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




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We use this protocol and it's working

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Abstract

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.

Guidelines

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.

Materials

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**

Formalin solution neutral buffered 10% **Sigma Aldrich Catalog #HT501128-4L**

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

DNA extraction kit of choice (e.g. DNeasy Blood & Tissue Kit (QIAGEN) **Qiagen Catalog #69504** , or

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

MOLECULAR BARCODING

Molecular laboratory equipped with:

Micropipettes

PCR reagents of choice (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.

Equipment

SimpliAmp Thermal Cycler

NAME

PCR

TYPE

Applied Biosystems

BRAND

A24811

SKU

<https://www.thermofisher.com/order/catalog/product/A24811> LINK

Any standard PCR thermocycler will suffice

SPECIFICATIONS



Gel electrophoresis apparatus

Agarose gel

TAE buffer TAE (Tris Acetate-EDTA) Buffer **Catalog #T6025**

Standard size ladder (100-1000bp) (e.g. 100 bp DNA Ladder - 500 gel lanes **New England Biolabs Catalog #N3231L**)

Protocol materials

Ethanol Absolute **Lennox Catalog #CRTSE0021612** Materials, Step 5

TAE (Tris Acetate-EDTA) Buffer **Catalog #T6025** Materials

Formalin solution neutral buffered 10% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #HT501128-4L** Materials, Step 5

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

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

Virkon aquatic **AgriDirect** Materials

DNeasy Blood & Tissue Kit (QIAGEN) **Qiagen Catalog #69504** Materials, Step 6

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

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

Safety warnings

- !** 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.

Before start

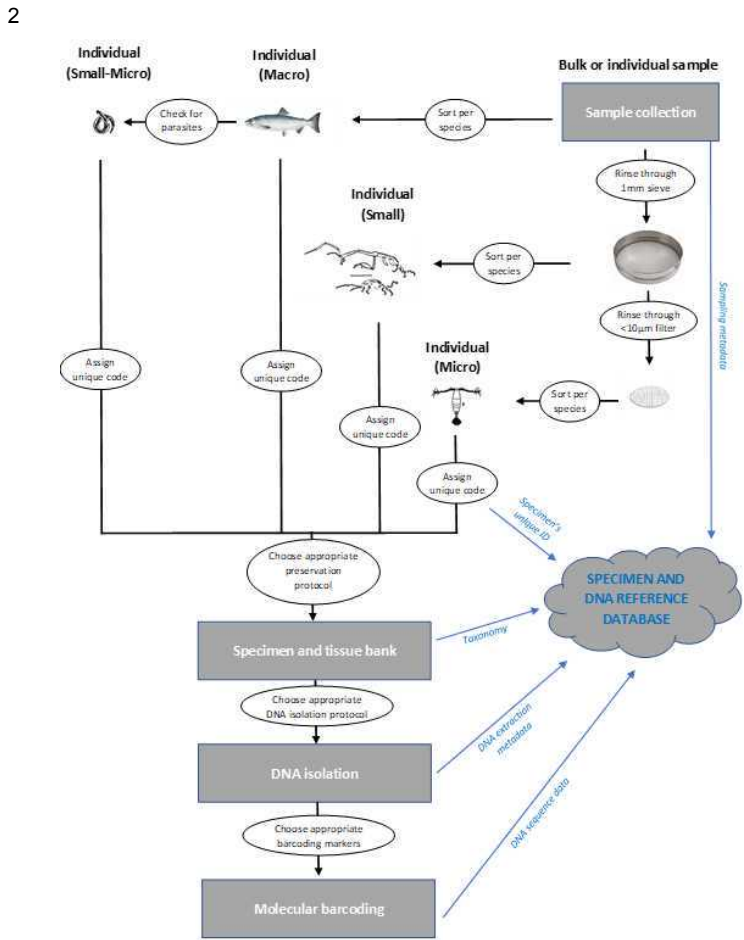
To minimize risk of cross-sample contamination, note that all re-usable materials (e.g. scalpels, scissors, tweezers) should be decontaminated to remove any traces of DNA. It is highly recommended to carry out these protocols in dedicated and PCR-free laboratories/rooms.

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

- 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



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

Collection of specimens and associated metadata

- 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).

CITATION

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*.

LINK

<https://doi.org/10.3897/mbmg.5.58056>

Specimen and tissue bank

- 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.

Safety information

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**).

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.

- 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.

Safety 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 mollusks and arthropods) and the
- ☒ DNeasy Blood & Tissue Kit (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™).

Equipment

Invitrogen™ Qubit™ 3 Fluorometer NAME

Accurately measures DNA, RNA, and protein using the highly sensitive fluorescence-based Qubit quantitation assays TYPE

Invitrogen™ Q33216 BRAND

Q33216 SKU

<https://www.fishersci.co.uk/shop/products/qubit-3-0-fluorometer/15387293> LINK

Equipment

NanoDrop™ One UV-Vis Spectrophotometer NAME

spectrophotometer TYPE

Thermo Scientific BRAND

ND-ONE-W SKU

<https://www.thermofisher.com/order/catalog/product/ND-ONE-W> LINK

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

Molecular barcoding

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Note

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
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	mIColintF-XT	GGWACWRGWTGRACWNTNTAYCCYCC	Leray et al 2013	jgHCO2198a	TANACYTCNGGRTGNCCRAARAAYCA	Leray et al 2013

A	B	C	D	E	F	G	H	I	J
3	Marine metazoans	COI	710	lgLCO1490	TITCIACIAAYCAYAARGAYATTGG	Geller et al 2013	lgHCO2198b	TAIACYTCIGGRTGICCRARAAYCA	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	AGGTGAARYWAAAGGTTTCWTAYTTAAA	Vasselon et al 2017	Diat_rbcL_R3	CCTTCTAATTTACCWACWACTG	Vasselon et al 2017

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Note

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** or

GoTaq® DNA Polymerase (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)

CITATION

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.

LINK

<https://doi.org/10.1186/1472-6785-13-34>

A	B	C
Reagent name and starting concentration	Amount per reaction (µL)	Final concentration (per reaction)
Molecular 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:

94°C-1min

94°C-30secs

45°C-60secs x5



72°C-60secs
 94°C-30secs
 54°C-90secs x45
 72°C-60secs

4°C-+∞

Assay 2 (COI - Leray et al 2013)

CITATION

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*.

LINK

<https://doi.org/10.1186/1742-9994-10-34>

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:

95°C-1min
 95°C-15secs
 46°C-15secs x40
 72°C-10secs
 72°C-3min
 4°C-+∞



Assay 3 (COI - Geller et al 2013)

CITATION

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.

LINK

<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)	
Molecular 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:

95°C-5min

95°C-1min

48°C-1min x40

72°C-1min

72°C-5min

4°C-+∞

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

CITATION

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)	

A	B	C	D
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:

95°C-5min

94°C-1min
 55°C-1min x40
 72°C-2min

72°C-7min

4°C+∞

Assay 5 (RuBisCO - Vasselon et al 2017)

CITATION

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*.

LINK

<https://doi.org/10.1016/j.ecolind.2017.06.024>

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	



A	B	C	D
GoTaq DNA Polymerase	0.15	0.04 U/ μ L	
Template DNA	2	<200 ng/rxn	
Total	20		

Thermal cycling conditions:

94°C-1min

95°C-30secs

46°C-30secs x40

72°C-30secs

72°C-10min

4°C- ∞

- 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.

Protocol

NAME
Sanger Sequencing

CREATED BY
Livia Sacchetto

PREVIEW

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.



Citations

Step 3

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-Tretyure 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. Metadata standards and practical guidelines for specimen and DNA curation when building barcode reference libraries for aquatic life <https://doi.org/10.3897/mbmg.5.58056>

Step 8

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ. 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. <https://doi.org/10.1186/1742-9994-10-34>

Step 8

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

Step 8

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

Step 8

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

Step 8

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