Environmental DNA (eDNA) Protocol (syringe technique)

The eDNA filtration protocol we followed was one created by the previous year's detection and monitoring of mussel species using environmental DNA group (by Taylor Shade, and Kelsey Ozment; class of 2016) which we revised after researching further and in order to maximize DNA yield. Here is the slightly revised and expanded version below.

Note: The syringe technique is ideal for water samples collected in sterilized media bottles and transported back to the lab for filtration.

Materials:

1) 25mm Swin Lock filter housing (from Whatman, LOT #7011782)
2) Ahlstrom Glass microfiber filters, 25mm (sterilized) (LOT #101804)
3) Sterilized collection bottles
4) Sterilized forceps
5) 30mL syringe with Swin Lock compatible screw
6) 1.5 mL sterilized centrifuge tubes
7) 1000mL Beaker

Visuals

From left to right (sterilized collection bottle used, Ahlstrom Glass microfiber box, Ahlstrom Glass microfiber filters)

Syringe set-up

Visual representation of step four in the following procedure

After conducting further research, we found that filtering on site was recommended in order to prevent contamination and maximize DNA yield. This is optional, but highly recommended as eDNA tends to yield smaller values. It is also recommended to collect at least 500 mL of water from your water body of interest as the more water filtrated the higher your chances are of getting a higher DNA yield.

Procedure

Filtration and Lysing

1. Before collecting water samples be sure to autoclave at least one forcep, the collection bottles for water samples, the filtration housing , and 1.5 mL centrifuge tubes. (syringes used were disposable and thus did not need to be autoclaved) Cycle fast vent 2 was used.
2. After all materials are gathered, dip your sterilized bottles into the body of water you are interested in and collect at least 500 mL. Be sure to cap the bottles closed in order to prevent contamination.
3. Follow the Swin Lock filter housing instructions for correct placement of filter inside the housing utilizing sterile forceps. Set this aside.
4. Remove cap from bottle and using a sterile 30 mL syringe , collect 30 mL of water. Attach the filter housing with the glass microfiber filter inside to the syringe. Hold the syringe (with the attached filter housing) over a 1000 mL beaker
5. Push the sample water through the syringe with the attached filter housing (filtration tool) and allow the water to drip into the beaker ensuring that there is no leakage out of the sides or syringe end of the filtration tool. If water flow is not fast enough gently push the plunger to increase the flow rate.
6. Once the syringe has emptied, remove the top part of the syringe and collect 30 mL of sample water into the syringe. Place the top part slightly back in and repeat step 5.
7. Complete these steps until at least 500 mL has been pushed through one filter. Be sure to record the amount of water filtrated.
8. Once finished filtrating the desired amount of water, remove the filter using sterilized forceps and place inside a 1.5 mL centrifuge tube that contains a mixture of 180 µL of ATL lysis buffer and 20 µL of Proteinase K (these two items can be found in the DNeasy Blood and Tissue Kit). Store the microcentrifuge containing the filter on the heating block at 55°C for 48 hours. This starts the lysing process which breaks cell membranes and exposes the DNA.

***Quick Notes: There is some leeway with the amount of time spent lysing the DNA in the heating block as we have left our DNA on the block for a day or two after the recommended 48 hours and still garnered results. Also we talked with a member of the Microbiology Department at GSEF, who told us that it is possible to lyse less than 48 hours; however, he did not recommend the minimum amount of time.**

DNA Purification

Materials

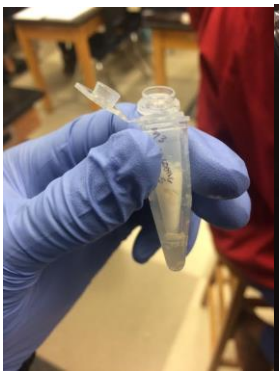
1) Sterile 0.5mL tube with hole at bottom

*Quick Note: the previous year's group drilled 1/16 inch wide holes at the bottom of the tube(s) utilized; however we found that using scissors to make the small incisions worked just as efficiently.

2) Sterilized forceps
3) Centrifuge
4) Vortex
5) DNeasy Blood and Tissue Kit

Visuals

From left to right (visual representation of step 1 final set-up, and centrifuge)




Procedure

1. In order to remove the DNA from the lysing centrifuge tube, remove the filter from the tube using sterilized forceps and push it up against the wall of the tube to remove any remaining fluid. Then place the filter into the sterile 0.5mL tube with a hole at the bottom, and place this tube into the 1.5mL centrifuge lysing tube holding the remaining fluid mentioned previously.
2. Place the 1.5mL tube mentioned previously in the centrifuge and spin for 30 seconds at 10,000rpm. This will allow any remaining liquid not squeezed out from the filter in the 0.5mL tube to spin down into the 1.5mL tube. This remaining liquid contains the DNA and will be used in the extraction process. You can now discard the 0.5mL tube with the filter (BE SURE YOU DO NOT THROW AWAY THE 1.5mL TUBE CONTAINING THE LIQUID).

***Quick Notes: After step 2 if you do not wish to continue with the procedure you can incubate your DNA overnight at 56°C. Be warned that once you begin step 3 you cannot stop until the full DNA purification procedure is done. The following procedure is copied from the Purification of Total DNA from Animal Tissues (Spin-Column Protocol), which is designed for purification of total DNA from animal tissues, including rodent tails. This is inserted for your convenience. For more detail on the purpose behind each step and what to do if certain complications arise, I advise that you refer to the original copy. The**

majority of materials for the rest of this procedure will be located in the DNeasy Blood and Tissue Kit.

3. Vortex your 1.5mL tube containing the DNA fluid for 15 seconds. Add 200µl Buffer AL to the sample, and mix thoroughly by vortexing. Usually the Buffer AL we use is pre-mixed and added together with ethanol to save time when using multiple samples. If ethanol has not already been added the then add 200µl ethanol (96-100%) and mix again thoroughly by vortexing.
4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2mL collection tube (provided). Centrifuge at 8,000rpm for 1 min. Discard flow-through (the liquid) and collection tube.
5. Place the DNeasy Mini spin column in a new 2mL collection tube (provided), add 500µl Buffer AW1, and centrifuge for 1 min at 8,000rpm. Discard flow-through and collection tube.
6. Place the DNeasy Mini spin column in a new 2mL collection tube (provided), add Buffer AW2, and centrifuge for 3 min at 14,000rpm to dry the DNeasy membrane. Discard flow-through and collection tube.

***Quick Note: In our particular case our centrifuge only went up to 13,000rpm, so we utilized that setting, thus if you find yourself in a similar situation use the highest rpm setting you have. Also for the following step (7) there is an option to use only 100µl of Buffer AE to increase the final DNA concentration in the eluate. This however also decreases the overall DNA yield.**

7. Place the DNeasy Mini spin column in a clean 1.5mL or 2mL microcentrifuge tube (not provided in the kit), and pipet 200µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 8,000 rpm to elute.

***Quick Note: If you decide to follow the original Protocol, it is advised that those using eDNA skip step 8 and simply end at step seven.**

Quantification Using Qubit Fluorimeter

***Quick Note: Once you get the hang of doing the full eDNA protocol or simply feel confident in your methods thus far you can skip the following step and save it for later to make the overall process quicker. It is recommended to do this step; however, to verify that you do in fact have DNA in your sample(s). Also be sure to use the High Sensitivity kit and not the Broad Assay Kit when dealing with eDNA.**

Materials

1) All materials will be found in the Qubit High Sensitivity Assay Kit
2) Qubit Fluorimeter

Visual

(This diagram pulled from the protocol was extremely helpful in the quantification process. Be sure to ignore the written numbers in black marker as this is scratch work. Essentially "n" represents the number of samples you have and you simply multiply that number in microliters times the number the diagram provides to get your amounts of each product. Below are the typed equations of the diagrams in case you find the diagram hard to read.)

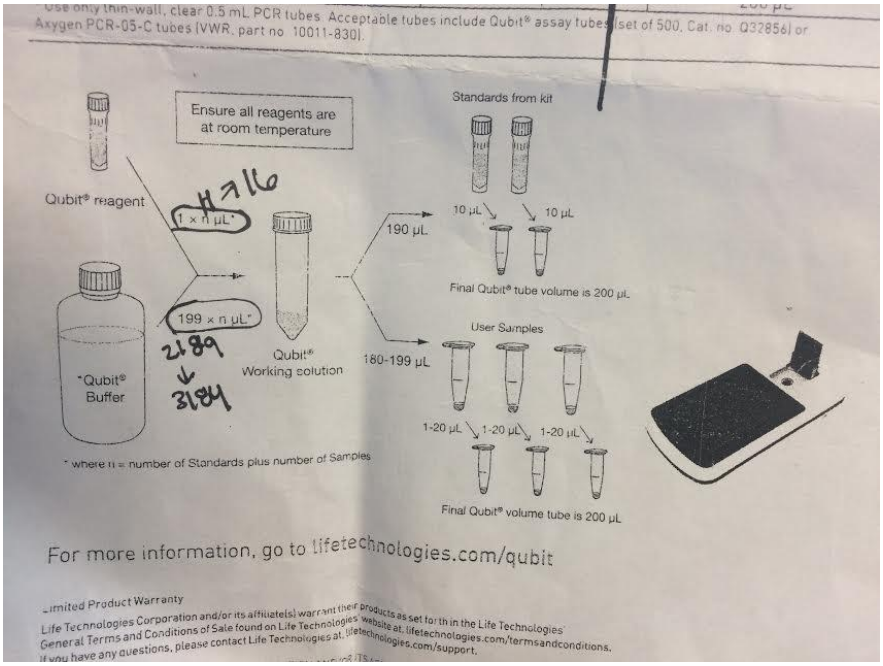


Diagram Explanation

1. Qubit reagent 1 x n µL
2. Qubit buffer 199 x n µL
3. The Qubit reagent and Qubit buffer are both pipetted into one sterile microcentrifuge tube, which is called the Qubit working solution
4. 180-199 µL of the working solution is added in combination with 1-20µL of DNA from your designated DNA sample into EACH of the final DNA sample tubes.
5. 190 µL of the working solution is added in combination with 10 µL of the designated standard into each of the final Standard tubes