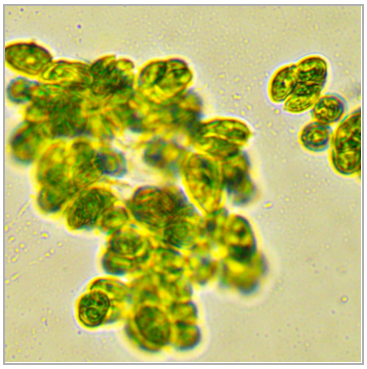

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We use this protocol and it's working

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Barcoding protocol for microalgae

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ABSTRACT

Here I describe two methods for barcoding microalgae.
One starts with extracted DNA. In that case, I use Promega GoTaq, and so far, it has performed well. If I am only barcoding, I prefer using Phire Direct PCR. as it doesn't require DNA extraction.

Both methods offer the possibility of using master mixes that include loading dye, which saves time and does not affect Sanger sequencing. Additionally, I use ExoSAP-IT as a cleaning method instead of a column-based kit. I am happy with the results, as I have successfully obtained sequences of nearly 1kb.

Regular PCR

1 PCR 18S/ITS

Use 1 ul of the extracted DNA.

	PCR reagent	1x reaction	___x reactions
	GoTaq® Green Master Mix (M712)	10 ul	
	Primer F 10 uM	0.4 ul	
	Primer R 10 uM	0.4 ul	
	DMSO	1 ul	
	Water	7.2 ul	
	DNA	1 ul	
	Total	20 ul	

Remember to add one negative reaction and one "for the pipette" (extra volume to compensate for pipetting error)

Run the PCR program (I use a touchdown PCR, starting at 63 C as annelaing temperature and reducing 0.5 C each cycle for 12 cycles. Then 25 cycles at 55 C)

Direct PCR using phire taq. No DNA extraction

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https://www.thermofisher.com/order/catalog/product/F160S

Use the Dilution & Storage protocol as follows:

1. Take a colony or an aliquot of culture (enough to see a small pellet in the tube) and place it in 20 µL of Dilution Buffer (use PCR tubes). You can crush the sample with a pipette tip by briefly pressing it against the tube wall.
2. Heat the sample to 95°C for 2 minutes and then store it at 4°C overnight before proceeding with PCR. Before usage, you can crush the sample with a pipette tip again. This step aids in breaking the cells.
3. If a larger amount of sample is used, increase the volume of Dilution Buffer to 50 µL. The solution should appear greenish.
4. Spin down the plant material, and use 0.5-1 µL of the supernatant as a template in a 20 µL

PCR reaction. The required volume of the supernatant may vary depending on the plant material used and the volume used for the dilution.

Table 1. Pipetting instructions

Component	20 µL rxn	50 µL rxn	Final conc.
2X Phire Plant Direct PCR Master Mix	10 µL	25 µL	1X
Primer A	X µL	X µL	0.5 µM
Primer B	X µL	X µL	0.5 µM
Plant tissue **Direct Protocol** **Dilution & Storage Protocol**	- 0.5 µL	0.5 mm punch/ sample of seed 1.25 µL	
H_2O	add to 20 µL	add to 50 µL	

*50 µL reaction volume is recommended for Direct Protocol.

Volumes

Table 2. Recommended cycling protocol

Cycle step	2-step		3-step		Cycles
	Temp.	Time	Temp.	Time	
Initial denaturation	98 °C	5 min	98 °C	5 min	1
Denaturation Annealing (see 6.2) Extension (see 6.3)	98 °C - 72 °C	5 s - 20 s ≤1 kb 20 s/kb > 1 kb	98 °C X °C 72 °C	5 s 5 s 20 s ≤1 kb 20 s/kb > 1 kb	35 - 40
Final Extension	72 °C 4 °C	1 min hold	72 °C 4 °C	1 min hold	1

Thermocycler program.

Check in agarose

3 I run 7 µl of the amplification reaction on 1.5% agarose gels to check for amplification and size. Since GoTaq already contains loading dyes, I only need to mix my sample with 2 µl of 1:1000 SyBr Safe dye. Then, I load the mixture onto the gel.

Enzymatic removal of PCR primers

4 ExoSAP-IT:
To use ExoSAP-IT, follow these steps:

1. Dilute the ExoSAP-IT enzyme in molecular-grade H2O at a ratio of 1:4 (0.25 µl enzyme in 0.75 µl water).
2. Add 1 µl of the diluted ExoSAP-IT mix to 1-2 µl of your PCR product. (For my application, I use 2 µl of the PCR product and 1 µl of the diluted enzyme mix, and it works well for me).
3. Place 1 µl of the mixture at the side of each well on a sequencing plate, then add your PCR reaction and mix by pipetting. Briefly spin down the plate to ensure all wells are properly filled. Since the PCR product is green, visual inspection is quick.
4. Seal the plate and run the enzyme protocol in the thermocycler. Due to the enzyme dilution, I

use slightly longer times: 30 minutes at 37°C, followed by 15 minutes at 80°C, and then hold at 4°C.

5 Add the appropriate sequencing reaction mix to each well. I use the BPC reaction mix, but with a 10 µM primer instead of 15 µM (as I don't keep a separate stock for sequencing). Additionally, I assume there might be some evaporation of my template after the ExoSAP-IT reaction. Instead of counting it as 3 µl (1 µl ExoSAP-IT + 2 µl reaction), I consider the template volume as 2 µl.

	Sequencing PCR	X1	___X
	BDT	0.5 ul	
	5X buffer	0.4 ul	
	Primer 10 uM	0.4 ul	
	DMSO	0.1 ul	
	H2O	2 ul	
	Template	2 ul (already in well)	

Remember to add one extra reaction "for the pipette" (extra volume to compensate for pipetting error)

Primers

6 Primers for sequencing the 18S barcoding region (primer pairs SSU1/C18G; or N18J/C18J)

	A	B
	SSU1-F	**TGGTTGATCCTGCCAGTAG**
	C18G-R	**TGGCACCAGACTTGCCCT**
	N18J-F	**CAATAACAGGTCTGTGATGCCCTTA**
	C18J-R	**TCTAAGGGCATCACAGACCTGTTATTG**