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ESTABLISHMENT OF A SPECIMEN/TISSUE BANK AND ASSOCIATED DNA REFERENCE DATA FOR eDNA ANALYSIS

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This protocol is intended to provide guidelines on the curation and establishment of a specimen/tissue bank and associated DNA sequence data to be used as reference material/data for subsequent environmental DNA (eDNA) analysis, with particular emphasis on marine non-indigenous and invasive species.

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This documents provides recommendations on the processing of marine samples (mixed or individual) for the collection, preservation and taxonomic identification of non-indigenous and invasive marine species.

SAMPLE COLLECTION

Materials will depend on the sampling method.
However, ensure that user safety and biosecurity are always taken into account prior to conducting field work.

SAMPLE PROCESSING (TISSUE BANK)

Ethanol
Absolute **Lennox Catalog #CRTSE0021612** Step 5
Formalin solution neutral buffered 10% **Sigma**
Aldrich Catalog #HT501128-4L Step 5
Disposable gloves
Labcoats
Eye protection equipment
2mL screw cap tubes
Pencil
Paper labels (preferably waterproof)
Tweezers
Scalpels
Scissors
Microscope (compound and/or dissecting)
Photo camera
Buckets
Sieve (1mm)
Filter membranes (10um)
Vacuum pump
Virkon aquatic **AgriDirect**

DNA EXTRACTION

Molecular laboratory equipped with:
Micropipettes
Heating block or oven
Microcentrifuge

☒ DNeasy Blood & Tissue Kit

DNA extraction kit of choice (e.g. (QIAGEN) **Qiagen Catalog #69504** Step 6 , or

☒ E.Z.N.A.® Mollusc DNA Kit (Omega Bio-tek) **Omega Biotek Catalog #D3373-01** Step 6)

MOLECULAR BARCODING

Molecular laboratory equipped with:
Micropipettes
PCR reagents of choiceed (e.g.

☒ Platinum II Taq Hot-Start DNA Polymerase (Invitrogen) **Thermo Fisher Scientific Catalog #14966001** or

☒ GoTaq® DNA Polymerase (Promega) **Promega Catalog #M3001**)

Thermal Cycler (PCR)

e.g.

SimpliAmp Thermal Cycler
PCR
Applied Biosystems **A24811**
Any standard PCR thermocycler will suffice

Gel electrophoresis apparatus
Agarose gel

☒ TAE (Tris Acetate-EDTA) Buffer **Contributed by users Catalog #T6025**

TAE buffer
Standard size ladder (100-1000bp) (e.g.

☒ 100 bp DNA Ladder - 500 gel lanes **New England Biolabs Catalog #N3231L**)

Make sure to establish a Risk Assessment to mitigate any adverse effect on users while carrying out any of these protocols. Useful information (e.g. Safety Data Sheets) should be sourced from the relevant suppliers who procured the materials.

To minimize risk of cross-sample contamination, note that all re-usable materials (e.g. scalpels, scissors, tweezers) should be decontaminated to remove any traces of DNA. It is highly recommended to carry out these protocols in dedicated and PCR-free laboratories/rooms.

Ensure that each protocol and list of materials is checked before starting any of the procedures. Specifically, make sure that all key materials(e.g. kits) have not been modified or discontinued by the relevant supplier.

Introduction

1 This document includes the following sections:

- A graphical representation of the overall process
- Collection of specimens and associated metadata
- Specimen and tissue bank
- Isolation of genomic DNA
- Molecular barcoding
- Acknowledgements

Graphical overview

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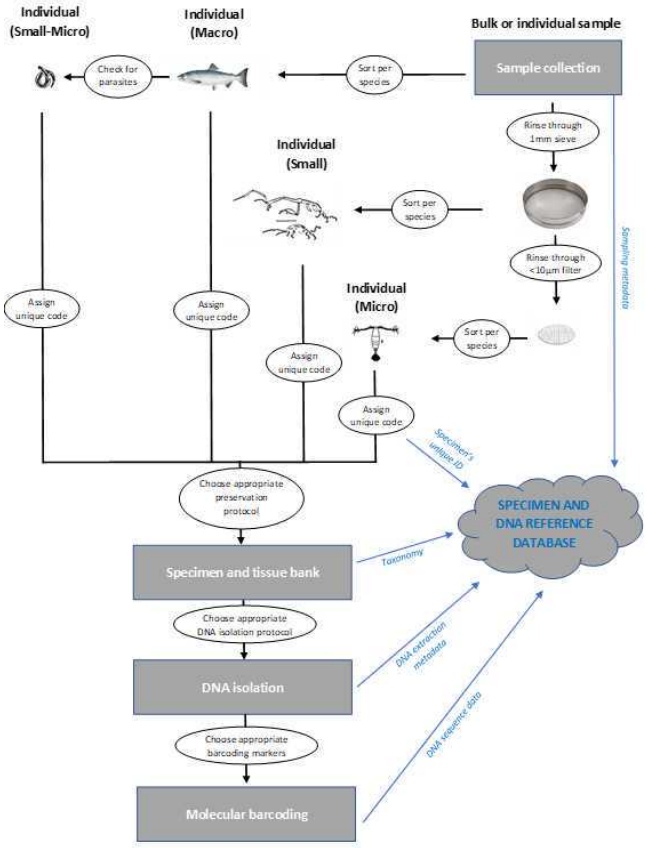

General workflow overview for the establishment and curation of a specimen and DNA reference database

Collection of specimens and associated metadata

3 Specimens can be collected following targeted surveys or opportunistically, using rapid methods (collection by hand, dredges, corers, traps, nets, etc.) or dedicated *in situ* approaches (e.g. SETL settlement plates; https://www.gimaris.com/Projects/SETL-project). When possible, whole specimens should be preserved for future downstream taxonomic identification.
As for metadata, the minimum information would include location (ideally with exact coordinates), date of collection, method of collection, photographs, and name/contact of person who collected the specimen. For an extensive list of recommended metadata, we recommend consulting guidelines provided in Rimet et al. (2021).

Rimet F, Aylagas E, Borja A, Bouchez A, Canino A, Chauvin C, Chonova T, Ciampor Jr F, Costa FO, Ferrari BJD, Gastineau R, Goulon C, Gugger M, Holzmann M, Jahn R, Kahlert M, Kusber W-H, Laplace-Treyture C, Leese F, Leliaert F, Mann DG, Marchand F, Méléder V, Pawlowski J, Rasconi S, Rivera S, Rougerie R, Schweizer M, Trobajo R, Vasselon V, Vivien R, Weigand A, Witkowski A, Zimmermann J, Ekrem T (2021). Metadata standards and practical guidelines for specimen and DNA curation when building barcode reference libraries for aquatic life. Metabarcoding and Metagenomics. https://doi.org/10.3897/mbmg.5.58056

Specimen and tissue bank

4 For samples containing multiple organisms (e.g. dredge, SETL), specimens should be sorted and separated into single species groups. If the goal of the survey includes small- to micro-organisms, any small (>1mm) organism should be collected by rinsing with clear seawater (or artificial seawater) any substrate or specimen through a 1mm sieve, whereas the flow-through water should be inspected with a microscope for the presence of micro-organism.

To avoid unintentional spreading of non-indigenous species and/or pathogens, make sure to treat any materials and liquids with appropriate disinfectants before disposal (e.g. ⊠Virkon aquatic **AgriDirect**).

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A unique specimen identifier code should be allocated to each specimen collected. The format and style of such code will depend on the intended database/repository.

5 Preservation conditions will depend on the organism and can vary substantially, however such conditions should be chosen with the purpose of (i) limit DNA degradation during storage and (ii) ensure that key taxonomic features are retained for subsequent identification. However, in the context of marine non-indigenous species, many taxa (including most invertebrates) can be preserved frozen (-20°C to -80°C), using 70-100%
☒Ethanol
Absolute **Lennox Catalog #CRTSE0021612** (for subsequent DNA analysis, but may lead to discoloration affecting taxonomic ID) and/or
☒Formalin solution neutral buffered 10% **Sigma Aldrich Catalog #HT501128-4L** (for subsequent taxonomic identification, but not recommended or DNA isolation).

WARNING: caution should be exercised when handling ethanol and formalin. Please consult the relevant Safety Data Sheets for further information.

H225 | Highly flammable liquid and vapour

H319 | Causes serious eye irritation

H317 | May cause an allergic skin reaction
H351 | Suspected of causing cancer

Isolation of genomic DNA

6 DNA isolation protocols should be adapted to each organisms following the manufacturer's recommendations. However, in the context of marine non-indigenous species, two DNA isolation kits have been used with highly successful rates, including the
☒E.Z.N.A.® Mollusc DNA Kit (Omega Bio-tek) **Omega Biotek Catalog #D3373-01** (particularly successful on
☒DNeasy Blood & Tissue Kit
mollusks and arthropods) and the (QIAGEN) **Qiagen Catalog #69504** (for most other animal species, algae and microorganisms).

Subsequent to DNA isolation, template DNA should be quantified using a Qubit Fluorometer (Invitrogen), whereas quality of extracts should be assessed using a small-volume spectrophotometer (Thermo Scientific™).

Invitrogen™ Qubit™ 3 Fluorometer
Accurately measures DNA, RNA, and protein using the highly sensitive fluorescence-based Qubit quantitation assays
Invitrogen™ Q33216 **Q33216**

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NanoDrop™ One UV-Vis Spectrophotometer
spectrophotometer

Thermo Scientific **ND-ONE-W**

Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit; Voltage: 12 V (DC); Wavelength Range: 190–850 nm

Molecular barcoding

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The choice of genetic markers to be used in molecular barcoding depends on the target organism as well as on the intended taxonomic resolution of the barcode. Here we provide a range of molecular markers and protocols that were successfully used to barcode a wide range of marine non-indigenous species.

A	B	C	D	E	F	G	H	I	J	K	L
Assay #	**Target Taxa**	**Target Gene**	**Amplicon Length Range (bp)**	**Forward Primer Name**	**Forward Primer Sequence (5'-3')**	**Forward Primer Source Reference**	**Reverse Primer Name**	**Reverse Primer Sequence (5'-3')**	**Reverse Primer Source Reference**		
1	Marine metazoans	COI	658	LoboF1	KBTCHACAAAYCAYAARGAYATHGG	Lobo et al. 2013	LoboR1	TAAACYTCWGGRTGWCCRAARAAYCA	Lobo et al. 2013		
2	Marine metazoans	COI	313	mlCOIintF-XT	GGWACWRGWTGRACWNTNTAYCCYCC	Leray et al 2013	jgHCO2198a	TANACYTCNGGRTGNCCRAARAAYCA	Leray et al 2013		
3	Marine metazoans	COI	710	jgLCO1490	TITCIACIAAYCAYAARGAYATTGG	Geller et al 2013	jgHCO2198b	TAIACYTCIGGRTGICCRAARAAYCA	Geller et al 2013		
4	Marine metazoans	16S rRNA	567	16sar-L	CGCCTGTTTATCAAAAACAT	Palumbi et al 2002	16sbr-H	CCGGTCTGAACTCAGATCACGT	Palumbi et al 2002		
5	(Freshwater) Diatoms	RuBisCO	312	Diat_rbcL_708F	AGGTGAARYWAAAGGTTCWTAYTTAAA	Vasselon et al 2017	Diat_rbcL_R3	CCTTCTAATTTACCWACWACTG	Vasselon et al 2017		

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The following PCR conditions are specific to the selected markers reported above and were optimized using ☒Platinum II Taq Hot-Start DNA Polymerase (Invitrogen) **Thermo Fisher Scientific Catalog #14966001**

☒GoTaq® DNA Polymerase

or (Promega) **Promega Catalog #M3001** . While the reported reagents and conditions were successful on a range of taxonomic groups, further optimization may be required for specimens that fail to amplify.

Assay 1 (COI - Lobo et al 2013)

Lobo, J., Costa, P.M., Teixeira, M.A., Ferreira, M.S.G., Costa, M.H., Costa, F.O. (2013). Enhanced primers for amplification of DNA barcodes from a broad range of marine metazoans. BMC Ecology. https://doi.org/10.1186/1472-6785-13-34

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A	B	C
Reagent name and starting concentration	**Amount per reaction (µL)**	**Final concentration (per reaction)**
Moleculr grade water	to 10 µL	-
5X Platinum™ II PCR Buffer	2	1X
10 mM dNTP mix	0.2	0.2 mM each
10 µM forward primer	0.2	0.2 µM
10 µM reverse primer	0.2	0.2 µM
Platinum™ II Taq Hot-Start DNA Polymerase	0.08	0.04 U/µL
Template DNA	1-2	<200 ng/rxn
Total	10	

PCR reagents and concentrations for Assay 1

Thermal cycling conditions:

94ºC-1min

94ºC-30secs
45ºC-60secs x5
72ºC-60secs

94ºC-30secs
54ºC-90secs x45
72ºC-60secs

4ºC-+∞

Assay 2 (COI - Leray et al 2013)

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents.. Frontiers in zoology.
https://doi.org/10.1186/1742-9994-10-34

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A	B	C	D
Reagent name and starting concentration	**Amount per reaction (µL)**	**Final concentration (per reaction)**	
Moleculr grade water	to 20µL	-	
5X GoTaq PCR Buffer	4	1X	
10 mM dNTP mix	0.4	0.2 mM each	
10 µM forward primer	1	0.5 µM	
10 µM reverse primer	1	0.5 µM	
GoTaq DNA Polymerase	0.15	0.04 U/µL	
Template DNA	2	<200 ng/rxn	
Total	20		

Thermal cycling conditions:

95ºC-1min

95ºC-15secs
46ºC-15secs x40
72ºC-10secs

72ºC-3min

4ºC-+∞

Assay 3 (COI - Geller et al 2013)

Geller J, Meyer C, Parker M, Hawk H (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys.. Molecular ecology resources.
https://doi.org/10.1111/1755-0998.12138

A	B	C	D
Reagent name and starting concentration	**Amount per reaction (µL)**	**Final concentration (per reaction)**	
Moleculr grade water	to 20µL	-	
5X GoTaq PCR Buffer	4	1X	
10 mM dNTP mix	0.4	0.2 mM each	
10 µM forward primer	1	0.5 µM	
10 µM reverse primer	1	0.5 µM	
GoTaq DNA Polymerase	0.15	0.04 U/µL	
Template DNA	2	<200 ng/rxn	
Total	20		

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Thermal cycling conditions:

95ºC-5min

95ºC-1min
48ºC-1min x40
72ºC-1min

72ºC-5min

4ºC-+∞

Assay 4 (16S rRNA - Palumbi et al 2002)

Palumbi S, Martin A, Romano S, McMillan W, Stice L, Grabowski G.. The Simple Fool's Guide to PCR, Version 2.0.

A	B	C	D
Reagent name and starting concentration	**Amount per reaction (µL)**	**Final concentration (per reaction)**	
Moleculr grade water	to 20µL	-	
5X GoTaq PCR Buffer	4	1X	
10 mM dNTP mix	0.4	0.2 mM each	
10 µM forward primer	1	0.5 µM	
10 µM reverse primer	1	0.5 µM	
GoTaq DNA Polymerase	0.15	0.04 U/µL	
Template DNA	2	<200 ng/rxn	
Total	20		

Thermal cycling conditions:

95ºC-5min

94ºC-1min
55ºC-1min x40
72ºC-2min

72ºC-7min

4ºC-+∞

Assay 5 (RuBisCO - Vasselon et al 2017)

Vasselon, V., Rimet, F., Tapolczai, K., & Bouchez, A. (2017). Assessing ecological status with diatoms DNA metabarcoding: Scaling-up on a WFD monitoring network (Mayotte Island, France). Ecological Indicators. https://doi.org/10.1016/j.ecolind.2017.06.024

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A	B	C	D
Reagent name and starting concentration	**Amount per reaction (µL)**	**Final concentration (per reaction)**	
Moleculr grade water	to 20µL	-	
5X GoTaq PCR Buffer	4	1X	
10 mM dNTP mix	0.4	0.2 mM each	
10 µM forward primer	1	0.5 µM	
10 µM reverse primer	1	0.5 µM	
GoTaq DNA Polymerase	0.15	0.04 U/µL	
Template DNA	2	<200 ng/rxn	
Total	20		

Thermal cycling conditions:

94ºC-1min

95ºC-30secs
46ºC-30secs x40
72ºC-30secs

72ºC-10min

4ºC-+∞

9 Successful amplification and confirmation of expected size of PCR products should be carried out by **agarose gel electrophoresis**.

(Optional) A **PCR clean-up** step can be included at this stage, but in many cases it is not necessary.

DNA sequences should be obtained by **Sanger sequencing** using the Forward and/or reverse primers.

Sanger Sequencing
by Lívia Sacchetto
PREVIEW RUN

Raw DNA sequence data should be inspected using any suitable software (e.g. MEGA, Geneious) and curated to ensure high quality (error-free) of final data.

Linking DNA sequence data to public repositories such as **GenBank** and **BOLD** is strongly encouraged.

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