

Molecular barcoding of protists

PREAMBLE. Molecular Barcoding has increasingly been employed to identify and/or confirm the identity of marine protists. The protocol outlined below is that employed routinely at the Culture Collection of Algae and Protozoa www.ccap.ac.uk.

Sample harvesting and DNA extraction

1. Centrifuge a 10 ml aliquot of a dense protozoan or algal cultures for 3 min at 2000 g. Discard supernatant and transfer the pellet into 2 ml safe-lock microcentrifuge tubes.

Although this approach is satisfactory for bacterially contaminated cultures, cultures should be uni-protistan and not have fungi or yeast contaminants present.

2. Add approximately the same volume of glass beads (acid washed, 425-600 µm, Sigma-Aldrich) to the tube. Plunge-freeze the sample in liquid nitrogen and insert into Qiagen TissueLyser adapters (or equivalent). Grind immediately for 1 min at 30 Hz. Repeat grinding step up to three times. Alternatively use a sterile pestle to grind the samples and repeat also up to three times.

For most protozoan cultures and algae with fragile/ non-rigid cell walls 1-2 grinding cycles is sufficient. Some Chlorococcales and taxa that produce cysts/ resting stages may require additional grinding cycles to release cellular DNA.

When extracting DNA from diatoms use mortar and pestle to grind the cells and not glass beads. From our experience this tends to work better and provide better quality DNA.

3. Extract genomic DNA using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), or equivalent following the manufacturer's instructions.

We have also noted that the Plant DNAzol Reagent (Gicco BRL Life Technologies) works very well with diatoms in particular (following manufacturer's instructions).

PCR and sequencing

4. Amplify the SSU and ITS rDNA using the primer pair EAF3/ITS055R. We recommend the Qiagen Taq Master Mix (Qiagen GMBH), or equivalent with a final volume of 50 µL plus template DNA and the general reaction composition of:

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Protists: Method has been successfully employed for a wide variety of algae and protozoa.

Apparatus: Microcentrifuge, TissueLyser/bead shaker, electrophoresis chamber, PCR machine, ABI 3730 capillary sequencer (Applied Biosystems), ABI GeneMapper (GenePool Sequencing Facility, Edinburgh, UK)

Solutions: Plant DNAzol Reagent (Gibco BRL, Grand Island, NY, USA), Taq PCR Master Mix Kit. For standard and specialised PCR applications with minimal optimisation (2010) Catalog no. 201203, (Qiagen, Hilden, Germany), Qiagen, QIAquick PCR Purification Kit (2012) Catalog no. 28106, (Qiagen, Hilden, Germany), DNeasy Plant Mini/Maxi Kit For purification of total cellular DNA from plant cells and tissues, or fungi (2012) Catalog no. 69106, Qiagen.

Additional information:

Marin et al. 1998. Phylogenetic relationships among the Cryptophyta: Analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages. *Protist* **149**: 265-276.

Marin et al. 2003. Phylogeny and Taxonomic Revision of Plastid-Containing Euglenophytes based on SSU rDNA Sequence Comparisons and Synapomorphic Signatures in the SSU rRNA Secondary Structure. *Protist* **154**: 99-145.

ASSEMBLE

Component	Volume/reaction
Taq PCR Master Mix (x2)	25 µl
dH ₂ O	24 µl
Template DNA	0.5 to 2 µl
EAF3 (10 µM)	0.5 µl
ITS055R (10 µM)	0.5 µl

For difficult templates, it might help to add 0.5µl high fidelity Deep Vent DNA Polymerase (New England Biolabs) to the mix.

- Run the PCR using the following thermal cycling program: 2 min, 95°C; 30x (1 min, 95°C; 30s, 55°C; 30s, 68°C).
- Check the PCR products by electrophoresis on a 0.8 / 1.5 % (w/v) agarose gel.
- Purify the PCR products using QiAquick PCR purification kits (Qiagen GMBH) or equivalent following the manufacturer's instructions. They are now ready for Sanger sequencing, using the primers described in the table below (primer conc. 3.2µM).

NB: when there is more than one band on the gel, you may use the QIAquick Gel Extraction kit (Qiagen). Load 90 µL of PCR product a 0.8% (w/v) agarose gel with the addition of GelStar and Loading-dye. Run the gel for 40 min at 100V. Excise the interesting bands from the gel and purify according to the manufacturer's instructions.

If necessary, attempt a second semi-nested PCR using e.g. primer combination EAF3 / BR, and GF / ITS055R respectively.

Table Oligonucleotide primers used for amplification and sequencing

Name	Base composition 5' – 3'	Direction	Position	Reference
Annealing position in rDNA of OK1-ZK (AB162912)				
EAF3	TCGACAATCTGGTTGATCCTGCCAG	forward	1-25	Marin et al. 2003
E528F	TGCCAGCAGCYGCGGTAATTCCAGC	forward	568-592	Marin et al. 1998
536R	GWATTACCGCGGCKGCTC	reverse	571-588	Marin et al. 1998
920F	GAAACTTAAAKGAA	forward	1460-1476	Marin et al. 1998
N920R	TTCCGTCAATTCCTTTRAGTTTC	reverse	1460-1482	Marin et al. 1998
BR	TTGATCCTTCTGCAGGTGAACCTGC	reverse	2102-2126	Marin et al. 1998
GF	GGGATCCGTTTCCGTAGGTGAACCTGC	forward	2088-2115	Marin et al. 1998
GR	GGGATCCATATGCTTAAGTTCAGCGGGT	reverse	2805-2833	Marin et al. 1998
ITS055R	CTCCTGGTCCGTGTTTCAAGACGGG	reverse	Beginning of LSU	Marin et al. 2003

- On receipt of data assemble and align manually using appropriate software e.g. Geneious Pro 4.7.5.