Protocol for DNA extraction from gauze filters

Such as the Metaprobe 2.0

Developed based on Maiello et al. Little samplers, big fleet: eDNA metabarcoding from commercial trawlers enhances ocean monitoring 2022. *Fisheries Research*. DOI: 10.1016/j.fishres.2022.106259

And as used in Kressler, Whitelegg et al. In Review. *Environmental DNA*.

The following protocol is designed for use with Metaprobe 2.0 passive eDNA samplers. The sampling unit of a metaprobe is one-10cm^2 piece of gauze rolled around one-1g piece of sterile cotton roving. Typically, there are three gauze rolls per metaprobe, which is why this protocol assumes volumes divisible by 3, with additional volume to account for 1 extraction blank.

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Before you start

- Wipe down the surfaces, oven, centrifuge, pipettes, door handles, everything, with 70% ethanol followed by 10% bleach, followed by 70% ethanol (hereafter, the "EBE" clean).
- Soak or thoroughly wipe down the centrifuge rotors in 10% bleach solution for no longer than 2 h (the rotor may corode if left too long), triple rinse, and dry.
- Ensure you have sufficient gloves, towels, tips, waste containers, tubes etc.
- Read the important notes on DNA extraction below
- Collect all consumables

Important notes on DNA extraction:

- It is important to do the ethanol-bleach-ethanol "EBE" clean because several of the extraction buffers contain chaotropic salts that react with bleach to form highly toxic compounds (basically mustard gas!). If formed this compound will appear yellow on the paper towels and may smell pleasant and garlicky.
- ALWAYS use filter tips.
- ALWAYS number every tube. ALWAYS replace and pick up tubes carefully as to not splatter liquid.
- Make aliquots of all your buffers and reagents because you could easily spill or contaminate them.
- For every extraction day in the lab, also do an extraction blank with just extraction reagents; otherwise treat it the same as the others.
- Full DNeasy kit instructions can be found at https://www.qiagen.com/us/resources/download.aspx?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en
- Photograph your lab book at the end of every day as a backup.
- Check the temp of the oven using the thermometer inside, or one that passes through the top of the oven (depending on the model), as the digital/external thermostat is inaccurate.

Consumables

- QIAGEN DNeasy Blood & Tissue Kit, mini spin columns
- Additional volumes of the following QIAGEN products
  - ATL buffer (4 x 20mL)
  - AL Buffer
  - Proteinase K
- Thermo Fischer Qubit kit
- Thermo Fischer Qubit tubes with caps
- 30 mL centrifuge/falcon tubes with conical base and caps
- 1.5 mL microcentrifuge/eppendorf *sterile* tubes
- 50mL conical base sterile tubes with caps

- 1000µL , 10L, and 20-200µL pipette filter tips
- Petri dishes, sterile
- Domestic chlorine bleach, e.g. Domestos in the UK, pre-mixed in a 10% solution with tap water
- Absolute ethanol (99.8%), both an aliquot and premixed in a spray bottle to a 70% solution with tap water
- Paper towels
- Disposable gloves
- Printable labels (cryo-babies)

Equipment

- 2 spray bottles for ethanol and bleach solutions
- Metal scissors, at least 3 pairs
- Metal long-nose tweezers, at least 3 pairs
- Pipettes: 1000µL, 1-100µL and 20-200µL
- Racks for microcentrifuge tubes and conical base tubes (recommend 1 per)
- Oven capable of reaching and holding 50º overnight
- Desktop centrifuge with rotor for 1.5mL eppendorfs
- -20°C freezer for long-term storage
- Desktop vortex

Extraction Steps

Day 1

1. Put gloves on. EBE clean the work surface, pipettes, and other equipment. Place samples to the side. Turn oven to 56ºC.
2. In a small plastic box make a 10% bleach bath and submerge the scissors and tweezers for at least 5 minutes. Then, remove the scissors and tweezers and place them on a clean sheet of paper towel to drip dry.
   NOTE: You will have to repeat this throughout the protocol depending on how many scissors/tweezers you have relative to samples, as each sample requires a clean pair of scissors and tweezers.
3. Prepare the extraction buffer master mix using extraction (ATL) buffer and proteinase K (20µg/µL). In a 30mL falcon tube with a conical base, vortex together 540 µL of extraction ATL buffer and 60 µL of proteinase K per sample. Vortex immediately and place in a tube rack. See Table 1. From the master mix, pipette 600µL of extraction buffer master mix into one sterile 30mL falcon tube, repeat for the total number of samples you have. Replace the cap on the master mix and the falcon tube.

   Table 1. Extraction buffer volumes based on sample size, including repeat-pipette draw

Number of Samples	ATL Buffer	Pipette volume (iterations)	Proteinase K	Pipette volume (iterations)	Total Volume
1	540µL	540µL (1)	60µL	60µL (1)	600µL
6	3,402µL	567µL (6)	378µL	378µL (1)	3,780µL
15	8,505µL	850µL (10)	945µL	315µL (3)	9,450µL
30	17,010 µL	945µL (18)	1,890µL	378µL (5)	18,900µL

   Volumes beyond 1 sample include 5% extra, for pipette error. Blue columns are the volumes divided into pipette-able volumes where the number in parentheses indicates the number of times you'd pipette that partial volume.

4. For each extraction day you must make an extraction blank. To make the blank, vortex 315µL of ATL buffer and 35µL of proteinase-K into a 1.5mL microcentrifuge tube. Label the microcentrifuge tube and place it in the oven to incubate overnight with the samples, ensuring it's upright. Treat the extraction blank as one replicate throughout the Day 2 extraction process.

5. To prepare to process gauze samples, lay out one sterile and open petri-dish per sample. Next to each dish place one set of scissors and tweezers. Each gauze will need a clean fresh set, so after processing one gauze fully, replace the scissors and tweezers into the 10% bleach bath you prepared in Step 1.
   Using fine tip tweezers remove the rolls of gauze from the tubes of ethanol and place them in a petri dish - *allow the gauze to drip momentarily back into the tube prior to fully removing the gauze*.
   Why? The gauze should be as dry as possible before submergence in the extraction buffer. This is too limit the total volume you extract on Day 2: more volume in the initial extraction phase significantly increases the volume of reagents AND the iterative passing of extraction elute through mini-spin column later in the protocol. So throughout Day 1 take your time and allow the cotton to drip and air dry.

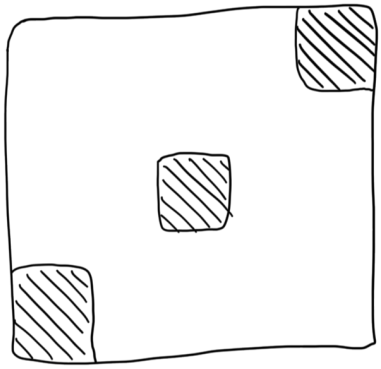

Figure 1. Identification of subsampling locations along 10cm^2 gauze, where hashed lines indicate a cut subsample.

6. Holding the gauze in place in the dish with the scissors, use the tweezers to pull the zip tie off the gauze roll. Dispose of the zip tie. Using the scissors and tweezers unroll the gauze within the petri dish, remove and dispose of the cotton. Repeat for all samples, cleaning scissors and tweezers as necessary.
   NOTE: At NO POINT should your hands, even gloved, touch the gauze. If they do, remove and replace your gloves. Allow the samples to sit in the petri dishes for 5-8 minutes, to allow some of the ethanol to evaporate.
7. Subsample the gauze rolls. Using each gauzes' scissors and tweezers, cut three small pieces (~ 2x2 cm) from each roll of gauze in a Petri dish, sampling the bottom and top corners and the centre of the gauze. See Figure 1. Using the tweezers, place all three cuts into the same falcon tube with extraction buffer and proteinase K. Label the tube according to the sample number with both a cryo-label on the side of the tube and the sample number written on the cap/lid. Spray scissors and tweezers with 70% ethanol solution away from samples, and replace into the bleach bath. Repeat until all samples are processed.
8. Incubate the cut samples in the falcon tubes with caps on overnight in an oven at 56ºC.

Day 2

PART 1: CAPTURING DNA IN THE SPIN COLUMN

9. Retrieve equipment: desktop vortex, paper towels, 70% ethanol and 10% bleach solutions, pipettes, pipette tips, microcentrifuge tubes, a 50mL conical tube, and gloves. Put gloves on. Wipe down surfaces following the EBE method. Place the bottle of AL buffer into the oven.
10. In a microcentrifuge tube rack, prepare four columns of 1.5mL tubes with caps, with one tube per sample per column plus one tube for the extraction blank *(I.E. If you have 8 samples you will have four columns of 8 tubes, for a total of 32 tubes)*. Label each tube cap with the sample number(s).
11. Remove samples and extraction blank from the oven.
12. Using a 1000µL filter tip and pipette, pipette the liquid from the falcon tubes into corresponding microcentrifuge 1.5mL tubes - use the first column of tubes you prepared, making sure to put the corresponding sample into it's 1.5mL tube. Close the caps and repeat for each sample and the blank.
    TIP: With the pipette draw engaged, push the gauze down with the pipette tip and then pull back slightly before drawing up liquid, this will help squeeze the liquid out of the gauze.
    NOTE: You should be able to collect between 500-700µL of supernatant depending on how wet the gauzes were when they went into the falcon tubes the night before. If you collect less that's fine.
13. Using the desktop centrifuge, centrifuge samples for 1.5 min at 13,000 g to collect debris. Transfer supernatant *(I.E. the clear liquid spun up by the centrifuge)* to the corresponding 1.5 mL microcentrifuge tube in the next column of your rack. Take care to not disturb the pellet *(I.E. the sediment type material at the bottom of the tube)*. Discard tube with debris. Close the caps and repeat for each sample and the blank.
    TIP: If in doubt whether you can pipette off the supernatant without disturbing the pellet, leave some supernatant behind. Ultimately we have to balance purity, efficiency, and precision.
14. Centrifuge samples again, but for 3 min at 14,800 g.

15. Transfer the remaining supernatant into the next new 1.5 mL microcentrifuge tube in your rack, taking care not to disturb the pellet and discard the tube with debris. Then, from the 1.5 mL micro centrifuge tube transfer 350 µL of supernatant into the next new 1.5 mL microcentrifuge tube. So you should have two columns of microcentrifuge tubes, with one column containing 350 µL of supernatant, and the other row containing the remainder. *NOTE: If the remaining supernatant is more than 350µL, that is fine however do NOT conduct a third draw of supernatant.*
16. Retrieve the AL buffer from the oven and procure an aliquot of ethanol from the main supply *(you can do this in a 50mL tube or a separate glass flask with a lid).* In a 50 mL centrifuge tube prepare a master mix of AL buffer and ethanol. You will need 350 µL of each for every sample, plus 10% extra. See Table 2 for workings per 1.5mL tube.
    NOTE: For metaprobe samples, you will typically have two-1.5mL microcentrifuge tubes with 350µL (or close to) supernatant. Therefore Table 2 will assume two-1.5mL tubes per ONE gauze sample, plus 1 extraction blank.

Table 2. Master mix volumes based on sample size assuming two-1.5mL replicate samples per metaprobe, including repeat-pipette draw volumes.

Number of Samples	AL Buffer	Pipette volume (iterations)	Ethanol (absolute, 99.8%)	Pipette volume (iterations)	Total Volume
1	350µL	350µL (1)	350µL	350µL (1)	700µL
6 +1	4,777.5µL	597µL (8)	4,777.5µL	597µL (8)	9,555µL
15 +1	11,392.5µL	712µL (16)	11,392.5µL	712µL (16)	22,785µL
30 +1	22,417.5µL	934µL (24)	22,417.5µL	934µL (24)	44,835µL

Volumes beyond 1 sample include 5% extra, for pipette error. Blue columns are the volumes divided into pipette-able volumes where the number in parentheses indicates the number of times you'd pipette that partial volume.

17. Into each tube of supernatant (with approx. 350µL), add 700 µL of Table 2 mastermix to your supernatant. Vortex each samples IMMEDIATELY (important). You will know you have mixed sufficiently when the liquid in the tube looks clear and smooth, and not bumpy or textured.
18. From the QIAGEN kit, remove one collection tube per sample and one spin column from the DNeasy kit for each sample, placing the spin column into the collection tube. Label the caps with the appropriate sample numbers.
    *NOTE: **NOT one per 1.5mL tube but ONE per sample**, I.E. if you had four samples and one extraction blank prepare 5 spin columns, even if you have nine-1.5mL tubes from Step 17)*
19. Add 525 µL from each 1.5mL tube of sample plus master mix from step 17 to the DNeasy spin column. Take care to pipette the contents gently to prevent salt contamination around the rim.
    TIP: Hold the pipette perfectly vertical, lower the tip just past the opening of the spin column and release the liquid one drop at a time. Then close the spin column cap carefully as to avoid jolting the liquid back up the sides of the column.
20. Close the caps of the spin column carefully to prevent splatter. Centrifuge for 1.5 min at 13,000 g.
21. Replace the tubes to the rack. Remove the column from the collection tube, holding it between fingers making sure not to touch the sides only the cap, and carefully pipette out the liquid from the collection tube with the 1000µL pipette, and discard this liquid by ejecting the full pipette tip. *Do not dispose of the collection tube.*
22. *Read this step entirely before proceeding.* You will need to repeat Steps 19-21 for each sampling replicate until all the supernatant + master mix from Step 17 has passed through the spin column in the centrifuge. After the final volume of sample is spun through the spin column, disregard step 21, discard the collection tube and contents, and place the spin column in a new collection tube.
    *NOTE: You **have to do this 525µL at a time, no more,** because that is the maximum volume of the spin column. You may pipette less than 525µL into the spin column if the volume from Step 17 that remains is less than 525µL.*

PART 2: WASHING COLLECTED DNA

Check: *You again have one tube per sample, plus one for the extraction blank. And you have the spin-columns in a clean collection tube. All samples are labelled on the caps/lids. Your gloves are clean and have no obviously liquid on them.*

23. To each sample/spin column, add 600 µL buffer AW1 to the spin column.
    TIP: Again, hold the pipette perfectly vertical, lower the tip just past the opening of the spin column and release the liquid one drop at a time. Then close the spin column cap carefully as to avoid jolting the liquid back up the sides of the column.
24. Centrifuge for 1.5 min at 13,000 g. Replace the spin columns and tubes to the rack, and use a pipette to remove contents of the collection tube and dispose of the pipette tip. Carefully replace the spin column, not letting it touch the sides or your gloves. *Apply the same technique as in Step 21.*
25. To each sample/spin column, add 600 µL buffer AW2 to the spin column and centrifuge for 1.5 min at 13,000 g. *Be careful to apply the same pipetting 'Tip' as in Step 23.*
26. Centrifuge for 1.5 min at 13,000 g. Replace the spin columns and tubes to the rack, and use a pipette to remove contents of the collection tube and dispose of the pipette tip. Carefully replace the spin column, not letting it touch the sides or your gloves. *Apply the same technique as in Step 21.*
27. Centrifuge the spin column and empty collection tube again, this time for 3 min at 14,800 g.
28. Place the AE elution buffer in the oven.

PART 3: RELEASING COLLECTED DNA

Prep: *In your microcentrifuge rack, prepare 1.5mL microcentrifuge tubes, one per sample plus the extraction blank, and cut the caps off each tube. Label each tube. Prepare a second column of 1.5mL microcentrifuge tubes, leaving the caps intact and closing them securely. Label each tube on the lid and with a cryo-laser label on the side of the tube - these are your final sample tubes so take care in writing the labels and on the caps. Use a permanent fine-tip marker.*

29. Transfer the spin column to the cap-less 1.5 mL microcentrifuge tubes.
30. Open the spin column cap for 2 min to allow ethanol to evaporate.
31. Retrieve the AE elution buffer. To each spin column, add 105µL of the pre-warmed AE elution buffer.
    TIP: Again, hold the pipette perfectly vertical, lower the tip just past the opening of the spin column and release the liquid one drop at a time. Then close the spin column cap carefully as to avoid jolting the liquid back up the sides of the column.
32. Close caps and incubate in the tube rack in the oven at 56ºC for 5 mins.
33. Remove samples from the oven. Turn the oven OFF.
34. Centrifuge the spin columns at 13,000 g for 1.5 mins to release the DNA.
35. Pipette the elute *(I.E. the liquid that has passed through the spin column and has collected in the bottom of the microcentrifuge tube)* back into the spin column and repeat step 34.
36. Pipette elute into final microcentrifuge tube. Close the caps securely, check labels are present and agree, and place into an opaque storage box in a -20ºC freezer.

Nanodrop/Qubit for concentration and purity

1. Nanodrop/Qubit 1µL and record the double stranded DNA (dsDNA) concentration (ng/µL) as well as the 260/280 and 260/230 ratios. Follow the protocols provided in Qubit kits.
2. Place samples in a dark sample box, and place in a -20°C freezer. Label the box with a piece of tape and write the project, date and your name, etc.
3. Clean the lab again following the EBE method.