II. At-Home Chelex Ant DNA Extraction

Reagents, Supplies & Equipment (for isolating DNA from 2 samples)

- Ants
- Permanent marker
- Boiling water
- Mug for hot water
- Aluminum foil
- Optional: tweezers, razor blade or scissors
- Ethanol in screw-cap tubes (4, 750 µL)
- Chelex solution in a 1.5-mL snap-cap tube (2, 100 µL)
- 2 Microcentrifuge tube cap locks
- 2 Plastic pestles
- 6 Toothpicks
- Whatman No.1 Chromatography paper discs (2, 3-mm diameter)
- 2 Microcentrifuge tubes (1.5 mL)
- Parafilm
- Ziploc bag
- Microcentrifuge tube storage box

Procedure

Any ants you barcode will provide data on the approximately 800 species across the United States and high quality sequences will be published in GenBank with you as an author.

Be careful not to cross-contaminate specimens. Carefully label tubes before each step and use different tweezers, scissors, and surfaces or thoroughly clean implements and surfaces for each sample.

1. Collect ants or use bait to attract them (a cookie, especially a Pecan Sandy, works well). Photograph the collection location and habitat and use the provided sample collection data sheet to record information to enter in the Sample Database. Whenever possible, collect several ants (at least two; up to ten is better) tentatively from one species and place in one of the tubes of ethanol. Store the tubes in the freezer as soon as possible. One ant will be used for DNA extraction and other(s) saved for taxonomic identification or to repeat extractions when necessary. Placing ants in ethanol euthanizes them. If collected when ethanol is not available, placing in a freezer will euthanize and preserve ants until ethanol becomes available. Do not use every tube of ethanol for ant collection, as more ethanol may be needed later.
2. To extract DNA, remove an ant from the ethanol using a clean toothpick and dry for at least 10 minutes on a clean surface. Ensure you label the surface with the sample number to avoid confusion – especially if working with multiple samples. Photograph the ant and upload the image

DNA Learning Center, Cold Spring Harbor Laboratory
1 Bungtown Road, Cold Spring Harbor, NY 11724
Email: micklos@cshl.edu

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file to the Sample Database. Extra tubes of ethanol are provided in case the ethanol in the original tubes is lost while removing ants. Replace the ethanol, if needed, and store the remaining ants in the freezer.

3. For each sample, label a 1.5-mL tube containing Chelex with the sample number. Label the cap and the side of the tube. During shipment, the small amount of liquid in the Chelex tube may have collected under the cap or on the sides of tube. Gently tap the bottom of the tube on a hard surface until the liquid and Chelex is at the bottom. There should be 100 µL of Chelex mixture, about the same level as the lowest gradation mark on the side of the tube. Check the level to ensure the mixture is at the bottom.

4. For most ants (~1/4" long body or smaller), place a whole ant in the appropriately labeled 1.5-mL Chelex tube. For ants significantly larger than this, use clean tweezers, razor blade, scissors, or sterile toothpicks to obtain a ⅛- to ¼-inch piece of ant tissue, preferably a large hindleg, - about the size of a grain of rice.

5. Use a plastic pestle to grind the ant tissue against the inner surface of the Chelex tube. Forcefully grind the tissue for at least 2 minutes. Use a clean pestle for each sample. Ensure the sample is ground into fine particles. Close the cap of the 1.5-mL Chelex tube. Save the pestles.

6. Prevent the cap from opening in the following steps by using a cap lock to secure the cap to the rim of the tube. Be sure that both the tube rim and cap are held within the cap lock so that steam can't force the cap open.

7. Boil water and fill a mug (for a few tubes) or pot (for many tubes).

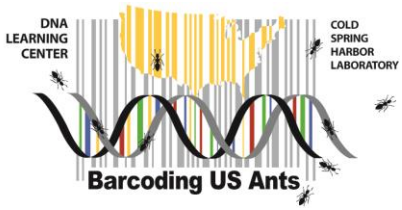
8. Cover the mug or pot with aluminum foil and punch holes to hold the tubes. Place the locked tubes through the aluminum foil so that the mixture in the bottom of the tube is below the surface of the hot water. Do not submerge the cap.

9. Let the tubes sit in the hot water for 10 minutes to break open cells and extract DNA. It is not necessary to keep the water boiling.

10. Remove the tubes from the hot water, being careful not to burn yourself. The resulting DNA extract will be used to amplify the barcode region and mailed to the DNALC. Check that the sample numbers on the tubes are still legible and relabel if necessary. Remove and save the cap locks.

11. Let the tubes sit upright for 10 minutes (or centrifuge 30 seconds in a balanced configuration, if a microcentrifuge is available) to allow the Chelex to settle on the bottom.

12. Use a fresh toothpick to add one 3-mm diameter disc of Whatman No. 1 Chromatography paper to the *clear supernatant at the top* of each DNA extraction. Avoid placing the disc in the Chelex. Allow the disc to soak in the extract for 1 hour.

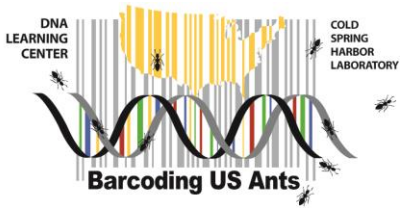


13. While the Whatman disc is soaking, label a clean 2" x 2" aluminum foil square with the sample identification number, the date, and your last name.
14. Use a clean toothpick to gently drag the disc out of the supernatant and up the tube wall. Ensure that little to no debris is attached to the disc. Use the toothpick to transfer the disc onto the aluminum and allow it to dry thoroughly (~10 minutes).
15. After the disc is completely dry, fold the aluminum foil around it to create a flat packet for mailing. Ensure the label is visible or relabel the foil.
16. If you are not setting up your own PCR reactions, continue to step 17. If you are setting up PCR reactions, let the tubes sit upright again for 10 minutes (or centrifuge 30 seconds in a balanced configuration, if a microcentrifuge is available), then transfer about half (20µL) of the clear liquid above the cloudy Chelex to an empty tube. Store these DNA tubes in the freezer (-20°C) until ready to set up PCR.
17. Use Parafilm to seal the caps of the tubes containing the remaining Chelex/DNA extract(s) and store in the freezer (-20°C) until mailing.
18. Place screw-cap tubes containing preserved ants or Parafilmed Chelex/DNA extract tubes into the microcentrifuge tube storage box for mailing. Label the box with your last name, the date, and "US Ants." **If you transferred half of the DNA to new tubes in step 16, keep those new DNA tubes for setting up PCR – you should mail the original Chelex/DNA tube.**
19. Place the foil-wrapped discs, pestles and cap locks into the Ziploc bag for mailing. Label the bag with your last name, the date, and "US Ants."

Mailing Instructions (if *not* doing amplification and gel):

Return several ants of each species for taxonomic identification. Use a separate tube of ethanol for each different ant species. Place the labeled screw-cap tubes containing ethanol and your ant specimens and the labeled Parafilmed tubes containing Chelex/tissue/DNA into the microcentrifuge tube storage box for mailing. Label the box with your last name, the date, and "US Ants." Place the foil-wrapped filter paper discs, pestles and cap locks into the Ziploc bag for mailing. Label the bag with your last name, the date, and "US Ants." Place the materials into an appropriate package and mail to:

Dave Micklos
DNA Learning Center
Cold Spring Harbor Laboratory
1 Bungtown Rd.
Cold Spring Harbor, NY 11724



III. Amplify Ant DNA by PCR

This protocol describes conditions for amplifying the *COI* barcode from ant DNA. For information on PCR primers and conditions for other organisms, see pages 19-20 of https://dnabarcoding101.org/files/using-dna-barcodes.pdf.

Reagents, Supplies & Equipment (for amplifying 2 samples)

- DNA extracted from ant(s) in part II
- Container with cracked or crushed ice
- Micropipettes and tips (2–100 µL)
- Microcentrifuge tube rack (or foil over a container)
- Permanent marker
- Thermal cycler
- 2 Ready-To-Go PCR Beads in 0.2-mL PCR tubes
- *COI* ant primer cocktail (50 µL; 23 µL per reaction)*
- PCR tube rack (empty yellow tip holder)

*Store on ice

1. Allow the tubes containing ant DNA to sit upright for 10 minutes (or centrifuge for 30 seconds) to ensure that any residual Chelex settles on the bottom of the tubes.
2. Obtain PCR tubes containing Ready-To-Go PCR Beads containing dehydrated *Taq* polymerase, nucleotides, and buffer. Label the tubes with your identification numbers.
3. Use a micropipette with a fresh tip to add 23 µL of the *COI* ant primer cocktail/loading dye mix to each bead tube. Allow the beads to dissolve for 1 minute at ambient temperature.
4. Place the PCR tubes on ice to prevent premature replication of unwanted primer dimers.
5. For each sample, use a micropipette with fresh tip to transfer 2 µL of the ant DNA (from step 1) from the top of the liquid directly into the PCR tube. Ensure that no DNA remains in the tip after pipetting. *It is important to avoid transferring any Chelex at the bottom of the ant DNA tube into the PCR tube because Chelex inhibits PCR.*
6. Keep your PCR tubes on ice until ready to begin thermal cycling.
7. Start the program listed below on the thermal cycler and pause the thermal cycler when the block reaches 95°C in the first step. Transfer your PCR tubes directly from the ice into the hot thermal cycler. Close the lid and resume the PCR program.

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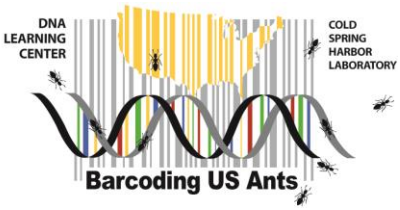

Primers	Profile
Invertebrate (ant) Invertebrate primer cocktail: (LCO1490 / HC02198 / FormCOId_F / FormCOId_R)	Initial step: 95° C 3 minutes 10 cycles of the following profile: Denaturing step: 95° C 10 seconds Annealing step: 45° C 30 seconds Extending step: 72° C 45 seconds 30 cycles of the following profile: Denaturing step: 95° C 10 seconds Annealing step: 50° C 30 seconds Extending step: 72° C 45 seconds Additional extending step: 72° C 1 minute One final step to preserve the sample: 4° C *ad infinitum*

INVERTEBRATE (ANT) PRIMER COCKTAIL

For use with ants:

Folmer et al. (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* (1994)3(5), 294-299. FormCOIdF and –R were developed at CSHL.

LCO1490	5'-**TGTAAAACGACGGCCAGT**GGTCAACAAATCATAAAGATATTGG-3'
HC02198	5'-**CAGGAAACAGCTATGAC**TAAACTTCAGGGTGACCAAAAAATCA-3'
FormCOId_F	5'-**TGTAAAACGACGGCCAGT**ATTCAACAAATCAYAAAGAYATYGG-3'
FormCOId_R	5'-**CAGGAAACAGCTATGAC**TAAACTTCIGGRTGWCCAAARAATCA-3'

If you are making your own primer cocktail rather than using mix provided by CSHL, mix the Invertebrate (Ant) primer cocktail in a 1.5-mL tube (FOR READY-TO-GO PCR BEADS)

- 640 µL of distilled water
- 460 µL of Cresol Red Loading Dye (see recipes above)
- 15 µL of 15 pmol/µL LCO1490 5' primer
- 15 µL of 15 pmol/µL HC02198 3' primer
- 5 µL of 15 pmol/µL FormCOId_F 5' primer
- 5 µL of 15 pmol/µL FormCOId_R 3' primer

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IV. Analyze PCR Products by Gel Electrophoresis

Reagents, Supplies & Equipment

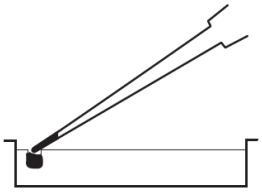
- PCR products from part III*
- Container with cracked or crushed ice
- Micropipettes and tips (1–100 µL)
- Microcentrifuge tube rack (or foil over a container)
- Gel-casting tray and comb
- Masking tape
- Gel electrophoresis chamber and power supply
- Microwave
- UV or LED transilluminator and eye protection
- Digital camera or photodocumentary system
- Permanent marker
- Latex gloves
- 2% agarose in 1X TBE (~50 mL per gel)
- 1X TBE buffer (~300 mL per gel)
- Empty 1.5-mL microcentrifuge tube (10)
- SYBR Green DNA stain (25 µL)
- 100-bp ladder (10 µL per gel)*
- PCR tube rack (empty yellow tip holder)
- Petri dish

*Store on ice

1. Seal the ends of the gel-casting tray with masking tape, or other method appropriate for the gel electrophoresis chamber used and insert a well-forming comb.
2. Melt the 2% agarose by repeatedly microwaving until just bubbling and then swirl. When completely melted, pour the 2% agarose solution into the tray to a depth that covers about one-third the height of the comb teeth.
3. Allow the agarose gel to completely solidify; this takes approximately 20 minutes.
4. Remove the seal from the ends of the tray and place the gel into the electrophoresis chamber. Add just enough 1× TBE buffer to cover the surface of the gel.
5. Carefully remove the comb and add additional 1× TBE buffer to fill in the wells and just cover the gel, creating a smooth buffer surface.
6. Use a micropipette with a fresh tip to transfer 5 µL of each PCR product (from part III) to fresh, labelled 1.5-mL microcentrifuge tubes. Store the remaining PCR reactions on ice in the PCR tubes.
7. *Do not add SYBR Green directly to the small PCR tubes containing the PCR product from part III; SYBR Green interferes with the sequencing reaction.* Add 2 µL of SYBR Green DNA stain to each

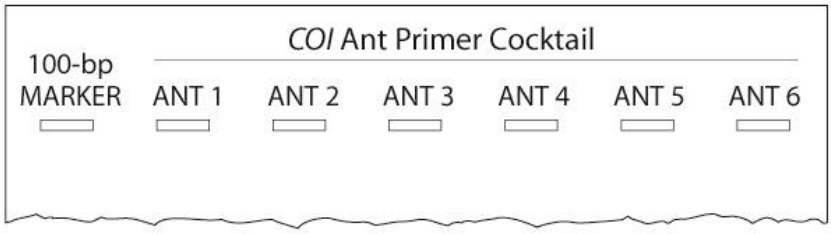
larger*1.5 mL tube* containing 5 µL of PCR product. Also add 2 µL of SYBR Green DNA stain to 10 µL of 100-bp marker in a separate tube.

8. Orient the gel according to the diagram below, so the wells are along the top of the gel. Use a micropipette with a fresh tip to load 5 µL of 100-bp marker into the far left well.



Expel any air from the tip before loading, making sure not to lose any liquid, and be careful not to push the tip of the pipette through the bottom of the sample well.

9. Use a micropipette with a fresh tip to load each sample from Step 6 in your assigned wells, similar to the following diagram:



The samples you load may not be exactly the same as those shown.

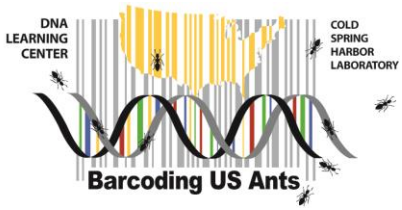
10. Store the remaining 20 µL of your PCR product on ice or at -20° C until ready to submit your samples for sequencing.

11. Run the gel for approximately 30 minutes at 130V. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.

12. View the gel using UV or LED transillumination. Photograph the gel using a digital camera or photodocumentary system.

Mailing Instructions (for amplicons, DNA and ants):

Return several ants of each species for taxonomic identification. Use a separate tube of ethanol for each different ant species. Place the labeled screw-cap tubes containing ethanol and your ant specimens and the labeled Parafilmed tubes containing Chelex/tissue/DNA into the microcentrifuge tube storage box for mailing. Label the box with your last name, the date, and "US Ants." Place the foil-wrapped filter paper discs, pestles and cap locks into the Ziploc bag for mailing. Label the bag with your last name, the date, and "US Ants." Ensure that your PCR tubes are clearly labeled with the sample identification numbers. Tape the PCR tubes inside of the petri dish and tape the dish closed. Label the dish with your last name, the date, and "US Ants." Place the materials into an appropriate package and mail to:



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