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DNA Barcoding Standard Operating Protocol Lichens at RBGE, Lab methods: DNA extraction v2025

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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
		<i>Date</i>	<i>Changes</i>	<i>Contributors</i>
	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
	1.5	December 2025	Revisions, addition of salting out protocol and the Aberystwyth team who shared it with us	Amanda L Jones, Gareth Griffith, Ruby Bye

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

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, Microlichens - Bento Lab

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 $<1\text{ mm}^2$ (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 750 μl 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 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.

3 DNA Extraction, Macro- or Microlichens - Salting Out Method

Note

This protocol is modified from the Griffith Lab (Aberystwyth, Gareth Griffith and Ruby Bye) based on Miller, S.A., Dykes, D.D. and Polesky, H., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic acids research*, 16(3): 1215.

3.1 Before starting: Buffers, reagents, and preparation

Salt lysis buffer: Tris 10 mM, NaCl 400 mM, EDTA 2 mM (make 30 or 50ml of this).

SDS: 2% w/v (c. 60 ul per sample)

NaCl: 5 M (c. 500 ul per sample)

Molecular grade isopropanol (c. 600ul per sample)

GlycoBlue (Invitrogen/Thermofisher) coprecipitant (optional)

Molecular grade ethanol

TE buffer: 1 mM Tris [pH 8.0], 1 mM EDTA

1.5 ml (samples including apothecia or <2mm²) or 2 ml tubes (larger samples ground with beads) (2 sets)

Make fresh 70% ethanol (1 ml per sample, plus extra). Store in refrigerator.

Take out an aliquot of isopropanol and store at room temp (c. 600 ul per sample).

Set heat block to 60°C (later, 37°C)

3.2 Method

1. Macerate tissue

- For larger samples, use tungsten beads in flat bottom 2ml tubes and a tissue homogenizer. Grind to fine powder (2 mins at appx. 20 Hz, rotate 180 degrees and homogenize for another 2 mins). Repeat if needed.
- For very small samples (see note, section 2 above), add a pinch of sand to each tube. Add a small amount of buffer (e.g. 20 ul) and grind each sample with glass or plastic micropestle.

2. Lyse cells

Add salt lysis buffer to make up to 515 ul.

Add SDS (57 ul = 2% final conc). Vortex briefly to mix. Pulse spin to get everything to bottom of tube.

Transfer tubes to heat block (60 °C x 60 min, shake/rotate at approximately 600 rpm).

3. Precipitate proteins

- Pulse spin (13,000 rpm) tubes if needed. Add saturated (5M) NaCl (400 µl, 0.7x volume). Vortex. The proteins will precipitate, the DNA remains in solution.
- Spin down (13,000 rpm x 5 min) to pellet the proteins.
- Transfer supernatant (c. 800 µl, containing the DNA), avoiding pellet to a new 1.5ml tube.
- Repeat spindown
- Transfer the supernatant (c. 750 µl), avoiding pellet to a new 1.5 ml tube (this is the final tube so label fully!)

4) Precipitate DNA

- Add room temp isopropanol (~525 µl, 0.7x volumes) to precipitate DNA, with GlycoBlue (1.5 µl). Vortex briefly.
- Leave at room temp x 5 min.
- Spin (13,000 rpm x 10 min) to pellet the DNA.
- Pour off the supernatant, taking care to retain the DNA pellet (which will be blueish from GlycoBlue).

5) Wash

- Add fresh, cold 70% ethanol (500 µl). Vortex briefly.
- Pulse spin to re-attach the pellet.
- Carefully pour off the ethanol, retaining the pellet.
- Repeat wash once more. Upright the tube onto a sterile tissue to drain.
- To evaporate residual ethanol, leave with lid open on 60°C heat block x 10 min.
- Re-dissolve DNA in TE buffer (30-100 µl) and heat on heat block 37°C x 10 mins, shake at 600rpm (check that pellet is dissolved!)

4 DNA extraction, lichenicolous species, parasites or tiny dissected samples - 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/>

Miller, S.A., Dykes, D.D. and Polesky, H., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic acids research*, 16(3), p.1215.