MBA DToL: DNA barcoding of macroalgae and microalgae, marine fungi and lichens V.2

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We use this protocol and it's working

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ABSTRACT

Version 1.5

Purpose: This SOP outlines the method used to DNA barcode macroalgae and marine protists (microalgae), fungi and lichens for the DToL project. The DNA barcodes will be generated for all
macroalgae and marine protists, fungi and lichens being collected by or cultivated at the MBA (as a Genome Acquisition Lab – GAL) and sent to Sanger for sequencing. Barcodes will act as markers to track the final genomes back to original samples, and to verify morphological identifications.

Summary
As part of the Darwin Tree of Life
(DToL) initiative, the MBA is tasked with the DNA barcoding of all macroalgal species and targeted marine protists (microalgae), fungi and lichens. DNA barcoding
complements the whole genome sequencing (WGS) undertaken at Sanger in two ways:
1. Sample tracking
2. To affirm morphological species identifications provided by experts

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1 Sequence generation

Note: the method has evolved during the project, and has therefore varied between samples. Initially, DNA was extracted from samples prior to PCR. Later, tissue-direct, Phire PCR was used to improve throughput and potentially lessen the impacts of inhibiters in samples. Both methods generate reliable sequence data, so the method chosen for a particular sample will not affect barcoding data. Both methods are detailed below.

1.1 Materials required

Here follows lists of equipment and consumables required for each stage of DNA barcoding. Standard laboratory equipment and consumables are omitted, unless particular items specified.

DNA extraction (if required)

NucleoSpin Plant II DNA extraction kit (Macherey Nagel) (macroalgae and lichens)
DNeasy Plant kit (Qiagen) (fungi)
DNeasy Plant kit (Qiagen) (protists)
2mm Zirconium beads (macroalgae)
Glass beads 100 µm – 2 mm
Bead beater (MP Bio)
3mm Tungsten carbide beads (macroalgae)
Dry ice
Liquid nitrogen (lichens and macroalgae)
DNase-free water (FisherScientific)
Nanodrop
Qubit HS 1x dsDNA kit
Plastics: 1.5ml microcentrifuge tubes, tips
Pestle and mortar (Lichens)

PCR (from extracted DNA)

Primers – see section 1.4 below for details of specific primer sets required per group.
Make up 100µM stock solution. Working aliquots are 1:10 dilutions to 10µM.
Molecular-grade water
GoTaq Polymerase kit (ProMega)
5x Green Buffer (GoTaq kit)
MgCl2 (GoTaq kit)
dNTP mix (10µM) – house stock
Bovine Serum Albumin (BSA) (20mg mL^{-1}) – aliquots stored in -20 freezer. Prepare by dissolving in molecular-grade water at room temperature

PCR (direct from tissue: used without prior DNA extraction)
Plastics: 0.2 ml and 1.5 ml microcentrifuge tubes, tips,
sterile scalpel blades and sterile cutting surfaces e.g. petri dishes, sterile
syringe needles.
Primers – see Section 1.4 below for details of specific primer
sets required per group. Make up 100 µM stock solution. Working aliquots are 1:10 dilutions to 10 µM.
Molecular-grade water
Phire Plant Direct PCR kit (Thermofisher F130WH)

PCR product visualisation, clean-up and quantification
DNA gel stain – GelRed or Ethidium bromide (depending on availability)
TAE buffer - 1%
Agarose
Hyperladder (Bioline: 50 bp and 1 kb)
GenElute PCR clean-up kit
Nanodrop
Qubit HS 1x dsDNA kit

1.2 Sample starting material and handling

Details of the collection, cultivation (where applicable) and vouchering of macroalgal, protist (microalgal), fungal and lichen samples for DToL WGS and DNA barcoding are described in taxon-specific SOPs.
Before commencing DNA extraction
and barcoding processes, samples should be prepared as follows (if DNA extraction required):

1. Macroalgae:
Fill bead-beating tube with approximately half a PCR tube of 2mm Zirconium beads and cool on cool rack or dry ice. Retrieve samples from -80°C freezer, keeping on dry ice. Carefully transfer the biomass (typically lentil-sized tissue) from the Fluid X tubes by tipping and brushing it into the bead-beating tube using a sterilised needle or pipette tip. Keep tubes cooled on dry ice to avoid thawing. Proceed with bead-beating and DNA extraction protocol.

2. Protists
and fungi: Retrieve samples from -80°C
freezer, keeping on dry ice. Resuspend pellet or scraped biomass in Fluid X tube with molecular-grade water and transfer to bead-beating tube (prefilled with a mixture of glass and zirconium beads 100 µm – 2 mm). Centrifuge at 4000 rpm for 5 minutes to pellet, remove supernatant and proceed with bead-beating and DNA extraction protocol.

1.3 Methods – DNA Extraction (if required)

DNA extraction protocols have been optimised for each taxon group processed at the MBA to ensure effective and efficient tissue lysis and homogenisation, and to generate high-quality, high-yield template DNA for PCR amplification of DNA barcoding loci.

Samples and labelling

Samples are typically processed in batches of 8-12, organised according to extraction needs. For example, tough brown macroalgae (phaeophytes) that are expected to require a lot of troubleshooting are processed in smaller batches.

During DNA extraction and barcoding processes, a simple numbering scheme is used to label tubes, rather than using the full DToL sample numbers. This is tracked and recorded in the lab book. Resultant DNA extracts should be labelled with the full DToL sample numbers.

DNA extraction of macroalgae (if required)

This is a modified version of the Nucleospin Plant II kit (Macherey Nagel) Protocol 5.1 Genomic DNA from plants. This should also be followed for lichens. Read manufacturer's instructions alongside these modifications thoroughly before commencing work. Refer to manufacturer's protocol Section 2.5 for choosing the optimal lysis buffer for your sample.

Before starting:

- Ensure the correct preparation of Wash Buffer PW2 and RNase A according to Section 3 of manufacturer's protocol.
- Preheat DNase-free water to 65°C.

1. Transfer tissue from FluidX tube to a bead-beating tube, pre-filled with approximately half a PCR tube (0.2ml) of 2mm Zirconium beads, using sterilised needle or pipette tip, if not done so already. Retain samples on dry ice to avoid thawing.
2. Grind in Bead Beater for 40s x 6 ms, and return to dry ice for 5 mins.

- If samples are not homogenised, repeat cycle 40s x 6 ms or if there is no visible degradation, follow 'Trouble-shooting cycles' as necessary.
- Trouble-shooting cycles:

Inspect samples. Add 2 x 3mm Tungsten carbide beads to samples that have not degraded at all.
Grind in Bead Beater for 40s x 8 ms, and return to dry ice for 5 mins.
Inspect samples. For those with no visible degradation, complete freeze-thaw cycles of freezing in liquid nitrogen for 30 s and thawing at 65°C.
Final cycle with 400 ml Buffer PL1 (step 2a) but not RNase A
If samples are not homogenised, repeat cycle 40s x 6 ms or if there is no visible degradation, follow 'Trouble-shooting cycles' as necessary.
3. Proceed with cell lysis using Buffer PL1 (step 4a) or alternatively Buffer PL2 (step 4b).
4a. Add 400µl Buffer PL1. Vortex the mixture thoroughly. Add 10µl RNase A solution and invert. Incubate the suspension for 1-2 hours at room temperature.

• Trouble-shooting: inspect samples, if they are particularly viscous, add another 400µl PL1 and 4µl RNase A. Vortex to mix.

4b. Transfer the resulting powder to a new tube and add 300µl Buffer PL2. Vortex the mixture thoroughly. Add 10µl RNase A solution and invert. Incubate the suspension for 1-2 hours at room temperature.

• Trouble-shooting: inspect samples, if they are particularly viscous, add another 300µl PL2 and 4µl

RNase A. Vortex to mix.
Add 75µl Buffer PL3, mix thoroughly and incubate for 5 mins on ice to precipitate SDS completely. If additional PL2 has been added, add a further 75µl Buffer PL3.
5. Centrifuge samples for 2 mins at 13,000 x g.
6. Place a NucleoSpin Filter (violet ring) into a new collection tube (2 ml) and load the lysate onto the column, taking care to avoid the pellet.
7. Centrifuge for 2 mins at 11,000 x g, collect the clear flow-through and discard the NucleoSpin Filter. If not all the liquid had passed the filter, repeat the centrifugation step.
8. Taking care to avoid any pellet, transfer the clear supernatant to a new 1.5ml microcentrifuge tube (not provided).
9. Add 450µl Buffer PC and mix thoroughly by pipetting up and down (5 times) or by vortexing
a. For samples that have additional lysis Buffer PL1 or PL2/PL3, add the same proportion of Buffer PC.
10. Place a NucleoSpin Plant II Column (green ring) into a new Collection Tube (2ml) and load a maximum of 650µl of the sample.
a. According to manufacturer's instructions, the maximum loading capacity of the NucleoSpin Plant II Column is 700µl but it is not possible to then close the lid. Loading between 600-650µl is recommended. For higher sample volumes, repeat the loading step, by loading lysate and centrifuge for 1 min at 11,000 x g.
11. Centrifuge for 1 min at 11,000 x g and discard the flow-through.
12. Add 400µl Buffer PW1 to the NucleoSpin Plant II Column. Centrifuge for 1 min at 11,000 x g and discard flow-through.
13. Add 700µl Buffer PW2 to the NucleoSpin Plant II Column. Centrifuge for 1 min at 11,000 x g and discard flow-through.
14. Add another 200µl Buffer PW2 to the NucleoSpin Plant II Column. Centrifuge for 1 min at 11,000 x g. Discard flow-through and centrifuge for 2 mins at 11,000 x g in order to removed wash buffer and dry the silica membrane completely.
15. Place the NucleoSpin Plant II Column into a new 1.5ml microcentrifuge tube (not provided). Pipette 50 □l DNase-free water (65□C) onto the membrane. Incubate the NucleoSpin Plant II Column for 5 minutes at 65□C. Centrifuge for 1 min at 11,000 x g to elute the DNA.
a. If a more dilute sample is desired, repeat this step with another 50µl DNase-free water (65□C) and elute into the same tube.
16. Using the Nanodrop, record DNA quantity and quality (noting ng/µl, 260/230 and 260/280 values), in Macroalgae Progress log in the Barcoding folder on the DToL shared drive.
a. If the DNA quality is poor and the sample is < 10 ng/µl or > 50 ng/µl, additionally assess quality using the Qubit HS dsDNA kit.
17. Store DNA in 1.5ml microcentrifuge elution tube at -20□C for short term storage whilst confirming identity using barcoding. Once sent for genome sequencing, transfer to storage at -80□C.

DNA extraction of protists (microalgae) (if required)
Follow recommendations outlined in the DToL for Protist Sample Preparation SOP, using a DNA extraction kit e.g. DNeasy Plant kit (Qiagen) and following the manufacturer's instructions. Record DNA yield and quality using Nanodrop and/or Qubit HS dsDNA kit in Protist/Microalgae Progress log in the Barcoding folder on the DToL shared drive. Store DNA in 1.5ml microcentrifuge elution tube at -20 □C for short term storage whilst confirming identity using barcoding. Once sent for genome sequencing, transfer to storage at -80 □C.

DNA extractions of fungi and lichens (if required)

For fungi, this follows the MBA Fungal Culture Collection DNA extraction protocol (dated 24.06.2020) using DNeasy Plant kit (Qiagen).

Lichen DNA extraction

If sample is very small, the DNA extraction protocol for macroalgae described earlier should be followed. Otherwise continue with liquid nitrogen grinding, as described below.

1. Pre-cool a pestle and mortar by pouring liquid nitrogen into the mortar. Pre-cool a spatula and an Eppendorf tube for the ground sample in liquid nitrogen.
2. Place the frozen sample in the dry ice – don't let it defrost. Pour on some liquid nitrogen and grind the sample in the liquid.
3. Continue grinding until the sample is a fine powder. Add more liquid nitrogen between grinding if needed.
4. Using the pre-cooled spatula, scrape the powder off the pestle and mortar and place it in an Eppendorf tube.
5. Add 400µl of PL1 buffer from the NucleoSpin Plant II DNA extraction kit (Macherey Nagel) and vortex sample. Continue DNA extraction by following steps 4-17 in macroalgae DNA extraction.

Fungi DNA extraction

1. If not already prepared, resuspend pellet or scraped biomass in FluidX tube with molecular-grade water and transfer to bead-beating tube, prefilled with a mixture of glass and zirconium beads (100 µm – 2 mm). Centrifuge at 4000 rpm for 5 minutes to pellet, remove supernatant.
2. Bead beat for 1 min at 10ms with 5 mins rest on ice, repeat three times.
3. Transfer lysate (liquid) into new sterile 1.5ml tube.
4. Centrifuge at max speed (13,000 rpm) for 5 mins, discard supernatant (liquid).
5. Add 400µl Buffer AP1 and 4µl RNase A. Vortex and incubate for 10 mins at 65□C. Invert 3 times during incubation.
6. Add 130µl Buffer P3. Mix and incubate for 5 mins on ice.
7. Centrifuge lysate for 5 mins at maximum speed (13,000 rpm).
8. Pipette lysate into QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 mins at maximum speed (13,000 rpm).
9. Transfer the flow-through into a new tube without disturbing the pellet (if present). Add 1.5 volumes of Buffer AW1 and mix by pipetting.
10. Transfer 650µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 8,000 rpm. Discard flow-through. Repeat this step with the remaining sample.
11. Place the spin column into a new 2 ml collection tube. Add 500µl Buffer AW2 and centrifuge for 1 min at 8,000 rpm. Discard flow-through.
12. Add another 500µl Buffer AW2. Centrifuge for 2 mins at maximum speed (13,000 rpm)

a. Remove the spin column from the collection tube carefully so that the column does not come into contact with flow-through.

13. Discard the flow-through and collection tube. Place spin column into a new 1.5 ml microcentrifuge tube.
14. Add 100µl Buffer AE (pipette directly onto DNeasy membrane) and incubate at room temperature for 5 mins.

a. Elution volume can be reduced (e.g. to 50µl) to increase yielded DNA concentration. This will

reduce overall yield.
15. Centrifuge for 1 min at 8,000 rpm.
16. Using Nanodrop, record DNA quantity and quality (noting ng/µl, 260/230 and 260/280 values), in Fungi Progress log in Barcoding folder on DToL shared drive.
17. Store DNA in 1.5ml microcentrifuge elution tube at -20 □C for short term storage whilst confirming identity using barcoding. Once sent for genome sequencing, transfer to storage at -80□C.

1.4 Methods – PCR amplification of DNA barcoding loci from extracted DNA

Specific sets of primer pairs optimised for different macroalgae, protists and fungi will be amplified by PCR to produce DNA barcode sequences.

Standard DNA barcoding PCR reactions

This is the standard PCR reaction used for all primer pairs across organisms. Taxon-specific instructions are also detailed below.

1. Prepare mastermix according to the following table:

Mastermix	Volume per reaction (50 µl)
5x Green Buffer	10 µl
MgCl2	4 µl
dNTP Mix (10 µM)	1 µl
Forward Primer (0.25 µM)	2 µl
Reverse Primer (0.25 µM)	2 µl
GoTaq Polymerase	0.25 µl
BSA (20 mg ml-1)	1 µl
Molecular Grade H20	up to 50 µl (depending on template volume)

2. Distribute required volume of mastermix into each PCR tube e.g. 49 µl if using 1 µl template gDNA. Add 1 µl template gDNA to each PCR tube, though this may vary. Add 1 µl of molecular-grade water to one PCR tube as a negative control.
3. Briefly (2 secs) spin down PCR tubes before placing in appropriately programmed thermocycler.

PCR reactions for macroalgae

Replicate PCR reactions are routinely undertaken for macroalgae due to PCR inhibitors present in DNA extracts. It may be necessary to pool products from 2-4 replicate PCR reactions (depending on yield). Pooling should be carried out at step 2 of the clean-up process (see PCR clean-up section).

Dilution of template gDNA is extremely important when working with macroalgae as it reduces the amount of PCR inhibitors. Dilute templates to 1:20 where possible. For samples with low yields (<5ng/µl), do not dilute and add up to a maximum of 3 µl template per reaction.

PCR primers and thermocycler parameters

Details of PCR primer pairs used for each taxon group and specific thermocycler programmes to be used are in the table below. References for primers and protocols are listed in Section 3. Loci/primers with an * denotes that the primer sets are still in development and TBC. Additionally, rbcL primer sets for macroalgal phaeophytes are yet to be determined.

DNA barcoding SOP - Table 1 .docx

PCR visualisation
This is the standard visualisation process for all PCR reactions.

1. Prepare 100 ml agarose gel by adding 100 ml of TAE buffer and 1 g of agarose to a conical flask. Swirl and microwave on high power for 1 min. Swirl and ensure all agarose particles are fully dissolved, if not, microwave for a further 20 secs
2. Add 3 µl of Ethidium Bromide or 10 µl of Gel Red to the cooling agarose gel mixture and swirl
3. Pour gel into appropriate mould and add combs. Leave to set for 20-30 mins
4. Remove combs and transfer set gel to electrophoresis tank. Load 5 µl of 100 bp Hyperladder to first well, 3-5 µl of PCR product to consequent wells, and 3-5 µl of the negative control to the final well.
5. Run gel at 110v for 45 mins
6. Photograph gel in UV hood and save image. Annotate gel image and save in the Barcoding folder on the DToL shared drive.

PCR failure
If bands are faint, repeat the PCR reaction with increased cycles and pool multiple (2-4) replicate reactions.
If no bands are present, assess the concentration and quality of the DNA extract. If <5 ng/µl, repeat PCR reactions using a larger template volume (up to 5 µl) or alternatively, replicate and pool PCR reactions. If the DNA is of poor quality, dilute template with molecular-grade water to 1:20 or 1:10 concentration (depending on the yield), and repeat PCR reactions with replicates and pool reactions. Pooling of PCR products should take place at stage 2 of the PCR clean-up protocol.

PCR clean-up
Below is the standard process for the clean-up of PCR reactions prior to sequence submission. This is based on the GenElute PCR clean-up kit manufacturer's instructions. (However, Macherey-Nagel NucleoSpin Gel & PCR Clean Up Kit may also be used, according to manufacturer's instructions).

1. Insert a GenElute plasmid mini spin column into the collection tube provided. Add 0.5 ml of the Column Preparation Solution to each mini spin column and centrifuge for 1 min at 13,000 rpm. Discard flow-through but retain collection tube
2. Add 5 volumes of Binding Solution to 1 volume of PCR product and mix gently by pipetting. Transfer the solution into the binding column. Centrifuge for 1 min at 13,000 rpm. Discard flow-through but retain collection tube
a. Pooling replicate PCR products: combine all products into a single 1.5ml microcentrifuge tube with corresponding volume of Binding Solution. Pass through the binding column in stages.
3. Replace binding column into the collection tube. Apply 0.5 ml of diluted Wash Solution to the column, and rotate the column to ensure even coverage of the Wash Solution. Centrifuge for 1 min at 13,000 rpm. Discard flow-through but retain the collection tube
4. Replace binding column into the collection tube. Centrifuge the column at 13,000 rpm for 2 mins without further addition of Wash Solution, to remove excess ethanol
5. Transfer column to a fresh 2 ml collection tube. Apply 50 µl of Elution Solution to the centre of the

column. Incubate at room temperature for 1 min
6. Centrifuge the column for 1 min at 13,000 rpm to elute the purified PCR product
7. Quantify yield of purified PCR product using both Nanodrop and Qubit HS dsDNA kit and record results in the relevant Progress log in the Barcoding folder on the DToL shared drive.
8. In general, purified PCR products are submitted undiluted with their concentrations disclosed. Source Bioscience can then dilute as necessary. However, where PCR product concentrations are > 50 ng/µl, dilute to a target concentration of 10-20 ng/µl with molecular-grade water. If concentrations of purified PCR products are < 5 ng/µl, there is insufficient PCR product for sequence submission. However, PCR product concentrations as low as 7 ng/µl have been successfully sequenced.
9. Transfer 20 µl of purified PCR product to a clean 1.5 ml microcentrifuge tube clearly labelled with DToL sample number on both lid and tube wall. Store in fridge if sequence submission expected within 2 days, otherwise store in -20□C freezer. Store the remaining product in -20□C freezer.

1.5 Alternative methods

Tissue-direct PCR amplification of DNA barcoding loci, without prior DNA extraction. This method uses the Phire Plant-direct PCR kit (Thermofisher). Please see manufacturer's instructions for the general protocol. We have successfully used this method on macroalgae (fresh, frozen and dried), microalgae (both fresh cultures and cell concentrates frozen at -80°C), and expect the protocol to work equally well on fungi and lichens (work in progress). In general, less sample yields better results. Tissue samples may be added direct to PCR tubes, but more consistent results are obtained by adding a sample of lysis mix instead – as described below.

Sample preparation for Phire tissue-direct PCR

1. Thaw bottle of Phire Dilution Buffer (this may be stored in aliquots and kept at room temperature for future use).
2. Pipette an aliquot (see 3a – 3c below for volumes) of Phire Dilution Buffer into a 200 µl tube for each sample.
3. Carefully (to avoid cross-contamination), transfer a tissue / culture sample into the tube of Phire Dilution Buffer (For details for specific sample types, see 3a – 3c below). If working from frozen samples, keep tubes on dry ice, work quickly and return samples to the freezer ASAP. Do not thaw the tube contents; simply collect a piece of frozen tissue. Samples may be collected and transferred using a sterile scalpel blade, sterile syringe needle and / or sterile pipette tips (NB avoid plastic tips for transferring dried macroalgae samples due to problems with static). Squash the sample in the Phire Dilution Buffer against the side of the tube using a sterile plastic tip. Some (especially dried) samples may not squash - place on ice ~ 20 minutes, then try squashing again. This should release enough DNA, even if the tissue doesn't appear at all squashed. Centrifuge tubes (1 min., 13,000 rpm) and use a sample of the S/N (usually 1 µl or a dilution of this in Dilution Buffer) as template for PCR. Samples can be stored in Phire Dilution Buffer at -20C for future use, allowing several PCRs to be performed without having to go back to the master samples.

a. Macro algae: samples vary a lot, so it's not easy to be consistent, but aim for equivalent of approx. 2 x 1 x 1 mm fresh / frozen tissue; 1 x 1 x 1 mm for dried, added to 20 µl Phire dilution buffer. (To use 1 µl S/N in 50 µl PCR reaction)

b. Micro algae: For a fresh culture: Mix 2 µl culture with 18 µl Phire dilution buffer. (To use 1 - 5 µl in 50 µl PCR reaction). For frozen cell concentrates, scrape ~ 1 µl from frozen sample and combine with 19 µl Phire Dilution Buffer (to use 1 µl / PCR reaction). It is also possible to pick from a colony grown on solid media into 20 µl Phire Dilution Buffer, mix, then use 1 µl for PCR.

c. Fungi & lichens: This has not yet been optimised, but it is anticipated that samples can be treated

like microalgae: taking samples from liquid cultures or scraping from a colony grown on agar.

Phire PCR reactions

1. Thaw required PCR primers and Phire PCR buffer (2x), then place on ice. NB This Phire buffer contains all PCR components – nothing further (e.g. dNTPs, MgCl2) is required.
2. Label PCR tubes for the reactions (including + and – controls). Use a simplified numbering scheme, but record how this relates to MBA DToL sample codes in lab book. Phire is a hot start polymerase, therefore reactions can be set up at room temperature, rather than on ice.
3. Make up PCR master mix and aliquot into PCR tubes. Method here is for standard reactions. Specific information / optimisation listed separately.

Mastermix	Volume per (50 µl) reaction
Water (Molec Grade)	19.6 µl *
2x Phire PCR Buffer	25 µl
Forward Primer (10 µM)	2 µl
Reverse Primer (10 µM)	2 µl
Phire DNA Polymerase	0.4 µl

Aliquot PCR mastermix into PCR sample tube: usually 49 µl / tube.
*This is for standard reactions (using 1 µl sample). If more sample is used (e.g. 5 µl for fresh microalgae cultures), adjust volume of water added in the master mix, and the volume of master mix used, to bring final reaction volume to 50 µl.

4. Add sample to each tube, making sure that negative controls (e.g. containing Phire Dilution Buffer or diluted culture media) are the last tubes set up. Positive controls may also be used, e.g. a sample of extracted DNA if available, or another sample previously shown to work.
5. Place tubes in pre-programmed thermal cycler.
Typical parameters: 98°C 5 min; 40 x (98°C 5 sec; χ°C 5 sec; 72°C 20 sec); 72°C 1 min; 10°C hold if necessary.
Annealing temperatures (χ) are typically 46-60°C and must be calculated for Phire polymerase (e.g. online Tm calculator, Thermofisher), rather than using standard Taq conditions.
PCR product visualisation: see 1.4.3 for agarose gel electrophoresis. Load 5-8 µl sample / well. Clean up of PCR product is carried out as described above for standard PCR.
If not enough PCR product is obtained, replicate reactions may be set up and the product combined before clean up performed.

If optimisation of Phire PCR is required:
Sample: adjust sample volume / concentration. Diluting sample 1 in 10 or 1 in 100 is a good place to start (even 1 in 1000). Problems are most likely due to too much, rather than too little sample.
PCR reagents: because degenerate primers are used, it may help to add more primer. The volume of each primer in a pair need not be equal – e.g. if one primer is more degenerate, it may help to add more of that one (3 or even 4 µl in 50 µl reaction).
Thermocycling conditions: alter annealing temperature (increased temperature increases specificity, but may also reduce yield) and / or cycle number (more cycles increase yield, but may bring up background): 40 – 43 cycles are typical for Phire PCR.

PCR visualisation.
See 1.4.3 for agarose gel electrophoresis. Load 5-8 ul sample / well.

1.6 Sequence submission

DNA barcode sequences are generated on the Sanger sequencing platform. This is currently outsourced to Source Bioscience (Nottingham & Cambridge, UK).
For submission, complete the appropriate online form with Source Bioscience. In addition to purified PCR products (5 µl / reaction at 10 ng / µl), 5 µl of primer (+ 10% coverage to allow for evaporation) per sequencing reaction must also be submitted. Primer concentrations should be 3.2 µM for submission.

2 Analysis and reporting (Informatics) - Section still in progress

1. Sequence editing
2. Contig assembly
3. Database matching
4. Reporting

This is to be drafted in the light of the generic Informatics SOP (see below) discussed at the Barcoding process progress meeting 15/12/20.

Here are some notes
For barcoding analysis I have been manually inspecting trimming them to a minimum of Q20 but where possible Q30. These are searched against the NCBI nucleotide database. The top hits are inspected as some of the hits have dubious identification. The best matches are then reported in the spreadsheet. The ones I consider to be good quality I have exported the consensus sequence as FASTAs and stored them on the DToL drive. The raw .seq and ab1 files are also stored on the drive. Some of the hits fail to distinguish to species level. These are indicated with a Species x/species y in the consensus sequence column in the spreadsheet. I indicate in the Sequence column whether further markers are required.

Sequencing failures:
If one of the reads (forward or reverse) has extremely poor quality, but the other is good quality (Q30), investigate matches to this read alone. If matches are <98 % identity, repeat PCR with different primer set. For protists, if not 100% identity, repeat PCR with alternative primer set.

If both reads are poor quality assess the quality of the PCR product sent. If low quality or low yield, repeat PCR and pool to improve yield. If the quality and yield was sufficient, repeat with an alternative primer set which may be more specific to the taxa, or select a different marker.

Additional notes (Jan 2021)
Consider using reference sequence (where available) to guide contig assembly. This could be beneficial for macroalgae, as well as protists and fungi. This will also help to delimit primer sequences for removal. The length of sequence can also be informed by this, though one should already have an idea of expected product size.

Place consensus sequence in an alignment, as this can also be informative, where data are available and identifications can be problematic.

If any of the sequence reads are re-edited and new consensus sequences are produced, ensure that the FASTA file stored on the server is the correct version. This will be submitted to ENA.

Databases for sequence matching

All are 'to be confirmed' but here are the current options:

Protists – SILVA + PR2 (+ BOLD)
Diatoms – Diat.barcode https://www6.inrae.fr/carrtel-collection_eng/Barcoding-database
Macroalgae – BOLD/NCBI
Marine Fungi – UNITE (ITS), taxonomy updated via NCBI from Index Fungorum and Mycobank; SILVA or PhyMyco-db for 18S, though both have issues with taxonomy updates. Ask Kew to confirm.

2.1 DToL DNA Barcoding Bioinformatic SOP (Generic template for revision by GALs)

1. Transfer DNA sequence data from sequencer
From a Sanger sequencing platform, this will be a set of .ab1 files.
2. If part of an automated pipeline: Collate all F sequences into a single .fastq format file using BioPython. Separately collate all R sequences into a single .fastq format file. If part of a manual pipeline: inspect sequences and quality trim.
4. Assemble F and R strands for each amplification.
Where F and R strands do not assemble, note in the metadata entry.
5. Query the appropriate sequence reference database. For invertebrates this will probably be BOLD.
6. Compare to expected species identification from the manifest.
7. Where the expected and test binomials agree, and are within the binning threshold, complete the DNA barcode metadata entry and approve the sample, and send to COPO.
8. For the remainder, identify the nature of the mismatches.

(A1) DB identification is a different species to expected identification, with high match value.
Interpretation: Wrong sample in well, or cross-contamination from another well.
Resolution: Check to see if DB species identification is present elsewhere on plate.
Action: If it is, repeat extraction and amplification of affected sample.

(A2)
Interpretation: DB or DToL identification is incorrect.
Resolution: Request that submitter/identifier check for traits that differentiate between the two species.
Action: If the submitter is confident in species ID, proceed with barcode manifest extension, and notify BOLD about possible misidentification.

(B1) DB identification is to the same species as expected identification, but with a poor match value (outside the binning threshold).
Interpretation: Bad sequencing or amplification error.
Resolution: Check sequence quality.
Action: Repeat sequencing if low quality.

(B2)

Interpretation: DToL specimen could be from a related species, not found on DB, or currently not recognized.
Resolution: Request that submitter/identifier check for traits that identify this species from sister species, and consider possibility of a cryptic species.
Action: Proceed with Barcode Manifest Extension submission to COPO, flagging as a potential issue.

(C) DB identification is a different species but same genus as expected identification, with a match value below bin threshold.
Interpretation: Test species not on the database.
Resolution: Check by searching database for species. If species is not present on DB, submit sequence to BOLD
Action: Submit to COPO.

	A	B	C
		Same species sequence present	***Same species sequence not present***
	Same genus sequence present	**PASS_SPECIES** if new sequence matches dbspecies > threshold and this match is > other species in same genus **FAIL_SPECIES** if sequence doesnt match species > threshold	**PASS_GENUS** if new sequence matches dbgenus as best hit, but not within threshold of any individual species within genus **FAIL_GENUS** if sequence has top matches to another genus > threshold
	Same genus sequence not present		**BARCODE DB is NOT INFORMATIVE** check that top match is from same Family and provisional **PASS** if it is close

3 DNA barcoding marker selection

Primer set and protocol references

Macroalgae
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Heywood JL, Sieracki ME, Bellows W, Poulton NJ, Stepanauskas R. Capturing diversity of marine heterotrophic protists: one cell at a time. ISME J. 2011;5(4):674-684. doi:10.1038/ismej.2010.155

Kucera H, Saunders GW. (2008) Assigning morphological variants of Fucus (Fucales, Phaeophyceae) in Canadian waters to recognised species using DNA barcoding. Botany, 86 (9). https://doi.org/10.1139/B08-056

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