Detection of Non-Indigenous Marine Species from Port Water Samples

Workflow

Authors:

Cátia Chaves, BGE Lab Technician BIOPOLIS Association (BIOPOLIS-CIBIO)

Filipa MS Martins, PhD Researcher BIOPOLIS Association (BIOPOLIS-CIBIO)

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Reviewed by:

Glenn Dunshea (NTNU)

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1 eDNA Extraction

This step uses Qiagen DNeasy PowerWater extraction method (with few modifications) to isolate eDNA from encapsuled (Sylphium® dual filters) water samples:

Consumables

Items	Quantity	Storage
Sylphium capsules filled with ATL buffer	3.5 mL per sample	-20°C
96% Ethanol	3.5 mL per sample	RT
5ml Syringes	1 per sample	RT
Proteinase K	40µL per sample	-20ºC
Qiagen DNeasy Blood and Tissue kit (250)	2	RT
96-well 0.2 mL PCR plate		RT

Day 0

Thaw and access each capsule state individually, record this information (e.g if it is empty, has leaks, needs more buffer or can proceed to day 1).

In cases where the capsule is empty or mostly empty (<2 mL):

- Clean the working space and material with disinfectant and ethanol and leave the UV light on for at least 15 mins;
- Thaw and clean the outside of the capsules using a disinfectant. Refill them with 2.5 mL ATL buffer and seal each cap with parafilm.
- Incubate in the oven at 37 ºC for 30 min.

Day 1

Preparation

- Set the oven at 56ºC
- Clean the working space and material with disinfectant and ethanol and leave the UV-light on for at least 15 mins;
- Use filtered pipette tips at all steps;
- For each 23-sample batch, include a negative control (PNC) by preparing a 15-ml tube with 2 mL ATL + 23 µL PK.

Procedure

1. Thaw the capsules and clean the outside of each capsule using a disinfectant solution. For samples prepared on Day 0, proceed to step 2.
2. Fill each capsule with proteinase K (20mg/mL) and shake it manually. Adjust the volume of PK based on the available ATL volume in each capsule, up to a maximum of 40µL.(e.g. for approximately 2mL of ATL add 23 µL of PK).
3. Seal each capsule cap with parafilm, and incubate at 56ºC overnight (ON) in the mechanical shaker.

Day 2

Preparation

- Set the thermoblock at 70ºC;
- Set the oven at 55ºC;
- Clean the working space and material with disinfectant and ethanol and leave the UV light on for at least 15 mins;
- Use filtered pipette tips at all steps;
- If AL buffer has precipitate, heat to 55°C for 5–10 min to dissolve.
- Follow the manufacturer's instructions to prepare the AW1 and AW2 buffers.
- AL buffer + ethanol (96%) could be used pre-mixed.

Procedure

4. Mix each capsule by hand and transfer its content into a 15 mL tube using a syringe, through the inlet side.
5. Add 96% ethanol and AL Buffer to each tube in a 1:1:1 ratio (e.g. 2,000µL ATL+PK : 2,000µL AL buffer: 2,000µL 96% ethanol). Vortex tubes immediately, for 20s, and short-spin them.
6. Capture and purify the eDNA following either option a) or b):

a) By centrifugation (based on the DNeasy Blood and Tissue standard protocol):

I. Load up to **650 µL of supernatant** onto a Mini Spin Column. Centrifuge at **6,000 x g for 1 min**.
II. Discard the 2-mL collection tube and replace it with a new collection tube (not provided). Repeat until all the supernatant has been processed.
III. Place the Mini Spin Column Filter into a clean 2-mL collection tube (provided).
IV. Add **500 µL of AW1 Buffer** and centrifuge at **6,000 x g for 1 min**. Discard the 2-mL collection tube, and place the Mini Spin Column in a new collection tube (provided).
V. Add **500 µL of AW2 Buffer** and centrifuge at **20,000 x g for 1 min**. Discard the 2-mL collection tube.

b) Using the QIAvac 24 Plus vacuum manifold as an alternative:

I. Place the Mini Spin Columns in the QIAvac system.

II. Load **650 µL of supernatant** onto a Mini Spin Column.
III. Turn on the vacuum pump at -80/-90 kPa.
IV. Repeat the previous steps until all the supernatant has been processed.
V. Add **500 µL** of **AW1 Buffer**.
VI. Add **500 µL** of **AW2 Buffer**.
VII. Turn off the vacuum pump once all the volume has passed through.

7. Place the DNeasy Mini Spin Column in a new collection tube. Centrifuge at **20,000 x g for 2 min** to completely dry the membrane. Discard the collection tube containing the flow-through.
8. Place the column in a clean 1.5 mL tube and add **100 µL of heated TE (at 70ºC)** to the centre of the column membrane.
9. Incubate for **10 min** at room temperature. Centrifuge at **6,000 x g for 1 min**.
10. Repeat steps 8-9 using the same 1.5 mL tube to obtain maximum yield.
11. Transfer **60µL** eDNA extract to a 96-well plate (working plate) and archive the remaining at -20ºC or -80ºC. Leave at least two empty wells per plate for the PCR negative control (PNC).
12. Quantify the samples by spectrophotometry. Dilute samples with EB buffer into a new 96-well plate (if needed).

2 Amplicon PCR

This step uses PCR to amplify template out of a DNA sample for an interest-specific region using primers with (or a mix of) custom overhang adapters attached:

Shifter = 0-6 bp (N)

Forward sequencing adaptor site — Shifter — Forward region-specific primer

5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNNNNNNNNNNNNNNNNN 3'

Reverse sequencing adaptor site — Shifter — Reverse region-specific primer

5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNNNNNNNNNNNNNNN 3'

Consumables

Items	Initial Concentration	Quantity	Storage
eDNA (*dil* 1:1)	*n.i.*	2 µL per sample	-15° to -25°C
Amplicon PCR Forward Primer	10 µM	0.3 µL per sample	-15° to -25°C
Amplicon PCR Reverse Primer	10 µM	0.3 µL per sample	-15° to -25°C
Qiagen Master Mix	5x	5 µL per sample	-15° to -25°C
H_2O		2.4 µL per sample	
96-well 0.2 mL PCR plate		1 plate	

Procedure

1. Set up the following reaction per sample:

	1X
eDNA (*dil* if needed)	2µL
Amplicon PCR Forward Primer (10 µM)	0.3µL
Amplicon PCR Reverse Primer (10 µM)	0.3µL
Qiagen Master Mix	5µL
H_2O	2.4µL
Total	**10µL**

2. Seal plate and perform PCR, in triplicate, for each sample in a thermal cycler using the following protocol:
   - 95ºC for 15 minutes
   - 40 cycles of:
     - 94ºC for 30 seconds
     - **Ta** for **TDa** seconds
     - 72ºC for 30 seconds
   - 60ºC for 10 minutes
   - Hold at 10ºC

Ta stands for annealing temperature and *TDa* for annealing time, which need to be adjusted to each of the 3 markers:

Target Group	Marker	Primer Sequences	*Ta*	*TDa*	Expected Amplicon size*
Metazoa	(tbd)	FWD: REV:	º	s	bp
Eukaryotes?	(tbd)	FWD: REV:	º	s	bp
Fish?	(tbd)	FWD: REV:	º	s	bp

** expected amplicon size = insert length + primer lengths + 6bp NNs (avg.) + 67bp overhangs*

3. Test the PCR success of all samples through electrophoresis of 2µL using 2% agarose gel.
4. Dilute PCR product 1:4 using ultrapure water.

3 Indexing PCR

This step uses PCR-based reactions to index samples using custom P5 and P7 adapters:

Index = 7 bp
Shifter = 0-6 bp (N)

	P5	Index	Forward sequencing adaptor site	Shifter	Forward region-specific primer
PCR2	5' AATGATACGGCGACCACCGAGATCTACAC	*xxxxxxx*	TCGTCGGCAGCGTC 3'		
PCR1			5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNNNNN	NNNNNNNNNNNNNNNNNNNN 3'

	P7	Index	Reverse sequencing adaptor site	Shifter	Reverse region-specific primer
PCR2	5' CAAGCAGAAGACGGCATACGAGAT	*xxxxxxx*	GTCTCGTGGGCTCGG 3'		
PCR1			5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNNNNN	NNNNNNNNNNNNNNNNNNNN 3'

Consumables

Items	Initial Concentration	Quantity	Storage
PCR 1 (*diluted* 1:4)	*n.i.*	2.8 µL per sample	-15° to -25°C
P5-P7 Index Primer Mix	10 µM	1.4 µL per sample	-15° to -25°C
KAPA HiFi Hot-Start Ready Mix	2x	7 µL per sample	-15° to -25°C
H_2O		2.8 µL per sample	
96-well 0.2 mL PCR plate		1 plate	

Procedure

1. Transfer 1,4 µL of each mixed combination of P5 and P7 indexing primers to a new plate.
2. Set up the following reaction per sample:

	1X
KAPA HiFi Hot-Start Ready Mix	7 µL
H_2O	2.8 µL
Total	**9.8 µL**

3. Mix components, spin down and distribute it in each well.
4. Add **2.8 µL** of the diluted PCR1.
5. Seal plate and perform PCR in a thermal cycler using the following protocol:
   - 95ºC for 3 minutes
   - 8 cycles of:
     - 95ºC for 30 seconds
     - 55ºC for 30 seconds
     - 72ºC for 30 seconds
   - 72ºC for 5 minutes
   - Hold at 10ºC
6. Test size shift of PCR1 and PCR2 amplicons (1.8µL) of 15% of samples (e.g. 4 sets of 4 samples selected from random rows) through electrophoresis in 2% agarose gel.
   Note: expected library size = expected amplicon size + 67bp overhangs

4 Library Pool

Clean-up

This step uses magnetic beads to purify the amplicon away from free primers and primer dimer.

Equipment and Consumables

Items	Quantity	Storage
Buffer EB (Qiagen)	25 µL per sample	15ºC - 25ºC
KAPA HyperPure Beads (Roche)	8 µL per sample	4ºC
Freshly prepared 80% ethanol (EtOH)	300 µL per sample	
Cell culture plate *(new)*	4 plates	
96-well PCR plate Non-skirted (VWR)	1 plates	
Reservoirs	1	
Magnetic Bead Extractor for 96 Well Microplates (V&P Scientific)	1	
Low-bind microplate (Optional)	1	

Preparation

- Bring the **KAPA HyperPure Beads** to room temperature for **30min** prior to usage.
- Prepare **fresh 80% ethanol**.
- Prepare a 50mL with EB Buffer and protect it from any direct light source;
- Short spin the Amplicon PCR plate to collect condensation.
- Sterilize, under UV light for about **15min**, four U-bottom 96-well plates, a falcon with freshly prepared 80% ethanol and EB Buffer.

Procedure

1. Distribute the appropriate volume of beads in one of the U-bottom 96-well plates (*U-plate 1*).

   Note: The volume of beads may depend on the ratio chosen, this varies according to library quality and size. A standard ratio of 0,8x is used, adding 8 µL of beads for 10 µL of sample.

2. Transfer the full PCR volume (10 µL) into the plate containing the KAPA HyperPure Beads, carefully pipetting the entire volume **up and down 10 times**.
3. Incubate at room temperature without shaking for **3 min**.
4. While waiting prepare three more U-bottom 96-well plates as follows: two plates with **150µL 80% ethanol** and one plate with **25µL EB Buffer**.
5. Gently place a 96-well PCR plate on the plate from step 2 and attach the magnetic bead separation extractor for **2min** or until the supernatant is cleared.
6. Carefully remove the extractor and submerge the beads into one of the plates with freshly prepared 80% ethanol (*U-plate 2*) for **30s**.
7. Carefully remove the extractor and perform a second ethanol wash (*U-plate 3*).

8 Allow the beads to air-dry for **6-7min**.

Note: Do not over-dry the beads, if they start to appear cracked immediately proceed to the next step.

9 Carefully immerge the beads into the EB buffer (*U-plate 4*) and release the PCR plate from the extractor.

10 Carefully resuspend the beads in EB buffer.

11 Attach the magnetic extractor to the PCR plate for **2min** or until the supernatant is cleared.

12 Carefully remove the magnetic extractor and seal the U-bottom plate (or transfer it to a new low-bind PCR plate)..

Pooling & Quantification

Consumables

Items
EB buffer (Qiagen)
96-well 0.2 mL PCR plate
KAPA Library Quantification Kit (Roche)
Tapestation High Sensitivity D5000 (Agilent)
Qubit HS (Invitrogen)
KAPA HyperPure Beads (roche)

Procedure

1 Quantify each library in the Nanodrop to estimate average library concentration (ng/µL).

2 Pool libraries equimolarly at 50ng by taking the corresponding µL from each library. The negative controls should be added at the maximum volume of any other single library (up to 20 µL).

Note: In cases where a sample does not have the volume to take 50ng, use the smallest common concentration available

3 Clean each marker pool with KAPA HyperPure Beads.

Note: The volume of beads may depend on the ratio choosen, this varies according to library quality and size. A standard ratio of 0.75x is used for libraries Xbp, adding 75 µL of beads for 100 µL of sample.

4 Quantify each marker library pool using all three methods: *KAPA Library Quantification Kit* for qPCR, Qubit and Tapestation.

5 Dilute each library pool using 10mM Tris (pH 8.5) to 4nM.

6 Verify the final concentration of each library pool using *KAPA Library Quantification Kit* in qPCR.