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DNA extraction protocol for DNA-metabarcoding of marine phytoplankton using Zymobiomics DNA minprep kit (Zymo Research; D4300)

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The DNA extraction protocol described in this document follows the standard protocol provided with the Zymobiomics DNA miniprep kit (https://files.zymoresearch.com/protocols/_d4300t_d4300_d4304_zymobiomics_dna_miniprep_kit.pdf) with a few modifications. The protocol is part of the project "DNA-metabarcoding of marine phytoplankton" funded by the Swedish Environmental Protection Agency and the Swedish Agency for Marine and Water Management.

Phytoplankton play a key role in global biogeochemical cycles and form the basis of marine food webs. Quantitative assessment of the abundance and biodiversity of phytoplankton is therefore a natural component of environmental monitoring programs. Until now such monitoring has largely been based on microscopic counting. This is a time consuming process and requires personnel with high skill for identifying the organisms, and the smallest cells can hardly be identified with this method. There is hence an urge for faster, more precise and reproducible ways of quantifying plankton species in environmental samples. High-throughput sequencing of taxonomic marker genes (metabarcoding) has emerged as a popular alternative for studying plankton diversity. However, also this method has its limitations, and in order to be robustly implemented in monitoring programs, protocols need to be optimised and validated. The main goal of the project is to evaluate high-throughput sequencing as a tool to investigate the diversity of phytoplankon in the seas surrounding Sweden, with special focus on non-indigenous species and harmful algae. Specifically we want to test effects of storage of samples, sample volume, replication and background DNA in the water (“eDNA”), develop and evaluate an improved method for identifying phytoplankton based on sequencing a longer region of the rRNA operon and compare results from metabarcoding with results from microscope-based methods. The tests will be conducted on water samples collected as part of the Swedish National Marine Monitoring programme. This will provide a large data set covering all major sea areas surrounding Sweden and allow us to compare the metabarcoding results with taxonomic identifications obtained using microscopy as part of the monitoring programme. This collaborative project will be conducted by a team of researchers with complementary expertise in plankton ecology, marine monitoring, molecular biology and bioinformatics.

illustrated_zymobiomics_miniprep_DNA_extraction_protocol_Version_0.1_20200815.pdf

DNA_flöde_Mikael_Hedblom_SMHI.png

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https://www.umu.se/en/research/projects/dna-metabarcoding-of-marine-phytoplankton/

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DNA, plankton, barcoding, 16S, 18S, extraction, biomass, biodiversity, phytoplankton, ocean, marine, Baltic Sea, Kattegatt, Skagerakk, Baltic

protocol ,

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Before you start make sure that you have the following equipment (not supplied by the kit manufacturer) described in Table 1.

TABLE 1. Equipment required for DNA extraction with Zymobiomics DNA miniprep kit (Zymo; Cat# D4300).

A	B	C
Required equipment	**Activity**	**Catalog number (if applicable)**
Sink	General wash	
Fridge (+4°C)	Store chemicals and samples	
Freezer (-20°C)	Storage of chemicals, short storage of filters etc.	

70% Ethanol	Sterilization of benches and equipment	
Glass beakers	Disposing leftover liquids and pipette filter tips	
Micropipettes in varying sizes (1000 µl, 200µl, 10µl) with pipette filter tips, e.g. Eppendorf Reference 2	Extracting and quality check for DNA	4924000916
Forceps	Handling filters	
Petri dishes	Handling filters	
Vortex with adapter, e.g. Vortex-Genie 2 and adapter Horizontal Microtube Holder	(1) Lyse cells. Shake tubes with filters and beads (tubes comes with the DNA extraction kit), (2) Mixing extracts solution during DNA extraction	SI-A256, SI-H524
Sterile Bench	Extract DNA for sensitive samples	
Eppendorf centrifuge (1.5/2 ml rotor), capable of 16,000 x g, e.g. Sorvall Legend Micro 17	Used during DNA extraction	75002430
Rack for Eppendorf microcentrifuge tubes	Used during DNA extraction	

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Eppendorf microcentrifuge tubes 1.5 ml	Used to capture (1) eluted DNA and (2) purify eluted DNA	0030108051
Qubit Fluorometer with dsDNA HS (high sensitivity) Assay Kit	Measure DNA concentration in extracts	Q33240, Q33231

Sample/Filter collection of biomass at sea 30m

1 Filter water samples onto 0.2 µm pore size Millipore filter (Merck Millipore; GSWP04700) attached to a filter funnel connected to a vacuum source. Filter a minimum of 500 ml collected seawater. 30m

500 mL Seawater

Filter up to 500 ml of seawater at a pressure less than 200mm Hg/267mbar/27kPa. Try to keep the filtration time below 60 minutes. Start the filtration as close to sampling as possible preferably within 60 minutes. If it is not possible to start filtration within 60 minutes then incubate the water sample at close to *in situ* temperature and take note of the incubation time.

2 Using two sets of sterile forceps, pick up the filter membrane at opposite edges and roll the filter into a 2 ml cryo tube (Avantor, 479-1262) with the top side of the filter facing inward. 1m

3 ⚠ 1m

Take note of station, date, sample ID, sampling time, time duration of filtration, filtered volume and any comments of potential use (such as filtration problems, colour of filter etc.).

4 Store the filters at minimum -20°C (short-term storage) or -80°C (long-term storage) no

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additional reagent for preservation is needed.

-20 °C Freezer storage

A preservation agent, DNA/RNA Shield™ (Zymo Research;R1103), can be used to simplify storage and downstream processing while increasing the possible storage time. In theory, filters stored in cryo tubes preserved with DNA/RNA Shield™ can be kept in room temperature for >1 year until processing. For more information see separate protocol here https://files.zymoresearch.com/protocols/_r1100-50_r1100-250_r1200-25_r1100-125_dna_rna_shield.pdf.

Laboratory preparations 20m

10m

5 ⚠

- Clean your work bench with 70% ethanol.
- Prepare sterilized forceps and petri dish on the bench.
- Prepare pipettes in varying sizes with filter tips.
- Take out beakers for discarding liquids and used tips.
- Turn on the microcentrifuge and set the machine to 13000 x g for 1 minute.
- Prepare Vortex with adapter plates and set timer for 10 minutes.

6 ⚠

Prepare spike-in genes using artificial 16S and 18S rRNA gene oligonucelotides (designed in-house and generated by Twist Bioscience). See sequences and sub-steps below:

>16SrRNA_gene_1447bp_ATGCfreq_E.coli
NR_024570.1_primerbindingsites_341F_515F_805R_806R
GGGGTGAACGTCCGGCGTGGGGTCACCTAGTAAGAGAGAGCGGCCGGGACATCAATAGA
CCGGCTGAGTGTTGGGATAGGTAGGTCGCGATTGTGTTAAGTTTTGGCCGCCGTGCTTGA
AGAAAGTCCCGGGGACAGGAGGAACCGACGGTGAGCGGTGGAGATGGCAACGCGGAGCG
CCCATGAAGGAGCCAGGACTGACTGGCTTGGGACGAGTAATCTACGTGATTTTACAGAAC
CCTCGTGGGCGCTAGAGAGGAGCATCCCGTGTCTGCGGCGCATCAGCAGTGAACGGTGTC
ACTGACCAGCATCACTGGCGTATGGCGCGGGGTCCTACGGGAGGCAGCAGACGGGCAGC
TCTCGATAACCGGCGGAAGGTGGTAGCCACGGACAGGATCAGAACAATTAGAAGTGCCGC
AGGTGGCCAAGTCCCCCGGACACAAGACGAGGCCGGAGGCCTGGTATATACACGTAGCT
AAGAAGAGCTCATCCAGACTGGGAACGGTGTGCCAGCAGCCGCGGTAACATCACCACAA
CGTATTCGGTCACAAATTGATCGGAGGGAGAAATCGTCCGCAGGATCTCAAACTTTAACT
AAGGACTAGTACTACATAGGCTCGAGAAGAGCTACCGTTTGCAGGGTCGCCGGGTACCGC
TTAACCATAAAAGATCCACTCAGGTAGCCGTCCAGTTTCCTCTGAAATGATGGGGCGAGA
AACACGGCTGGGCGTTATACGAGTGCTTTAGAATATGAGGAGAGACAGGGGTATATTCAA

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GGATTAGATACCCTGGTAGTCCCGATCTCGATCTACGTCTGGACGTGACGTGTACATTCT
GGTCGAAACGGTACAAAGATCGTTTGGAGATGGAGGGGCGCGCATAAACATGAGTCAGCG
CACCTACTACACTGCATTGACGACGAGTCAAGCGGGTTGGAACCGACCCAGGACCGTGTG
CTCTGGGACATGCCTAGACAGGAAGGTGACGTATAGGAGACGTCGAGGGGGAGATATAGC
CGTGGGTCAAAGCTAATCGTACCCCGCCTTCCGCTAGCTCATAATTTGCTTCCTGGGACC
AAGCCGCTGAGGGATTACGGTATCTAGTTGCTCGGCGACTTTGGGAATAGCCACACTGAT
CGAGGTGAGAGGAAGGGAAGTATGTAAAGTTTGTCTTAGGGTACCGGCAGGTGGCGCAAT
CTTCTCCTTGCGAAGGAAGGGGGGACCGAGCATGGGTCAGAATGACTCCGGGGCGTAGGG
TACACAAGGGTGCAGCGACCAGGGCCGCGCGAATTGTTGAATGCTATGTATTATATCGAG
TGTGCGTTGGCAGCGAACGCTAAGACGTGTCGGAGCCTGCAGCTGTGAAGTTTAACCGTG
ACACCTATACAATGTTCAGAATGGGGGGTTAGTTATAGATGGTAGCCCGTTTGCAACGGT
TCCTCGCGAATC

>18SrRNA_gene__ length1261_primersites_563f/V4fw-1563(18)_TAReuk454FWD-1564(20)_TAReukREV3-1991(18)_V4rev-2017(18)_1132r-2157(18)_V9dropfw_2205(22)_V9fw-2207(18)_V9droprev-2675(19)_V9rev-2676(18)

AAGAGAGCCATGGCGTGAAGTAGCGGGATGAGTTACCCTCCATACTAGTAAATACGGCGT
GTTTTCAAATGCCCCTGCCAACCTACATAGATGGGACTAAGCCAGCAGCCGCGGTAATTC
CCTTCGTTATCGTCACGGAGAGAACTGCCTTTAGCGATCTGTCTAGAGACCCGCCAAATA
TAAGCCTTGGGGTTATTAACAAAGACGCCAAACACTAGTGAATATGACAGTGAAGGGGTG
TGAGATAGCTTTACTGGGTGAGAAAACACTCGTTAAAAAGAATTAGACCGGATAATCCCC
GAGGGGCCGTAGGCATGGACTTGTCGTTGCCACCGAGCATAGCGGTTTCGAAATAGCCGA
GATGGGCACTGGCGAATTAACCCACTGGTTTATATGGATCCGATGGGTTCACTTAATAAG
CTCGTACCAGGGATGAATAAAGCGTTACGAGAATTATAAACATGGAGTTCCTATTGATTT
GAGGTTAATACCGAACGGGAACATTTGTCGATCATGCTTCACATAGAGTTTAATCAAGAA
CGAAAGTACAGTCTTCGAAGTGGATTAGATACCGCCGACCTAGTGACGCGAATATATCGA
TGACGATCTCCTATAACGGGAGGTCTCGTTAACTGACTGTGTATGGTTGTCCGTGTTTCGA
ATTAGGAGAAGGTTAAAGACTGTGATCGTGATAAATTTAAAGGAATTGACGGGCTTTGAT
TAGTAGTTCCCGTTACCTCAATTCAATTCGAATCAACGCGGGGAAAGTCGCCGGCCAATA
TCAGCTTTTATTGATTAGTGGGGTATTAGATTGGCAGACTGAATAGCCAACAGAGTGCAG
TTATTTCCGTTACTTGATACTCGCTGCGACTTACCGGGGCTTGGTAAGTGCACCATAACA
GTGCACGTTTGCTCATCTACATATCAAAGTTTTGGTCTTTTGTCTGCTGGCTGATGCTGTA
AAGCTCGTACGACGGGCCCAGTCTACAGAACGGCTTTGTCTAAGTACTGAGGCAGGGAGC
ATGACTTGCCGAAGTGATGTAGAGTGGAATGTCGCCATCCGTGACAGTATAATGGGGCGA
GGAGAGGACTTCGCTGGGTATCTTAGTCACAGAAGAATGGCTCGGTTTTTACTTGATGAT
TTGGCCAGTATGAAACGCTCACGCCCTGTTTAGATTCTTGGAGCGCAGTATGTTAATCTA
GTCCACATCCTTTTGTACACACCGCCCGTCGTTCTTCATTGGTGTTTGGTGGGTAGTGTAA

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The sequences are random but based on the GC content of a consensus 18S of the PR2 sequences and 16S on the E.coli sequence. Both contain primer positions for several primer pairs (specified in the fasta header) that produce amplicons with a length to be expected from real samples. If needed it is easy to add other primer motifs.The oligonucleotides are delivered as a dry pellet with a concentration of 1000 ng. The goal concentration of spike-in genes is to reach around 5% of total 16S and 18S rRNA gene sequences in the final extracted sample of genomic DNA. Using results from metagenomic data across a whole season (both low and high biomass) the calculated amount of 16S and 18S rRNA gene material to be added to samples, regardless of season or biomass collected, is presented in Table 2.

TABLE 2. Calculated amount of spike-in 16S and 18S rRNA gene oligonucleotides, based on metagenomic data, to add in order to reach approximately 5% concentration in a given sample of extracted genomic DNA.

A	B	C
Spike-in gene concentration	**16S spike DNA to add (ng)**	**18S spike DNA to add (ng)**
5 %	0.00204	0.00729

6.1 Add 100 µl sterile filtered autoclaved MQ, DNase/RNase Free Water or TE buffer to the dry pellet bringing the stock solution to a concentration of 10 ng/µl.

100 µL MQ

6.2 Then, in sequence through continued serial dilution in steps of x 10 dilutions, prepare working solutions with the final concentration of the 16S and 18S spike-in DNA to 0.0002 ng/µl and 0.0007 ng/µl, respectively, note the x 10 dilution for the final concentration compared to Table 2.

6.3 Aliquot and freeze working solution in batches at -20°C until processing.

-20 °C Freezer storage

Laboratory procedure 13m

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7 Thaw filters in the sampling tubes at room temperature and add the filter cut into small pieces to a **ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm)**. 5m

Room temperature Filter thaw

What's happening? Cutting the filter membrane into the ZR BashingBead™ Lysis Tube allows for efficient bead beating and homogenization in proceeding steps.

8 Rinse the sampling tube with 750 µl and add the **Lysis Solution** into the **ZR BashingBead™ Lysis Tube**. 1m

750 µL ZymoBIOMICS Lysis Solution

Note: For samples stored and lysed in **DNA/RNA Shield™ Lysis buffer**, do not add **ZymoBIOMICS™ Lysis Solution** and proceed to step 9.

9 Add 10 µl of the prepared dilutions of **16S and 18S spikes**, and cap tightly. 30s

10 µL 16S and 18S spikes

10 ⚠ 10m

Secure in a **Vortex-Genie 2** fitted with a 2 ml **tube holder assembly (adapter)** and process at maximum speed for 10 minutes.

3200 rpm, Room temperature , 00:10:00

Note: Vortex for 10 min at maximum speed (2850Hz) on a Vortex-Genie 2 is a specific selected step and slight modification from the standard ZymoBIOMICS protocol.

What's happening? The mechanical action of bead beating will break apart the surface of the filter membrane that contains trapped cells and aids in cell lysis. Use of the vortex adapter will maximize homogenization by holding the tubes equal distance and angle from the center of rotation.

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11 1m

Centrifuge the **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)** in a microcentrifuge at 13,000 x g for 1 minute.

13000 x g, Room temperature, 00:01:00

What's happening? The supernatant is separated and removed from the filter membrane and beads at this step.

12 1m

Transfer up to 400 µl supernatant to the **Zymo-Spin™ III-F Filter (red mark)** in a **Collection Tube** and centrifuge at 8,000 x g for 1 minute. Discard the **Zymo-Spin™ III-F Filter**.

400 µL supernatant

8000 x g, Room temperature, 00:01:00

What's happening? Any remaining beads, proteins, and cell debris are removed at this step. This step is important for removal of any remaining contaminating non-DNA organic and inorganic matter that may reduce the DNA purity and inhibit downstream DNA applications.

13 1m

Binding preparation: Add 1,200 µl of **ZymoBIOMICS™ DNA Binding Buffer** to the filtrate in the **Collection Tube** from Step 12. Mix well.

1200 µL ZymoBIOMICS™ DNA Binding Buffer

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14 1m

Transfer 800 µl of the mixture from Step 13 to a **Zymo-Spin™ IICR Column** in a **Collection Tube** and centrifuge at 10,000 x g for 1 minute.

10000 x g, Room temperature, 00:01:00

What's happening? The DNA is selectively bound to the silica membrane in the Spin Filter basket and the flow through containing non-DNA components is discarded.

15 Discard the flow through from the **Collection Tube** and REPEAT STEP 14. 2m

16 Place the **Zymo-Spin™ IICR Column** in a **NEW Collection Tube**. 30s

17 1m

Add 400 µl **ZymoBIOMICS™ DNA Wash Buffer 1** to the **Zymo-Spin™ IICR Column** and centrifuge at 10,000 x g for 1 minute. Discard the flow-through.

400 µL ZymoBIOMICS™ DNA Wash Buffer 1

10000 x g, Room temperature, 00:01:00

What's happening? ZymoBIOMICS™ DNA Wash Buffer is an alcohol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt and other contaminants while allowing the DNA to stay bound to the silica membrane.

18 1m

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Add 700 µl **ZymoBIOMICS™ DNA Wash Buffer 2** to the **Zymo-Spin™ IICR Column** in a **Collection Tube** and centrifuge at 10,000 x g for 1 minute. Discard the flow-through.

700 µL ZymoBIOMICS™ DNA Wash Buffer 2

10000 x g, Room temperature, 00:01:00

What's happening? ZymoBIOMICS™ DNA Wash Buffer 2 ensures complete removal of ZymoBIOMICS™ DNA Binding Buffer and ZymoBIOMICS™ DNA Wash Buffer 1 which will result in higher DNA purity and yield.

19  1m

Add 200 µl **ZymoBIOMICS™ DNA Wash Buffer 2** to the **Zymo-Spin™ IICR Column** in a **Collection Tube** and centrifuge at 10,000 x g for 1 minute.

200 µL ZymoBIOMICS™ DNA Wash Buffer 2

10000 x g, Room temperature, 00:01:00

What's happening? The second spin removes residual ZymoBIOMICS™ DNA Wash Buffer 2. It is critical to remove all traces of wash solution because the ethanol can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

20  1m

Transfer the **Zymo-Spin™ IICR Column** to a clean **1.5 ml microcentrifuge tube** and add 50 µl **ZymoBIOMICS™ DNase/RNase Free Water** directly to the center of the column matrix and incubate in room temperature for 1 minute. Centrifuge at 10,000 x g for 1 minute to elute the DNA.

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50 µL ZymoBIOMICS™ DNase/RNase Free Water

Room temperature 1m

10000 x g, Room temperature, 00:01:00

Note: Adding 50 µl of ZymoBIOMICS DNase/RNase Free Water is a specific selected step and slight modification from the standard ZymoBIOMICS protocol.

What's happening? Placing ZymoBIOMICS™ DNase/RNase Free Water (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As ZymoBIOMICS™ DNase/RNase Free Water passes through the silica membrane, the DNA that was bound in the presence of high salt is selectively released by ZymoBIOMICS™ DNase/RNase Free Water which lacks salt. ZymoBIOMICS™ DNase/RNase Free Water contains no EDTA. If DNA degradation is a concern, sterile TE may also be used instead of ZymoBIOMICS™ DNase/RNase Free Water for elution of DNA from the Spin Filter.

21  3m

Place a **Zymo-Spin™ III-HRC Filter** in a **NEW Collection Tube** and add 600 µl **ZymoBIOMICS™ HRC Prep Solution**. Centrifuge at 8,000 x g for 3 minutes.

600 µL ZymoBIOMICS™ HRC Prep Solution

8000 x g, Room temperature, 00:03:00

22 Place the prepared **Zymo-Spin™ III-HRC Filter** in a clean **1.5 ml microcentrifuge tube**. Make sure to mark the tube (both lid and side) clearly with a unique ID using a permanent marker resistant to freezing. 30s

23  3m

Transfer the eluted DNA (Step 20) to the **Zymo-Spin™ III-HRC Filter** and centrifuge at exactly 16,000 x g for 3 minutes.

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16000 x g, Room temperature, 00:03:00

What's happening? TheZymoBIOMICS™ HRC Prep Solution includes PCR Inhibitor Removal technology enabling PCR from the most PCR prohibitive environmental samples rich in humic and fulvic acids, tannins, melanin, and other polyphenolic compounds.

24 Freeze extracted gDNA at -20°C or -80°C. Measure the concentration of the extracted DNA 30m using **Qubit Fluorometer**; see separate protocol for Qubit provided by the manufacturer. The filtered DNA is now suitable for PCR and other downstream applications.

-20 °C Freezer storage

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