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DNA extraction and PCR amplification of petB gene of marine Synechococcus

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We use this protocol and it's working

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Abstract

This protocol was described in the paper "Nested PCR Approach for *petB* Gene Metabarcoding of Sorted Marine *Synechococcus* Populations". The objective was to create a nested PCR approach for amplifying the *petB* (encoding the cytochrome b_6 subunit of the cytochrome b_6f complex) marker gene from marine *Synechococcus* populations. The protocol can be used with DNA templates from samples obtained by traditional filtration methods as well as flow cytometry sorting, which often have low nucleic acid concentrations. We suggested that our protocol would be of interest to those applying flow cytometry for cell physiology measurements, such that these studies can also obtain the community composition simultaneously.

This protocol has two parts:

1) The steps used to extract the DNA from filtered marine seawater samples and flow cytometry sorted samples.
2) Standard and nested PCR amplification reactions of the *petB* marker gene.

Materials

- Ethanol 70%
- Nucleospin Plant II Mini kit **Macherey-Nagal** **Catalog #**MN740770.50
- NucleoSpin Plant II Midi kit **Macherey-Nagal** **Catalog #**MN740771.20
- Liquid nitrogen
- nuclease free water
- BSA molecular biology grade 20 mg/ml **New England Biolabs** **Catalog #**B9000S
- 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems** **Catalog #**KK2602
- FrameStar® 96 Well Semi-Skirted PCR Plate Roche Style **Catalog #**4ti-0951
- 96-well plate adhesive sealing film

Thermocycler
Spin down or centrifuge
UV hood
Pipettes
Sterile filtered pipette tips
2mL PCR clean microcentrifuge tubes

Protocol materials

- Nucleospin Plant II Mini kit **Macherey-Nagal** **Catalog #**MN740770.50
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- Liquid nitrogen
- 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems** **Catalog #**KK2602

Troubleshooting

Primers

1

	A	B	C
	Primer name	**Sequence**	**Reference**
	petB-F	5'-TACGACTGGTTCCAGGAACG-3'	Mazard et al. (2012)
	petB-R	5'-GAAGTGCATGAGCATGAA-3'	Mazard et al. (2012)
	petB-50F	5'-CAGGACATYGCTGAY-3'	Ong et al. (submitted)
	petB-634R	5'-GCTTVCGRATCATCARGAAG-3'	Ong et al. (submitted)

Primers used in this protocol to amplify the *petB* (encoding the cytochrome b_6 subunit of the cytochrome b_6f complex) marker gene of marine *Synechococcus*. Overhang sequences were specified by the sequencing facility and appended to the 5' end of respective primers.

References:
Farrant, G. K., Doré, H., Cornejo-Castillo, F. M., Partensky, F., Ratin, M., Ostrowski, M., Pitt, F. D., Wincker, P., Scanlan, D. J., Iudicone, D., Acinas, S. G., & Garczarek, L. (2016). Delineating ecologically significant taxonomic units from global patterns of marine picocyanobacteria. Proceedings of the National Academy of Sciences of the United States of America, 113(24), E3365–E3374.https://doi.org/10.1073/pnas.1524865113

DNA extraction of filtered samples

2 1.5 - 2L of seawater was filtered through a 0.22µm pore-size Sterivex filter. DNA extraction from Sterivex filters require the following kits:

Nucleospin Plant II Mini kit **Macherey-Nagal Catalog #**MN740770.50

NucleoSpin Plant II Midi kit **Macherey-Nagal Catalog #**MN740771.20

Refer to https://github.com/deniseong/marine-Synechococcus-metaB/tree/main/6_DNA%20extration%20protocol for step-by-step protocol.

3 After DNA extraction, store samples in -80 °C until PCR.

DNA extraction of flow cytometry sorted cells

4 *Synechococcus* cells from seawater samples were sorted through flow cytometry and collected in Eppendorf tubes containing Tris-EDTA lysis sorting buffer (10 mM Tris pH8.0, 1 mM EDTA pH8.0 and 1.2% Triton x-100).

5 Remove the tubes with cells from the -80 °C and place them at a Room temperature in a water bath.

6 Once thawed, place the tubes in Liquid nitrogen for a few seconds until frozen.

7 Repeat steps 5 and 6 two more times.

8 After DNA extraction, store samples in -80 °C until PCR.

Set-up before PCR

9 Clean all surfaces and pipettes with 70% ethanol before working.

Use a separate set of pipettes for loading mastermix in the UV hood and loading DNA template. Ideally, pipettes used to prepare mastermix stays in the UV hood and is never used for DNA. The other set stays on the bench.

10 Use filtered pipette tips to avoid contamination.

11 Prepare mastermix in UV hood. Place PCR clean microcentrifuge tubes, PCR clean plates, seals, pipettes, pipette tips and tube racks in the UV hood.

Clean with 70% ethanol and UV for 15 minutes.

12 Store all PCR reagents (e.g. polymerase, primers, nuclease-free water, BSA) at -20 °C until use. Before using all PCR reagents, ensure that the reagent is completely thawed.
Only open PCR reagents in the UV hood. Use only filtered pipette tips to transfer the reagents. Ideally, PCR reagents are used in aliquots.

13 Dilute primers to 10 µM in clean UV hood.
For primers at 100µM, add 10µl of primer to 90µl of nuclease free water.

14 Ensure DNA template is completely thawed before adding to mastermix.

To reduce DNA degradation in the sample, place the samples in an eppendorf tube rack over ice when loading DNA template.

Standard PCR of filtered samples

15 In UV hood, prepare the mastermix in a PCR clean microcentrifuge tube.

Each sample is performed in duplicate reactions (2 × 25 µL).
For each PCR, include at least one positive control and one negative control.

Each reaction is conducted to a **final volume of 25 µL**:

- 12.5 µL
  2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602
- 0.75 µL 10 µM petB-F (0.3 µM final concentration)
- 0.75 µL 10 µM petB-R (0.3 µM final concentration)
- 0.125 to 0.5 µL 20µg/µL BSA (0.1 to 0.4 µg/µL final concentration)
- DNA template (maximum 10µL of template, 1.3 ng/µL average final concentration)
- nuclease free water to a final volume of 25 µL per sample

Always prepare extra reactions, as some solution will be lost during pipetting.

To calculate the total amount of mastermix volume per N samples, multiply above reaction volumnes by X, whereby

X = 2 x N + (number of positive controls) + (number of negative controls) + (number of spare reactions)

16 Dispense mastermix into PCR wells in UV hood.

Add nuclease free water into negative controls inside UV hood.

Add DNA template of samples and postive controls outside UV hood.

Seal PCR plates/tubes and spin down.

17 Load PCR plates/tubes in thermocycler using the following thermal conditions:

	A	B	C	D
	Temperature (°C)	Time	Cycles	
	94	5 min	1x	Initial Denaturation

	A	B	C	D
	94	30 s	30x	Denaturation
	55	30 s	30x	Primer Annealing
	72	45 s	30x	Extension
	72	6 min	1x	Final Extension
	4	∞	1x	Hold

Thermocycler settings for PCR (petB-F and petB-R primer set).

Hold at 4 °C until collection of PCR plates/tubes.

18 Pool duplicate samples.
Store in -20 °C .

19 Perform gel electrophoresis to check results of PCR.

Nested PCR of filtered and sorted samples

20 Two sequential PCR reactions are conducted for nested PCR. A nested PCR is necessary to amplify the *petB* gene of flow cytometry sorted *Synechococcus* populations.

For the first PCR reaction, prepare the mastermix in a PCR clean microcentrifuge tube in UV hood.

Each sample is performed in duplicate reactions (2 × 10 µL).
For each PCR, include at least one positive control and one negative control.

Each reaction is conducted to a **final volume of 10 µL**:

- 5 µL 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602
- 0.3 µL 10 µM petB-F (0.3 µM final concentration)
- 0.3 µL 10 µM petB-634R (0.3 µM final concentration)
- 0.05 µL 20µg/µL BSA (0.1 µg/µL final concentration)
- DNA template (maximum 4µL of template, 0.54 ng/µL average final concentration for filtered samples or volume corresponding to approximately 160-400 sorted *Synechococcus* cells)
- nuclease free water to a final volume of 10 µL per sample

Always prepare extra reactions, as some solution will be lost during pipetting.

To calculate the total amount of mastermix volume per N samples, multiply above reaction volumes by X, whereby

X = 2 x N + (number of positive controls) + (number of negative controls) + (number of spare reactions)

21 Dispense mastermix into PCR wells in UV hood.

Add nuclease free water into negative controls inside UV hood.

Add DNA template of samples and postive controls outside UV hood.

Seal PCR plates/tubes and spin down.

22 Load PCR plates/tubes in thermocycler using the following thermal conditions:

	A	B	C	D
	Temperature (°C)	Time	Cycles	
	94	5 min	1x	Initial Denaturation
	94	30 s	30x	Denaturation
	59	30 s	30x	Primer Annealing
	72	45 s	30x	Extension
	72	6 min	1x	Final Extension
	4	∞	1x	Hold

Thermocycler settings for first round of nested PCR (petB-F and petB-634R primer set).

Hold at 4 °C until collection of PCR plates/tubes.

23 Pool duplicate samples.
Store at -20 °C .

24 For the second round of PCR reaction, prepare the mastermix in a PCR clean microcentrifuge tube in UV hood.

Each sample is performed in duplicate reactions (2 × 25 µL).
For each PCR, include at least one positive control and one negative control.

Each reaction is conducted to a **final volume of 25 µL**:

- 12.5 µL
  2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602
- 0.75 µL 10 µM petB-29F (0.3 µM final concentration)
- 0.75 µL 10 µM petB-R (0.3 µM final concentration)
- 2.5 µL first round PCR product **after pooling duplicates**
- 8.5 µL nuclease free water to a final volume of 10 µL per sample

Always prepare extra reactions, as some solution will be lost during pipetting.

To calculate the total amount of mastermix volume per N samples, multiply above reaction volumes by X, whereby

X = 2 x N + (number of positive controls) + (number of negative controls) + (number of spare reactions)

25 Dispense mastermix into PCR wells in UV hood.

Add nuclease free water into negative controls inside UV hood.

Add DNA template of samples and postive controls outside UV hood.

Seal PCR plates/tubes and spin down.

26 Load PCR plates/tubes in thermocycler using the following thermal conditions:

	A	B	C	D
	Temperature (°C)	Time	Cycles	
	94	5 min	1x	Initial Denaturation
	94	30 s	30x	Denaturation
	55	30 s	30x	Primer Annealing
	72	45 s	30x	Extension
	72	6 min	1x	Final Extension
	4	∞	1x	Hold

Thermocycler settings for second round of nested PCR (petB-29F and petB-R primer set).

Hold at 4 °C until collection of PCR plates/tubes.

27 Pool duplicate samples.
Store in -20 °C .

28 Perform gel electrophoresis to check results of PCR.