
2. Use to the following address for creating the shipping labels:
    - 2205 Tech Dr, Hogan 1-500, Evanston, Illinois, 60208, United States

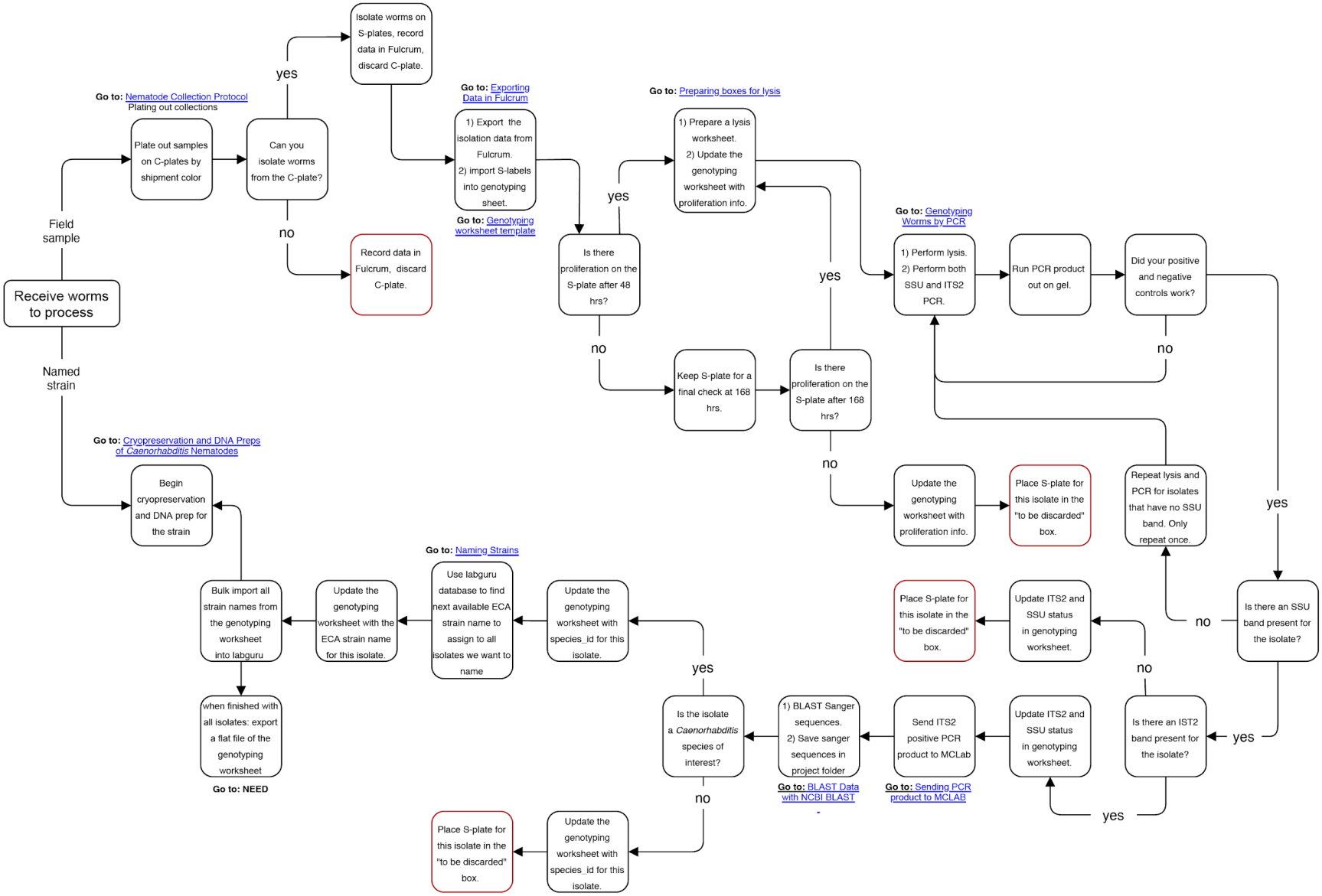
(847) 467-4264

3. Be cognizant of the shipping dates/time of your chosen FedEx location. Hawaiian FedEx locations are limited. Also, shipments leave the Hawaiian islands Monday through Wednesday, so please ship on those days.
4. Track your package.
5. Let the lab members know the shipment is on the way. Provide the tracking number.

Nematode Workflow Chart

This workflow can be used as a reference in coordination with this protocol. These steps will take the lab member through the wild collections intake process, from plating the field sample to beginning the cryopreservation protocol.

- Link for editing file: https://app.diagrams.net/#G1qrPv8ltD8ZzUFarDj_JpDof7xl0BqDzm
- Path to PDF with active links: ~/Dropbox/AndersenLab/Protocols/LabProtocols/PicsForProtocols/NematodeCollections Protocol/nematode_intake_workflow_v3.pdf


Field sample
Receive worms to process
Named strain
Go to: Nematode Collection Protocol
Plating out collections
Plate out samples on C-plates by shipment color
Can you isolate worms from the C-plate?
yes
no
Isolate worms on S-plates, record data in Fulcrum, discard C-plate.
Record data in Fulcrum, discard C-plate.
Go to: Exporting Data in Fulcrum
1) Export the isolation data from Fulcrum.
2) import S-labels into genotyping sheet.
Go to: Genotyping worksheet template
Is there proliferation on the S-plate after 48 hrs?
yes
no
Go to: Preparing boxes for lysis
1) Prepare a lysis worksheet.
2) Update the genotyping worksheet with proliferation info.
Keep S-plate for a final check at 168 hrs.
Is there proliferation on the S-plate after 168 hrs?
yes
no
Go to: Genotyping Worms by PCR
1) Perform lysis.
2) Perform both SSU and ITS2 PCR.
Run PCR product out on gel.
Did your positive and negative controls work?
yes
no
Repeat lysis and PCR for isolates that have no SSU band. Only repeat once.
Update the genotyping worksheet with proliferation info.
Place S-plate for this isolate in the "to be discarded" box.
Is there an SSU band present for the isolate?
yes
no
Update ITS2 and SSU status in genotyping worksheet.
Place S-plate for this isolate in the "to be discarded" box.
Is there an IST2 band present for the isolate?
yes
no
Update ITS2 and SSU status in genotyping worksheet.
Send ITS2 positive PCR product to MCLab
Go to: Sending PCR product to MCLAB
1) BLAST Sanger sequences.
2) Save sanger sequences in project folder
Go to: BLAST Data with NCBI BLAST
Is the isolate a Caenorhabditis species of interest?
yes
no
Update the genotyping worksheet with species_id for this isolate.
Place S-plate for this isolate in the "to be discarded" box.
Update the genotyping worksheet with species_id for this isolate.
Use labguru database to find next available ECA strain name to assign to all isolates we want to name
Go to: Naming Strains
Update the genotyping worksheet with the ECA strain name for this isolate.
Bulk import all strain names from the genotyping worksheet into labguru
when finished with all isolates: export a flat file of the genotyping worksheet
Go to: NEED
Go to: Cryopreservation and DNA Preps of Caenorhabditis Nematodes
Begin cryopreservation and DNA prep for the strain

Materials for Plating Out Collections

1. Beaker with 95% ethanol
2. Spoon
3. Paper towels
4. Ethanol spray bottle
5. C-labels that match the C-labels on bags used in the collections (refer to the Introduction to review C-label distribution).

Plating Out Collections

1. Open the shipment and keep the contents in a separate location from other shipments (*e.g., put a collection on the racks first, top to bottom, then use Bench #2 at D7).* Assign a unique number and color to the shipment (*e.g.*, shipment 1 is green, shipment 2 is orange).
2. Print a Wild Collections Shipment Log with the information about the separate shipments and keep it near the benches with the samples.
    - This data sheet includes the project name, distinct shipment numbers, dates the shipment was sent and received, important shipment notes, and shipment color designations.
3. Look in the stack of labels labeled '*For 10 cm plates*' to find the C-labels matching the samples in the shipment. Add the matching C-labels to the tops of 10 cm plates (1 label per plate). **When collections are large, we found that organizing each bagged sample with the matching C-labelled 10 cm plate on top of it made it easier to plate out the samples* (**Figure 4**).

Figure 4

4. Remove a spoon from the ethanol beaker and wipe off with a paper towel. Transfer ~1 Tbsp of sample from the collection bag onto the 10 cm plate with the matching C-label. Add the sample around the bacterial lawn in a crescent or ring shape. Do not cover the lawn (**Figure 5**). This is a C-plate. Place the spoon back in the beaker with 95% ethanol.

Figure 5

5. Use a colored Sharpie to add the shipment color designation to the lid of each C-plate on the label away from the QR-code (**Figure 6**).

Figure 6

6. Parafilm plates with visible mites to prevent spread.
7. When setting out the plated sample, make sure you remember which plates will be ready first (*e.g.*, left side of bench to right side; top rack to bottom rack).
8. Keep C-plates organized by shipment at room temperature for at least 24 hours then move to the next step. **Note: ~600 10 cm plates can fit on an empty bench.*

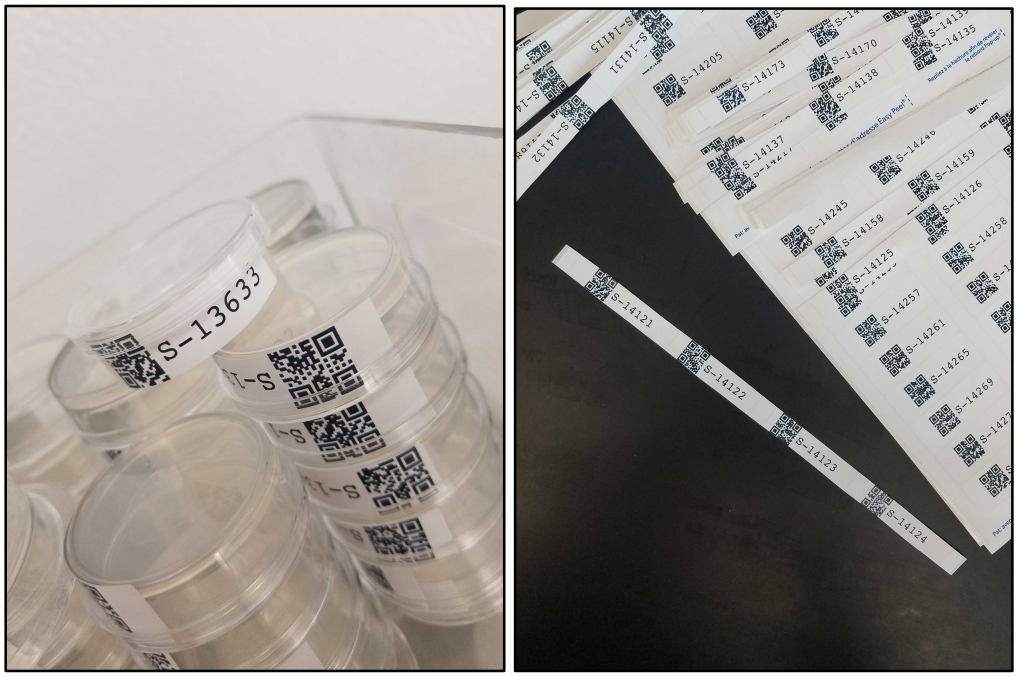
Isolating Nematodes from Collections

Process collection shipments one at a time. For example, all collections from shipment 1 of *your_project* must be plated out, isolated, synched, and exported before starting shipment 2. Follow the protocol below to accomplish these steps.

Materials for Isolation

1. Dissection scope(s)
2. Printed S-labels (located at Bench D7)
3. S-plates (3.5 cm plate)
    - The S-plates should be labelled (label on the side of the plate) prior to receiving the shipment. S-labels need to be trimmed to fit the 3.5 cm plates. Use a scissors to cut out the label into strips, making a better fit for the side of the 3.5 cm plate.

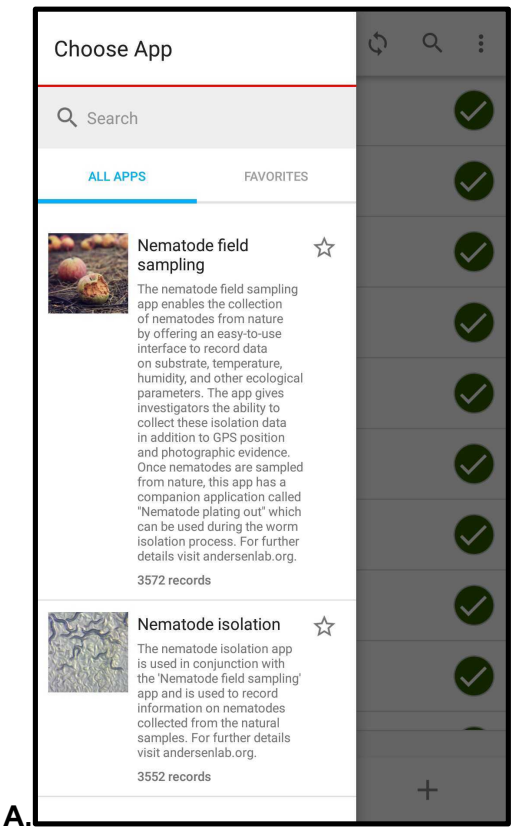
Figure 7



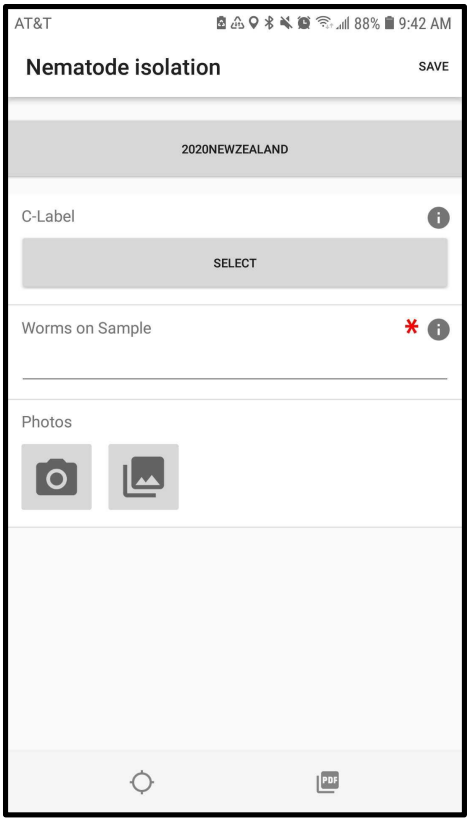
4. Worm pick(s)
5. Bunsen burner
6. Large biohazard waste bag(s) to catch excess sample
7. Parafilm

Nematode isolation

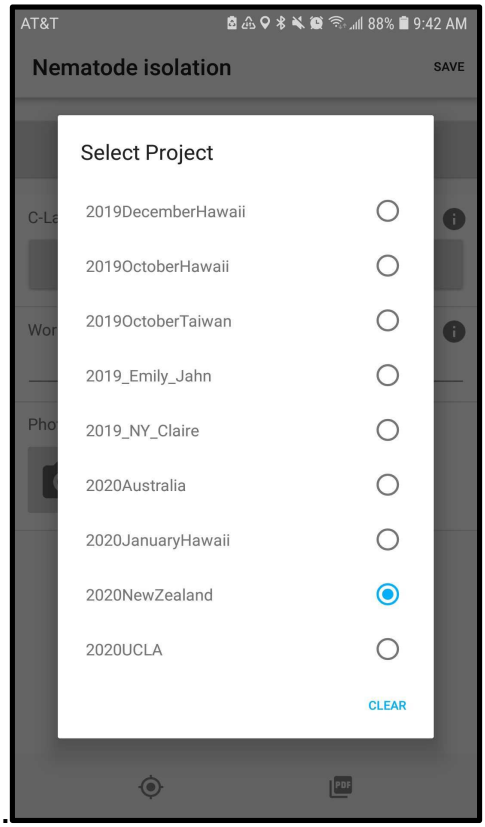
Figure 8 (reference photos are from Android phone)


Choose App
Search
ALL APPS
FAVORITES
Nematode field sampling
The nematode field sampling app enables the collection of nematodes from nature by offering an easy-to-use interface to record data on substrate, temperature, humidity, and other ecological parameters. The app gives investigators the ability to collect these isolation data in addition to GPS position and photographic evidence. Once nematodes are sampled from nature, this app has a companion application called "Nematode plating out" which can be used during the worm isolation process. For further details visit andersenlab.org.
3572 records
Nematode isolation
The nematode isolation app is used in conjunction with the 'Nematode field sampling' app and is used to record information on nematodes collected from the natural samples. For further details visit andersenlab.org.
3552 records

A.


AT&T
88% 9:42 AM
Nematode isolation
SAVE
2020NEWZEALAND
C-Label
SELECT
Worms on Sample
Photos

B.

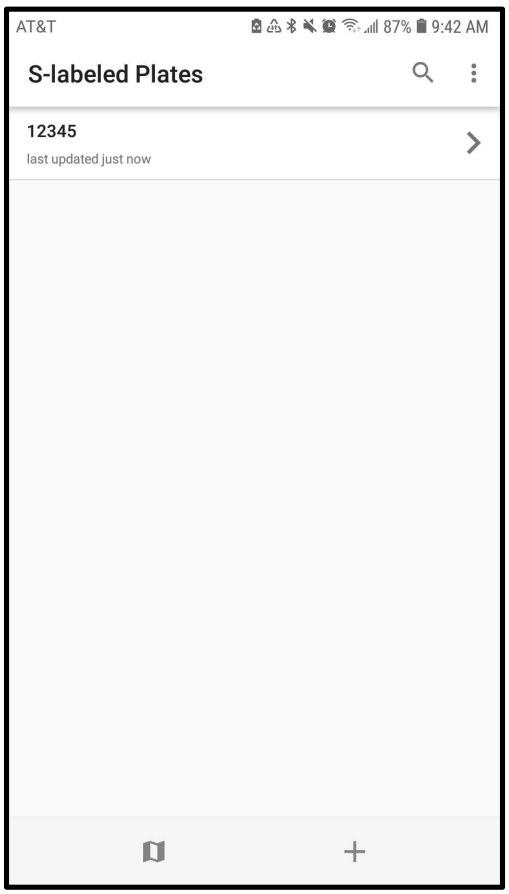

AT&T
88% 9:42 AM
Nematode isolation
SAVE
Select Project
2019DecemberHawaii
2019OctoberHawaii
2019OctoberTaiwan
2019_Emily_Jahn
2019_NY_Claire
2020Australia
2020JanuaryHawaii
2020NewZealand
2020UCLA
CLEAR

C.


4:42
55°
Select Record
C-1542
1
2
2
3
3
4
5
5737 records

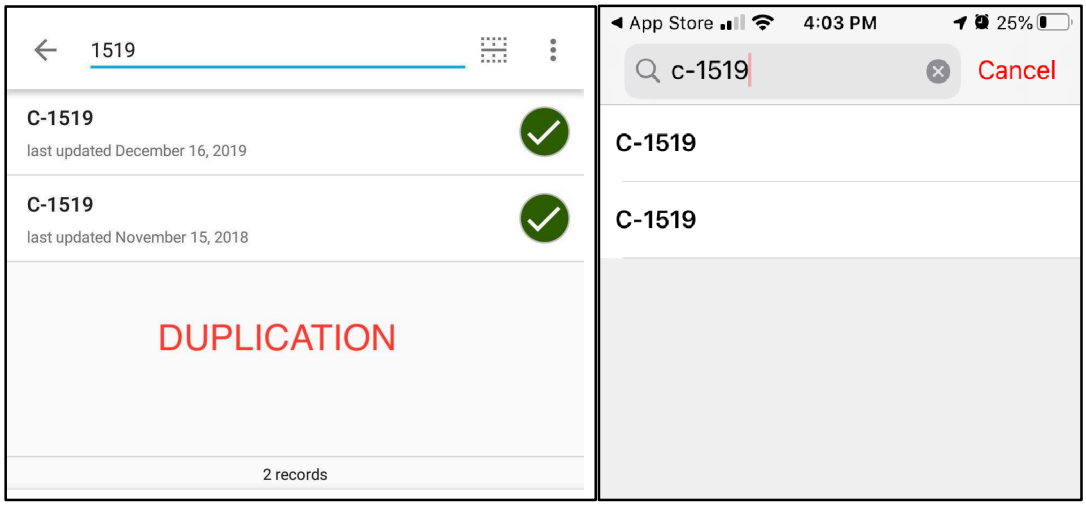
D.

E. 

F. 

1. Open Fulcrum and choose *Nematode isolation* from the menu (**Figure 8A**).
2. Tap the '+' icon to make a new isolation record in your project (**Figure 8B**). The project name associated with this isolation is displayed in the box at the top center. If the project name does not match your project, tap the project name to change it. The projects which appear are the projects in which you have access (**Figure 8C**). Tap the correct project to select it. **If you do not see the correct project, you need to ask Erik for access.*
3. Tap the 'Select' button under the 'C-Label' field to find the C-label associated with the sample from which you are isolating nematodes (**Figure 8D**).
    a. Tap the magnifying glass in the top right corner (Android phones) or the bottom center (iOS) to enter the C-label code.
    b. Tap the scan icon to scan the C-label QR code on your C-plate with your device camera. Once the QR code is scanned, a C-label record will appear in the 'C-Label' field.
4. If one record appears, then move on to Step 5. If two or more records of the same C-label appear (**Figure 9**), that C-label has been used multiple times. Unfortunately, the C-label has either been duplicated across different projects or within the same project, perhaps both. If you find a C-label duplication, then follow the steps in the duplication procedure below.

Figure 9 - Duplicated C-labels - Left - Example from an Android phone; Right - Example from an iPhone

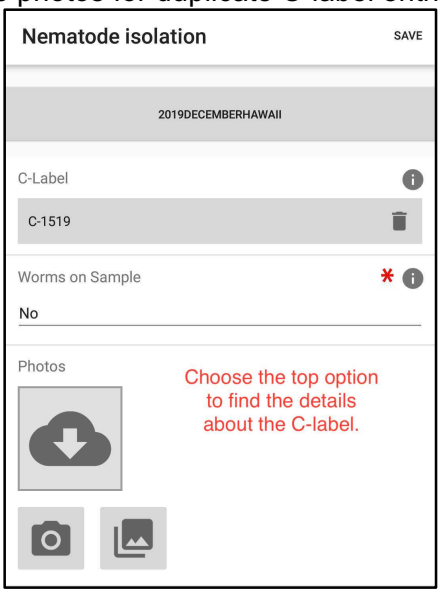


Duplication (Android)

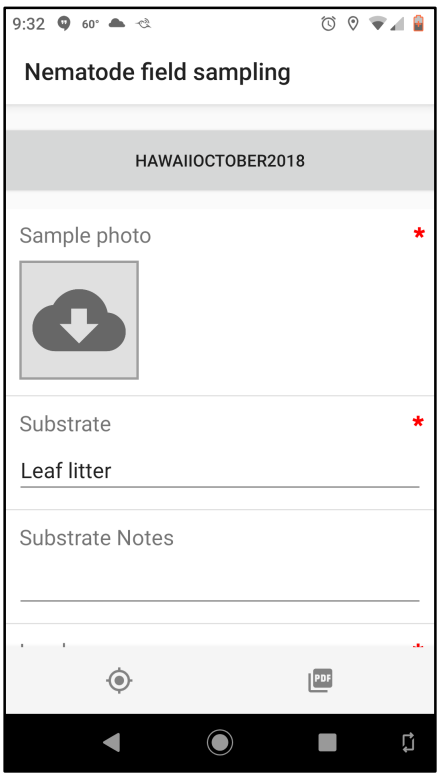
- Choose the top C-label (**Figure 9**, left) and that C-label will be filled in to your Nematode Isolation entry (**Figure 10A**).

Figure 10 - Android reference photos for duplicate C-label entries.

A.



B.



- Click on this C-label to reveal the specifics of that collection (**Figure 10B**) in Nematode field sampling. The gray bar at the top indicates the field collection to which this C-plate belongs. If the collection is different than the one you are processing (*e.g.*, 2018OctoberHawaii vs. 2019DecemberHawaii), click on the back arrow at the bottom of the screen and then press on the garbage can icon next to the C-label label. Repeat Step 3 to go back to the list of multiple C-labels. Choose the next C-label on the list to see if it matches the information for the collection you are currently processing. Repeat this process until you identify a C-label that matches the current C-plate.
- If the collection listed for the C-label is the same as the collection you are currently processing and the substrate matches what you see on the plate, this is most likely the correct entry for your plate. However, to make sure that this is the correct entry, press the back arrow at the bottom of the screen and then press on the garbage can icon next to the C-plate label. Repeat Step 3 to go back to the list of multiple C-plate labels. Go through each of the remaining C-labels to make sure none of the others also belong to the current collection.
- If you find that the C-label you are processing has two records in the same collection and both entries have the same substrate, you may be able to determine which record is correct by looking at the date collected and making sure it matches with the shipment for this plate. Exit Nematode Isolation and go to Nematode Field Sampling. Search for the C-label and look at the creation date. For example, if one of the C-plates was collected on the first day of the trip, it would be in shipment one. If you are currently processing shipment three, you would not choose the record that was created on the first day.

- If you still need help, please ask Robyn.
- If you match the C-label correctly, go to Step 5.

Duplication (iOS)

- Choose the top C-label in the list (**Figure 9, right**), the app will return to the Nematode isolation record screen with a C-label in the place of the selection button (**Figure 11A**).
- Click on this C-label. The next screen will say 'Nematode field sampling' and will provide the collection information for this instance of the C-label (**Figure 11B**). Examine the collection information and ensure it matches the sample you are isolating from *(e.g.* the C-1519 record displayed in (**Figure 11B**) matches the 2018OctoberHawaii project and the substrate was Leaf litter).

Figure 11 - iOS reference photos for duplicate C-labels

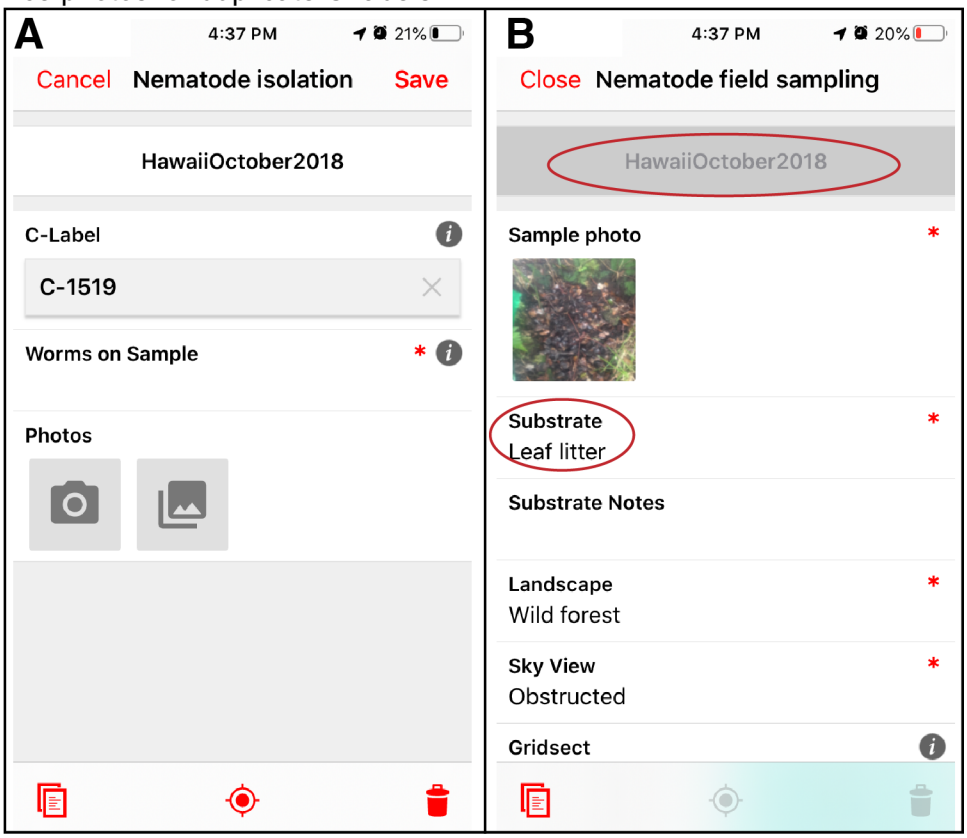


Figure 11. Duplication iOS. (**A**) Screen shot of the Nematode isolation record with the top instance of C-1519 in the C-Label field. (**B**) Screen shot of the Nematode field sampling record for C-1519. This screen appears when the user taps the C-1519 in the Nematode isolation record.

- If you are certain the current instance of the C-label matches your sample, great! Tap Close in the upper left (**Figure 11B**) to return to the isolation record (**Figure 11A**) and proceed to step 5 below. However, you may want to go through the remaining C-labels to make sure none of the others also

belong to the current collection. If there is another C-plate in your same collection, see IMPORTANT note below.
    - If the current instance of the C-label does not match your sample or you are uncertain you will need to check the other instance(s). Tap Close in the upper left to return to the isolation record. Tap the grey X to the right of the selected C-label to remove it from the isolation record (**Figure 11A**). Click "Yes, Remove" in the popup dialogue. Click the select button again and search for the C-label again as you did above. This time select the bottom instance of the C-label and confirm that it matches your sample.
    - If you match the C-label correctly, go to Step 5.
    - **IMPORTANT:** If both instances of the C-label belong to the same collection project (**duplication within collection**), you will need to use substrate information to match the correct instance to your sample. If the substrate info is not informative, exit the *nematode isolation* app and open the *field sampling* app. Search for the C-label in question. Look at where and when each record was generated. You might be able to select the correct record based on this information, especially if samples were sent over multiple shipments.
    - If you still need help, please ask Robyn.
    - If you match the C-label correctly, go to Step 5.

5. Tap on the camera icon in the 'Photos' field to open the device camera and use it to take a photo of the sample on the C-plate with the QR code and shipment color visible (**Figure 8E**). Tap 'Done' to return to the Isolation screen.
6. Invert the C-plate over the biohazard waste bin and gently tap the back of the plate to dislodge all of the sampled substrate. This step makes it easier to find and isolate nematodes.
7. **Prevent mite contamination in the lab!** Keep the lid on your C-plate closed whenever possible (*e.g.*, while you search for nematodes under the dissecting scope).
8. Tap on the 'Worms on Sample' field to record the presence of nematodes on sample (**Figure 8D**).
    a. Yes - worms are present
    b. No - no worms are present
    c. Tracks - only tracks are observed, no visible worms.
9. If nematodes are present, choose 'Yes.' Next, tap on the 'Approximate number of worms' field to record the population size on the C-plate at the time of isolation (**Figure 8D**).
10. If no nematodes are present, choose 'No.' Parafilm any C-plate you will discard in the biohazard bin. Then, move on to **Step 14**.
11. Isolate up to six nematodes from your C-plate and transfer each to a single S-plate by picking **one nematode onto each S-plate**.
    a. **IMPORTANT:** Please keep the S-plate(s) used for this step organized together in a neat stack away from other S-plates until they are entered into Fulcrum in **Step 13**.
    b. Isolate healthy, gravid, adults if possible. However, isolate other stages if adults are not found. Gravid females or hermaphrodites give us the best chance to isolate those nematodes. If virgin females (stages younger than adults or unmated adults) are chosen, then we will not isolate outcrossing species. Nematodes in nature are likely outcrossing, even if they are hermaphrodites, so we need to be aware that isogenizing might be necessary.
    c. Prioritize isolation of *Caenorhabditis*-like nematodes unless told otherwise.
        - Reference for pictures and procedures is here

12. Tap on the 'S-labeled Plates' field to enter the S-plate(s) you used for this isolation.
    a. **IMPORTANT:** Please ensure the S-plate(s) you scan are the ones onto which you picked nematodes and none are duplicated!
    b. Tap on the '+' in the lower right.
    c. Tap on the 'S-Label' field.
    d. Tap on the 'Scan' button to open the device camera. Use it to scan the S-label QR code on the S-plate you want to enter. Ensure that the S-label code matches the code you see on the plate. If it matches, tap on 'Done'. If it does not, tap on 'Cancel' and rescan until it matches, then tap on 'Done'. Sometimes QR codes of nearby plates are accidently scanned.
    e. **IMPORTANT:** Save your entry with the 'Save' button on top right, if you do not save you will lose your entry.
    f. Tap on the '+' to add more S-labeled plates if necessary until all nematodes picked from the C-plate are entered.
    g. Once you are done adding S-labeled plates, tap on the '<' button on the upper left to go back to the isolation record screen.
13. Tap on the 'S-labeled Plates' field again to ensure that all the S-label plates assigned to the isolation record are correct and NOT duplicated. If you see a problem use the 'Edit' button on the upper left to edit them. Use the '<' button on the upper left to go back to the isolation record screen.
14. When animals are picked to S-plates, you will parafilm the C-plate and discard it in the biohazard bin.
15. Tap the 'Save' button on the upper right once the isolation record is mistake-free.
    a. **IMPORTANT:** Please review the record one last time for any mistakes. If you need to cancel an isolation record because mistakes cannot be resolved click 'Cancel' in the upper left. This step will open a dialog asking if you are sure you want to discard without saving. If you do, click 'Yes, Discard'.
16. Parafilm the S-plates you just entered and set aside.
17. Sync to upload all the data to Fulcrum.
18. Stop all isolations once the last sample for a shipment is picked.
19. Sort all the S-plates from the shipment into alphanumeric order then place the S-plates into cardboard boxes. Make sure the S-plates are lid-side down and parafilmed. Stack up to four S-plates into one position in the box. Ensure the lowest number of S-plate is on the bottom (*e.g.* S-12345 is beneath S-12346).
20. Label the box with the project name, shipment number, shipment color, date and time of the last isolate, and a unique box number.
21. Store the labelled boxes at room temperature. These isolates will be checked for proliferation at 48 hours and again at 168 hours if necessary.

Exporting S-plates from Fulcrum by shipment

IMPORTANT: If a project consists of multiple shipments, **DO NOT** perform isolations from other shipments until you export the S-labels from the previous shipments.

1. Sign into the Fulcrum website and select the *Nematode isolation* app.
2. Click 'exporter' from the left hand side of the screen.
3. Click to select your project.
4. Make sure *Nematode isolation* is checked.
5. Click 'Next.' Wait for it to download.
6. The zip file will include a file titled 'nematode_isolation_s_labeled_plates.csv'.
7. Open the 'nematode_isolation_s_labeled_plates.csv' file and sort it by the 'created_at' column in ascending order (the earliest record will be on top).

8. Locate the ‘s-label’ column on the far right and select all S-labels with a ‘created_at’ date/time in the range of the shipment you are exporting S-labels for.
    - **IMPORTANT:** Make sure S-labels are not duplicated. If you see duplicates, this problem means that the S-label was accidentally scanned twice. Alert Robyn so they can help resolve this issue in Fulcrum before continuing.
9. Open the wild_isolate_genotyping_template google sheet.
10. Right click the 'genotyping template' tab on the lower left and select 'Copy to new spreadsheet'. Click 'Open spreadsheet' to set up a new genotyping google sheet for this collection project.
    - **IMPORTANT:** Name this sheet with the fulcrum project name followed by 'wild_isolate_genotyping'.
    - For example, '2020FebruaryAustralia_wild_isolate_genotyping'
    - Only one genotyping sheet will be used per project.
11. Paste the S-labels you copied from the 'nematode_isolation_s_labeled_plates.csv' 's_label' column into the genotyping google sheet column titled 's_label'.
    - **NOTE:** If you are adding S-labels from another shipment, paste them below the existing S-labels.
12. Fill in the genotyping google sheet columns below with the shipment information and 'isolation_box_number' for all S-labels in the shipment.

shipment_number	**shipment_sent_date**	**shipment_received_date**	**shipment_color**	**isolation_box_number**

13. Check the ‘s_label_repeat_error’ column for ‘1’s. A value of ‘1’ in this column means the S-label is duplicated somewhere on the genotyping sheet.
    - **IMPORTANT:** If duplications are discovered, investigate why they occured and correct them before moving forward.
14. If no duplicates are present, the S-plates from this shipment are ready to move on to the next step.
15. While you are waiting to check for proliferations on the S-plates from this shipment, you can begin isolating nematodes from the next shipment (see, Isolating Nematodes from Collections).

Check for Proliferation

1. Check for proliferating animals on S-plates 48 hours after isolation (use the date and time of last isolation on the box to guide your timing). Proliferating nematodes are characterized by multiple animals on the plate.
2. If an S-plate is proliferating, enter `1` in the proliferation_48 column on the genotyping google sheet then move the S-plate to a box labelled 'shipment X, 48 hr proliferation, box 1'.
3. Place a maximum of 88 S-plates in a proliferation box then start filling a new box labelled 'shipment X, 48 hr proliferation, box 2'.
    - **IMPORTANT:** Do not toss the non-proliferating S-plates, you will check them again in Step 5 below after 168 hours post-isolation. You can consolidate these S-plates in numeric order in boxes labelled 'shipment X, 48 hr non-proliferating, box X'. Just be sure to note when the 168 hours check should occur on the box.
4. Move on to the Lysis step below for proliferating S-plates at 48 hours (See, Lysis).
5. Check the S-plates that were not proliferating at 48 hours post-isolation again at 168 hours post-isolation. If an S-plate is proliferating, enter '1' in the proliferation_168 column on the

genotyping google sheet then move the S-plate to a box labelled 'shipment X, 168 hr proliferation, box X'.

6. Discard the S-plates that have no proliferation after 168 hours.

Lysis

1. Use the data filter tool in google sheets to print lysis worksheets for the S-plates in the proliferation boxes. *The purpose of the lysis worksheets is to provide team members with the correct positions for S-labels in lysis strip tubes at the bench.*
    a. Open the genotyping google sheet for your project and select all cells by typing Cmd+A.
    b. Click on Data > Create a filter. This step will add a filter button to each column header.
    c. Use the filter buttons to display only the S-plates you intend to perform genotype by lysis, PCR, and Sanger sequencing. For example, if you intend to lyse all S-plates from shipment 1 with proliferation at 48 hours you would:
        i. Click the filter button in the 'shipment_number' column and select '1'.
        ii. Click the filter button in the 'proliferation_48 column' and select '1'.
    d. Once the genotyping google sheet has been filtered, review the list of S-labels displayed to ensure that you have only the ones you want to print on a worksheet.
    e. In the 'strip_tube_number' column of the genotyping google sheet, enter a unique number every 11 rows. *The strip tube numbers for a project should be entered in successive order starting at 1 and never duplicated.*
    f. In the 'strip_tube_position', enter 2 through 12 for each strip tube number. **We use 12-tube strip tubes for lysis. The first position (strip_tube_position 1) will be a control. The controls will not be added to the lysis worksheets, only strip_tube_positions (2-12). N2 should be in position 1 of every even numbered strip tube as a positive control. No worms should be in position 1 of every odd numbered strip tube as a negative control.*
    g. Print a lysis worksheet for each proliferation box you intend to lyse.
        i. Filter the genotyping google sheet further to include just the S-labels in one proliferation box you intend to lyse, then select the columns 's_label' through 'lysis_notes'.
        NOTE: Each proliferation box holds up to eight strip tubes of S-plates.
        ii. Click on the print icon in the upper left.
        iii. In the upper left of the new screen, click on the dropdown menu in the 'Print' field and select 'Selected Cells …'.
        iv. Click the 'Next' in the upper right, then use the dialogue to print the lysis worksheet for the proliferation box.
        v. Repeat steps i-iv to print a lysis worksheet for each proliferation box.
    h. Put the printed lysis worksheet(s) on top of the appropriate proliferation box(es) and move on to Step 2.
2. Prepare enough 12-well strip tubes for all the samples you intend to lyse.
    a. Label a strip tube with a unique 'strip_tube_number' assigned in the lysis worksheet. This label should be written on the cap strip and the strip tube to avoid confusion if they are separated.
    NOTE: Position 1 of each strip tube will always contain a control.
        i. EVEN strip tubes have a positive control (N2 worms) in position 1.
        ii. ODD strip tubes have a negative control (no worms) in position 1.
        iii. **IMPORTANT:** Do not repeat strip tube numbers for different shipments in a project. Make sure the strip tube numbers continue in ascending order.
    b. Arrange up to eight strip tubes in order and place in a 96-well plate rack.

3. Make up enough lysis buffer for all of your samples.
    a. General recipe: 98 µl 2X buffer and 2 µl Proteinase K are mixed together and **keep on ice during use**.
    b. For 96 samples, you would need 800 µl of master mix. Mix together 16 µl of 20 mg/ml Proteinase K and 784 µl of 2X buffer. Scale as necessary.
4. Pick nematodes into the lysis buffer one strip tube at a time:
    a. Arrange the S-plates for that particular strip tube in order using the printed lysis worksheet as a guide.
    b. **Don't forget the controls!** The controls are not printed on the lysis worksheet.
        i. EVEN strip tubes will have a positive control (N2 worms) in position 1.
        ii. ODD strip tubes will have a negative control (no worms) in position 1.
    c. Uncap one strip tube and add 8 µl of lysis buffer to each cap with a repeat pipettor. Add the lysis buffer to one strip of caps at a time. Otherwise, the lysis buffer will evaporate the longer it is left at room temperature and uncovered.
    d. Pick 3-5 animals from the source plates (S-plate or N2 stock plate) into the appropriate cap positions (confirm positions with the lysis worksheet).
    IMPORTANT: Work as quickly as possible and do not leave the caps on the microscope light to avoid evaporation of the lysis buffer.
    e. Record notes for any S-plate with fewer than five worms picked to the lysis in the lysis_notes section of the lysis worksheet.
    f. When you are finished loading worms into each position of the strip tube, place the cap strip back on the strip tube. Match the marked cap (position 1) with the marked tube (position 1).
    g. Once capped, place the strip tube in an empty 200 µl tip wafer and briefly spin down your samples so the material is now in the tube, not the cap.
    h. Place the strip in the -80ºC freezer until it is completely frozen (at least 10 minutes)
    i. Repeat steps 4a-4h until all strips are completed.
5. Place the strips in a thermocycler and run the worm lysis program: (Lid 105°C; volume 8 µl).

Time	Temperature	Number of cycles
1 hour	60°C	1
15 min	95°C	1
Hold	12°C	1

6. When the lysis program is done, spin down your samples briefly. Store the lyses at -80°C for up to one week. Include a label with proliferation box number, strip tube number range, date, and your initials.
7. Update the genotyping google sheet columns below with lysis information from the lysis worksheet.

lysis_date	**lysis_notes**

8. Move on to PCR when ready.

PCR - SSU and ITS2

This section will provide instructions on how to perform two separate PCRs (SSU and ITS2) for each lysed S-plate.

SSU PCR: The SSU PCR amplifies a 500-bp fragment of the 18S rDNA (small subunit) gene. It uses the small subunit (SSU) primer set shown below. This PCR is used to check the quality of the template DNA. The PCR should amplify for nearly all nematode species. If the SSU PCR fails to amplify, this result suggests that the lysis quality is poor and the lysis should be repeated for this S-plate.

SSU primer set
oECA1271 = forward primer TACAATGGAAGGCAGCAGGC
oECA1272 = reverse primer CCTCTGACTTTCGTTCTTGATTAA

ITS2 PCR: The ITS2 PCR amplifies a 2,000-bp fragment of the ITS2 region (Internal Transcribed Spacer) between the 5.8S and 28S rDNA genes. It uses the ITS2 primer set shown below. The ITS2 PCR product is sent to MCLAB to be sequenced. ITS2 sequences are used to identify nematodes in the *Caenorhabditis* genus to the species level by sequence similarity.

ITS2 primer set
oECA1687 = forward primer CTGCGTTACTTACCACGAATTGCARAC
oECA202 = reverse primer GCGGTATTTGCTACTACCAYYAMGATCTGC

1. Perform both SSU and ITS2 PCRs at the same time. *Running both PCR types at the same time prevents degradation of lysis because the sample is only thawed once.* Use the filtering tool in the genotyping google sheet to view only the S-labels on which you plan to perform PCR. Update the pcr_date, pcr_plate_number, and pcr_well columns in the genotyping google sheet.
    a. The pcr_plate_number is the same for ITS2 and SSU PCRs even though these are separate reactions with separate plates. They will be distinguished with 'SSU' or 'ITS2' labels.
    b. Assign a pcr_plate_number to eight or fewer strip tubes (one strip tube per row of the 96-well PCR plate, arranged in ascending order, *e.g*. lowest strip tube number on top). *Note: The strip tubes should already be arranged in a 96-well plate holder in ascending order in the -80C freezer.*
    c. Assign a pcr_plate_well to each S-label in the strip tubes. The strip tubes are arranged in ascending order with the lowest strip tube number assigned to row A and the highest number in row H. Position 1 of all strip tubes is assigned to column 1. Therefore, strip tube number 1, position 1 will be assigned to PCR plate number 1, well A01.
2. Label 96-well PCR plate(s) to accommodate the samples on which you will perform PCR.
    a. Each PCR plate should be labelled with the following information: project name, PCR type, PCR plate number, and date of PCR (*e.g.*, 2020FebruaryAustralia_SSU_1_20200304).
    b. Label the plate with the strip tube numbers that will be loaded into each row.
3. Remove the lysis material from the -80°C freezer and thaw the strip tubes containing the lysis material on ice.
4. While the lysis is thawing, prepare ITS2 and SSU master mixes in separate tubes on ice.

NOTE: Prepare 100 reactions of PCR master mix for each 96-well plate to allow for pipetting error. Use a 15 or 50 mL conical to hold the master mix if you are making large volumes.

SSU PCR Master Mix	Working Concentration	Volume to Add	Final Concentration
oECA1271	4 µM	4 µL	0.4µM
oECA1272	4 µM	4 µL	0.4µM

10X PCR buffer	10X	4 µL	1X
2.5 mM dNTPs	2.5 mM	3.2 µL	0.2 mM
Taq	-	.2 µL	-
Lysed worm solution	-	2 µL	-
dH20	-	22.6 µL	-
Total reaction vol.		40 µL	

ITS2 PCR Master Mix	Working Concentration	Volume to Add	Final Concentration
oECA202	4 µM	4 µL	0.4µM
oECA1687	4 µM	4 µL	0.4µM
10X PCR buffer	10X	4 µL	1X
2.5 mM dNTPs	2.5 mM	3.2 µL	0.2 mM
Taq	-	.2 µL	-
Lysed worm solution	-	2 µL	-
dH20	-	22.6 µL	-
Total reaction vol.		40 µL	

5. Put the empty, labeled 96-well PCR plate(s) on ice.
6. Dilute the primers. The primer stock is 100 µM. You will need a working concentration of 4 µM.
7. Make sure your dNTPs are at a working concentration of 2.5 mM.
8. When preparing the PCR master mix, the order of adding regents is as follows: dH20, 10X buffer, primer 1, primer 2, dNTPs, and Taq *(lysed worm solution will be added to each strip tube after the master mix is aliquoted)*. After mixing the first five reagents, spin down the master mix in the centrifuge, then add Taq.
9. Aliquot the master mix. We use the sterile, single-use troughs with V-bottoms to hold all the master mix. Use a 12-well multichannel pipette to add 38 µL of master mix to each well needed in the 96-well PCR plate on ice.
10. Spin down the thawed lysis strip tubes to remove lysis material from the caps.
11. Carefully remove the lids of all the strip tubes that will be loaded into the first PCR plate. Place the caps in an empty tip wafer rack to keep them separated and in order.
12. Use a low-volume multichannel pipette (either 12-well or 8-well) to add 2 µL of lysis to the appropriate well in the PCR plate.
    a. Gently pipette the lysis up and down once before removing the 2 µl. Check the tips to make sure they contain the lysis before the transfer.
    b. **IMPORTANT:** Change tips before you pipette from the next row/column!
13. Place caps back on lysis if needed.
14. Cover the PCR plate with PCR adhesive foil (Cat No. 60941-126) and use the roller to create a tight seal. Keep the plate you just finished on ice until the PCR block reaches a minimum of 72°C.
15. Repeat steps 11 - 15 until all the PCR plates are loaded.

16. Briefly spin down the PCR plates
17. Place the SSU PCR plate(s) in a thermocycler and run the following program: SSUPCR

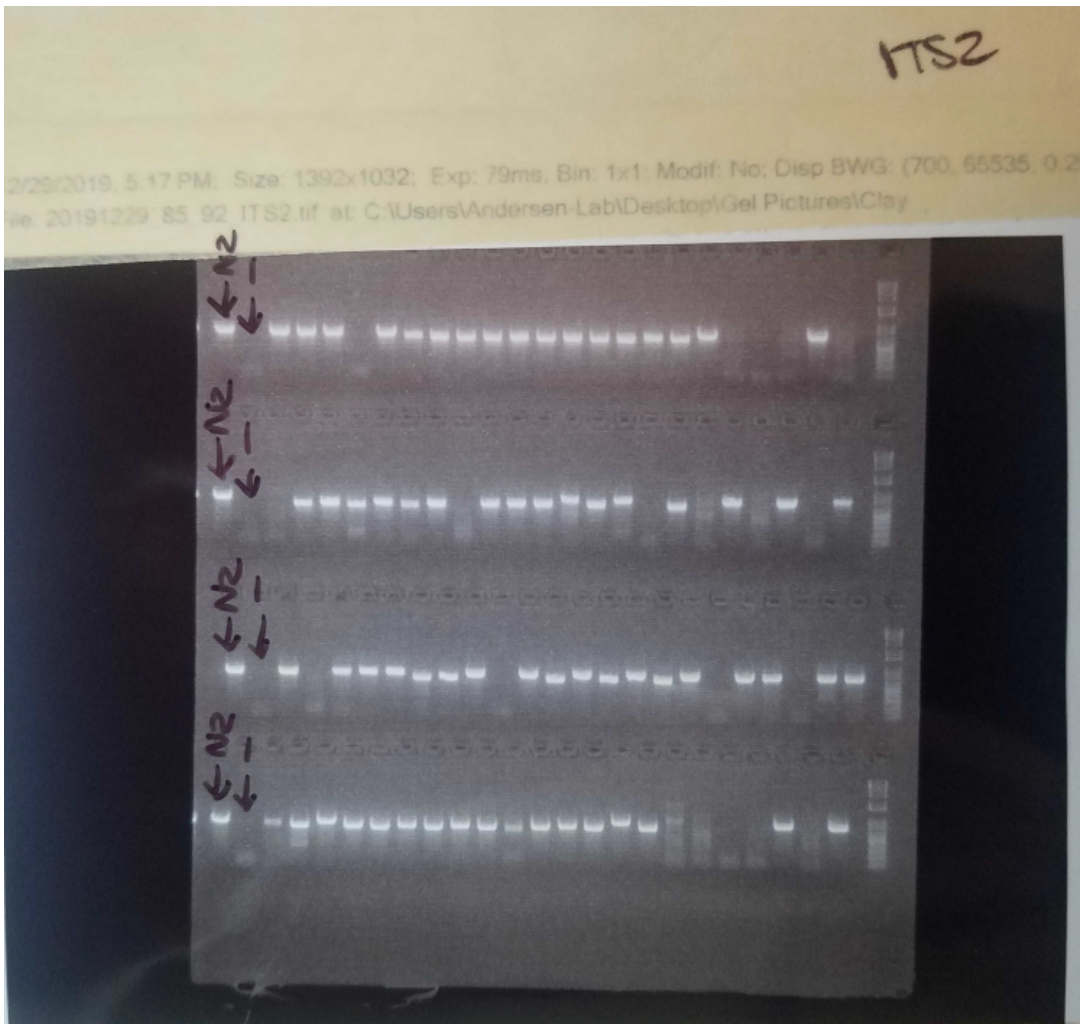
SSU PCR Steps	Time	Temperature
1	2 min	95°C
2	20 s	95°C
3	60 s	55°C
4	30 s	72°C
5	Got to step 2, repeat 35 times	
6	5 min	72°C
7	hold	12°C

18. Place the ITS2 PCR plate(s) in a thermocycler and run the ITS2 program.

ITS2 PCR Steps	Time	Temperature
1	3 min	95°C
2	15 s	95°C
3	15 s	60°C
4	2 min	72°C
5	Got to step 2, repeat 34 times	
6	5 min	72°C
7	hold	15°C

19. During the PCR, pour an 1.5% agarose gel: (one gel holds 96 samples)
    a. Take a 500 mL glass bottle from the EtBr station.
    b. Measure 1.5 g of agarose on a weigh boat.
    c. Pour agarose into the bottle.
    d. Add 100 mL of 1X TAE into the bottle.
    e. Swirl slightly to mix.
    f. Microwave 2 minutes.
    g. Use the oven mits to carry agarose to the hot plate.
    h. Let mixture cool to 60 degrees; add a stir bar to the mixture and set on the hot plate. Turn dial to level 6 for about 25 minutes.
    i. Add 5 µL of 10mg/ml Ethidium bromide to the mixture before pouring into the gel mold and adding the combs.

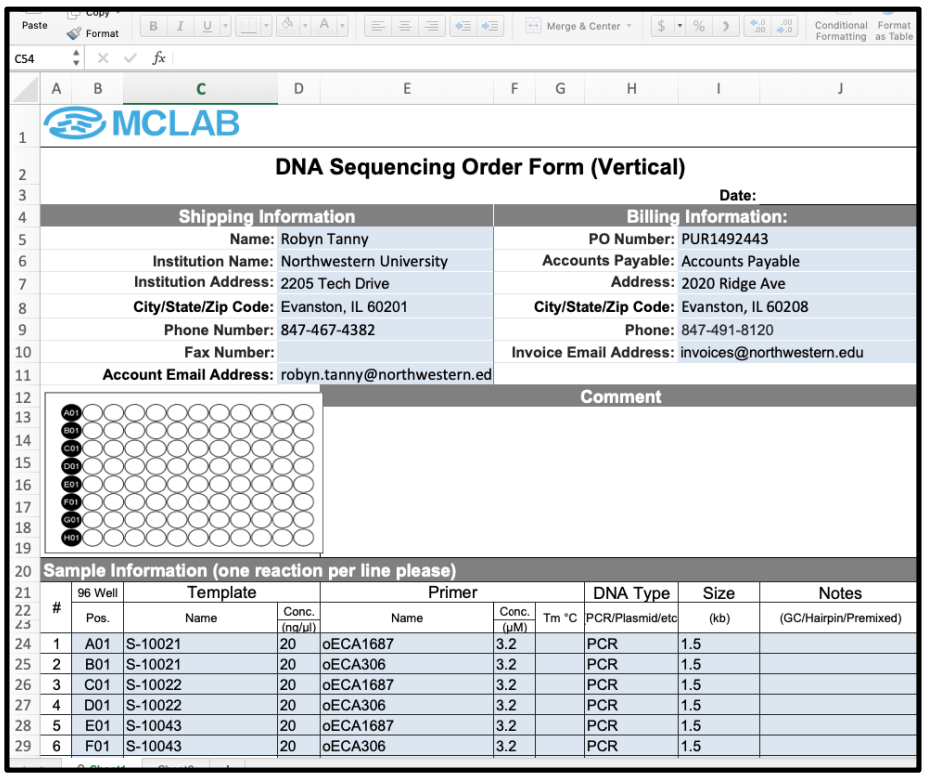
20. When the PCR is finished, add 6X loading dye to a trough. Use the 8-well multi-channel pipette to add 2 µL of 6X loading dye to each well of a new 96-well plate. This plate will be used to load the samples into the gel. Each row in the plate can correspond to one of the strip tubes. Make sure to note which row on the plate is for which strip tube.
21. When the PCR is done, briefly spin down the samples for a 'short spin' on the large centrifuge.
22. Use a 12-well multi-channel pipette to add 5 µL of each sample to the appropriate well of the 96-well plate.
23. Load the 1 KB plus ladder (Thermo Scientific, SM0243).
    a. The ladder is aliquoted into the first position on the left hand side of the gel for each row. **For 96 samples, four rows will be made; four wells will have ladders.*
    b. 8 µL of 1 kb ladder is used.
24. Use a 12-well multi-channel pipette to add the samples to the gel. ***Note that Row A from the PCR plate will be interspersed with Row B, Row C will be interspersed with Row D, etc.*
25. Run the PCR products out on a gel at 120 V for about 20 minutes.
    a. Be sure to include positive and negative controls.
    b. Don't let the gel run too long! The samples will run over and you will have to re-do the gel.
    c. Both SSU and ITS2 PCR products are run on gels. Keep them separate for organizational purposes.
26. Record the gel_number and gel_position in the genotyping google sheet. The gel_position refers to the row (A - D), and then the well where the sample is located (*e.g.* gel 1, A03).
27. Record which S-plates yield ITS2 and/or SSU PCR products in the pcr_product_its2 and prc_product_ssu columns of the genotyping google sheet. Mark the presence of a band with a '1'; mark a '0' for no band.
    a. Save an image of your gel in your file on the imaging computer. Use the following format: Project_PCR_type_gel_number_date (*e.g.* 20200508_2020FebruaryAustralia_ITS2_4).
28. Ensure you have updated the **ssu_pcr_date** to **neg_ctrl_its2** columns in the genotyping Google sheet before moving to the next step.
29. Note for band length: Everything larger or the same size as the N2 positive control is Caenorhabditis; everything with smaller bands are non-Caenorhabditis (see image below).



Sending PCR product to MCLAB

1. For each sample that is ITS2 and SSU positive, send the ITS2 product to MCLAB for sequencing. To determine which samples those are, filter the genotyping Google sheet to show which samples have ITS2 bands (pcr_product_its2).
2. Go to the MCLAB website and login with the username and password found in 'Best Practices'.

Figure 12



MCLAB

DNA Sequencing Order Form (Vertical)

Date:

Shipping Information		Billing Information:	
Name:	Robyn Tanny	PO Number:	PUR1492443
Institution Name:	Northwestern University	Accounts Payable:	Accounts Payable
Institution Address:	2205 Tech Drive	Address:	2020 Ridge Ave
City/State/Zip Code:	Evanston, IL 60201	City/State/Zip Code:	Evanston, IL 60208
Phone Number:	847-467-4382	Phone:	847-491-8120
Fax Number:		Invoice Email Address:	invoices@northwestern.edu
Account Email Address:	robyn.tanny@northwestern.ed		

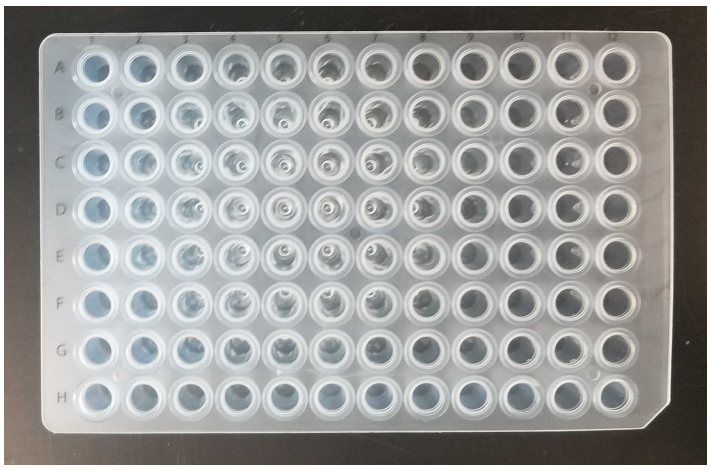
Comment

Sample Information (one reaction per line please)

#	96 Well	Template		Primer			DNA Type	Size	Notes
	Pos.	Name	Conc. (ng/µl)	Name	Conc. (µM)	Tm °C	PCR/Plasmid/etc	(kb)	(GC/Hairpin/Premixed)
1	A01	S-10021	20	oECA1687	3.2		PCR	1.5	
2	B01	S-10021	20	oECA306	3.2		PCR	1.5	
3	C01	S-10022	20	oECA1687	3.2		PCR	1.5	
4	D01	S-10022	20	oECA306	3.2		PCR	1.5	
5	E01	S-10043	20	oECA1687	3.2		PCR	1.5	
6	F01	S-10043	20	oECA306	3.2		PCR	1.5	

   a. Login and click 'Order sequences' in the top-right corner.
   b. Enter the quantity of samples you are sending.
   c. Add to cart.
   d. Fill out the order form (Figure 12) with the shipping information, current PO Number, billing information, and sample information (those samples visible after filtering the Google genotyping sheet for ITS2-positive plates).
      - *Make sure you save as an Excel document.
   e. In the “Download and upload order form” page of MCLab, choose the “Select” button to find your form. “Upload” the order form (it will appear in the file(s) uploaded box).
   f. Add the purchase order number (PO).
      - Make sure the PO number is the current PO for MCLAB.
   g. Accept terms should be checked.
   h. Submit order.
3. Print a physical copy of the same order form to include in the shipment.
4. On the filtered Google genotyping sheet, fill in the **sequencing_well** positions in alphanumeric order (*e.g.* A01-H01). Print this *wild_isolate_genotyping_worksheet sheet* to use when aliquoting samples into strip tubes for MCLab.
5. Arrange a 0.2 mL 96-well PCR plate so that A-H is on the left hand side and 1-12 on the top of the plate to explain the MCLab well position (*e.g* A01) (**Figure 13**). The MCLab order form begins at A1, so you will move down the number columns to fill wells with samples.

Figure 13



6. Set out your PCR product plates. **You will now find the samples you want to send to MCLab by looking at the wild_isolate_genotyping_worksheet you printed.*
7. Find the strip_tube_number and strip_tube_position of the samples with ITS2 bands from the printed, filtered wild_isolate_genotyping_worksheet.
8. Aliquot 12 µL of sample into the well indicated on the genotyping_sheet (*e.g.*, S-12607, strip tube number 1, strip tube position 2, MCLab well position A1).

2020JanuaryHawaii_wild_isolates_genotyping

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collection_received_date

	C	D	H	I	R	S	T	U	V	W
1	isolation_box_number	s_label	strip_tube_number	strip_tube_position	MCLab Sequencing Positions	notes	manual_blast_notes	ECA_dirty	ECA_clean	possible_new_caeno_sp
2	1	S-12607	1	2	A1	Proliferations (48hrs) were checke		ECA2498	ECA2499	
3	1	S-12608	1	3	B1	This shipment was picked from the		ECA2500	ECA2501	
8	1	S-12664	1	5	C1			ECA2502	ECA2503	
10	1	S-12666	1	6	D1			ECA2504	ECA2505	
22	1	S-12705	1	9	G1			ECA2506	ECA2507	
24	1	S-12717	1	10	H1			ECA2508	ECA2509	
28	1	S-12756	1	11	A2			ECA2510	ECA2511	
29	1	S-12766	1	12	B2			ECA2512	ECA2513	
30	1	S-12775	2	2	C2			ECA2514	ECA2515	
33										
34										

9. For each sample to send for ITS2 sequencing, add 10 µl of 3.2 µM oECA306 (CACTTTCAAGCAACCCGAC) primer into a single microcentrifuge tube to accompany the samples. Add two extra samples worth of oECA306 to account for error. **For example, if you are sending 10 samples, add 120 µL of the primer. Do not add the primer to your sample. Parafilm the primer microcentrifuge tube.*
10. Print a FedEx label using the “best practices” protocol to find the login and password.
11. Find a box (look in room Room 4539). Package the contents with care (*e.g.* bubble wrap) by parafilming the plate of samples to an empty 200 µl tip wafer and placing in an appropriate-sized sealable bag. Wrap all contents in the box in bubble wrap.
12. Bring package to Shipping (Mailroom Packages MG92) before 4:30 p.m.

BLAST Data using the NCBI BLAST server

1. Download the Sanger sequence results from MCLAB as soon as it is available.
    a. Login with credentials from the ‘Best Practices’ document.
    b. Click the ‘Download Sequences’ button in the upper left of the screen.
    c. Click on the .zip files in the list that contain your sequences to download them.
2. Move the .zip files to the collection project folder on the AndersenLab dropbox: ~/Dropbox/AndersenLab/Collections/<year>/<project_id>.
3. Unzip the files and manually BLAST the data.
    a. On a Mac, open the Terminal.
    b. Go to the collection project folder with the .zip files.

`cd ~/Dropbox/AndersenLab/Collections/<year>/<project_id>`

c. Unzip the .zip files.
`unzip '*.zip'`

d. Move all sequencing files into a subfolder named sequences under the collection project folder. Each batch of sequences should be held in their own subdirectory. For example, you could have two sequencing batches held in the sequencing directory.

```
<project_id>
      |_sequencing
            |_sequence_batch1
                        |_S-14041_oECA306_A01.seq
                        |_S-14042_oECA306_B01.seq
                        |_…
            |_sequencing_batch2
                        |_S-14156_oECA306_A06.seq
                        |_S-14041_oECA306_D08.seq
                        |_...
```

e. Go to the sequencing subdirectory.
`cd ~/Dropbox/AndersenLab/Collections/<year>/<project_id>/sequencing`

f. Create a FASTA file from all sequences. `for dir in */; do cd $dir; for file in *.seq; do echo ">"$file; cat $file; done >>../all_seqs.fa; cd ..; done`
The code above will create a merged FASTA file named "all_seqs.fa" from all the .seq files in the sequencing directory.

g. If you prefer to create merged FASTA files for each subdirectory follow steps below for each of the desired subdirectories.
   i. Go into a subdirectory in the collection project folder
   `cd ~/Dropbox/AndersenLab/Collections/<year>/<project_id>/sequencing/<your sub directory>`
   ii. Create a FASTA file from all sequences in the subdirectory with the command `for file in *.seq; do echo ">"$file; cat $file; done >> all_seqs_<your sub directory>.fa`
   iii. Change back into the sequencing subdirectory `cd ..`
   iv. Repeat steps i - iii for all subdirectories in the sequencing folder.

h. On a browser, navigate to the BLAST website

i. Click on the 'Choose file' button and select the `all_seqs.fa` or `all_seqs_<your sub directory>.fa` file you just created.

j. Click the 'BLAST' button to begin the BLAST search.

4. Update the genotyping Google sheet with the BLAST results for each S-label.
   a. Use the filter tool to make updating the genotyping google sheet easier. Click on Data > Create a filter. This step will add a filter button to each column header. Filter the sequencing_plate column to select the sequencing plates you are updating the BLAST results for.
   b. Use the dropdown menu on the NCBI Blast results page to check the results for each S-label sequence that was BLASTed one at a time (**Figure 14 Blast Results**).
      i. **Check for no blast hits**. A sequence ID in the dropdown prefixed with * has no blast hits. For these S-labels, enter 'no hit <current date>' in the manual_blast_notes column of the genotyping google sheet.
      ii. **Check for a possible new *Caenorhabditis* species**. Click the link on the top hit to visualize the alignment (**Figure 14 Blast Results**). If the top hit is (1) a *Caenorhabditis* species, (2) the alignment contains more than

5 mismatches in the center of the sequence, and (3) the query coverage is >50%, this suggests the isolate may be a new *Caenorhabditis* species (**Figure 15 Blast Alignment**). For these S-labels enter, the species of the top blast hit in the species_id column, enter a 1 in the possible_new_caeno_sp column, and 'possible new Caeno sp.' into the manual_blast_notes column along with percent identity, (e.g. 'possible new Caeno sp. 89% identity').

iii. For S-label sequences that BLAST to a *Caenorhabditis* species enter the full genus and species name of the top BLAST hit in the species_id column. For example, '*Caenorhabditis elegans*'.

iv. For sequences that BLAST to a non-*Caenorhabditis* species, enter only the genus of the top blast hit followed by 'sp.' in the species_id column. This notation means the isolate is an unknown species within the named genus. For example, '*Oscheius* sp.'.
NOTE: ITS2 sequence can not be used to reliably identify isolates to the species level outside of the *Caenorhabditis* genus.

c. Enter 1 in the make_strain_name column of the genotyping google sheet if species_id = 'Caenorhabditis elegans', 'Caenorhabditis briggsae', or 'Caenorhabditis tropicalis', OR possible_new_caeno_sp = 1. Leave the make_strain_name column blank otherwise. If there is a reason why the strain cannot be named, enter that reason in the reason_strain_not_named column. **NOTE: S-labels with a 1 in the make_strain_name column will be named and added to the Andersen Lab strain inventory, (see Naming Wild Strains).**

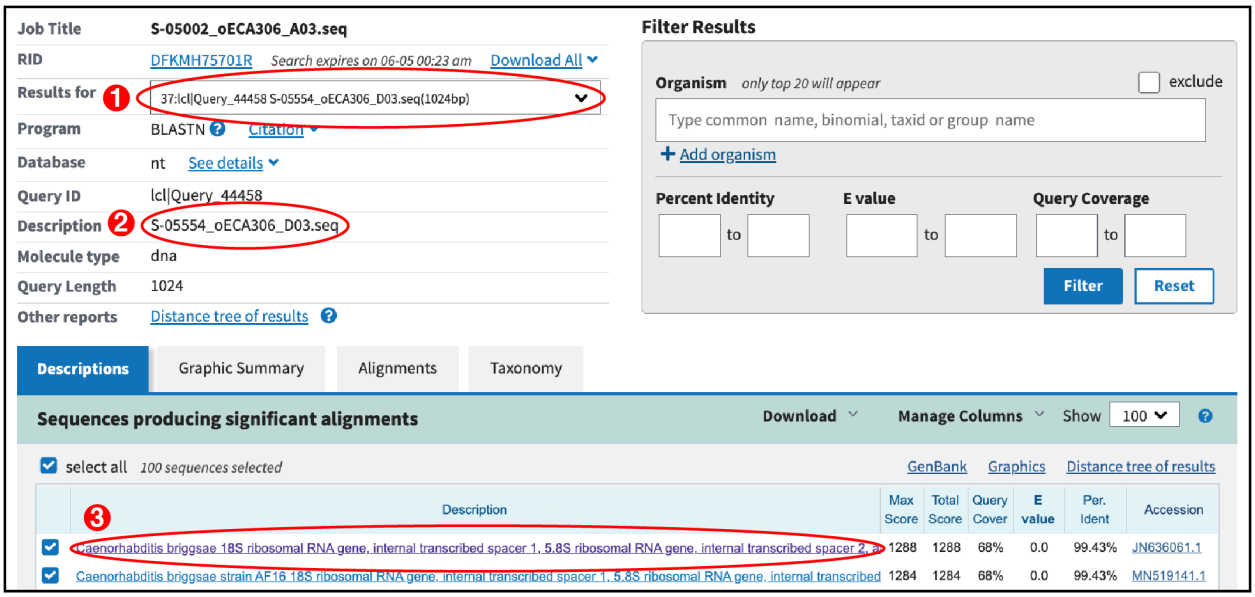

Figure 14. NCBI BLAST results page. (1) The dropdown menu used to view the BLAST results for all sequences. (2) The description of the current sequence selected from the dropdown. In this case the results for S-label S-05554 are shown. (3) The top BLAST hit for S-05554 is shown. The purple text indicates the link to visualize this alignment has been clicked. Please be sure to inspect the alignments by eye to identify possible new *Caenorhabditis* species, see step 4bii above.

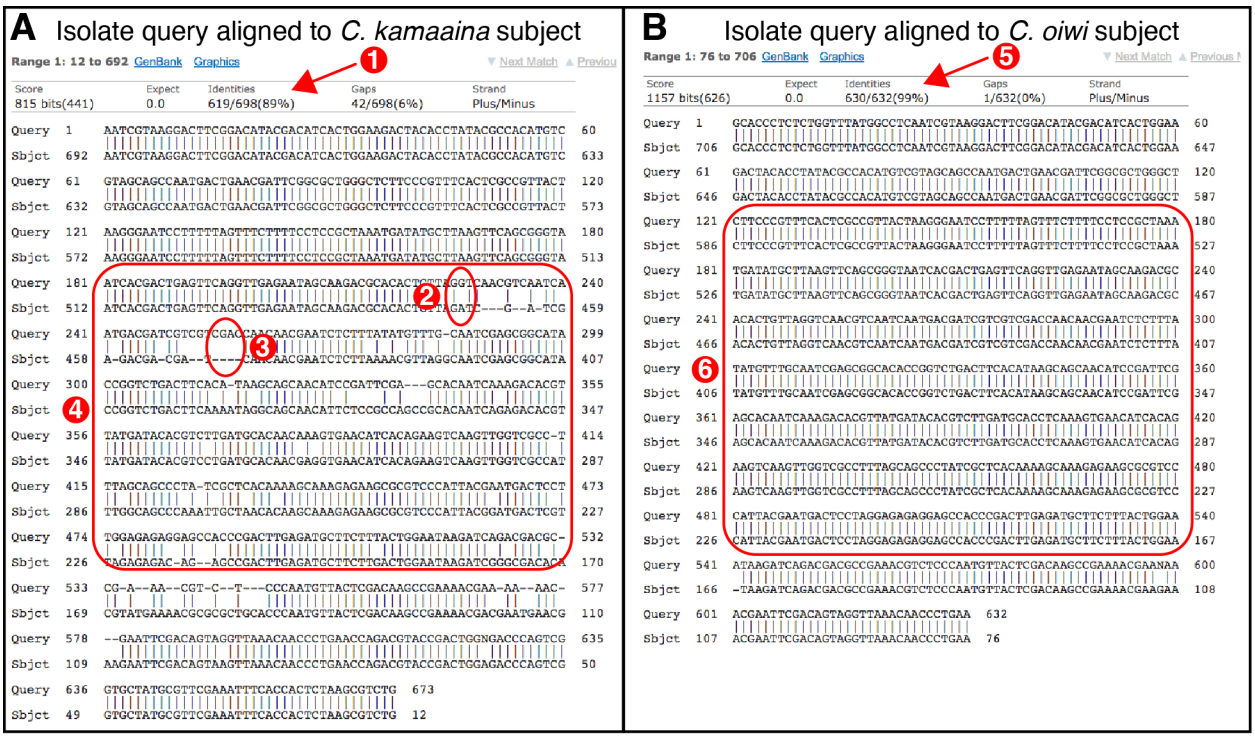

Figure 15. NCBI Blast alignment visualization examples. (**A**) An example of an isolate's ITS2 query sequence aligned to a *C. kamaaina* subject sequence. (1) The percent identity of the alignment (89%), which is low for a top blast hit. (2) A mismatch between the query and subject sequence (G to A). (3) A four base pair gap in the subject sequence made by the alignment algorithm, gaps in the query or subject indicate poor alignment. (4) A generalized region in the center of the alignment with many mismatches and gaps. A region like this suggests that the query sequence might come from a new *Caenorhabditis* species. This is an actual alignment example of a new species, *C. oiwi*, that was discovered by the Andersen lab in 2017. (**B**) An example of a good alignment between an isolate's ITS2 query sequence and a subject sequence. (5) The percent identity of the alignment (99%), which usually means the query sequence comes from an isolate of the same species as the subject. (6) A central region of the alignment with perfect identity. A region like this suggests that the query isolate is very likely the same species as the subject.

Naming Wild Strains

If an isolate is identified as *C. elegans*, *C. briggsae*, *C. tropicalis*, or a possible new *Caenorhabditis* species, the S-plate will be given a strain name and put into the cryopreservation pipeline (*Cryopreservation protocol*). Before any wild strain can be processed, it must be given a strain name and entered into Labguru. Before assigning a name, check your S-plates to ensure that you can recover the strain. Wild isolates can be sensitive and are sometimes lost at this stage.

1. Arrange your data by S-plate number.
2. Open the LaguruUpload excel template and **duplicate the sheet to make a new copy.** Name the new copy with the following format: YYYYMMDD_ProjectName_LabguruUpload.
3. Fill out the following columns on the sheet (please note - some of these fields are redundant, but **BOTH** must be filled out): *Name, Genotype, Organism, Species, Source*

Lab, *Submitted By*, and the *Notes.* The S-label goes in the *Notes* field. **Make sure you copy appropriately so that the correct species is with the correct S-plate!**

4. Email Robyn the filled-in import sheet. She will assign ECA strain names, import your sheet to Labguru, and send you back the sheet with the strain names filled in.
5. Enter the strain names from the import sheet into the genotyping google sheet of your project.

Labels for the Wild Collections Intake Process:

1. Make three sets of labels for each shipment:
    a. Make seven clear labels for each strain: 1 initial chunk plate, 1 bleach plate, 1 L1s plate, 1 freezing plate, three DNA prep plates (DYMO; label type: ol1930).
        i. The label should include the S-plate and strain name (*e.g.* S- 5555; ECA4375).
    b. Make five freezing labels for each strain (Fisher 15930A).
        i. Each label should indicate the strain name, the approximate date frozen, and your initials (*e.g.* ECA4375, 4/17/2020 CMB).
    c. Make four DNA prep tube labels (DYMO cat#9138-4000) for each strain using the DYMO printer. The DYMO printer is accessed at the imaging computer. Manually enter the strain name you want printed. *No copy and paste option is available; you must enter the strain name four times for a complete set of four stickers.*

Processing collection data with easyFulcrum in R

1. Create a repository (repo) in the Andersen Lab Github page named after the collection project name in Fulcrum.
    a. Click on the green “New” button to create a new repository.
    b. Name the repo with the naming convention <Year><Month><Location>, *e.g.*, 2021JuneHawaii.
    c. In the description, add a short note about when the sampling was conducted, who participated in collections, and where the sampling occurred.
    d. Select the “Public” option.
    e. Initialize the repo with a README file, .gitignore, and a license.
        i. Check the box “Add a README file”.
        ii. Check the box “Add .gitignore” and select the “R” .gitignore template from the dropdown menu.
        iii. Check the box “Choose a license” and select the “MIT license” from the dropdown menu.
    f. Click the “Create repository” button on the bottom of the page. This step will take you directly to the “main” branch of the new repository in your browser.
2. Clone the new repository to the desired location on your local machine.
    a. Click on the green “Code” button in the new repository page.
    b. Copy the “https” URL displayed to your clipboard.
    c. Open terminal and navigate to the directory you wish to clone the project directory to using the ‘cd’ command. `cd <your path>`
    d. Use the following command to clone the repository directory
        i. `git clone <your repository URL>`
3. Open Rstudio and Install the *easyFulcrum* package to process the collection.
    a. If necessary install the *devtools* package in R with the command `install.packages("devtools")`

    b. Install the easyFulcrum package in R with the command `devtools::install_github("AndersenLab/easyfulcrum")`
4. Create the necessary directory structure within the project repository using the *makeDirStructure()* function from the *easyFulcrum* package.
    a. In R, enter the following command `easyfulcrum::makeDirStructure(startdir = "<your local repository path>")`
5. Export the Fulcrum data for your collection project using the Fulcrum export tool. If you do not have access to the data export tool on Fulcrum ask Robyn or Erik to export the project data for you.
    a. When exporting a project select the following options:
        - Click only the desired project
        - include photos
        - include GPS data
        - field sampling
        - isolation
6. The data will be exported as a `.zip` file. After the data is exported and uncompressed, the `.csv` files must be moved to `<your local repository path>/data/raw/fulcrum`, and the field sampling photos in `.jpg` format are moved to `<your local repository path>/data/raw/fulcrum/photos`
7. Process the collection with the *easyFulcrum* package following the *easyFulcrum* vignette beginning at the "Reading, processing, and joining Fulcrum results" section.
    a. Open a new R script file in Rstudio then save it as `<your local repository path>/scripts/<your project name>_process_collection.R`
    b. Follow the *easyFulcrum* vignette to process the collection further using the `nematode` profile where necessary.
        i. Please use the `easyfulcrum::procPhotos2` function rather than `easyfulcrum::procPhotos` function shown in the vignette.
        ii. Upload the processed collection photos to the google bucket. This step will allow you to visualize substrate photos in your project report made with the `easyfulcrum::generateReport` function
            1. Follow this link to the google bucket.
                a. If you do not have access, request access from Erik.
            2. Click on the "Create Folder" button and name the folder with the project name.
            3. Click the newly created project folder and make a subfolder titled "sampling_thumbs".
            4. Click into the "sampling_thumbs" folder then add the .jpg files from `<your local repository path>/data/processed/fulcrum/photos/C_labels/thumbnails` to the google bucket by dragging them into the white space in your web browser. The .jpg files will be uploaded to `elegansvariation.org/photos/isolation/fulcrum/<your project name>/sampling_thumbs`
8. When you have processed the collection be sure to push the files to the remote repository on github.
    a. In terminal, navigate to your project directory with the command `cd <your local repository path>`
    b. Stage all changes in the repository using the command `git add .`
    c. Make a commit with a message to document the changes with the command `git commit -m "your short message here"`

d. Push the changes to the remote repository using the command `git push`