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DNA/RNA extraction and qPCR protocol to assess bacterial abundance in the sponge Halichondria panicea

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Abstract

This protocol summarizes experience and recommendations regarding DNA and RNA extractions from the sponge *Halichondria panicea* and qPCR to quantify bacterial abundance.
We have used both bacterial and sponge DNA and RNA for subsequent qPCR. Further, bacterial 16S rRNA amplicon sequencing was performed on DNA and sponge transcriptomes were sequenced successfully from extracted RNA.

Guidelines

work under clean bench
sterilize
contamination

Materials

Kits:

- DNA extraction sponge tissue: DNeasy PowerSoil Kit (Qiagen, Netherlands)
- DNA extraction bacterial pellet (inoculum for recolonization): Blood+Tissue Kit (Qiagen, Netherlands)
- RNA extraction sponge tissue and bacterial pellet: RNeasy Mini Kit (Qiagen, Netherlands)
- iScript cDNA synthesis kit (Bio-Rad)
- Marcherey-Nagel Nucleo Spin Clean-up kit (Marcherey-Nagel Düren, Germany)
- DNA-free DNA removal Kit (Thermo Fisher Scientific, USA)
- Qubit DNA and RNA HS Kits (Thermo Fisher Scientific, USA)

Chemicals and reagents:

- DNase/RNase-free water
- +RNase AWAY®
- RNAlater
- Ethanol
- beta-mercaptoethanol (14.3 M)
- SUPERase-IN (Thermo Fisher Scientific, USA)
- nucleic acid stain for gel electrophoresis (e.g. GelGreen, Millipore, USA)
- Maxima SYBR Green 2x Master Mix (Thermo Fisher Scientific, USA)
- tRNA solution (10 ng/µl Sigma Aldrich, Germany)

Consumables and other material:

- forceps
- scalpel
- sterile plastic petri dishes
- 2 ml polypropylene tubes
- Lysing matrix-E tubes
- 1.8 ml Cryovials DNase/RNase free

Equipment:

- Autoclave
- Finescale
- Fume hood
- PowerLyser 24 (MoBio)
- heating block / incubator at 37°C
- Biosafety cabinet (UV sterilization)
- Centrifuge - for 2 polypropylene tubes and PCR plates
- PCR cycler
- Bluelight table
- CFX96 real-time detection system (Bio-Rad, Germany)
- Nanodrop
- Qbit

- qPCR software Bio-Rad CFX Manager Software (version 3.1)

Safety warnings

RNA: work under fume hood (beta-mercaptoethanol)

DNA extraction

1 **Extract DNA from samples fixed in RNAlater (stored at -80°C) or flash frozen (stored at -80°C)** 3h

Qiagen PowerSoil Kit
Input: 70-200 mg tissue
Process max.24 samples at a time, depending on experience

1.1 Follow manufacturers protocol (lysis in PowerBeadTubes and PowerLyzer Bead-Based Homogenizer at 3500 rpm, 2× 30 sec bead beating with 45 sec pause in between)

1.2 We recommend to immediately take a 5 µl aliquot for quality and quantity control (Nanodrop, Qbit, PCR to check contamination presence of 16S DNA). DNA extracts can be frozen at -20°C or for long-term storage at -80°C

2 **Extract DNA from bacterial recolonization pellets flash frozen (stored at -80°C)** 3h

Qiagen Blood+Tissue kit
Input: cell pellet
Process max.24 samples at a time, depending on experience

> Note
>
> No good results were achieved with the Qiagen PowerSoil Kit for cell pellets.

2.1 Follow manufacturers protocol (incubation with proteinase K at 56°C for 30 min)

2.2 We recommend to immediately take a 5 µl aliquot for quality and quantity control (Nanodrop, Qbit, PCR to check contamination presence of 16S DNA). DNA extracts can be frozen at -20°C or for long-term storage at -80°C

3 **Dilute DNA extracts for qPCR** 1h

Measure DNA extracts of all samples with qbit

3.1 Dilute each extract to a concentration of 6 ng/µl with DNase/RNase-free water. Per qPCR reaction we will use 5 µl template, so the total DNA used per qPCR reaction is 30 ng

RNA extraction

4 **Extract RNA from sponge samples fixed in RNAlater (stored at -80°C)** 4h

- Clean all surfaces and instruments with ethanol and subsequently RNase-Away
- The manufacturers protocol for the RNeasy Mini Kit have been optimized for Halichondria tissue. For more details please refer to the manufacturers protocol
- Work clean and quickly to prevent RNA degradation due to long waiting times. We therefore recommend to not process more than 6-12 samples (depending on the experience of the person) at once

Safety information

beta-mercaptoethanol is highly toxic. Work under the fume-hood only and dispose contaminated material properly. Use nitril gloves.
Prior to each extraction effort, prepare fresh aliquots from concentrated beta-mercaptoethanol stock (6 µl beta-mercaptoethanol + 594 µl RLT-buffer provided with the extraction kit)

4.1 Thaw tissue samples on ice and meanwhile label lysis tubes (label on the side, since during powerlysing step labelling on the lid might disappear!)

4.2 Remove first sample from the cryovial (leave others on ice) and cut on a sterile petri dish with sterile forceps into pieces

4.3 Tare the lysis tube and load with ~80 mg tissue. Place tube on ice and proceed with the next sample (go back to step 4.2). Use a new petri dish and scalpel to prevent cross-contamination.

4.4 Add 600 µl beta-mercaptoethanol/RTL buffer under the hood and disrupt cells in homogenizer for 30 sec at a speed of 3000

4.5 Centrifuge for 10 min at maximum speed

4.6 Remove supernatant carefully without touching the pellet and transfer it to a 2 ml collection tube. From here on, work at RT

4.7 add 1 volume (~370 µl) of 70 % molecular grade ethanol and mix well by pipetting. Proceed immediately to next step

4.8 transfer up to 700 µl including any precipitate to RNeasy Spin column placed in a 2 ml collection tube (provided in kit). Close the lid and centrifuge for 30 sec at 10,000 rpm. Discard flow-through and re-use collection tube. Repeat this step if the sample exceeded 700 µl

4.9 Add 700 µl RW1 buffer and centrifuge for 30 sec at 10,000 rpm. Discard the flow-through

4.10 Add 500 µl RPE buffer and centrifuge for 30 sec at 10,000 rmp. Discard the flow-through

4.11 Add 500 µl RPE buffer and centrifuge for 2 min at 10,000 rpm. Carefully remove the spin column and place it in fresh 2 ml collection tube. Centrifuge for 1 min at max speed to remove any remaining buffer

4.12 Place the spin column in a fresh 1.5 ml Eppendorf tube and add 60 µl EB buffer. Take care to place it directly onto the spin column membrane. Close the lid and incubate for 10 min at RT. Centrifuge for 1 min at 10,000 rpm to elute RNA

4.13 Keep extracts always on ice! Proceed immediately with RNase inhibition and nuclease treatment.
Already preheat incubator or heating block to 37°C (incubator needs much longer to pre-heat than heating block)

Safety information

important! The DNase treatment was challenging for *H. panicea* extracts. For other sponge species (*Aplysina aerophoba, Dysidea avara*) we have successfully used the TURBO™ DNase (AM2238) kit for DNA removal, but it did not work for *H. panicea*: DNA remained and RNA was degraded, even if we tested different batches of the kit. We also tried unsuccessfully the on-column kit RNase-Free DNase Set (79254).
Finally, **good results** were obtained with the DNA-free™ DNA Removal Kit (AM1906) to remove DNA from *H. panicea* RNA extracts.

4.14 Under the clean bench: add 3 µl SUPERase-IN (0.05 volume of RNA) to 60 µl RNA. Mix gently

4.15 Add 6 µl (0.1 volume of RNA) 10x DNAse I buffer and 1.2 µl DNAse and mix gently

4.16 Incubate for 20 min at 37°C, mix frequently

4.17 Add 6 µl resuspended DNAse I inactivation reagent and mix well

4.18 Incubate for 2-5 min at RT, mix every minute by shortly vortexing

4.19 Centrifuge for 1.5 min at 10,300 rpm and transfer supernatant (RNA) to a fresh tube. Take care to not transfer any inactivation reagent (white)

4.20 We recommend to immediately take a 5 µl aliquot and a 2 µl aliquot, and freeze all samples at -80°C.
Use the 5 µl aliquot to assess quality and quantity (Nanodrop, Qbit, PCR to check contamination with DNA) and the 2 µl aliquot for capillary electrophoresis (e.g. Experion).
To avoid thaw-freeze cycles, the main RNA sample can be split in two equal parts, so that one sample can remain frozen until analysis at -80°C if it is intended for e.g. transcriptomics

cDNA synthesis

5 **cDNA synthesis**

Use iScript kit and follow manufacturers guidelines. Only thaw reverse transcriptase directly before adding to master mix and keep on ice!

Use 500 ng RNA per reaction and random primers

5.1 Dilute cDNA 1:5 with TE buffer and use this dilution for qPCR

5.2 Store cDNA dilutions at -20°C

Primers and standard curves

6 **Primers**

	A	B	C	D	E
	Specificity	**Primer**	**Sequence**	**Fragment length (bp)**	**Reference**

	A	B	C	D	E
	eubacterial 16S rRNA	E1052f	TGCATGGYTGTCGTCAGCTCG	141	Wang&Qian 2009
		E1193r	CGTCRTCCCCRCCTTCC		
	Ca. H. symbioticus specific 16S rRNA (DNA and cDNA)	Hal_symF	CGCGGATGGTAGAGATACCG	148	this study
		Hal_symR	TGTCCCCAACTGAATGCTGG		

Table: qPCR primers to quantify total bacterial 16S rRNA and *Ca.* Halichondribacter symbioticus. The amplified gene products for the *Ca*. H. symbioticus primers have been sequenced to confirm specificity.

Note

Important: dilute primers in TE buffer
- work under the clean bench and use sterile packed equipment only
- especially eubacterial 16S rRNA primers are prone to contamination. It is recommended to prepare aliquots in advance and thaw a fresh aliquot for each qPCR plate
- prepare 50 µM stocks in TE buffer as 10 µl aliquots and freeze at -20°C. Only use once

7 Standard curve preparation

Prepare one DNA and one cDNA standard curve. It is recommended to prepare larger volumes in order to use the same standard curve for a complete experiment, or even several experiments

7.1 Run 50 µl PCRs with primer pair intended for qPCR with DNA from different sponge individuals, e.g. 8 × 50 µl

7.2 Prepare electrophoresis gel with nucleic acid stain (e.g. GelGreen) with large pockets to load 50 µl/well

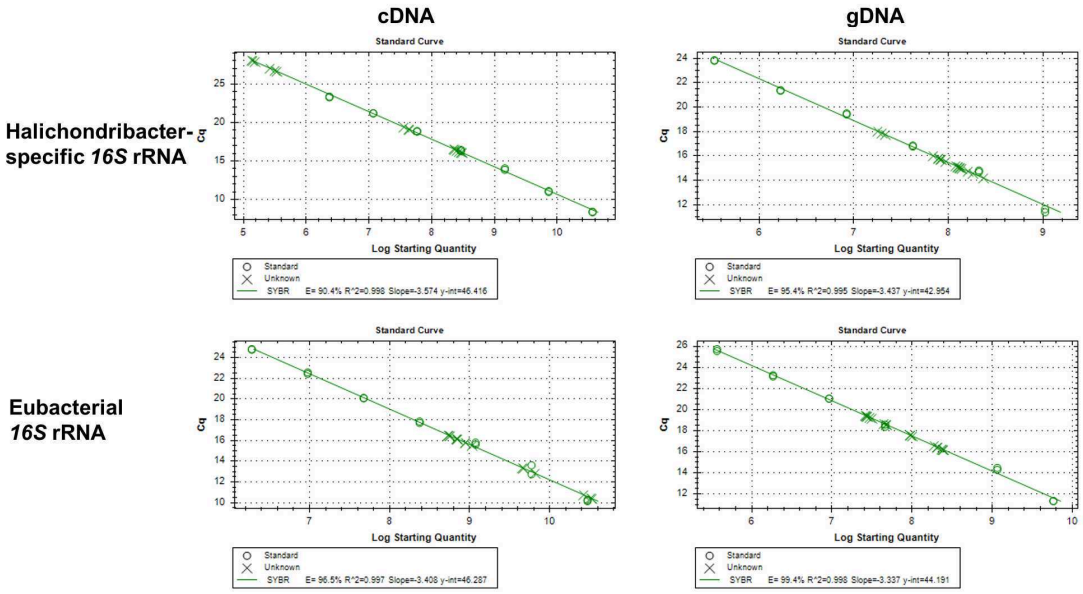
7.3 Cut out gel bands under blue light table with sterile scalpel. Clean up gel bands (Marcherey Nagel Nucleo Spin Clean-up kit) according to the manufacturers protocol and pool PCR products

7.4 Prepare dilution series:
- Probably, the concentration of the PCR products is way too high for qPCR. Start by preparing a 1:10 dilution by adding 200 µl PCR product + 1800 µl tRNA water

- Then proceed with 1:10 (200+1800 µl) or 1:5 (400+1600 µl) dilutions

7.5 Measure highest standard in QBIT (high sensitivity kit) and calculate concentration for dilutions (~ 1-5 ng/µl)
calculate copy numbers http://cels.uri.edu/gsc/cndna.html

7.6



Example standard curves for primer-pairs used with cDNA and gDNA. The efficiencies should be between 90-110 %. During the experiments, the efficiencies were consistent.

qPCR

8 **Recommended plate set-up**

Note

Important:

have standard curves on each plate

run different primers for a sample within the same plate (= don't run separate plates for total 16S and Halichondribacter)

include negative controls on each plate: just qPCR mix and purified water instead of DNA to control for contamination

9

qPCR preparation

Work under the clean bench.

Prepare master mix for each primer pair:

	A	B	C
	Template	5 µL	gDNA: Total 30 ng per reaction, cDNA: 1:5 dilution of cDNA from 500 ng RNA
	Primer F (50 µM)	0.16 µL	Total 400 nM per reaction
	Primer R (50 µM)	0.16 µL	Total 400 nM per reaction
	qPCR mix (2x)	10 µL	
	H2O	4.68 µL	
	Total	20 µL	

Table: qPCR master mix

9.1 Work in 96-well plate. Pipet 15 µl master mix per well

9.2 Add 5 µl template per well (use new pipet tip for each well)

9.3 Seal plate with optical adhesive foil (check which brand fits your plates and qPCR machine) and spin down plate briefly in plate centrifuge

9.4

Run plate immediately, or store plate at 4°C in the dark and run within 24 h

	A	B	C
	72°C	1 min (plate read)	
	96°C	2 min	40 x
	94°C	30 sec	
	60°C	30 sec (plate read)	
	60-90°C	0.5 interval, 5 sec Melt curve	

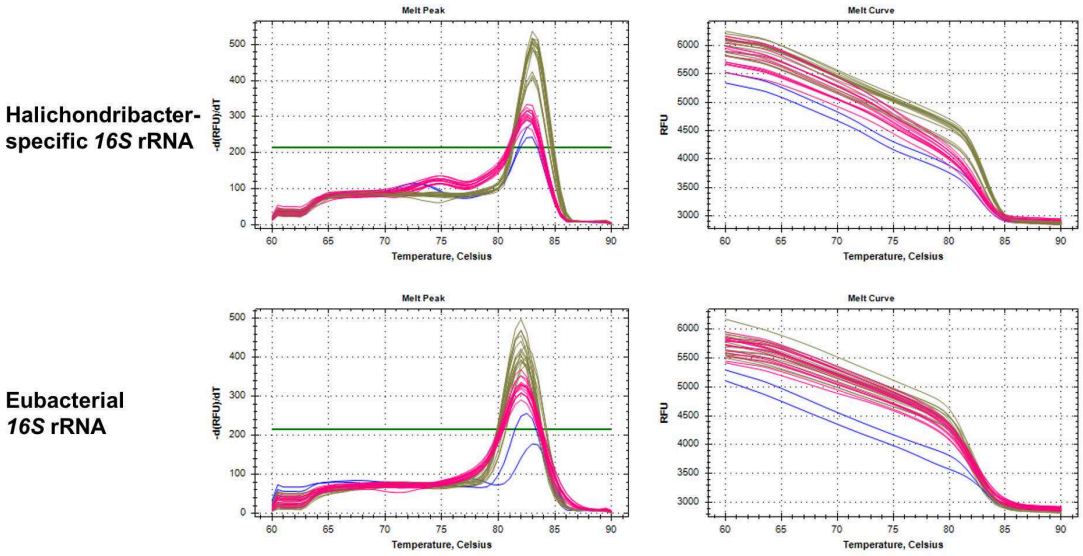
Table: qPCR conditions

9.5 Run gel electrophoresis with some qPCR products to check for correct amplification (1 band of correct product length) at least once per experiment

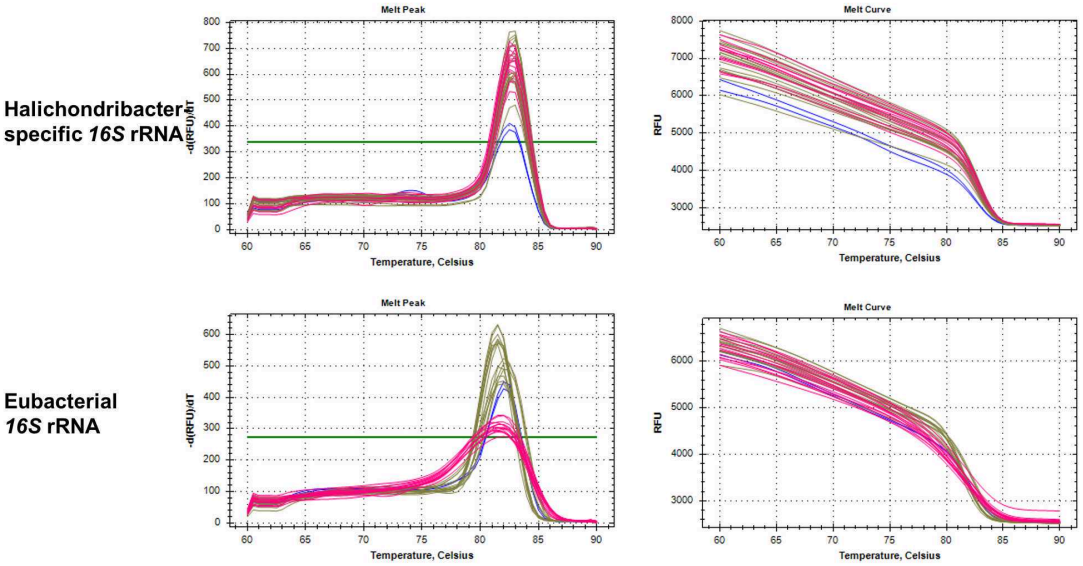
9.6

Figure: Example gel electrophoresis of qPCR products. Five replicate products and two negative controls were run per primer pair (eubacterial and Halichondribacter-specific *16S* rRNA gene).
Specificity of Halichondribacter-primers was confirmed by extraction of the gel band and sequencing of the PCR product.
Regularly check qPCR products on a gel to confirm specificity and amplification of one product only.

9.7



Example melt curves for qPCR products from cDNA. Always check melt curves for correct amplification of one qPCR product, and the overlap of melt curves from standard curve samples and experimental samples.



Example melt curves for qPCR products from gDNA. Always check melt curves for correct amplification of one qPCR product, and the overlap of melt curves from standard curve samples and experimental samples.