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DNA Barcoding Standard Operating Protocol, Plants and Lichens at RBGE, Lab methods: amplicon sequencing with ONT



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External link: <https://github.com/Gesners/petithebi>

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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 taxa within the scope of the Darwin Tree of Life project (DTOL). The guidance specifically refers to the laboratory processing of extracted DNA from Darwin Tree of Life plant and lichen collections that 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 PCR pooling, library prep and ONT sequencing on a Flongle flow cell of DNA extractions from plant and lichen samples for the Darwin Tree of Life project (and for other in-house DNA barcoding projects) at the Royal Botanic Garden Edinburgh. This protocol is based on one that was developed by Nishii and Möller to sequence traditional PCR-based molecular markers using Oxford Nanopore Technologies (ONT) MinION or Flongle devices. This protocol uses ONT's Native Barcoding Kit 96 V14 (SQK-NBD114.96), but may be possible with another kit, e.g. Rapid Barcoding Kit 24 V14 (SqK-RBK114.24). The kit was chosen for its wide application possibilities. Some alterations/modifications from original ONT protocols have been made.

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) for the Darwin Tree of Life Project.

Definition: amplicon sequencing from any taxon

Guidelines

Including: Bryophyta, Marchantiophyta, Anthocerotophyta, Lycopodiophyta, Polypodiophyta, Pinophyta, Cycadophyta, Ginkgophyta, Gnetophyta, Magnoliophyta, lichenized fungi

Note

This SOP is complementary to other RBGE Darwin Tree of Life SOPs for sample collection, preparation, DNA extraction and PCR amplification of barcode loci.

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

Marker choice

For plant and fungal accessions that are required for DToL identification/sample tracking purposes:

- Bryophytes (liverworts, hornworts and mosses) are routinely amplified for plastid markers *rbcL*, *psbA-trnH* and *trnL-trnF*, and nuclear marker *ITS2*.
- Ferns and lycophytes are routinely amplified for plastid markers *rbcL* and *trnL-trnF*.
- Seed plants are routinely amplified for plastid markers *rbcL* and *trnL-trnF* and nuclear marker *ITS2*.
- Lichens are routinely amplified for nuclear marker *ITS*.
- The plastid markers *matK* and *rpoC1* are subsidiary markers that can be used for all land plants.

PCR amplification.

- Routinely use PCR enhancers TBT-PAR (Samarakoon et al., 2013) or CES (Ralser et al., 2006) for consistent amplification.
- Set up PCRs in strips rather than plates, due to a high rate of failure of samples around the margins of plates (probably through poor lid seals).
- **For plants, per reaction:** 1x PCR buffer, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, 1x additive, 1 μM of each primer, 2.5 U taq polymerase and c. 1 ng template DNA.
- **For lichens, per reaction:** 1x PCR buffer, 0.2 mM each dNTPs, 2.4 mM $MgCl_2$, 1x additive, 0.4 M of each primer, 0.05 U/ μl taq polymerase and c. 2-5 ng template DNA

Best practice

Sequence your negative controls.

PCR failure (before library prep for sequencing):

If there is no band on the gel for a given amplification, the PCR should be repeated. At least one repeat PCR should be carried out for all poor / failed amplifications in a batch. If over c. 20% of samples have failed for a marker that is usually successful (e.g. rbcL), the entire batch of PCRs should be discarded and the PCR repeated, to save time cherry-picking successful samples.

If PCR of an extraction fails across both/all loci, DNA may be quantified on an agarose gel and by fluorometry, or just discarded, and a repeat DNA extraction made from the silica-gel dried tissue sample. In some cases, extraction from tissue removed from the herbarium sample may be more successful (e.g. if ins

If PCR just fails for one or two loci, it may be repeated using an alternative additive (TBT-PAR or CES) and/or with a 1:25 dilution of the original DNA.

If there are multiple bands, particularly for variable-length markers (usually ITS2 and psbA-trnH), continue with ONT sequencing, as reads from both bands should form contigs.

Sequencing failure

Check the PCR gel images to estimate the quality of the PCR product (band tightness and brightness).

Note

For samples that frequently fail using Sanger sequencing, e.g. due to coamplification of endogenous fungi, ONT offers a solution in that both the target sequence and the contaminating sequence can often be recovered.

General considerations:

Use filter tips for shared reagents.
Note the defrosting/storage requirements for each reagent (e.g., mixing with pipette, defrosting on ice/room temp, etc.).
Be particularly careful with enzymes — these are sticky, expensive and should be treated gently (not vortexed).

	A	B	C	D
		<i>Date</i>	<i>Changes</i>	<i>Contributors</i>
	1.0	2023	First draft	Kanae Nishii, Michael Möller
	2.0	2024	Simplification, with removal of PCR comparison software; removal of most Qubit steps to reduce time and	Laura Forrest

	A	B	C	D
			cost. Addition of spectrophotometry for rough normalization. Reduction of reaction sizes for library prep to ¼ reaction	

Previous Version History, RBGE DTOL DNA Barcoding Standard Operating Procedure

Working SOP, checked by experts



Materials

In the following list, general equipment and consumables that are typically available in molecular biology laboratories, such as benchtop centrifuges, water baths, heating blocks, orbital shakers, 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, microcentrifuge tubes and tube racks, are generally omitted.

Water: Molecular biology grade Sigma water

Template quantification

- **DNA gel stain:** SYBR®Safe (Invitrogen).
- **Tris-Borate-EDTA** (TBE) buffer (kept at 10x or 5x stock; 1x working solution).
- **Agarose**, molecular grade.
- **Loading solution:** 30% glycerol, 0.25% bromophenol blue, Millipore water.
- **DNA ladder:** 1 Kb Plus DNA ladder (Invitrogen): to make working solution aliquots, combine 100 µL stock, 250 µL Sigma gel loading buffer and 650 µL Sigma water.

Template clean-up

- **AMPure XP Bead-Based Reagent**, 60 ml [AMPure XP Beads](#) | [PCR & NGS Library Cleanup \(mybeckman.uk\)](#)
- **80% ethanol**
- **magnetic rack** for strips
- set of new **PCR strips** for cleaned sample
- **plastic pipetting reservoir** for 80% ethanol,
- **0.1X TE buffer** (dilute stock, e.g., 100 µl 1X buffer / 900 µl water),
- **Qubit DS DNA HS** reagents and tubes.

Library preparation

Note

At RBGE most library prep reagents are stored in the bottom drawer of the under-bench freezer in lab 32; when stocks run out, there may be replacements in the large free-standing consumables freezer in lab 32. Flongles are in the large free-standing consumables fridge in lab 32.

- **Native Barcoding Kit 96 V14 (SQK-NBD114.96)** [Native Barcoding Kit 96 V14 \(nanoporetech.com\)](#)
- **NEBNext Ultra II End Repair/dA-Tailing Module**, 96 rxns [NEBNext Ultra II End Repair/dA-Tailing Module](#) | [NEB](#)
- **NEBNext Ultra II Ligation Module**; 96 rxns [NEBNext Ultra II Ligation Module](#) | [NEB](#)

End prep

- **Ultra II End-prep Reaction buffer**
- **Ultra II End-prep Enzyme mix**

Barcode ligation

- **Native barcode** (NB01-96)
- **Ultra II Ligation Master Mix**
- **Ligation Enhancer**
- **EDTA**

Sample pooling and clean-up

- **AMPure XP Bead-Based Reagent**, 60 ml [AMPure XP Beads](#) | [PCR & NGS Library Cleanup \(mybeckman.uk\)](#)
- **80% ethanol**
- **magnetic rack** for strips
- set of new **PCR strips** for cleaned sample
- **plastic pipetting reservoir** for 80% ethanol,
- **0.1X TE buffer** (dilute stock, e.g., 100 µl 1X buffer / 900 µl water),
- **Sigma water**,
- 1.5 ml **LoBind** tubes.

Adaptor ligation and clean-up

- **Native Adapter** (NA),
- **NEBNext Quick Ligation Reaction Buffer** (5X)
- **quick T4 DNA Ligase**
- **Short Fragment Buffer** (SFB),
- **Elution Buffer** (EB).
- **AMPureXP beads**
- **magnet rack** for 1.5 ml tube.
- 1.5 ml **LoBind** tube
- Either: Flongle Flow Cell (R10.4.1), pack of 12 (FLO-FLG114); supplied with Flongle Sequencing Expansion (EXP-FSE002) [Flongle Flow Cell \(R10.4.1\) \(nanoporetech.com\)](#).
- Or: MinION Cell <https://store.nanoporetech.com/us/flow-cell-r10-4-1-2025.html>

Before start

For RBGE lab users, as early as possible, register your intent to use a Flongle in the Flongle log (Teams, Lab users, MinION_Flongle users), so we can keep track of availability.

On the day of your ONT run, make sure you check the computer, and restart it with any updates several hours before you intend to use it.

GENERAL OVERVIEW

1 PRIOR TO RUN

1. Perform PCR for each locus. (PCR Reaction volumes of 15–20 μ l should be sufficient to obtain 200 fmol for the MinION run (for 1000 bp fragment, this corresponds to c. 130 ng)).
2. Gel electrophoresis and relative quantification from gel image.
3. Pool amplicons for each sample/specimen (one pool per specimen, containing e.g., rbcL, trnL-trnF, psbA-trnH and ITS2 amplicons).
4. AmpureXP bead clean the amplicon pools – use the excel sheet to calculate the quantity of beads needed (minimum of 1.3X the pool volume).
5. OPTIONAL: Nanodrop the amplicon pools, and use the concentrations to perform a rough normalization.
6. Library End prep – to optimize ligation of the barcode adaptors to the amplicons.
7. Ligation of the sequencing Barcodes (which will allow the sequence reads to be separated back into reads for each specimen).
8. Pool the samples then bead clean.

DAY OF RUN

9. Sequencing adaptor ligation and bead clean.
10. Computer updates and Flongle pore scan.
11. Prepare sequencing mix, prime and load the Flongle.
12. Start the MinION/Flongle sequencing run.
13. Data analysis.

PCR success/quantification

2 Gel electrophoresis and relative quantification from gel image

Perform gel electrophoresis with existing protocols:

Run 3 μ l PCR product and 3.5 μ l 1kb+ ladder on a 1% agarose TBE gel, and take an image of the gel.

Using the gel image, roughly score PCR success for each amplicon on an excel sheet, and use the relative brightness of products to estimate how much of each to add to the amplicon pool.

Note

Do not store PCR products too long in the fridge before the next steps, as products in strips can dry out.

3 **Normalise and pool (PCR products from multiple loci per sample)**

Pool amplicons for each sample (taking into account approx. concentration and fragment size) to give a total volume of c. 20 µl.

(Skip this step if you have a single amplicon per sample.)

4 **AmpureXP bead clean (I)**

Note

Use the beads and the amplicons at room temp.

AMPureXP beads may be light sensitive — take sensible precautions - keep in the fridge when not in use; keep out of direct light where possible.

1. Prepare 80% ethanol (400 µl per sample — use a disposable plastic reservoir) (20 ml for 48 samples = 16 ml ethanol and 4 ml sigma water).
2. Vortex AMPureXP beads well, then add at least 1.3x vol beads to samples (26 µl for a 20 µl sample). For speed and simplicity, add the maximum number of µl for each strip, rather than adjusting the bead volume for every sample. Re-vortex beads after the end of every strip.
3. Mix beads and samples by vortexing (with quick spin down if needed); visually check that the layers of liquids have mixed through.
4. Leave for > 8 minutes (or stop for lunch 😊); vortex again half way through if beads look like they're settling out.
5. Move strips to magnetic rack, wait > 1 minute or until beads pellet and liquid is clear; sometimes rotating the strips on the magnet or using the block magnet helps get a sharper pellet.
6. Remove and dispose of supernatant, using multichannel with 200 µl tips.
7. With the strips still on the magnetic rack, quickly add 200 µl 80% ethanol (use multichannel) — if you are too slow, the ethanol will drip.
8. Leave ethanol covering pellets for > 30 seconds (do not mix).
9. Remove as much ethanol as possible without disturbing the pellet, using a multichannel with 200 µl tips.
10. Repeat ethanol wash (i.e., steps 7-10).
11. Remove as much ethanol as possible (use a P10 or P20) and air dry for a few minutes (not too dry — pellet surface should be slightly wet/shiny not starting to crack).
12. Working off the magnet, add 12 µl resuspension buffer (0.1X TE buffer) (mix with multichannel 10-15 times, light spin and leave for a few minutes to elute).
13. Move the strips back to the magnet, pellet the beads, and transfer 11 µl cleaned PCR product to new strips for library prep. Dispose of the old strips with beads in them.

5 **Normalization II (optional)**



1. Check the concentration of the cleaned pooled amplicons using the spectrophotometer setting on the deNovix, using 1.5 μl sample for each reading; blank with 0.1X TE. Wipe clean with tissue between readings.
2. Roughly normalize samples relative to each other using 0.1X TE, so that there are none that are significantly higher than all the others (do not normalize to the lowest concentration). (The ideal concentration is 11 ng/ μl , but nanodrop readings are unreliable so do not attempt to get to this concentration...)

Note

200 fmol (ca. 130 ng for 1000 bp amplicons) is the recommended input for Native Barcoding Kit 24 V14, for full reaction MinION runs.

We use half, third or quarter reactions for End prep and Barcode ligation steps in this protocol.

The NEB calculator can calculate relative mol from ng (<https://nebiocalculator.neb.com/#!/dsdnaamt>)

Library Preparation

6 End Prep

Note

Perform all reactions on ice.

We do not use the diluted DNA control sample.

Starting material: cleaned/normalised samples in strips

	A	B	C	D	E
Reagent		Volume (half reaction), μl	Volume (third reaction), μl	Volume (quarter reaction), μl	Master Mix, μl
amplicon pool		6.25	4.17	3.124	n/a



	A	B	C	D	E
	ultra II End-prep Reaction Buffer	0.875	0.483	0.4375	
	ultra II End-prep Enzyme mix	0.375	0.25	0.1875	
	Total volume	7.5	4.9	3.75	--

Volumes for master mix calculations for library end prep

1. Put the appropriate volume of the amplicon pool into new strips.
2. Make a master mix of End-prep buffer and End-prep enzyme (x µl per sample, plus c. ½ to 1 reaction extra, depending on the total number of samples).
3. Add the appropriate µl master mix to each amplicon pool in the strips.
4. Set **thermal cycler lid to 75°C** and incubate at 20°C for 15 mins, then 65°C for 15 mins.

Note

At RBGE, the programme is set in 'Main' 'endp' (e.g., on the 'Moomin Tree' thermocycler).

7 Barcode Ligation

This uses the **NEBNext ultra II Ligation Module**.

Note

The original ONT protocol uses NEB Blunt/TA Ligase Master Mix, but this protocol is modified to use NEBNext ultra II Ligation Module. We use either a half, third or quarter reaction.

Perform all reactions on ice.

Native barcodes - note that there may be barcodes that have already been totally used up; spin the barcode plate down if needed.

Ligation enhancer is extremely sticky (and expensive); only insert tip of pipette into liquid and pipette very slowly).

EDTA - tube supplied in NEB kit; defrost, mix and transfer into a strip before use.

Starting material: end-prepped samples in strips



	A	B	C	D	E
	Reagent	Volume (half reaction), μl	Volume (third reaction), μl	Volume (quarter reaction), μl	Master Mix, μl
	End-prepped DNA	7.5	5	3.75	n/a
	Native barcode	1.25	0.833	0.625	n/a
	ultra II Ligation Master Mix	4.2	2.8	2.1	
	Ligation enhancer	0.14	0.09	0.07	
	Total volume	13.09	8.727	6.545	--

Volumes for master mix calculations for barcode ligation

1. Make a master mix of Ligation Master Mix and Ligation Enhancer using the volumes in the table (e.g., for a quarter reaction, 2.17 μl per sample plus c. $\frac{1}{2}$ to 1 reaction extra, depending on the total number of samples).
2. Add barcodes to each sample (e.g., for a quarter reaction, 0.625 μl), first making a hole in the foil lid of each well in the row with a green pipette tip (use the multichannel), then pipetting barcodes into the corresponding sample strip using a 10 μl multichannel. Reseal holes with sticky foil, re-number rows, and note which barcodes have been used on the box. Make sure you record which barcodes you have added to which samples!
3. Add master mix to each well (e.g., for a quarter reaction, 2.17 μl). Mix by pipetting.
4. Incubate at 20°C for 20 minutes, **heated lid OFF**.
5. Add EDTA to stop the reaction (half reaction 0.5 μl , third reaction 0.33 μl , quarter reaction 0.25 μl) using 10 μl multichannel, mix by pipetting, then spin down.

Note

At RBGE, the EDTA is in the nanopore box kit in the freezer — in a strip

Pool and clean up barcoded samples

- 8 1. Pool the barcoded ligated samples (put approx. 2 μl - 3 μl of each sample into a single 1.5 ml LoBind tube).



2. Remove 50 μ l of the above pooled sample and put it in a new 1.5 ml LoBind tube.
3. Perform AMPureXP bead cleaning (as previously), adding 1.4 vol. beads (e.g., 65 μ l for 50 μ l sample – it's also possible to use this step to concentrate your samples by starting with more than 50 μ l and increasing the volume of beads, but not increasing the elution volume).
4. Ethanol washes - use 500 μ l of 80% ethanol instead of 200 μ l, to better cover the beads.
5. Following the bead clean, elute in 32 μ l sigma water, pipetting off 30 μ l.

Adaptor ligation and clean-up

9 Adaptor ligation

Note

Do this the day you will be loading the flongle.

Keep reagents on ice, perform reactions on ice.

For 1000 bp amplicon sequencing, we use Short Fragment Buffers (SFB). The AMPureXP bead volume is increased to obtain short fragments c. 200-1000 bp.

Starting material: 15-30 μ l barcoded/pooled sample

	A	B	C
	Reagent	Volume (full reaction), μ l	Volume (half reaction), μ l
	Pooled barcoded sample	30	15
	Native Adapter (NA)	5	2.5
	NEBNext Quick Ligation Reaction Buffer (5X)	10	5
	Quick T4 DNA Ligase	5	2.5
	Total	50	25

Note**Before use:**

NEBNext Quick Ligation Reaction Buffer (5X): vortex, spin down.
quick T4 DNA Ligase: do not vortex - very sticky and expensive.

1. Add the above reagents to the tube with the 30 μ l barcoded sample, mix by pipetting 10-20 times between each addition.
2. Incubate in thermal block (for tubes), with heated lid off, for 20 minutes at 20°C.

10 Clean-up

1. Add 80 μ l (full reaction) or 40 μ l (half reaction) re-suspended AMPureXP beads to the barcoded amplicon sample (1.6X vol.).
2. Mix by pipetting and leave for 10 minutes at room temperature.
3. Spin down the sample and pellet on magnet rack (tube hinge to the back).
4. Remove supernatant.
5. Add 125 μ l SFB (which acts like ethanol here), flicking gently to resuspend beads, and quick spin down. Place tube on magnet. Once beads pellet, remove SFB supernatant.
6. Repeat SFB wash once more.
7. Pipette off any residual supernatant with a P10 or P20.
8. Add 15 μ l (full reaction) or 10 μ l (half reaction) Elution Buffer (EB), and resuspend pellet/beads by pipetting.
9. Incubate at 37°C for 10 minutes.
10. Pellet the beads on the magnet and move the library (supernatant) to a new LoBind tube.
11. Perform Qubit to check library concentration (do two and take the average).
12. The Flongle requires 3-4 ng/ μ l. Dilute sample to c. 4 ng/ μ l. Repeat Qubit. Dilute again if still too high, and repeat Qubit until close to correct concentration. [Preparing enough master mix for two standards and for two rounds of qubit, i.e., c. 1200 μ l, is realistic, and saves having to prepare standards twice]. Flongle final input library is 5-10 fmol (in 5 μ l elution buffer EB) – c. 3-4 ng/ μ l.
13. Library is ready to load. For same-day use, the library should be stored on ice or at 4°C. (Longer term storage should be at -80°C.)

Note

For a MinION run, make up the library to 12 μ l at 10-20 fmol (for 1000 bp amplicons, 12 μ l at 15 fmol is about 10 ng/ μ l concentration).

For Qubit HS DNA fluorometer:

1. Make a master mix of 199 μ l HS buffer plus 1 μ l HS reagent for each tube (two tubes per sample, plus two standards); add approx. $\frac{1}{2}$ extra reaction to allow for pipetting error.
Using Qubit tubes, add 190 μ l master mix and 10 μ l standard (at room temperature) to the standard #1 and standard #2 tubes. For each sample, add 199 μ l master mix and 1 μ l sample.
2. Vortex, and leave to sit for a few minutes. Spin down if there are bubbles.
3. On the deNovix, run standards (set a new standard curve), then measure samples, save as a .csv. Calculate the average concentration from the two readings for each sample.

Flongle checks

11 Prep and considerations

- **Important: The morning of the run, check for updates, complete updates and restart (don't snooze or delay).**
- Log in as a local user (do not log in to ONT account to use MinKNOW); change 'active hours' in settings (to max 18 hr before starting run); turn off any news, One Drive, etc.
- Plug in MinION, remove dummy, insert Flongle adapter and flow cell (once plugged in, do not unplug!).
- Ensure the device is flat.
- Do a pore scan (it takes a while to come to temperature); ideally there should be at least 50 available pores—record this info. If the flongle was delivered less than a month ago and there are fewer than 50 pores, it can be replaced at no cost to us, so let STS know ASAP.
- While pore scan is completing, thaw Sequencing Buffer (SB), Library Beads (LIB), and Flow Cell Flush (FCF) (Flongle expansion kit) and Flow Cell Tether (FCT) (sequencing kit), vortex, spin down and store on ice. Write the number of uses on the Flongle expansion kit.

Note

Report the flow cell ID and pore number on the RBGE Flongle log. One of the simplest ways to do this is to write the number of pores with a Sharpie on the foil flow cell packet, and keep the packet until the data is recoded on the log.

Flongle library and run

12

Note

NEB has reported that contaminants seeping out of plastic tubes affect the Flongle flow cell system. To avoid this, Sequencing Buffer (SB), Library Beads (LIB), and Flow Cell Flush (FCF) are provided in glass vials in the Flongle expansion kit.

Reagent	Volume
Sequencing Buffer (SB)	15 µl
Library Beads (LIB)	10 µl
DNA library	5 µl
Total	30 µl

1. In a new LoBind tube, mix 117 µl FCF with 3 µl FCT—mix by pipetting.
2. In another LoBind tube, make the final library and store on ice:
3. Vortex the loading beads immediately before use.
4. Prime the flow cell: peel back the seal tab to expose the sample port (stick the seal tab to the MinION lid), ensure there is no air gap at the end of the pipette tip and dispense just under 120 µl with the P200 (twist the top of the pipette rather than pushing down, to dispense very slowly into flongle port - to avoid flushing too rapidly and damaging pores).
5. Load the sequencing mix: with P200, mix well immediately before dispensing as above (by twisting not pushing and ensuring no air is introduced). NB: if you do get some bubbles, go ahead with the run; there's nothing to lose at this point!
6. Replace seal sticker to original position.
7. Place light blocker on flow cell.
8. Close the device and set up sequencing run.

13 Sequencing run

1. Click 'Start Sequencing' on the start page to set up the sequencing parameters.
2. Name the experiment (e.g., Flongle1_amplicon_2024_Feb_16, sample ID (barcodes you used: Flongle1_bc1-26_28-44_46-50), type of flow cell.
3. Continue to **kit**, and enter flow cell ID (6 digits printed on flow cell, e.g., AAC 758) and enter the kit used (this will usually be 96 barcodes unless you have just used the first 24 barcodes).

4. Continue to **run** options, run until 'flow cell is' 'end of life' to get maximum data.
5. It should be possible to use "Super accurate base calling" at RBGE, as the PC is powerful enough.
6. Continue to **analysis**—'basecalling on', 'barcoding on'.
7. Retrieve data from :/C/data/name of experiment (Flongle1_amplicon...)
8. If the run has stopped prematurely, with pores still active, try running again.
9. Report your run on the RBGE Flongle log sheet – number of ligations, number of pores, amount of data generated, run time, and any comments that might be useful.

Note

At RBGE: copy data to Z drive (once finished) and put on Data Heap using our data management folder-naming conventions; there is also an external hard drive plugged in for you to back up your run.

Data Analysis

14 Data analysis

1. Run the data through the Petithebi pipeline (<https://github.com/Gesners/petithebi>) on the CropDiversity server.
2. The pipeline outputs consensus files for each barcode that can then be BLASTed against NCBI to work out what loci they are from.
3. Loci will contain either all or part of their PCR primer sequences, and sometimes parts of adaptor sequences too. They may also have stuck to other loci during the barcode ligation step, generating concatenated reads of more than one locus. Loci may be in forward or reverse orientation. Programmes like Sequencher can be used to sort, trim and flip sequences into the right orientation, so that the reads can be uploaded to BOLD.
4. Quality is difficult to estimate – generally very high read numbers confer greater reliability, but some contigs with only 20+ reads can match the relevant taxon on GenBank with 100% sequence identity. Also, more than one copy of a marker can be present in the data – BOLD does not handle multiple copies of a marker for a specimen, so upload the copy with the highest number of reads.



Note

At RBGE: Add the EDNA number and morphological ID to each fasta sequence before the BLAST step (e.g., >EDNA24-0065638|Sphagnum_beothuk_consensus_barcode15.trimmed_2_0(5323)), and then when you have the BLAST results, add the locus or add an indication that the sample either does not match anything in GenBank, or has matched to a contaminant (e.g., >EDNA24-0065638|ITS2_Sphagnum_beothuk_consensus_barcode15.trimmed_2_0(5323); >EDNA24-0065638|NOMATCH_Sphagnum_beothuk_consensus_barcode15.trimmed_2_0(5323)).

General notes

15

Note

Potential tweaks (based on an NHM protocol)

- Doubling the incubation timings of end-prep (use a shaking block);
- Potentially draw out a bit of storage buffer prior to loading;
- Final library of 500 ng DNA in 25 ul (20 ng/ul).

Protocol references

This protocol is based on: Kanae Nishii, Michelle L Hart, MK Akhil, Laura L Forrest, Santhosh Nampy, Subhani Ranasinghe, Aaron Jeffries & Michael Mölller, "Validating Oxford Nanopore Technologies amplicon sequencing as an alternative to Sanger sequencing for generating plant molecular markers" (submitted 2025, New Zealand Journal of Botany), and uses the Petithebi bioinformatics pipeline (<https://github.com/Gesners/petithebi>) developed by Kanae Nishii.



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