JAN 18, 2023

Protocol Citation: Laura L Forrest, Michelle Hart 2023. DNA Barcoding Standard Operating Protocol, Plants and Lichens at RBGE, Lab methods: PCR and Sequencing. **protocols.io** https://protocols.io/view/dna-barcoding-standard-operating-protocol-plants-a-cmvcu62w

Protocol status: Working
We use this protocol and it's working

Created: Jan 17, 2023

Last Modified: Jan 18, 2023

PROTOCOL integer ID: 75396

Keywords: DToL, RBGE, Plant Barcoding, Darwin Tree of Life, Lichen, rbcL, nrITS, psbA-trnH, ITS2, Sanger sequencing

DNA Barcoding Standard Operating Protocol, Plants and Lichens at RBGE, Lab methods: PCR and Sequencing

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

In 1 collection

Laura L Forrest[1], Michelle Hart[1]

[1]Royal Botanic Garden Edinburgh

Laura L Forrest
Royal Botanic Garden Edinburgh

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 amplification and Sanger sequencing of DNA extractions from plant and lichen samples for the Darwin Tree of Life project at the Royal Botanic Garden Edinburgh.

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: Land plants (Embryophyta) and lichens
Including: Bryophyta, Marchantiophyta, Anthocerotophyta, Lycopodiophyta, Polypodiophyta, Pinophyta, Cycadophyta, Ginkgophyta, Gnetophyta, Magnoliophyta, lichenized fungi

Excluding: non-lichenized fungi

GUIDELINES

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

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

Marker choice
For plant accessions that are required for DToL identification/sample tracking purposes:

- Bryophytes (liverworts, hornworts and mosses) are amplified for plastid markers rbcL and psbA-trnH and nuclear marker ITS2.
- Ferns and lycophytes are amplified for plastid marker rbcL.
- Seed plants are amplified for plastid marker rbcL and nuclear marker ITS2.
- Lichens are amplified for nuclear marker ITS.
- The plastid marker trnL-trnF is a subsidiary marker 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

PCR visualization

- We save our gel images in a DToL Gel folder on the RBGE DNA server, then copy them into electronic lab books.

PCR failure (Before sending for sequencing):
If there is no band on the gel for a given amplification, the PCR should be repeated.

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.

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

If there are multiple bands, particularly for variable-length markers (usually ITS2 and psbA-trnH), PCR may be repeated with alternative primer pairs (where available), or at a higher annealing temperature. If this happens repeatedly then the DNA can be re-extracted from the silica-gel dried tissue, to rule out sample contamination, or the sample may pass barcoding with just a single DNA barcoding locus.

At least one repeat PCR should be carried out for all poor / failed amplifications in a batch.

If over 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.

Sequencing failure (after sending for sequencing):
If a sample that was sent for sequencing has generated a low quality read, or failed to sequence:
First check the reads to rule out microsatellites close to the primers causing the failure,
If that is not the reason, check the PCR gel images to estimate the quality of the PCR product (band tightness and brightness).

- If the PCR band quality in the jpg is high and microsatellites were not the problem, sequencing can be reattempted from the stored, cleaned PCR product.
- If the PCR band quality in the jpg is low, PCR can be repeated as above (PCR failure section).
- If microsatellites are causing the problem, unidirectional sequence can be accepted if it is of high quality, or alternative primers can be tried if there are any available.
- Unreadable sequences for ITS2 can also be due to co-amplifying endogenous fungi, particularly in liverworts, so failures in these cases are to be expected and will not be rectified by redoing the extraction or the PCR; occasionally PCR with alternative primers rectifies this, but time constraints mean that this is usually not feasible.
- If sequence reads contain more than one peak at any position (except for where wobbles are likely to be real i.e. in diploid or concatenated markers, in this case the nuclear ribosomal repeat region ITS), indicating contamination, PCR can be repeated from the extracted DNA if only a single marker is affected (therefore the double peaks are likely due to contamination during PCR or sequencing), or the extraction should be repeated if all markers are similarly affected (therefore the double peaks are likely due to contamination at the DNA extraction stage).

Note

Where a high quality unidirectional sequence read exists, the read is usually accepted for DToL purposes and uploaded to BOLD, as waiting for the missing sequence to get a bidirectional read could cause a month or more delay to the sample. However, the sample may still be rerun at a later date and the read data on BOLD subsequently updated.

	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

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.

PCR

dNTPS: combine stock solutions of dATP, dCTP, dGTP and dTTP into 500 µl aliquots of all four dNTPs at 2 mM each dNTP (e.g. if each stock dNTP is 100 mM, make a 1:50 dilution by combining 920 µL water and 20 µL each dNTP to make 1 ml working solution).

Primers, desalted: make up 100 µM stock solution. Working aliquots are a 1:10 dilution to 10 µM. Avoid excessive freeze-thaw cycles by making several aliquots of 200-500 µL, and keeping very frequently-used working aliquots in the fridge.

	A	B	C	D	E	F
	Locus	Primer name (as given in BOLD) / common primer name	Taxon	Primer direction	Primer sequence	Primer reference
	rbcL	rbcLa_f	land plants	forward	ATGTCACCAC AAACAGAGA CTAAAG	Kress, Erickson. 2007. PLOS ONE 2(6): e508. https://doi.org/10.1371/journal.pone.0000508

	A	B	C	D	E	F
	rbcL	rbcLajf634R	land plants	reverse	GAAACGGTCTCTCCAACGCAT	Fazekas et al. 2008. PLOS One 7 e2802
	rbcL	M745R	bryophytes	reverse	CTTCACAWGTACCTGCRGTAGC	Lewis et al. 1997. Mol. Phylogenet. Evol. 7(3):377-93. doi: 10.1006/mpev.1996.0395
	rbcL	rbcL-aar	land plants	reverse	CTTCTGCTACAAATAAGAATCGATCTC	Kress, Erickson. 2007. PLOS ONE 2(6): e508. https://doi.org/10.1371/journal.pone.0000508
	psbA-trnH	psbA501F	bryophytes	forward	TTTCTCAGACGGTATGCC	Cox in Forrest, Crandall-Stotler. 2004. Monographs in Systematic Botany, Missouri Botanical Garden, 98, 119-140
	psbA-trnH	trnHR	land plants	reverse	GAACGACGGGAATTGAAC	Sang et al. 1997. Amer. J. Bot. 84(9): 1120-1136
	ITS2	ITS2.seqF	bryophytes	forward	AACAACTCTCAGCAACGG	Olsson et al. 2009. Bryologist 112: 447-466
	ITS2	ITS.4bryo	bryophytes	reverse	TCCTCCGCTTAGTGATATGC	Stech et al. 2003. Australian Syst. Bot. 16: 561-568
	ITS2	ITS_S2F	seed plants	forward	ATGCGATACTTGGTGTGAAT	Chen et al. 2010. PLoS ONE 5, 1–8; Chiou et al. 2007. Planta Medica 73, 1421–1426
	ITS2	ITS_S3R	seed plants	reverse	GACGCTTCTCCAGACTACAAT	Chen et al. 2010. PLoS ONE 5, 1–8; Chiou et al., 2007. Planta Medica 73, 1421–1426
	ITS	ITS1-F/ITS1F	lichens	forward	CTTGGTCATTTAGAGGAAGTAA	Gardes, Bruns.1993. Mol. Ecol. 2(2):113-8. doi: 10.1111/j.1365-294x.1993.tb00005.x.
	ITS	ITS4	lichens	reverse	TCCTCCGCTTATTGATATGC	White et al. 1990. In: PCR Protocols: a guide to methods and applications. (Innis et al., eds). Academic Press, New York, U.S.A.: 315-322
	trnL-trnF	trnLUAAc/trnLC	land plants	forward	CGAAATCGGTAGACGCTACG	Taberlet et al.1991. Pl. Mol. Biol. 17: 1105-1109.
	trnL-trnF	trnFGAAf/trnLF	land plants	reverse	ATTTGAACTGGTGACACGAG	Taberlet et al. 1991. Pl. Mol. Biol. 17: 1105-1109.

DNA barcoding primer details

PCR additives

- **TBT-PAR**, 5x: 750 mM trehalose, 1 mg/mL nonacetylated bovine serum albumin (BSA), 1% Tween-20, 8.5 mM Tris hydrochloride, pH 8.0.

CITATION

Samarakoon T, Wang SY, Alford MH (2013). Enhancing PCR amplification of DNA from recalcitrant plant specimens using a trehalose-based additive.. Applications in plant sciences.

LINK

https://doi.org/10.3732/apps.1200236

Note

To make 10 mL of 5x TBT-PAR (Samarakoon et al. 2013):

Make up a 20 mg/mL BSA solution (store in 0.5 mL aliquots in the freezer).
Make (and freeze 1 ml aliquots of) 10% Tween-20 detergent.
Prepare 8.5 mL of 10 mM Tris-HCl buffer (8.415 ml water plus 85 µl 1 M Tris-HCl).

Dissolve 2.835 g trehalose in 6 mL 10 mM Tris-HCl buffer; adjust the total volume to 8.5 ml, and add one 0.5 ml aliquot of BSA, and one 1 ml aliquot of Tween-20.

Aliquot into 2 ml eppendorfs and freeze until required.

- **Combinatorial Enhancer Solution** (CES), 5x: 0.54 M betaine, 1.34% dimethyl sulphoxide (DMSO), 10 µg/µl BSA.

CITATION

Ralser M, Querfurth R, Warnatz HJ, Lehrach H, Yaspo ML, Krobitsch S (2006). An efficient and economic enhancer mix for PCR.. Biochemical and biophysical research communications.
LINK
https://doi.org/

Note

To make 50 ml of 5x CES (modified from Ralser et al. 2006), combine 27 ml of 5 M betaine, 3.35 ml of DMSO and 125 µl of 20 mg/ml BSA with 19.525 ml water. Aliquot and freeze.

- **Betaine** (5 M): 27g Betaine inner salt monohydrate made up to 40 ml with molecular biology grade Sigma water or Millipore filtered water.
- **Trehalose** (2 M): 3.78g D-(+)-Trehalose dihydrate made up to 5 ml with molecular biology grade Sigma water or Millipore filtered water.
- **DMSO**: as supplied.
- **BSA**: stock at 20 mg/mL.

Water: Molecular biology grade Sigma water or Millipore filtered water.

Polymerase:
It is useful to keep stocks of:

- Low-cost unspecialized taq polymerase (e.g. **BIOTAQ™ DNA polymerase** from Bioline) for rbcL, psbA-trnH and ITS, with buffer and $MgCl^2$.
- Specialized polymerases for matK amplification and other problematic PCRs, e.g. **Platinum® Hot-Start Taq DNA polymerase** (Invitrogen™), **Phusion® High Fidelity DNA polymerase** (New England Biolabs/ Thermo Scientific®), with buffers and $MgCl^2$.

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

- **ExoSap-IT** (GE Healthcare).

DNA sequencing

- **ABI BigDye® Terminator Cycle Sequencing kit** (either version 1.1 or 3.1) plus BigDye® Terminator v1.1, v3.1 5x sequencing buffer.
- **BigDye® Enhancing Buffer BDX64** (MCLAB): big dye dilution buffer.
- **Primers:** take aliquots of the PCR primers.

Before starting - pre-PCR lab

1

1. Set up a laminar flow hood in a pre-PCR lab with tips, strips, sharpie etc. Get a freezer rack out the freezer.
2. Take PCR reagents out of the freezer and allow to defrost. Keep the polymerase in the freezer rack. Once reagents have defrosted, put them in the freezer rack to keep cool. Take water aliquot out of the fridge and put it on the freezer rack
3. Take the DNA out of the fridge, and sort the tubes for amplification into rows of 8 in a new rack; leave empty rows between the rows of tubes, to allow for easier handling of the tubes when setting up the PCR.
4. Label (strip number, locus, date) sufficient strips to have a well for each DNA sample to be amplified plus an extra well for the negative control.

PCR mix

2 **Create a Master Mix for each locus.** Per reaction, add:

FOR LAND PLANTS (20 µl total volume reactions):

2 µL PCR buffer (at 10x), 2 µL dNTPs (each at 2 mM), 0.6 µL $MgCl^2$ (at 50 mM), 4 µL TBT-PAR or CES additive (both at 5x), 2 µL forward primer, 2 µL reverse primer (each at 10 µM), 0.25 µL BIOTAQ (at 5 units/µl) and 6.5 µL Sigma PCR-grade water

FOR LICHENS (12.5 µl total volume reactions):

1.25 µL PCR buffer (at 10x), 1.25 µL dNTPs (each at 2 mM), 0.6 µL $MgCl^2$ (at 50 mM), 2.5 µL TBT-PAR (at 5x), 0.5 µL forward primer, 0.5 µL reverse primer (each at 10 µM), 0.13 µL BIOTAQ (at 5 units/µl) and 4.77 µL Sigma PCR-grade water

Multiply quantities by [no. of DNA extractions to be amplified plus 1.5-3 extra (to allow for pipetting errors and a negative control)].

Vortex briefly to mix then centrifuge briefly to get the contents to the bottom of the tube.

Note

Primer sequences and references are given in a table in the Materials section.

- **rbcL:** rbcLa_F (forward), rbcLajf634R (reverse) (two alternative reverse primers to try in case of poor amplification: M745R, rbcL_aaR)
- **psbA-trnH:** psbA501F (forward), trnHR (reverse)
- **ITS2 (vascular plants):** S2F (forward), S3R (reverse)
- **ITS2 (bryophytes):** ITS2.seqF (forward), ITS.4bryo (reverse)
- **ITS (lichens):** ITS1F (forward), ITS4 (reverse)

3 Using a repeater pipette:

LAND PLANTS: Aliquot 19 µl of master mix into the bottom of each well in the strips.
LICHENS: Aliquot 11.5 µl of master mix into the bottom of each well in the strips.

Pipette 1 µl of the DNA extraction onto the upper side of each well (avoiding touching any master mix if you are using the same tip to add the DNA into PCRs for multiple loci); do not add DNA to the negative control.

Put lids on the strips, and briefly centrifuge to spin the DNA down into the master mix.

Polymerase Chain Reactions - PCR lab

4 **Take the strips through to a PCR lab and put into a thermocycler.**

Standard PCR parameters (plants):

- **rbcL:** 2 min initial denature at 94°C followed by 35-40 cycles with a 1 min denature at 94°C, 1 min anneal at 51°C and 90 sec extension at 72°C, with a final extension of 72°C for 5 min.
- **psbA-trnH:** 3 min initial denature at 94°C followed by 2 cycles with a 45 sec denature at 94°C, 45 sec annealing at 50°C and 1 min extension at 72°C, then 30 cycles with a 45 sec denature at 94°C, 45 sec

anneal at 45°C and 1 min extension at 72°C, with a final extension of 72°C for 5 min.

- **ITS2:** 4 min initial denature at 95°C followed by 30 cycles with a 1 min denature at 94°C, 1 min annealing at 55°C and 45 sec extension at 72°C, with a final extension of 72°C for 5 min.

Standard PCR parameters (lichens):

- **ITS:** 4 min initial denature at 94°C followed by 30 cycles with a 45 sec denature at 94°C, 1 min 30 sec anneal at 55°C and 90 sec extension at 72°C, with a final extension of 72°C for 10 min.

Make a note of the thermocycler and block, and of the name of the PCR programme.

5 After the cycle is complete, put the strips into a fridge in a post-PCR lab until you are ready to run them on a gel.

Gel visualization - post-PCR lab

6

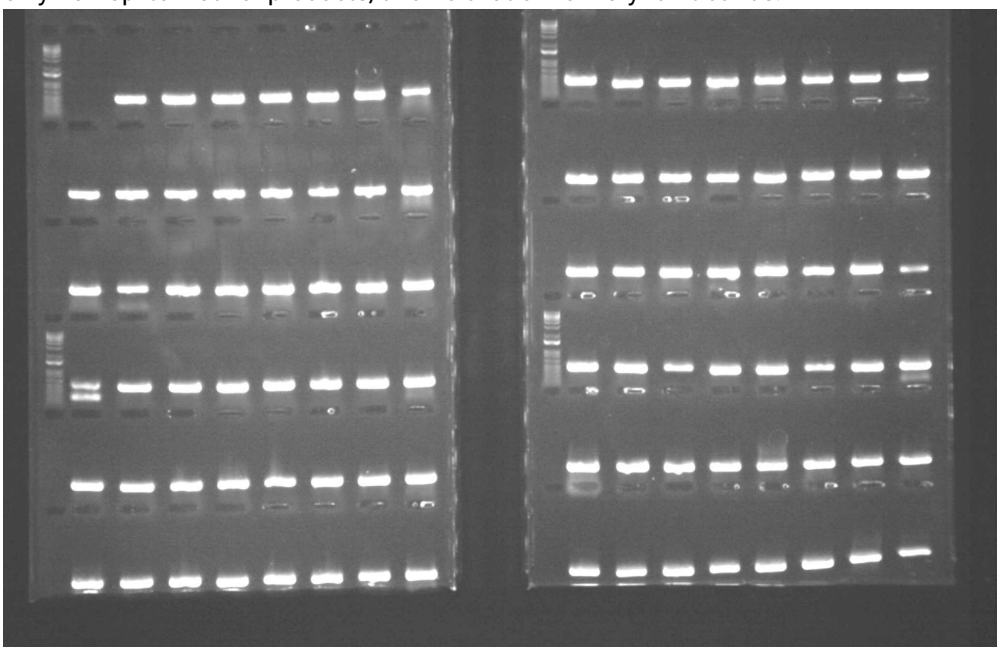
1. Make up 100 ml of 1% agarose 1x TBE-buffer gel (1 g agarose in 100 ml buffer) by mixing together in a conical flask and microwaving until the agarose is completely melted, then adding 5 µl SYBR Safe gel stain. Leave to cool for c. 20 mins or cool under running water.
2. Slot in gel ends and pour the gel into the 96-well gel rig, add comb, and leave to set for at least half an hour.
3. Add approx. 3.5 µl of each PCR product to 1.5 µl of a glycerol/bromophenol blue loading dye in a plastic plate, using an electric Rainin multichannel.
4. Remove the gel comb and ends, and cover with 1x TBE running buffer.
5. Load the samples using a Rainin manual multichannel pipette. Also add 3.5 µl of 1 kb ladder every third row.
6. Run at 80 volts for 40 mins to an hour.
7. Turn off and disconnect the power box.
8. Tip off excess buffer, carry the gel to the light box, and visualise under blue light, following the instructions for the gel imaging system.
9. The gel image jpg should be labelled with the locus name and date, exported and saved.
10. Either dispose of the gel or store for reuse. Empty the gel tank and rinse all the parts with tap water.
11. Short term, store the PCR products at 4°C in a post-PCR lab. If it is likely to take more than a couple of days to get around to cleaning and processing them, they can be stored in a -20°C freezer, to reduce the chance of the reactions drying out. If the PCRs have failed, immediately dispose of the strips in a plastic recycling bin.

Note

- Pipette tips for aliquotting loading dye can be replaced in a labelled tip box and reused multiple times.
- Pipette tips for gel loading can be replaced in a tip box and reused multiple times.
- Agarose gels can be stored in a conical flask in a dark cupboard, melted down and re-poured twice (after this they start smelling fishy). Add another 2-3 µl SYBR Safe gel stain if the gel has been stored for more than 2-3 days.

PCR clean-up - post-PCR lab

7 **Sample dilution:** Depending on the brightness of the PCR band on an agarose gel when compared to a standard, e.g. 3.5 µl of NEB's 1kb DNA ladder – successful barcode PCRs following the above protocols usually have VERY bright bands. For a good PCR product, dilute with 30 µl of Sigma water per well (using an electronic repeat 1000 µl multichannel). It may be necessary to add 45 µl water to extremely bright products, conversely only 10-15µl to weaker products, and no dilution for very faint bands.



Gel image showing ITS2 amplification - PCR product subsequently diluted with 40 µl Sigma water

Note

This step is important! Our current sequencing service has far more sensitive machines than the old service, and failure to dilute strong PCR product will cause signal leakage between capillaries, making the sequences unusable.

8 **ExoSAP-IT:** After the samples are diluted, clean them using the commercial ExoSap-IT (Affymetrix) mix.

1. Set up your tips etc. in a laminar flow hood in a post-PCR lab. Get a cool rack from the freezer, and put your ExoSap-IT in it to keep it cold.
2. Add 2 µl of ExoSap-IT to each PCR product using a 10 µl repeater multichannel pipette, putting the ExoSap-IT near the top of each well to reduce the risk of carrying over contamination.
3. Reseal lids.
4. Spin down strips.
5. Run the incubation in a thermocycler. PCR clean-up incubation parameters: 37°C for 30 min for optimal enzymatic activity, followed by 80°C for 15 min to deactivate the enzymes, then storage at 8-12°C.

6. Cleaned PCR product is stored short-term in labelled racks (project / date/ locus/ cleaned) in the post-PCR fridge. The strips are subsequently discarded, following successful sequencing, into the plastic recycling bin.

Note

- Aliquot a tube of the ExoSap-IT into a strip, and store at -20°C, so that it can be added to the PCR product using an electronic multichannel repeater pipette. Do not attempt to aliquot less than 2 µl this way, as the reagent is viscous and small volumes will not always leave the pipette tip.
- The addition of 1-2 µl of ExoSap-IT to a 10-20 µl PCR is generally more than sufficient (manufacturer's recommendations are addition of 2 µl ExoSap-IT to 5 µl PCR product).

Sanger sequencing reaction - post-PCR lab

9 **Sequencing PCR (in a total volume of 10 µl)**:

1. Label a PCR plate, or strips if you have fewer than c. 72 samples.
2. Make up one tube of master mix for each primer. Per sample, that is: 1.5 µL BigDye® Terminator sequencing buffer, 0.125 µL BigDye®, 0.32 µL primer (at 10 µM), 0.875 µL BDX-64 cycle sequencing additive, made up to 8 µl with 5.18 µL Sigma water.
3. Distribute 8 µl master mix, using a repeater pipette, to the bottoms of the wells. Do not leave gaps in the plate or the strips unless you will be able to infill them later.
4. Add the cleaned PCR product near to the top of wells, using a repeater multichannel pipette set to deliver two loads of 2 ul (so you can use the same pipette tip for the forward and reverse reactions).
5. Seal the plate with a foil lid labelled in a standard format (e.g. RBGE_dateletter, i.e. RBGE_210628A, etc, and with a note to say "sample for clean-up and sequencing, lane names in attachment"), or put lids on strips.
6. Spin down strips or plates, and transfer to one of the BioRad PCR machines in the PCR lab for cycling using the BDXsq programme:

Sequencing thermocycling parameters, following BDX-64 manufacturer's instructions: a 3 min initial denature at 96°C then 30 cycles with 10 sec denature at 96°C, 5 sec annealing at 50°C and 2 min extension at 60°C.

Note

- BDX-64 cycle sequencing additive reduces the amount of BigDye in each 10 µl reaction to 0.125 µl and cuts the length of the sequencing PCR; further dilutions of BigDye may be possible for material that routinely gives high quality DNA sequences.
- Sequence with the primers that were used for amplification.
- **Sequencing thermocycling parameters for samples without BDX-64, following BigDye manufacturer's instructions:** 25 cycles with a 30 sec denature at 95°C, 20 sec annealing at 50°C and 5 min extension at 60°C.

After the sequencing reaction is complete, store the plates/strips in the post-PCR lab at 4°C until they can be submitted for a sequencing run.

Note

At RBGE an **RBGE-DToL Sequencing form** (available from the User folder on the RBGE DNA server) must be completed for each batch of sequencing and emailed to sequencing@rbge.org.uk. Samples are sent first class post to DNA Sequencing & Services at the University of Dundee (as either half or full plates; it is not possible to send one or two samples) for clean-up and Sanger sequencing on an ABI 3730.

Sequence reads are usually returned within c. 2-3 days from posting date, and are downloaded as zipped folders by plate from the sequencing provider web site.