# Green Crab diet DNA Master Lab Protocol

Compiled by Mary Fisher for Fisher et al. (in review). Changes to company-provided kit protocols are primarily based on European green crab DNA metabarcoding work shared by Georgina Cordone and Bettina Thalinger (Cordone et al., 2022), and eDNA protocols developed by University of Washington Kelly Lab members and shared by Eily Allen, Megan Shaffer, Erin D’Agnese, and Maya Garbor-Yonts. Specific attributions are detailed at the beginning of each protocol.

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## Preventing Contamination

The laboratory work for Fisher et al. was completed in (a) an ecology wet lab (for crab dissection), and (b) four separate spaces within the University of Washington’s Center for Environmental Genomics lab: (1) a DNA extraction fume hood; (2) a DNA-free fume hood to prepare PCR master mixes and indexes; (3) a pre-PCR bench to work with DNA extracts; and (4) a post-PCR bench to work with PCR product. Cleaning protocols were derived from practices used by the UW Kelly Lab.

Before starting any protocol after crab dissection:

* Wipe down the bench space with 10% bleach and 70% ethanol, unless otherwise noted in the protocol
* Wipe down pipettes with 70% ethanol. Sterilize pipettes with UV light.
* Wipe down the other non-electronic surfaces of shared lab equipment with 70% etOH - particularly the interior of extraction centrifuges. For pre-PCR equipment, UV surfaces that come into direct contact with, or cover, samples.
* Pre-PCR plasticware should be autoclaved and UV’d prior to use.
* I usually wipe down the outside of any kit buffers with 10% bleach / 70% etOH and UV the container. I also bleach and put aside a permanent marker for pre-PCR use.

When working at a pre-PCR bench or under the pre-PCR fume hood:

* Change or bleach gloves every time you need to touch something that has not been UV-sterilized and/or is not under the fume hood. Essentially, treat everywhere except your immediate workspace as a “post-PCR” zone, even in the pre-PCR workroom. You may want to wipe down a permanent marker with 10% bleach and UV it, if you will need to take notes during a pre-PCR protocol.

## Crab Dissection

### Summary

Dissection technique based on the method used by Georgina Cordone (Cordone et al., 2022), with some modifications. Purpose is to obtain green crab gut contents with minimal green crab DNA contamination.

### Materials

* Two plastic lunch trays
* Dissection kit - scalpel, tweezers, scissors, etc.
* Extra scalpel blades
* 1000uL pipette + filter tips
* 1.5-2mL centrifuge tubes with screw caps (or something else to store gut contents)
* Plastic petri dish if weighing stomachs
* Plastic tube wrack
* Alcohol burner (or other flame source)
* Gloves, kim wipes
* 100% etOH
* DI H2O
* Bleach

### Protocol

#### Preparation:

The following can be completed at any point before dissections:

1. Sterilize 1.5mL or 2mL microcentrifuge tubes (and 10x10 tube box):
   1. Tubes autoclaved on vacuum dry cycle – if using an autoclave, make sure that the tubes are autoclavable! Some non-brand Falcon tubes may lose their shape
   2. Tubes UV’d under a fume hood for 15 minutes (caps off)
   3. Fill each tube with at least 40uL 100% ethyl alcohol. *Set aside one tube as a tare, for the stomach fullness protocol.*
   4. *Fill one tube with 400uL 100% ethyl alcohol as a tare for the stomach fullness protocol*.
2. Sterilize 15mL or 50mL centrifuge tubes with UV light, under fume hood for 15 minutes. Fill tube with 100% ethyl alcohol and label “100% etOH” with initials and date (flushing ethanol)

The following should be completed the day of dissections:

1. If working with frozen (-20C or lower) crab, move the crab (1) into the refrigerator 5-7 hours before dissection, or (2) onto the bench top 45 minutes - 1 hour before dissection
2. Write crab IDs on a printed [Dissection Data Sheet](https://docs.google.com/document/d/1xdaeuV1FXEQ3JFPeEPcun0vY8sOLbfHa/edit?usp=sharing&ouid=100545905198498902708&rtpof=true&sd=true)
3. If not already prepared, fill lab wash bottles with:
   1. bleach (20%) + DI H2O,
   2. ethanol (70%) + DI H2O
   3. ethanol (95-100%)
4. Label the top and sides of the microcentrifuge tubes with the ID numbers of the crabs that will be dissected. Include the date on the side of the tube. You may want to put aside a few extra, unlabeled tubes in case you take a back-up sample (see step 14)
5. Cut parafilm for the planned number of tubes that will be used.

#### Dissection:

1. Wipe down the lab bench and two plastic dissection trays with 50% bleach and 70% ethanol from the lab wash bottles. Clean off the pipette with 50% bleach and 70% ethanol (\*this was an old pipette about to be retired. Do not bleach pipettes intended for continued lab use\*)
2. Fill three beakers: one with 20% bleach / 50% DI H2O, one with DI H2O, and one with 100% etOH
3. Light bunsen burner or ethanol burner
4. Sterilize dissection tools: Put a clean pair of scissors, a pointer, and two pairs of forceps into the 50% bleach beaker. Take the sanitized scissors and scalpel out of the bleach; swish them first in DI water, then ethanol, burning off excess ethanol. Place the sanitized tools on a new kim wipe on the dissection tray
5. Get the crab from the refrigerator and place it on the first dissection tray
6. Put on a fresh pair of gloves
7. Loosen or open the cap of the microcentrifuge tube
8. Loosen the cap of the 100% etOH-filled centrifuge tube
9. Cut open the crab carapace with scissors, starting at the attachment of the right hind leg and continuing just above the top half of the carapace (approximate location marked in red); be particularly careful cutting the carapace at the front of the crab, where you leave the gill chamber and enter the main body of the crab with internal organs. The cardiac stomach is directly behind the eyes. Then gently remove the thin, colorful layer attached to the bottom of the carapace by scraping it with a pointer, so that the top of the carapace comes off of the crab and the internal anatomy is visible.

A crab with a red outline

Description automatically generated

1. Put a fresh glove on the hand you will pipette with. Do not touch the crab or anything else on the bench after you put on this new glove!
2. Put a new filter pipette tip on the pipette and extract gut contents through the crab’s mouth
   1. Press the pipette plunger down
   2. Put the pipette through the mouth of the crab, between the bottom edge of the mandibles, and navigate to the stomach
   3. Let the pipette plunger slowly release
3. Eject gut contents into the microcentrifuge tube.
4. If first aspiration is unsuccessful, or you are collecting back-up samples: Put a new filter pipette tip on the pipette, and draw up 400uL of 100% ethanol from the etOH-filled centrifuge tube.
5. Inject ethanol into the stomach of the crab; if you have not punctured the wall, you should be able to see the stomach inflate. Then let the plunger slowly release, extracting gut contents mixed with ethanol. Place gut contents into a new microcentrifuge tube; label the microcentrifuge tube with the crab ID and “etOH,” so that you know this gut sample has ethanol in it.
6. Remove the entire cardiac stomach of the crab. Start by gripping the esophagus just behind the eyes with the forceps, to close but not break it. Gently cut away the front of the stomach from the crab using scissors. As the stomach begins to come away from the rest of the organs, use the scalpel to sever the back of the stomach where the two gill chambers are closest to each other
7. Place the newly separated stomach onto a clean petri dish and set aside for later.
8. Make relevant notes on the [Dissection Data Sheet](https://docs.google.com/document/d/1xdaeuV1FXEQ3JFPeEPcun0vY8sOLbfHa/edit?usp=sharing&ouid=100545905198498902708&rtpof=true&sd=true)
9. Rinse the dissection tray in the sink; clean the tray with 20% bleach and 70% ethanol from lab wash bottles.
10. Repeat #**4-19** for each crab. I usually do 5-6 crab at one time, and then move on to the gut fullness protocol.
11. At the end of the day, parafilm all microcentrifuge tubes and transfer to -20C freezer.

## Stomach Fullness Dissection

### Summary

Extension of the crab dissection protocol, for rough wet and dry weights of stomach contents. Purpose is to have some internal data to corroborate the semi-qualitative 0-7 scale of stomach fullness.

### Materials

* Plastic lunch tray
* Dissection kit - scalpel, tweezers, scissors, etc.
* Plastic petri dish (1 for tare)
* Scale
* Extra scalpel blades
* Clean tin weigh boats
* 95-100% etOH
* Gloves, kim wipes

### Protocol

#### Preparation

1. With a permanent marker, label each of the clean tin weigh boats with the crab IDs

#### Weighing (Day 1)

1. Prior to Step 21 of the Crab Dissection Protocol, weigh the tubes full of stomach contents.
   1. Tare the scale with the appropriate tube (1.5mL tube with only 40uL or 400uL of 100% ethyl alcohol, set aside during preparation for the crab dissections)
   2. Record the weight of the 1.5mL tube on the Dissection Data Sheet
2. Tare the scale with a clean plastic petri dish.
3. Weigh each of the full stomachs set aside during the Crab Dissection Protocol, and record the weights on the Dissection Data Sheet.
4. For each stomach:
   1. Record the weight of the appropriately labeled clean tin weigh boat on the Dissection Data Sheet
   2. Cut open the stomach and flush the contents into the weigh boat with 95-100% ethyl alcohol.
   3. Set aside the now empty stomach for 5-7 minutes to let the ethyl alcohol evaporate (time should be consistent across all stomachs)
   4. Place the weigh boat with stomach contents under the fume hood to dry overnight (or, at least 12 hours).
   5. Tare the scale with the clean petri dish. Record the weight of the empty stomach on the Dissection Data Sheet.

#### Weighing (Day 4)

1. Record the weight of each tin boat with dry stomach contents on the Dissection Data Sheet.

## DNA Extraction

*Note -* I used the QIAGEN DNeasy© Blood & Tissue Kit (individual “mini” tubes, 50 samples) by default, but some DNA extracts did not amplify even after a second extraction on the extra subsample. When this was the case, and when I had enough stomach sample left, I re-extracted from the same sample using the QIAGEN DNeasy© PowerSoil© Pro Kit.

Before following either protocol below, please read the appropriate handbook in full: Qiagen’s DNeasy® Blood & Tissue Handbook, or DNeasy® PowerSoil© Pro Handbook.

### Blood & Tissue Kit

#### Summary

Qiagen’s Purification of Total DNA from Animal Tissues (Spin-Column Protocol) from the Handbook, with adjustments from the WSU Goldberg Lab (2015), Bettina Thalinger (Cordone et al. 2022), and myself. Purpose is to extract DNA from subsamples of digesta.

#### Protocol

#### Preparation for Day 1

1. Autoclave more 1.5mL / LoBind tubes
2. Clean out the extraction lab bench, and clean and UV all necessary equipment and pipette tips.
3. Re-sterilize (with UV) any aliquots of Buffer ATL (but \*not\* pro-k), along with new 1.5-mL LoBinds
   1. (\_\_\_\_samples + 1 negative control) x 1 = \_\_\_\_\_\_\_\_1.5-mL LoBinds
4. Clean out and fill an ice bucket
5. At the pre-PCR bench, wipe down each 1.5mL tube with digesta with 70% EtOH before moving to a UV-d tube rack. Have one UV-d rack ready for moving the samples around the lab, and another for working with the samples under the hood (do not remove the second rack from the hood)
6. Turn on the shaker-incubator to 56C

#### Day 1 (Part 1)

1. Before working with the digesta samples, aliquot Buffer ATL and Proteinase-K
   1. 20uL x (\_\_\_\_\_\_\_\_\_ samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_uL ProK
   2. 180uL x (\_\_\_\_\_\_\_\_\_\_samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_\_uL Buffer ATL
2. For samples with solid digesta above the 200uL mark on the tube: Gently vortex the digesta to resuspend any solids, and then subsample into new 1.5mL tubes using the 1000uL pipette.
3. Centrifuge the digesta samples / subsamples at 1,000 × g for 10 min to aggregate the solid material
4. Once solid material is aggregated at the bottom of the tube, siphon off the supernatant (stomach liquid / etOH) using a combination of the 1000uL and 20uL pipette. The supernatant can be returned to the original sample tube (for those subsampled) or saved in a new tube (for those not subsampled).
5. All samples should have 100% etOH added at time of dissection. Place samples into the speed vac, with one negative control. You can add an equivalent amount of 100% etOH to the negative control to keep track of evaporation (20-40uL)
6. Turn on speed vac to Low, No Heat. Start a timer
   1. *I usually do 10 min in the speed vac at a time, then check samples*
7. Once samples appear to be dry, record the time they are taken out of the speed vac on the [extraction worksheet](https://docs.google.com/spreadsheets/d/1kchoy4tLL2LIXpUHkmwGVUyn4EYI_Tks/edit?usp=drive_link&ouid=100545905198498902708&rtpof=true&sd=true)
8. Weigh the dried samples on a tared scale and record the weight of the dried subsample on the extraction worksheet

#### Day 1 (Part 2)

1. Add 180uL ATL and 20uL ProK to each tube (**Qiagen, Steps 1 & 2**). If needed, use the pipette tip to dislodge the dried digesta pellet from the bottom of the tube.
   1. If a sample has a large amount of dried digesta, I add an additional 5-10uL ProK and note this in the extraction spreadsheet.
2. Tightly seal each tube.
3. Mix each tube by vortexing, then briefly spin down.
4. **(Qiagen, Step 2**) Incubate spin columns at medium speed in rocking incubator at 56C overnight. If samples have a lot of solid material, may want to incubate on platform for closer to 14-15 hours
5. You can do some of the prep steps for Day 2 before leaving for the evening.

#### Preparation for Day 2

1. If using a new kit, add 100% etOH to Buffer AW1 and Buffer AW2 concentrates according to labels on buffers
2. UV tubes for the day
   1. (\_\_\_\_samples + 1 negative control) x 2 = \_\_\_\_\_\_\_\_1.5-mL LoBinds \*\*Label these tubes with sample IDs. One set will be if samples have remaining digesta, and one set will be for elution
   2. (\_\_\_\_ samples + 1 negative control) x 3 wash steps = \_\_\_\_\_\_\_\_ collection tubes
   3. 50mL falcon tubes for 100% etOH, AW1, AW2 aliquots
   4. 1.5mL LoBind tubes for Buffer AE and Buffer AL
3. Aliquot 100% etOH and buffer
   1. 200uL etOH x **(** \_\_\_ samples **+** 1 negative control**) = \_\_\_\_\_\_**uL x 1.2 = \_\_\_\_\_\_uL 100% etOH
   2. 200uL AE/AL x **(** \_\_\_\_\_ samples **+** 1 negative control**) =\_\_\_\_\_\_**uL x 1.2 = \_\_\_\_\_\_uL AE/AL
   3. 500uL AW1/AW2 x **(** \_\_\_\_\_ samples **+** 1 negative control**) =\_\_\_\_\_\_\_\_\_**uL x 1.2 = \_\_\_\_\_\_\_\_uL AW1/AW2
4. Create receptacle for Qiagen kit waste.
5. Turn on the small incubator / heat block to 70C
6. But Buffer AE in the small incubator / heat block. If Buffer AL has precipitate, put it in the small incubator / heat block as well. (See Qiagen protocol p. 33)

#### Day 2

1. Remove the samples from the incubator, spin them down, and allow them to return to room temperature.
2. For samples with extra solid material after incubating overnight: centrifuge the sample at 1,000 × g for 10 min to aggregate the solid material. Remove as much of the supernatant as possible and add it to a new 1.5-mL LoBind tube. You can add 100 µL Buffer ATL to further dilute it if necessary (Brandl et al. 2019, DOI: 10.1080/00028487.2015.1131745)
   1. Record how much extra material was not digested in the [extraction worksheet.](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20Extraction%20Worksheet.xlsx) These can be saved at -20C and re-extracted with additional ProK if needed.
3. (**Qiagen, Step 3**) Add 100uL Buffer AL. Mix thoroughly by vortexing each sample immediately.
   1. Incubate at 70C for 10 minutes
4. (**Qiagen, Step 3**) Add 200uL etOH (96-100%). Mix thoroughly by vortexing each sample immediately.
5. (**Qiagen, Step 4**) Pipet mixture into DNeasy Mini spin column, in a 2mL collection tube.
   1. Centrifuge at ≥6000 x *g* (8000rpm) for 1 min.
   2. Discard the flow-through and collection tube.
6. (**Qiagen, Step 5**) Place the spin column in a new 2 mL collection tube. Add 500 µl Buffer AW1.
   1. Centrifuge at ≥6000 x g (8000rpm) for 1 min.
   2. Discard the flow-through and collection tube.
7. (**Qiagen, Step 6**) Place the spin column in a new 2 mL collection tube. Add 500 µl Buffer AW2
   1. Centrifuge at 11,000rpm for 3 min.
   2. Centrifuge for another 3 min at 11,000 rpm
   3. Discard the flow-through and collection tube.
8. (**Qiagen, Step 7**) Transfer the spin column to a new, \*labelled\* 1.5-2 mL Lo-Bind microcentrifuge tube.
9. (**Qiagen, Steps 7-8**) Elute the DNA in two steps
   1. Add 75uL pre-heated Buffer AE to the center of the spin column membrane.
   2. Incubate for 5 min at room temperature (15–25°C).
   3. Centrifuge for 1 min at ≥6000 x g (8000rpm).
   4. Add 75 µl Buffer AE to the center of the spin column membrane.
   5. Incubate for 1 min at room temperature (15–25°C).
   6. Centrifuge for 1 min at ≥6000 x g (8,000 rpm)
10. Store the extracted DNA at -20C.
    1. I usually put the spin columns into a new collection tube and store them at 2-8C until you’ve had a chance to amplify and visualize a subset of the samples, to make sure the extraction worked.

### PowerSoil© Pro Kit

#### Summary

QIAGEN DNeasy© PowerSoil© Pro Kit Quick-Start Protocol with some additional preparation at the start of the protocol. Purpose is to extract DNA from subsamples of digesta that were not successful with the Blood & Tissue Kit.

#### Protocol

#### Preparation

1. Autoclave more 1.5mL / LoBind tubes
2. Clean out the extraction lab bench, and clean and UV all necessary equipment and pipette tips.
3. Re-sterilize (with UV) any aliquots of buffers, along with new 1.5-mL LoBinds
   1. (\_\_\_\_samples + 1 negative control) x 1 = \_\_\_\_\_\_\_\_1.5-mL LoBinds
4. Clean out and fill an ice bucket
5. At the pre-PCR bench, wipe down each 1.5mL tube with digesta with 70% EtOH before moving to a UV-d tube rack. Have one UV-d rack ready for moving the samples around the lab, and another for working with the samples under the hood (do not remove the second rack from the hood)
6. UV tubes for the day
   1. (\_\_\_\_samples + 1 negative control) x 2 = \_\_\_\_\_\_\_\_1.5-mL LoBinds \*\*Label these tubes with sample IDs. One set will be if samples have remaining digesta, and one set will be for elution
   2. (\_\_\_\_ samples + 1 negative control) x 3 wash steps = \_\_\_\_\_\_\_\_ collection tubes
   3. 50mL falcon tubes for solution aliquots
   4. 1.5mL LoBind tubes for Solution C6
7. Create a receptacle for kit waste.
8. If Solution CD6 has precipitated, put it on the small incubator / heat block at 60C (Qiagen Quick-Start, p. 1)

#### Part 1

1. Before working with the digesta samples, aliquot buffers
   1. 800uL x (\_\_\_\_\_\_\_\_\_ samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_uL CD1
   2. 200uL x (\_\_\_\_\_\_\_\_\_\_samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_\_uL CD2
   3. 600uL x (\_\_\_\_\_\_\_\_\_\_samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_\_uL CD3
   4. 500uL x (\_\_\_\_\_\_\_\_\_\_samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_\_uL EA, C5
   5. 100uL x (\_\_\_\_\_\_\_\_\_\_samples + 1) = \_\_\_\_\_\_uL x 1.1 = \_\_\_\_\_\_\_uL C6
2. For samples with solid digesta above the 200uL mark on the tube: Gently vortex the digesta to resuspend any solids, and then subsample into new 1.5mL tubes using the 1000uL pipette (See Qiagen Quick-Start, p. 1)
3. Centrifuge the digesta samples / subsamples at 1,000 × g for 10 min to aggregate the solid material
4. Once solid material is aggregated at the bottom of the tube, siphon off the supernatant (stomach liquid / etOH) using a combination of the 1000uL and 20uL pipette. The supernatant can be returned to the original sample tube (for those subsampled), saved in a new tube (for those not subsampled), or thrown away.
5. All samples should have 100% etOH added at time of dissection. Place samples into the speed vac, with one negative control. You can add an equivalent amount of 100% etOH to the negative control to keep track of evaporation (20-40uL)
6. Turn on speed vac to Low, No Heat. Start a timer
   1. I usually do 10 min in the speed vac at a time, then check samples
7. Once samples appear to be dry, record the time they are taken out of the speed vac on the [extraction worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20Extraction%20Worksheet.xlsx)
8. Weigh the dried samples on a tared scale and record the weight of the dried subsample on the extraction worksheet

#### Part 2

1. (**Qiagen, Step 1**) Spin down the PowerBead Pro Tubes briefly so that all of the beads have settled at the bottom.
2. (**Qiagen, Step 1**) Add 600-800μl of Solution CD1 to the 2mL tubes containing the samples. Record how much CD1 was added in the [extraction worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20Extraction%20Worksheet.xlsx)
3. (**Qiagen, Step 1**) Transfer the digesta+CD1 to a PowerBead Pro Tube. Vortex briefly to mix.
   1. Incubate at 65C for 10 minutes (if cells are difficult to lyse; Qiagen DNeasy PowerSoil Pro Kit Handbook, www.qiagen.com/HB-2495)
4. Complete **Qiagen QuickStart Protocol Steps 2 – 17**. For Step 16, Add 75uL of Solution C6.
5. Store the extracted DNA at -20C.

I usually put the spin columns into a new collection tube and store them at 2-8C until you’ve had a chance to amplify and visualize a subset of the samples, to make sure the extraction worked.

## PCR 1: BF3/BR2 primers

Before following the protocol below, please read the appropriate handbook in full: QIAGEN® Multiplex PCR Plus Handbook.

#### Summary

Based on the QIAGEN® Multiplex PCR *Plus* Kit for the 2x Multiplex PCR Master Mix, with changes according to Bettina Thalinger (Cordone et al., 2022). Purpose is to conduct the first PCR with BF3/BR2 primers to amplify part of the COI Folmer fragment.

#### Protocol

##### ***Preparation***

1. Create the layout for the PCR plate with sample IDs and the controls, using the [PCR Layout Worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20PCR%20Layout%20Worksheet.xlsx). Include at least one negative and one positive control.
2. Calculate the amount of each PCR reagent required with the [PCR Protocol Spreadsheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20PCR%20protocols.xlsx)
3. Check the thermocycler program against the protocol in the spreadsheet linked in 2
4. Wipe down the pre-PCR fume hood and pre-PCR lab bench with bleach (20%) and etOH (70%). Clean and UV all necessary equipment and pipette tips, including:
   1. 2 x 1.5mL tube for Rxn Mix, PCR water
   2. PCR water,
   3. 96-well plastic PCR plate for thermocycler
   4. PCR strip tube caps (2 x \_\_\_\_ strips + 1 = \_\_\_\_)
5. While waiting for UV sterilization, fill two chill buckets (one for PCR reagents, and one for samples) and make sure that the thermocycler is programmed appropriately (reference: [PCR Protocols Spreadsheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20PCR%20protocols.xlsx)). Take Multiplex PCR Plus Master Mix (MM), 10uM aliquot of primers, and sample working aliquots / controls out of the freezer to thaw on the appropriate bench.

##### ***PCR Reaction***

*Under the pre-PCR UV Hood:*

1. Aliquot the total amount of PCR water required for the protocol into a microcentrifuge tube. Then add the appropriate amount of PCR water to the PCR plate for each negative control.
2. Vortex gently and spin down primers in the pre-PCR UV hood. Invert and spin the MM to mix.
3. Create the Reaction Mix in a 1.5mL tube according to the [PCR Protocol Spreadsheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20PCR%20protocols.xlsx). Invert to mix, and spin down when complete.
4. Add the appropriate amount of Reaction Mix to each PCR tube or well. Mix by pipetting up and down 5-10 times before transferring for the first well in each column.
5. Cover the PCR plate with strip caps.
6. Bring the PCR plate with Reaction Mix to the pre-PCR bench.

*At the Pre-PCR bench:*

*If working with >6 samples, complete steps 9-12 for six samples at a time. Keep the remaining samples in a rack on ice.*

1. Vortex gently and spin down the thawed sample aliquots / controls
2. Add the appropriate amount of sample DNA to each PCR tube or well.
3. Mix by pipetting up and down 5-10 times before and after transferring.
4. Tightly seal the PCR plate with strip caps. Vortex gently / invert to mix. Spin it down.
5. Place the PCR plate into the thermocycler and start the program.
6. Return the sample aliquots to the fridge for now.

##### ***Visualizing PCR Product***

1. Run every PCR sample on a 1.5% agarose gel to visualize the PCR product. I aliquoted 3uL of each PCR sample and 1uL of the Loading Dye separately, so that I didn’t have to bring the PCR plate into the gel room.
   1. Combine 1uL Loading Dye and 3uL DNA sample

and 3uL DNA ladder

* 1. Run the gel at … 100V for 30 min (1%)

100V for 40 min (1.5%)

1. Return the sample aliquots that successfully amplified / controls to the -20C freezer.
2. Put the PCR plate in the refrigerator (if conducting the bead clean-up within the next few days) or the freezer.

## 

## Bead Clean-up 1

*Note* - I usually do 40-50 samples at a time, so that each 96-well plate is split into two consecutive (but separate) bead cleans. I’ve found that with a 20uL PCR product volume, the beads dry out too fast to do a full 96-well plate in a single clean. I used 0.85x for the first clean because I had trouble maintaining the post-clean DNA concentration.

#### Summary

Protocol based on the UW Kelly Lab protocol, the Agencourt AMPure XP Quick Reference for 96 Well Format, and the University of Oregon’s Evaluation of NGS Beads for DNA Size Selection. Purpose is to use AMPure Beads to clean the PCR 1 product.

#### Protocol

##### ***Preparation***

1. Prepare the bead / elution plate layout, and calculate the volume of beads / elution buffer needed, using the [Green Crab PCR Layout Worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20PCR%20Layout%20Worksheet.xlsx)
2. Remove the AMPure beads from the fridge **at least 1hr before Step 4** and get to room temperature. Always keep the beads at the pre-PCR bench
3. Wipe down the pre-PCR fume hood, pre-PCR lab bench, and post-PCR lab bench with bleach (20%) and etOH (70%). Clean and UV all necessary equipment and pipette tips, including:
   1. A strip tube and 2mL microcentrifuge tube, for beads and elution buffer
   2. Two 96-well plates and one set of strip tube caps per PCR plate layout. Optional: a third 96-well plate to set aside bead waste; if something goes wrong in the bead clean, DNA may be recovered from the bead waste.
4. Make fresh stock of 70% ethanol (1.5 times as much as is needed) at the pre-PCR bench

2 x 200uL 70% etOH x \_\_\_\_\_\_\_ samples = \_\_\_\_\_\_\_\_\_\_\_total uL 70% etOH

\_\_\_\_\_\_\_\_mL 70% etOH = \_\_\_\_\_\_\_\_mL 100% etOH + \_\_\_\_\_\_\_\_mL PCR water

##### ***Bead Clean-up***

*Pre-PCR Bench*

1. Aliquot Qiagen Buffer EB for elution at the pre-PCR bench, into a UV’d strip tube.

30uL x \_\_\_\_\_\_\_samples x 1.2 = \_\_\_\_\_\_\_\_\_uL Buffer EB

1. Gently shake the AMPure XP bottle to resuspend any magnetic particles that may have settled. Pipette into 2 mL tube.

\_\_\_\_\_\_\_uL PCR product x 0.85 = \_\_\_\_\_\_\_\_uL beads

\_\_\_\_\_\_\_uL beads x \_\_\_\_\_\_\_ samples = \_\_\_\_\_\_\_\_\_\_\_total uL beads

*Bead Clean-up: Post-PCR Bench*

1. Transfer 20uL PCR product into a new 96-well plate. *This ensures that there is exactly 20uL product for each sample going into the bead clean-up*. *If there is <20uL product, record the exact amount and calculate a new bead volume.*
2. Add 0.85x beads (see table) to samples using a single channel pipette. Vortex 2 mL tube of beads every 3 wells.
3. **AMPureXP Step 3:** After all beads are added, use multichannel to pipette up and down 20x to mix beads. Let sit on bench at room temperature for **7 minutes**.
4. **AMPureXP Step 4:** Place samples onto magnet for **3 minutes (or until clear)** to separate the beads from the solution. The solution should be clear before proceeding to the next step.
5. **AMPureXP Step 5:** Aspirate the cleared solution from the reaction plate (using single channel pipette set to slightly higher volume than total volume of product and beads - go SLOW) and put the discards into (1) a second 96-well plate, or (2) the second half of the bead plate. This step should be performed while the purification plate is situated on the magnet. Do not touch the magnetic beads, which have formed a spot on the side of the well.
6. **AMPureXP Step 6:** Add **160 µL** of 70% ethanol wash solution (made fresh) using multichannel to each well of the reaction plate and incubate for **30 seconds** at room temperature.
7. Aspirate the ethanol out (with multichannel set to 180uL) and discard.
8. Repeat Steps 8 & 9 for a total of two washes. Perform these steps with the reaction plate situated on the magnet. Do not disturb the magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
   1. For the second aspiration, remove any extra ethanol with a single channel pipette (set to 10uL). *If you choose to double check that all ethanol has been removed (single channel pipette set to 4uL), only air-dry the beads for 1 minute in Step 11.*
9. Leave the plate to air-dry for **1-2 minutes** on a bench top to allow residual ethanol to evaporate completely. Check beads every ~30sec to make sure no cracking is occurring, which can cause loss of DNA.
10. Remove the plate from the magnet.
11. **AMPureXP Step 7:** Add 30 uL Buffer EB to each well (using multichannel). Pipette up and down 20x to mix *after buffer has been added to all wells*. Let sit at room temperature for **7 min** on bench.
12. **AMPureXP Step 8:** Put on magnet and let sit for **4-5 min** or until liquid is clear.
13. **AMPureXP Step 8:** Remove 28 uL total elute and put in a new 96-well PCR plate. Cover the plate with close-fitting strip tube caps.
14. Store the plate at 2-8C for a few days; for long-term storage, keep samples at 20C. Optional: cover the bead plate with plastic film and store at 2-8C until after the cleaned PCR product has been quantified.

Bead Concentration Table.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Marker** | **PCR1 bead ratio** | **PCR1 volume product** | **PCR1 volume beads** | **PCR2 bead ratio** | **PCR2 volume product** | **PCR2 volume beads** |
| **BF3/BR2**  Opt 1 | 0.85 | 20 | 17 | 0.75 | 20 | 15 |
| **BF3/BR2**  Opt 2 | 0.8 | 20 | 16 | 0.8 | 20 | 16 |

##### 

##### ***Quantify Cleaned PCR Product***

1. Use the Quant-iT™ dsDNA High-Sensitivity Assay Kit and Quick Reference protocol to quantify the cleaned PCR product.

**Step 5:** Add 2 μL of each unknown DNA sample to separate wells. Using a multi-channel 200uL pipette, carefully mix each well by pipetting up and down 10x. It is important not to introduce bubbles or allow the mixture to splash while mixing.

## 

## PCR 2: Indexing

Before following the protocol below, please read the appropriate handbook in full: QIAGEN® Multiplex PCR Plus Handbook.

#### Summary

Protocols from Emily Curd and Zack Gold (CALeDNA “Methods for Researchers”), updated for Nextera adapters by Eily Allen & Erin D’Agnese. Purpose is to attach the Nextera indices to the cleaned PCR 1 amplicon. Either 10ng or 11.25uL of cleaned PCR 1 product is needed.

#### Protocol

##### ***Preparation***

1. Use the [Green Crab PCR 2 Calculations Worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20PCR%202%20Calculation%20Spreadsheet.xlsx) to (tab-1) determine the volume of amplicon / PCR water that will be added for each PCR 1 sample, and (tab-2) assign indices to each PCR 1 sample.
2. Create the layout for the PCR plate with sample IDs and the controls, using the [PCR Layout Worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20PCR%20Layout%20Worksheet.xlsx). I use separate pieces of paper for the 5x, 8x, 12x PCR samples / plates, and then cut the page in half so that the layouts that record the volume of PCR water / indices can be brought to the pre-PCR benches.
3. Calculate the amount of each PCR reagent required with the second tab in the [PCR 2 Protocol Spreadsheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20dDNA%20PCR%20protocols.xlsx)
4. Wipe down the pre-PCR fume hood, and post-PCR lab bench with bleach (20%) and etOH (70%). Clean and UV all necessary equipment and pipette tips, including:
   1. Two 2mL microcentrifuge tube for the Ready Mix and PCR water aliquots
   2. 96-well plates and strip caps
   3. Mini tube racks and new strip tube caps for the indexes
5. Check the thermocycler parameters against the indexing protocol in the protocol spreadsheet.
6. Remove the PCR1 product from the freezer and set on bench in the post-PCR room to thaw.

***Pre-PCR fume hood***

1. Add the appropriate volume of PCR water to each well in the 96-well plate or strip tubes.
2. Bring the plate / strip tubes to the post-PCR room.
3. Take the aliquot of the Kapa HiFi Hot Start Ready Mix, and the primer plate, out of the freezer to thaw. You can leave the primer plate on ice instead of the benchtop if you have a lot of samples to transfer in step 5.

***Post-PCR Bench***

1. Gently vortex the cleaned PCR1 product, and spin down.
2. Add the appropriate amount of PCR1 product for each sample into the 96-well plate or strip tube. Pipette up and down 5-10x while transferring; if transferring volumes of < 3uL, pipette up and down 15x while transferring.
3. Cap the plate or strip tubes, spin down, and put them aside on the benchtop.

***Pre-PCR Fume Hood***

1. Gently mix the Ready Mix by inverting it several times, and spin it down.
2. Aliquot the total Ready Mix into a sterilized 1.5mL microcentrifuge tube.
3. Add the Ready Mix to the sterilized PCR plate that will be used in the thermocycler. Pipette up and down 5-10x at the start of each new column.
4. Spin down the strip tubes with the indices in them.
5. Add each index to the appropriate strip tube well. Once all of the wells in a PCR plate column have received indices, put on the lids immediately (or close lids as you go, if attached to strip tube). Replace the strip tube caps on the indices with new, sterilized caps.
6. Put the Ready Mix and indices back in the freezer; or, if setting up more reactions, put on ice on the benchtop.
7. Bring the capped strip tubes with the Ready Mix + Indices to the post-PCR room. If using strip tubes with separate caps, bring the extra sterilized caps.

***Post-PCR Bench***

1. Using a multi-channel set to 11.5uL, add the PCR1 product + water to the PCR plate with the Ready Mix + Indices. Take off the caps of one PCR plate column at a time, and replace it immediately.
2. Gently mix and spin down the strip tubes. Put in the thermocycler.

##### ***Visualizing PCR Product***

1. Run every indexed PCR 2 sample on a 1.5% agarose gel to ensure that the indices attached. I aliquoted 3uL of each PCR sample and 1uL of the Loading Dye separately, so that I didn’t have to bring the PCR plate into the gel room.
   1. Combine 1uL Loading Dye and 3uL DNA sample

and 3uL DNA ladder

* 1. Run the gel at … 100V for 30 min (1%)

100V for 40 min (1.5%)

1. Return the sample aliquots that successfully amplified / controls to the -20C freezer.
2. Put the PCR plate in the refrigerator (if conducting the bead clean-up within the next few days) or the freezer.

## 

## Bead Clean-up 2

Repeat **Bead Clean-up 1** using 20uL of PCR 2 product with 0.75x beads (15uL beads per PCR sample). There is a separate tab on the [Green Crab PCR Layout Worksheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/Green%20Crab%20PCR%20Layout%20Worksheet.xlsx) to create layouts / calculate reagents for the second bead clean-up.

## 

## Pooling

Use the [Pooling Calculations Spreadsheet](https://github.com/mfisher5/Green-crab-dDNA/blob/main/doc/lab_templates/DNA%20Metabarcoding%20Pooling%20Spreadsheet.xlsx) to determine the volume of each sample to pool. Pool samples into a UV-sterilized 1.5mL microcentrifuge tube.