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# DNA Barcoding Standard Operating Protocol Lichens at RBGE, Lab methods: DNA extraction

Forked from DNA Barcoding Standard Operating Protocol, Plants and Lichens at RBGE, Lab methods: DNA extraction

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**We use this protocol and it's working**

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## Abstract

This is part of the collection **DToL Taxon-specific Standard Operating Procedures for the Plant Working Group (protocols.io)**. The SOP collection contains guidance on how to process the various land plant and lichen taxa within the scope of the Darwin Tree of Life project. The guidance specifically refers to the tissue samples needed for DNA barcoding (which takes place at the Royal Botanic Garden (RBGE)). Every specimen is submitted for DNA barcoding first before potentially being sent to the Wellcome Sanger institute.

This DNA barcoding SOP outlines DNA extractions from lichen samples for the Darwin Tree of Life project (DToL) at the Royal Botanic Garden Edinburgh (RBGE).

DNA barcoding is used as part of the species identification process AND sample tracking (to check that the genome sequence corresponds to the material that was sent and that there have been no sample mix-ups).

**Definition**: Lichens

**Including**: Lichenized fungi

**Excluding**: All non-lichenized fungi

## Guidelines

**Including**: Marchantiophyta (liverworts), Bryophyta (mosses), Anthocerotophyta (hornworts), vascular plants, lichens

Note

Previous versions of the Plant Working Group SOPs can be found here:

**RBGE DToL Sample collection Standard Operating Procedure Vascular Plants**
**RBGE DToL Sample collection Standard Operating Procedure** Bryophytes
**SOP RBGE Plant DNA Barcoding sample submission**
**Darwin Tree of Life DNA barcoding of vascular and non-vascular plants and of lichens - Standard Operating Procedure**

| | A | B | C | D |
|---|---|---|---|---|
| | | ***Date*** | ***Changes*** | ***Contributors*** |
| | 1.0 | August 2020 | First draft | Laura L Forrest, Michelle L Hart |
| | 1.1 | January 2021 | Revisions | Laura L Forrest |
| | 1.2 | June 2021 | Lichens added | Laura L Forrest |
| | 1.3 | October 2023 | Lichens revised | Amanda L Jones |
| | 1.4 | January 2024 | Revisions | Amanda L Jones |

**Previous Version History, RBGE DToL DNA Barcoding Standard Operating Procedure**

**Working SOP, checked by experts**

## Materials

**Lichen DNA Extraction at RBGE** uses general equipment and consumables that are typically available in molecular biology laboratories, e.g. freezers, benchtop centrifuges, water baths, heating blocks, vortexers, thermocyclers, gel tanks and gel trays, agarose, UV or blue-light trans-illuminators, laminar flow hoods, fume hoods, water purification systems, autoclaves, micropipettes, tips, and microcentrifuge tubes and tube racks. Additionally, mini-pestles and acid-washed sand may be used for manual grinding of lichen tissues.

**Note**

Mini-pestles can be cleaned for reuse by rinsing them clean of lichen debris in water, sonicating to remove small fragments, and then washing for c. 1 min in either 0.4 M HCl or a bleach solution to remove any remaining DNA, rinsing well with water and autoclaving. We store these in sets of 8-16 in 50 ml Falcon tubes.

## DNA extraction (Qiagen DNeasy plant mini kits)

1 **Sampling.**

Lichen barcoding for the Darwin Tree of Life (DToL) project involves extraction of DNA from lichen material that has been flash-frozen or dried and then frozen, along with the required metadata, following the collecting and submission SOPs available in the collection **DToL Taxon-specific Standard Operating Procedures for the Plant Working Group.** Field collection and sampling of lichens, both in the field and in the lab, follows the **Collections Standard Operating Protocol: Lichens.** Different extraction protocols are used depending on lichen morphology and specimen size.

Note

All lab samples for DNA extraction are renumbered in consecutive order, with a short simple numbering scheme, rather than using the **DToL sample numbers**, as the numbers have to be transcribed onto several plastic tubes during the extraction process and this is least prone to error. Note the numbers both in a lab book and by adding a sampling slip containing the DNA number and the collection number of the specimen to the individual specimen packet.

Current **RBGE DToL DNA extraction numbering series** for barcoding are prefaced with **B** (mosses), **L** (Liverworts), **F** (ferns and lycophytes), **A** (seed plants) and **X** (lichens).

These temporary tube numbers are entered into the **EDNA_submissions_sheet_211209.xlsx** and recorded in the **EDNA database**. They are also tracked in the **RBGE DToL barcoding** google sheet.

In order to best troubleshoot contamination issues downstream, where possible avoid placing samples from the same genus, and particularly from the same species, into consecutive tubes.

2 **DNA extraction** using acetone rinse with REDExtract-N-Amp$^{TM}$

Note

So far, this method works well for macrolichens/foliose and fruticose lichens, or apotheciate crusts using c. 3 mm$^2$ of clean lichen material. Homogenization is not necessary for this technique, but apothecia should be thinly sliced to increase surface area. We use extraction and dilution solutions (c. 1/3 reactions) from REDExtract-N-Amp$^{TM}$ Plant PCR kits. Methods have been modified from those used in McCune Lab, Oregon State University (pers comm.).

**Acetone rinse (to reduce lichen secondary compounds)**

1. Place the lichen fragment(s) in a 1.7 ml tube.
2. Soak the sample in c. 50 µl acetone for at least 30 min, ensuring that the tissue is completely covered by the acetone.
3. Pipette off the acetone into a labelled 500 µl tube.
4. Leave the lichen tubes and the acetone tubes open in a fume hood to air dry (c. 10 min for lichen tubes, and 30 min-1 hour for acetone tubes).
5. Once dry, either pass the acetone sample tube on for thin layer chromatography (TLC), or store it with the lichen herbarium specimen in case TLC will be carried out in the future. Carry on with DNA extraction of the lichen sample tubes.

**DNA extraction**

1. Aliquot REDExtract-N-Amp$^{TM}$ reagents into 1.5 ml aliquots and store at -20°C until needed.
2. Preheat a heat block to 95°C (c. 20 min) and defrost aliquots of REDExtract-N-Amp$^{TM}$ extraction and dilution solutions.
3. Add c. 30 µl extraction solution to each lichen sample to cover.
4. Vortex, spin down and ensure lichen tissue is well submerged.
5. Incubate for 10 min at 95°C (keeping an eye on the lids, as they may pop open).
6. Add 30 µl dilution solution to neutralize inhibitors (the same volume as extraction buffer).
7. Store at 4°C until needed.

Note

Lichen tissues may be hydrophobic and require extra agitation for submersion in extraction buffer. Depending on the type and condition of material, samples may absorb more extraction buffer (and expand) requiring a greater volume of liquid to cover.

DNA stocks can be stored long term in the freezer. Removing the lichen material from the stock may improve preservation (but we have not experimented with this yet). DNA stocks can be banked by pipetting (avoiding the lichen) into barcoded tubes.

**Dilution of DNA stock for PCR and sequencing**

In PCR strips, dilute DNA stocks with molecular grade water to a 1:10 dilution (e.g. 3 µl DNA + 27 µl water) for standard samples. Smaller (just visible/scant samples) can be diluted 1:5 (3 µl DNA + 12 µl water).

3 **DNA extraction** using Bento Dipstick DNA Extraction Kit

Note

This extraction method is particularly suited to microlichens (leprose/granular sterile crusts and very small specimens). It reduces the potential for introduction of contaminants/PCR inhibitors and small tissue fragments into the final DNA stock. Sample material as small as <1 mm $^2$ (just visible) to 3 mm$^2$ can be picked directly into 1.7 ml tubes for DNA extraction. Since we often target more than one gene region, a DNA stock is made for later use (rather than using dipsticks directly in a PCR mix). These methods have been modified from **Bento Dipstick extraction protocol**.

1. Add a pinch of sterilised sand and a small amount of extraction buffer (c. 20-40 µl) to lichen sample tubes; homogenize well with a micro pestle.
2. Add additional buffer incrementally (for a total of c. 300 µl for very small samples—recommended adding up to 500 µl, depending on the amount of material); homogenize further as needed.
3. Prepare a wash buffer tube for each lichen sample. Recommended volume is 750ul in a 1.7 ml tube (500 µl for small samples).
4. Prepare 25 µl TE buffer (Tris 10 mM EDTA 1 mM) in a labelled 0.5 ml tube for each sample (to make a DNA stock).
5. On a clean piece of paper or foil, organize 2 dipsticks for each sample.
6. With the 3 prepared tubes for each sample lined up (DNA extract, wash buffer, TE buffer):
    a. Dip the cellulose end of a dipstick into the DNA/extraction buffer 3 times to capture DNA.
    b. Dip the dipstick into the wash buffer 3 times (pressing the cellulose end into the side of the tube to
    squeeze off excess wash buffer).
    c. Dip the dipstick into the TE buffer for c. 10 seconds, agitating gently, to release the DNA and make a
    stock.
    d. Repeat the process for the second dipstick.
7. Spin down eluted DNA and store at -20°C long term, or 4°C for immediate use.

Note

To release maximum DNA, incubate the dipsticks in TE buffer for as long as possible--ideally, to the point just before the cellulose end breaks off the stick. If a cellulose end does break off, simply remove it from the the tube using a pipette tip. Crude DNA extracts can be kept in the freezer and then gently defrosted and warmed (in a water bath) for future use. However, we have not experimented with this yet.

4 **DNA extraction** with direct PCR

Note

In exceptional circumstances, direct PCR has generated barcodes for problematic specimens which are either extremely small, contaminated with lichenicolous fungi, or difficult to isolate
from a mixed species sample. Likewise, in cases where a lichen parasite is the target, this method may enable extraction of parasite from host tissue, which is difficult to isolate using other methods without culturing.

1. Prepare a 0.2 ml tube with a 20 µl PCR mix for each specimen (ideally 2 tubes per specimen).
2. Working under a stereo microscope and with sterilised blades/needles, make sections of target lichen tissue areas (e.g. slices of apothecia/perithecia).
3. Working on a slide (and under higher magnification), make a dissection of the section to isolate target tissue for direct PCR (e.g. hymenium only).
4. Using a sterile pipette tip or needle (moistened in sterile water or PCR mix), pick up a minute amount of target material. Add the piece of lichen to the PCR tube, ensuring the material arrives into the tube (this may require examination of the tube under the microscope).
5. Carry out PCR.

5 **Storage**

**Frozen lichen sample material -**This should be kept frozen in labeled batches, with specimens only left at room temperature long enough to sample for DNA extraction.

Note

Once successful sequences have been obtained for each batch, frozen specimens and the relevant sample DToL manifest and EDNA accession numbers are passed on to herbarium staff for long term curation.

**TE-preserved lichen material -** Occasionally, fresh lichens are preserved in TE buffer and frozen for future DNA extraction. If any lichen thallus is left after the extraction process, return the tubes to the freezer drawer, in case extractions have to be repeated.

**DNA -** Long term banking into a freezer is only necessary after all PCR from that extraction set is complete, including rePCRs of problematic material.

Note

Short-term, we store barcoding DNA in labelled 1.7 mL elution tubes, at 4°C. DToL tubes are arranged taxonomically in 96-sample racks (i.e. by the temporary extraction numbers: A = seed plants; F = ferns and lycophytes; B = mosses; L = liverworts, X = lichens, each in numerical order in different racks).

Once good quality barcode DNA sequences have been obtained for all samples within a set of tubes, the DNA is transferred, in the same order, to the RBGE DNA bank, in barcoded fluidX tubes that have been scanned in their 96-tube racks. These are stored in a -80°C freezer. The tube and plate barcode details are entered into the relevant database (at RBGE, this is our in-house DNA database, EDNA).

## Protocol references

Bento Dipstick DNA Extraction Kit (protocol)
**https://bento.bio/resources/datasheets/dipstick-dna-extraction-kit/**