Environmental DNA protocol development guide for biomonitoring

© University of Canberra 2022

Ownership of intellectual property rights

Unless otherwise noted, copyright (and any other intellectual property rights) in this publication is owned by the University of Canberra.

Creative Commons licence

All material in this publication is licensed under a Creative Commons Attribution 4.0 International Licence except content supplied by third parties and logos

Inquiries about the licence and any use of this document should be emailed to EcoDNA@Canberra.edu.au.

Citation

De Brauwer M, Chariton A, Clarke LJ, Cooper MK, DiBattista J, Furlan E, Giblot-Ducray D, Gleeson D, Harford A, Herbert S, MacDonald AJ, Miller A, Montgomery K, Mooney T, Noble LM, Rourke M, Sherman CDH, Stat M, Suter L, West KM, White N, Villacorta-Rath C, Zaiko A & Trujillo-Gonzalez A (2022). *Environmental DNA protocol development guide for biomonitoring*. National eDNA Reference Centre, Canberra.

ISBN 978-1-74088-532-4

Disclaimer

The Australian Government, acting through the Department of Agriculture, Water and the Environment, has exercised due care and skill in preparing and compiling the information and data in this publication. Notwithstanding, the Department of Agriculture, Water and the Environment, its employees and advisers disclaim all liability, including liability for negligence and for any loss, damage, injury, expense or cost incurred by any person as a result of accessing, using or relying on any of the information or data in this publication to the maximum extent permitted by law.

Acknowledgements

This publication was developed as a collaborative project, funded by the Australian Government Department of Agriculture, Fisheries and Forestry, and led by Alejandro Trujillo-Gonzalez (ATG) of the University of Canberra and Maarten De Brauwer (MDB) of CSIRO. Anastasija Zaiko's contribution was supported by the New Zealand Ministry of Business, Innovation and Employment funding (CAWX1904 — A toolbox to underpin and enable tomorrow's marine biosecurity system). We would like to acknowledge and thank all co-authors who helped develop and write the guidelines, as well as the 53 experts from across Australia and New Zealand who provided feedback during various stages of the process. The authors thank the four external expert reviewers of the guidelines for their valuable feedback. ATG and MDB would also like to thank Biotext for their services and Paula Doyle for her invaluable role as knowledge broker.

Editing and design: Biotext Pty Ltd

UNIVERSITY OF CANBERRA

NATIONAL eDNA REFERENCE CENTRE

CSIRO

Australian Government

Department of Agriculture, Fisheries and Forestry

Contents

Glossary

Assay: The laboratory workflow from DNA extraction to sequence outputs. Often refers to the target gene and taxonomic group (e.g. 16S_Fish, 18S universal, COI).

Endogenous control: Endogenous controls refer to either exogenous DNA (i.e. DNA that is spiked in) or endogenous DNA (i.e. DNA that is naturally occurring) that can be targeted in environmental samples as positive controls to monitor method success. Exogenous DNA templates can be generated from custom-synthesised DNA fragments, DNA extract, plasmids, and be added to samples during any stage of the eDNA workflow after sample collection. Endogenous controls use the fact that DNA is ubiquitous in the environment, such that every environmental sample will contain DNA from multiple sources. Within this context, a generic primer assay can be designed to amplify abundant, non-target DNA that will be simultaneously sampled, captured, extracted and amplified with the target species' DNA (Furlan & Gleeson 2016).

Environmental DNA / RNA (eDNA / eRNA): DNA or RNA directly extracted from environmental samples (soil, sediment, water, etc.) without any knowledge of the original organism. DNA carries genetic material, while RNA transfers information within cells to produce specific proteins and is only shed by physiologically active (living) organisms.

High-throughput sequencing (HTS): A technique able to determine the nucleotide composition of millions of nucleic acid sequences. Different platforms of sequencing are available including sequencing by synthesis (e.g. Illumina), single molecule real time (e.g. PacBio), and nanopore (e.g. Oxford Nanopore Technologies).

Inhibitory substances: Substances in a sample or extract that reduce assay sensitivity and increase the risk of false negative results in a PCR test.

Library (also Reference library, Reference database): Database with DNA sequences of specific species.

Limit of detection (LOD): The lowest concentration of target DNA that can be detected with a defined level of confidence (usually a 95% detection rate).

Limit of quantification (LOQ): The lowest amount of DNA in a sample that can be quantitatively determined with a stated precision, under stated experimental conditions.

Metabarcoding: Simultaneous taxonomic identification of Operational Taxonomic Units (OTUs) or Amplicon Specific Variances (ASVs) in eDNA samples with millions of sequences, generated by PCR amplification using one of the HTS techniques.

Monitoring: The systematic collection of data over time to detect changes in a system (Gerber et al 2005). Data can include information on a range of factors such as environmental, ecological, biological and social.

Polymerase chain reaction (PCR): A molecular technique that allows the exponential amplification of a target fragment/region of DNA from a mixture of DNA fragments. The desired fragment is selected from the other fragments in the mixture by specific primers (small single-strand oligonucleotides) complementary to the desired sequence.

Primer: Short DNA fragments used in PCR amplification that bind adjacent to the target region or gene.

Quantitative PCR (qPCR): A variant of PCR. The main difference is that qPCR is able to quantify how many fragments of DNA are amplified during each step in the reaction, leading to quantitative data.

Sequencing: Determining the order of nucleotides in DNA or RNA; this can be done using a variety of methods.

Introduction

The use of environmental DNA (eDNA) and RNA (eRNA) methods is a rapidly advancing field that provides fast, cost-effective, non-invasive methods to identify the presence of target species. These methods can be used, for example, to screen for pest species as part of biosecurity measures and risk management, to screen for threatened species as part of development requirements, or for biodiversity monitoring. In an operational context, high-quality standard assurance requirements are needed to ensure that assays and protocols deliver reliable results across multiple applications and purposes.

What is the aim of these guidelines?

The *Environmental DNA protocol development guide for biomonitoring (EP guide for biomonitoring)* provides harmonised quality control and minimum standard operating procedures. This document is complemented by the *Environmental DNA test validation guidelines*, which has been published in parallel with the *EP guide for biomonitoring*. The *EP guide for biomonitoring* provides information to create standard operating procedures for eDNA/eRNA projects, whereas the eDNA test validation guidelines focus on advice for the development and use of eDNA and eRNA assays.

This document is a comprehensive guide for the development and use of eDNA/eRNA test protocols, as recommended and curated by experts, stakeholders and end users in Australia and New Zealand. The *EP guide for biomonitoring* covers protocols for both single species (qPCR) and multi-species (metabarcoding) projects. Differences between approaches are highlighted in relevant sections throughout the guidelines. The guide is designed to support a consistent and best-practice approach to eDNA/eRNA

testing to help detect species of interest. This approach ensures that surveillance and resource managers are provided with robust scientific evidence to support decision making.

Environmental RNA-based methods differ primarily from eDNA in the molecules they target. The different function of RNA in living organisms means that its detection is more likely to reflect metabolically active organisms. Current eRNA applications exploit its rapid degradation to quantify temporally recent or metabolically active communities (Pokon et al. 2017, Yates et al. 2021), providing important information to assess the active presence of species in an environment.

Note on the use of the terms eDNA and eRNA: Protocols for the use of eDNA and eRNA-based methods have considerable overlap; we therefore use the term 'eDNA' throughout the text when protocols are applicable to both eDNA and eRNA methods. Specific eRNA protocols are discussed separately when different protocols are required.

Who are they for?

The *EP guide for biomonitoring* provides clear best-practice benchmarks to help researchers develop eDNA-based protocols encompassing complete workflows for reliable eDNA analysis. It also provides information for end users requesting eDNA/eRNA projects.

The potential applications for eDNA are varied and eDNA-based methods have been applied in a wide range of fields, including biosecurity surveillance, biodiversity monitoring, endangered species research, risk management, and emergency response. The use of these guidelines for protocol development is strongly recommended, but not mandatory.

For researchers

The *EP guide for biomonitoring* details key steps in creating standard operating procedures for eDNA-based applications. This document also provides general principles and considerations to guide project development, and information on other important project areas such as communication and ethics.

For end users

The *EP guide for biomonitoring* provides quality assurance for any contracted eDNA work. The guide tells end users what services and standards can be

expected and may also be used to inform staff collecting samples or inform the development of other areas of the project.

How have they been developed?

The *EP guide for biomonitoring* and the *Environmental DNA test validation guidelines* were developed in a collaborative process with input from eDNA experts and end users from across Australia and New Zealand. Initial draft frameworks were developed and led by members of the Southern eDNA Society Standards and Best Practices Committee, after which multiple consultation rounds with experts, end users and stakeholders from private entities and public agencies were held to adapt the frameworks to meet Australian and New Zealand needs. Three consultation periods with eDNA experts, private stakeholders, government officials and end users were held in 2021–22 to ensure the guidelines were fit for purpose and met the highest quality standards in the field.

Updates

We recognise that eDNA methods and resources are still rapidly evolving. It is anticipated that the *EP guide for biomonitoring* will be updated and expanded over time, with a review and update as required.

The guidelines will be reviewed and updated by the Australian National eDNA Reference Centre, with input from leading experts in the field of environmental DNA.

Principles for conducting an eDNA project

The *EP guide for biomonitoring* provides best-practice guidance, while recognising that 'best practice' is broad and depends on:

- purpose of the test (e.g. the detection of high-impact pests may require a more sensitive detection threshold than is used for low-impact species)
- environmental constraints (e.g. sample type - water, soil or scats; integrity)
- target constraints (e.g. mobile or sedentary target, concentration)
- project constraints (e.g. budget, sample shipping, turnaround time requested)
- current knowledge gaps (e.g. in taxonomic groups or DNA reference sample data).

Best practice will therefore involve various considerations, taking 6 general principles into account:

1 Ensure processes are fit for purpose
2 Test and validate processes
3 Ensure good chain of custody and documentation
4 Understand the limitations of results
5 Ensure good communication
6 Recognise First Nations peoples' ownership and stewardship.

Principle 1: Ensure processes are fit for purpose

The fundamental steps of conducting an eDNA project do not differ substantially from other research or monitoring projects. As with other methods, projects need a clear goal and experimental design tied to the project goal, samples must be collected and processed, and results must be interpreted and communicated to a target audience or end users (Figure 1).

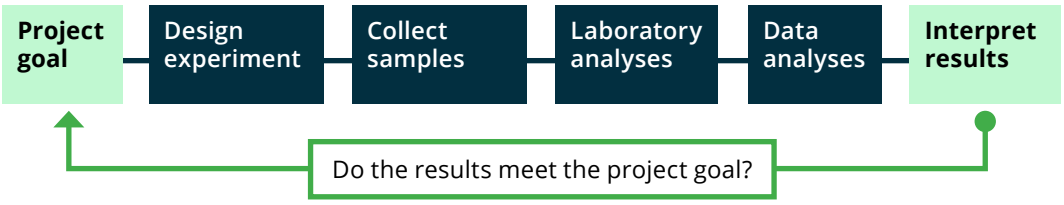


Figure 1 Steps of an eDNA project, and how they must consider the project goal if they are to be fit for purpose

It is important that processes at each of these steps are fit for purpose. Considerations include:

- project goal and experimental design (see Experimental design) – match all aspects of the experimental design to the purpose of the project
- sample collection and preservation (see Collect samples and Preserve samples for processing) – design and provide appropriate guidelines and workflows to suit the project purpose and the constraints of the site; train any non-experts involved in collecting samples
- recording all sample metadata, from collection through to project completion
- laboratory analyses (see Extract and isolate DNA/RNA and Ensure quality control and purification of extracts) – laboratory processes and methods must be optimised for eDNA and carried out using strict standard operating procedures
- data analyses (see Analyse extracts) – data analyses must be able to clearly identify positive, negative, indeterminate and unreliable results
- documenting all of the above processes (including sample storage and preservation, and laboratory protocols, optimisations and troubleshooting) for reporting to the end user
- result interpretation (see Interpret and communicate results) – interpretation of resuts must take into account any limitations and be assessed and compared with the project goals; conclusions should be communicated as clearly as possible, with reference to the project goal; any caveats around the results or interpretation should be clearly communicated in the report
- follow-up and confirmation of positive results.

Suggested considerations for each stage of the project workflow are provided in Table 1.

Table 1 Guiding questions for consideration in the design and implementation phases of an environmental DNA project

Workflow stage	Guiding questions
Study design	• Is the work basic science or applied (e.g. environmental biomonitoring)? • What is your study goal (e.g. presence or absence; diversity; quantification)? • What tools and methods are available and what are yet to be developed? (e.g. sampling methods, assays, analysis pipelines)? • What is the best analytical approach to address the research goal (e.g. DNA-based or RNA-based, metabarcoding vs qPCR)? • Which taxa will you target? • What characteristics of the target organism/communities may affect the sampling method (e.g. life form, size, expected abundance/occupancy, behaviour)? • What characteristics of the ecosystem may affect the sampling method (e.g. terrestrial/aquatic; lentic/lotic/marine; how variable is the environment)? • Is the scale of inference for your sample type appropriate to your questions? • Can you compare complementary data types (e.g. traditional vs eDNA)? • Does your sampling/replication scheme provide good statistical power? • Who are the end users and what will they do in response to eDNA results? Are they involved in project design?
In the field	• What type of sample (water, soil, air) is needed? • What metadata will you collect? • How many replicates will you collect? • Does your sampling protocol minimise or control for – contamination (e.g. positive and negative controls)? – any known biases (e.g. inhibitors, sample volume)? • How will you track samples (e.g. barcodes)? • What is the most appropriate sample collection method for the established goal/ecosystem/target organisms based on the characteristics of the target species/community and ecosystem? • How will samples be preserved and transported?

continues

Table 1 *continued*

Workflow stage	Guiding questions
In the laboratory – sample handling phase	• What extraction method (physical vs chemical)? • How much sample volume or weight? • How many samples? • How many controls and of what sort? • Is species-specific detection or metabarcoding more appropriate? • Is a species-specific assay available? Does it need validation? • Do you need to generate reference sequence data? • Are any technical replicates needed? • For eRNA analyses, will you use co-extraction or separate extraction? • For qPCR/ddCPR, choose the assay for the target species (e.g. is it validated for your region, checked for cross-reactivity with non-target taxa)? • What storage is needed? – How long will samples be stored before processing? – What are the most appropriate conditions for sample type? – What preservation should be used? – How will products (DNA/RNA extracts, PCR amplicons) be stored and for how long? • How can multiple freeze–thaw cycles for products be minimised? • Is contamination control implemented at different steps (extraction blanks, PCR blanks, etc.) and included in the analyses?
In the laboratory – DNA processing phase	• What sequencing platform/qPCR instrument (96/384 well) will you use? • What locus and primers will you use? • What read length will you use? • Do you need paired-end sequencing? • Have you included appropriate quality assurances? (e.g. internal control, mock community, qPCR, bioanalyser tracer) • Does your sampling protocol minimise or control for – contamination (e.g. positive and negative controls)? – any known biases (e.g. primer bias, coverage, taxonomic resolution)?

continues

Table 1 *continued*

Workflow stage	Guiding questions
At the keyboard	• How complete is the reference database? • How complete is the taxonomic knowledge for the group? Are there risks of taxonomic uncertainty that could affect how results are interpreted? • Do you have adequate sequencing coverage across samples? • Are you using appropriate choices for software tools and parameters? • Are your conclusions upheld when using alternative parameters and workflows? • Are you including appropriate quality filtering of your data? • Is the specificity of the test confirmed? • What is the LOD/LOQ of the test? • Are all the internal controls / QA controls valid? • Is sequencing data of adequate quality (sequence depth per sample and error rates adequate, and contamination negligible)? • Which sequencing database will you use? How complete and up to date is it? • How much ambiguity is in the taxonomic assignment (i.e. can your data provide required taxonomic resolution with high confidence?)

Source: adapted from Deiner et al. 2017.

Principle 2: Test and validate processes

Processes should be tested and validated to ensure robust results. Consider doing a pilot project, which may help to determine sample sizes and protocols.

Validation requirements will depend on the study purpose. For more information and detailed guidelines, see the *Environmental DNA test validation guidelines*, published in parallel with this document. Other scales and guidelines include:

- eDNA Validation Scale: edna-validation.com
- Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS, Bruce K (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA 3(4):823–836, doi:10.1002/edn3.189.

- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55(4):611–22, doi:10.1373/clinchem.2008.112797.

Processes or results can also be validated by crosschecking with other reputable laboratories.

Principle 3: Ensure good chain of custody and documentation

Good chain of custody and documentation (see Collect samples) ensures results can be trusted. Documentation of processes can help to ensure processes are robust and consistent and are followed correctly. Reliable tracking systems ensure the reliability of metadata collected in the field.

Principle 4: Understand the limitations of results

The limitations of eDNA results will be project-specific, and depend on the environment, target species and assays used. Limitations of results should be considered during experimental design and – most importantly – analysis and interpretation phases.

Limitations of results will be closely linked to the purpose of the project and thus to the stringency required (see Purpose and stringency). Projects with high stringency requirements (e.g. legal evidence), must take into account and communicate the limitations of results (see Interpret and communicate results).

In some cases, eDNA testing can be used for large-scale surveillance, to enable triage and better use of resources for targeted traditional surveys. Data obtained from eDNA methods might not necessarily confirm the presence or absence of a species, but positive eDNA tests are appropriate criteria to identify the potential presence of target species and trigger confirmatory efforts.

When eDNA methods are used to study communities using metabarcoding, limitations such as incomplete reference databases or unresolved taxonomy issues must be taken into account when interpreting results.

Principle 5: Ensure good communication

Clear communication, especially with service providers of the project and with project members without genetics expertise, is an essential part of project protocols, and will support best-practice processes. Clear communication is particularly important with:

- End users (see Interpret and communicate results). Clear communication throughout the project will help to ensure expectations are managed and the results meet user needs. It is important to emphasise limitations and explain uncertainties in results to ensure data are not misinterpreted.
- Collection teams (see Collect samples). Collection teams may not be trained as molecular scientists and will need clear, non-technical guidance about how to collect, preserve, store, transport, and document samples. In-person training may improve sample collection standards.

Principle 6: Recognise First Nations peoples' ownership and stewardship

Collection of environmental samples in Australia and New Zealand requires recognition of the relationship that First Nations peoples have with nature. Following the release of the global biodiversity framework from the United Nations Convention on Biological Diversity in 2021, and acknowledging the rights of First Nations peoples with respect to traditional knowledge associated with genetic resources, these guidelines highlight the following:

- There are international and national efforts to recognise the rights of Indigenous peoples with respect to the traditional knowledge associated with genetic resources.
- These guidelines recognise the need for fair and equitable sharing of the benefits accrued from gathering genetic resources and their associated digital information. Sharing strategies should be, among other things, efficient, feasible and practical. They should aim to be effective and not hinder research and innovation, while being mutually supportive of other access and benefit-sharing instruments.
- There are a wide range of views about the modalities and methodologies of a potential solution for the fair and equitable sharing of benefits arising from digital sequence information from genetic resources.

With these points in mind, and aligned with recommendations made by the Convention on Biological Diversity, these guidelines recommend that project designers discuss projects with appropriate First Nations communities and provide transparency on project requirements and outcomes. Discussions with Traditional Custodians about the collection and use of environmental samples must be flexible - listen to, adapt and incorporate recommendations from the Traditional Custodians of study sites. It is also recommended that project outcomes are shared with First Nations communities and, when possible, the Traditional Custodians are recognised and acknowledged in online repositories that store genetic data.

Environmental DNA/RNA test protocols

Environmental DNA/RNA test protocols outline standard operating procedures for field officers and users to collect, extract and analyse environmental samples for eDNA/eRNA. These protocols encompass the entire eDNA workflow and offer users standard guidelines for each stage, with appropriate controls and measures for independence. The protocols can be used as templates to develop standard operating procedures (SOPs) for specific projects and purposes. They align with and meet the minimum requirements of the:

- Development of National Diagnostic Protocols – Procedures for Authors Reference Standard (SPHD RS No. 2), approved by the Subcommittee on Plant Health Diagnostics (SPHD, 2019)
- Australian and New Zealand standard diagnostic procedures – Guide for Authors (ANZSDP, 2021).

Each of the following steps includes important considerations for quality assurance and reliable implementation and analysis of eDNA/eRNA testing:

1 Experimental design

Careful, detailed experimental design is an essential investment to ensure projects are effective and meet end-user needs. eDNA analyses provide powerful molecular tools with a wide range of biomonitoring applications, but a well-thought-out experimental design is essential to guarantee high-quality outcomes. Poorly designed experiments or surveys are more likely than not to compromise results, regardless of the quality of assays, laboratory protocols and bioinformatics pipelines.

- Experimental protocols should be designed in a way that complements the principles for conducting projects (see Principles for conducting an eDNA project).

Standard operating procedures should implement suitable sampling regimes determined during initial experimental design (Table 2). This initial step should assess:

- Purpose of test (see Purpose and stringency). Is the test intended to be used for a purpose that requires highest-level standards (e.g. legal purposes), survey biodiversity, or for routine biodiversity monitoring? What level of detection is meant to be achieved? What level of uncertainty is acceptable?
- Responsibility. Who is responsible for ensuring experimental design is fit for purpose?
- Spatial extent. How many samples should be collected from how many sites to achieve suitable representation of the environment and presumed distribution of the target species?
- Temporal extent. Are you collecting samples in a single session, or multiple sessions over time? Will the time of sampling affect the presence or abundance of target species (e.g. day, night, season)?
- Collection method. What collection method should be used to collect samples within the target environment?
- Replication. What volume of sample and how many replicates should be collected to improve detection probability?
- Preservation. What method is the most appropriate to preserve samples until eDNA/eRNA extracts can be obtained?
- Controls. Which controls will be implemented during different stages of the workflow?
- Confirmation. What method can be used to confirm positive results?

- For long-term programs, conducting a pilot study to inform proper study design, cost effectiveness, detection probabilities, and spatial distribution of eDNA of target species can be helpful. If relative abundance is included in the purpose, pilot studies and calibration with existing methods is essential.

The experimental design of a project should be done in consultation with the end users of the data to ensure the project workflow is fit for purpose. Before the project begins, researchers should communicate clearly and honestly about how and if eDNA can meet the project goals. This includes clearly explaining what eDNA testing is capable of and informing end users about the constraints and limitations of eDNA methods. Managing expectations around constraints such as abundance data, poorly amplifying taxa, and so on, is essential to ensure the eDNA project will meet end-user needs.

Table 2 Factors to consider when designing an eDNA/eRNA study

Factor	Considerations	Approach
Purpose	End-user goal	Projects with different purposes (e.g. routine biomonitoring vs biosecurity) might require different levels of stringency and controls
	Consideration of other potential sources of DNA (e.g. could the DNA originate from elsewhere?)	
Ethics and permits	Permits might be required to collect samples (e.g. in national parks)	Consult with appropriate authorities
	Outcomes of surveys might have upstream implications for First Nations peoples	Consult relevant First Nations representatives
Budget	Budget might not be sufficient to achieve intended purpose	Realistic estimates to meet user needs
Spatial and temporal extent	Single site vs large scale, single time point vs long-term sampling	Design for maximum reproducibility and transferability
	Consider using statistical (probabilistic) models and simulations to test your study design in silico	Consider minimum number of sites to reach adequate spatial coverage

continues

Table 2 *continued*

Factor	Considerations	Approach
Environmental factors	Temperature, UV exposure, water movement, turbidity, chlorophyll a, biochemical oxygen demand, dissolved oxygen, pH	Different conditions require specific methods and materials
Medium	Water, sediment, soil, grain, scat, stomach content, air	Different media require specific field and laboratory protocols
Controls	Controls should be implemented at different workflow stages	Different purposes and workflows might require different controls
Target restrictions	Biology and behaviour of the target, likely levels of DNA in the environment, risk of cryptic species Differential amplification between target taxa	Consider potential issues that will affect the sampling strategy, analysis, and interpretation of the data
Fieldwork restrictions	Facilities, distance to processing laboratories, contamination sources and controls, extraction and preservation options Sample tracking options	Consider all needs and potential issues
Laboratory processing	Choice of sample collection method (e.g. filtration, sediment core) Choice of preservation method Choice of DNA extraction method	Consider benefits and drawbacks of different sample collection methods at each stage of the project Consider logistics and effects of preservation method on sample quality and extraction protocol Consider logistics and effects of extraction method
Assay	Assays fit for purpose	Validated assay specific to target species Metabarcoding primers validated to detect taxa of interest

continues

Table 2 *continued*

Factor	Considerations	Approach
Replication	Need adequate replication for different environments or purpose	Higher replication needed for certain purposes requiring higher levels of stringency (e.g. surveys with potential legal implications) More biodiverse regions need higher sample replication
Staff training needs	Collection teams might not be experts Chain of custody, ensure each link in chain knows protocols	Ensure all relevant staff members are adequately trained

Purpose and stringency

Defining the intended purpose of a study should be the first step in designing any eDNA project. A thorough understanding of the purpose of eDNA testing is essential; it will form the framework around which the rest of the experimental design is built. The purpose of the study will help to guide decisions around the level of required stringency and what level of risk can be tolerated when taking management decision based on eDNA data (Table 3). Projects with legal or high-cost ramifications –such as for biosecurity regulation purposes – are likely to need higher standards and stricter protocols than, for example, citizen science projects that aim to monitor biodiversity.

Table 3 A matrix for assessing the quality or stringency of an eDNA project

	Quality or stringency		
Aspect of project	**High (desirable)**	**Medium (minimum requirements)**	**Low (insufficient)**
Experimental design	Power analysis done and sufficient sample size to answer question	Robust design and some replication	Ad hoc sampling without replication
Assay design	Proven assay with known specificity and sensitivity	Validated assay	Assay has not been tested

continues

Table 3 *continued*

Aspect of project	Quality or stringency		
	High (desirable)	**Medium (minimum requirements)**	**Low (insufficient)**
Sample collection	Using appropriate equipment and methods to avoid contamination, conducted by staff with relevant training and expertise	Using appropriate equipment and methods to avoid contamination, conducted by staff who have received sufficient training	Equipment used not sufficient to avoid contamination, staff with limited or no training
Quality assurance and control	Positive and negative controls during collection, extraction and processing	Negative controls during collection and extraction	No or limited controls throughout the project
Analysis: qPCR	In-depth analysis and validation of assays, reporting analytical specificity, sensitivity and limits of detection. Samples are analysed with multiple technical replicates, and each analysis includes positive and negative controls to assess method success	Reporting presence or absence. Assay may not be validated and may have undergone minimum analytical sensitivity and specificity testing. Samples are analysed using single technical replicates, and each analysis includes positive and negative controls to assess method success	Reporting presence or absence only Assay testing conditions are not reported and analyses are completed in the absence of positive and negative controls
Analysis: metabarcoding	In-depth analysis, species richness estimates, occurrence probability modelling, error analysis included	Reporting presence or absence and species richness estimates	Reporting presence or absence only

continues

Table 3 *continued*

Aspect of project	Quality or stringency		
	High (desirable)	**Medium (minimum requirements)**	**Low (insufficient)**
Communication	Clear and sustained communication with end users throughout each phase of the project	Some communication with end users at initial and final phases	No communication with end users
Optional extra validation: parallel traditional surveys	Parallel surveys with traditional methods targeting same taxa	Data available from past parallel surveys	No parallel surveys

Source: adapted from Jerde 2019.

Permissions and ethical considerations

Environmental DNA fieldwork might require research or collection permits, depending on the place where samples are collected. Before the start of an eDNA project, project leaders should check with the relevant bodies to find out which permits are required. For example, some states require permits to import samples from interstate, and research activities in national parks require specific permits to be in place before the start of sample collecting.

Environmental DNA is a non-destructive sampling method (for large organisms), which negates the need for most of the human or animal ethic approvals typical for other methods. At the time of writing, we are not aware of any institutions that require ethics approval for sampling eDNA from water, sediment, air or soil samples. We do, however, strongly recommend consulting up-to-date regulations before commencing any new eDNA research project.

Surveys will sometimes take place in culturally important locations or detect culturally significant species. Co-design and co-implementation of projects is important. First Nations peoples should be engaged during the project design phase to ensure that methods consider cultural sensitivities and relevant permissions are received (see Principle 6). Furthermore, there is a chance that samples might inadvertently detect human DNA, which might be culturally sensitive for some Traditional Owner groups (Handsley-Davis et al. 2021). Honest and clear communication with Traditional Owners is essential to avoid conflict and legal implications. In New Zealand, research practices

should consider the Wai 262 principles (Waitangi Tribunal 2012). It is strongly recommended that project outcomes are shared with Traditional Owner communities. The understanding of implications surrounding this issue is evolving and it is likely that new regulations will be developed in the future. It is the responsibility of project leaders to stay informed and conform to the most recent legislation on this matter. If potential concerns exist, it is advisable to consult with experts at the start of a new eDNA project.

2 Collect samples

Environmental DNA can be collected from many environments and substrates, so sampling protocols can differ substantially between projects. A few general principles should be followed when collecting eDNA samples. The main considerations are collecting an adequate number of samples for analysis, avoiding cross contamination, implementing appropriate preservation methods, and ensuring an appropriate chain of custody. Strict collection processes will preserve the integrity of your samples and improve testing accuracy.

Logistics around sampling for eDNA can be complicated and should be considered early in the project. Topics include:

- spatial and temporal variations in eDNA concentration in the environment (e.g. spawning events and migrations)
- fieldwork conditions
- sample collection method
- available infrastructure at the sampling locations
- training of collection teams.

Sampling should follow SOPs that are fit for purpose. Custom SOPs might have to be designed to suit project needs or to ensure interoperability with other projects within the same organisation.

Collection process

The purpose of the research and experimental design will affect the collection process. Different substrates, collection, and preservation methods require different field logistics and yield different results (Koziol et al. 2018). As such, they cannot be compared directly, which should be taken into consideration for long-term or ongoing projects. Common substrates used to collect eDNA include water, sediment, scat, and stomach content. However, eDNA can be collected from other substrates such as sweeping or vacuum samples, pollen

and grain. Preservation and collection methods will depend on available resources and transport time to the laboratory.

In designing and describing the collection process, first establish the best-practice approach to achieve the study purpose. Based on this best-practice plan, describe the potential considerations and limitations specific to the project. The sampling protocol should include a description of responsibility for each step in the process, and a detailed description of preservation and storage methods (see Preserve samples for processing).

Sample collection is often done by non-experts, including consultants and community members who might be unfamiliar with the rigorous contamination protocols required when collecting eDNA. In these cases, the sampling plan should be designed for non-specialists, with full details provided and written in non-technical language. Anticipate potential problems in the sampling plan (e.g. a particular site is inaccessible, silty water clogs filters) and provide backup plans to the sampling team to ensure that useful data are still collected. Training videos or hands-on training may also be necessary. If possible, test your sampling plan and training resources by requesting non-technical staff to follow the instructions and identify which points in the protocol cause confusion. Providing opportunities for feedback from trainees and updating standard operating procedures accordingly can help to clarify the process.

Sample size

Deciding on a suitable sample size (number of samples and volume of samples collected) for a project will vary depending on the purpose of the study, the type of environment, the characteristics of the target species/communities, and the known abundance of target species (Stauffer et al. 2021). The probability of detecting targeted DNA in any environment depends on both the collection method and the concentration and dispersion of DNA at those sites (Furlan et al. 2019). However, several studies (e.g. Deiner et al. 2018; Li et al. 2018; Coutant et al. 2021) have shown that targeted eDNA approaches can be quite robust to technical choices, within common ranges. The concentration of DNA in turn depends on the biology, ecology and behaviour of the target species (e.g. shedding rates, mobility, abundance) (Harrison et al. 2019).

Sampling frameworks can be based on available methods used to assess eDNA assay sensitivity (Furlan et al. 2016) or published work on similar taxa and environments. A pilot study may be needed to determine minimum sample size for long-term monitoring projects, particularly in novel habitats or with target taxa for which limited empirical eDNA data exist.

Avoiding cross contamination

Reducing the risk of contamination should be a primary concern when collecting samples, regardless of the type of sample being collected. Common sources of contamination include environmental contamination from other biological materials, poorly decontaminated sampling equipment, and cross contamination between samples.

It is critical to establish clean and consistent field collection protocols that reduce the probability of contamination. Decontamination of equipment and other field gear prior to sampling events is essential for ensuring sample independence (Goldberg et al. 2016). Consider single-use equipment and containers for sampling, and clean and sterilise equipment between sampling as well as before and after sampling. Decontaminating equipment is best done using >3% sodium hypochlorite (bleach) to destroy any traces of DNA/RNA. Field equipment supplies and field consumables must not be stored in areas with high copy number DNA settings, such as PCR laboratory or tissue handling areas.

Additional measures to reduce contamination risk should include diligent use of gloves, presterilised equipment, avoiding potential sources of contamination (e.g. not interacting with other biological materials during sampling), and including negative controls in the workflow.

Negative field controls (DNA-free samples collected using the same protocol and equipment, preserved and processed in the same way as field samples) are critical for detecting contamination (Goldberg et al. 2016). Negative controls should be collected before and after sample collection at each site to identify potential sources of contamination.

Once collected, samples should be stored appropriately to avoid cross contamination until they can be returned to the lab. Samples must not be stored in areas with high copy number DNA settings, such as PCR laboratory or tissue handling areas.

It should be noted that contamination can occur at any stage of the process. Consistently implementing laboratory processes that reduce contamination risks is therefore important. Such general processes can include

- segregating laboratory activities based on DNA content and risk potential
- introducing workflow automation where possible
- choosing consumables that reduce contamination risk.

Chain of custody

The chain of custody links the samples collected in the environment to the final processing and analysis. A high-quality chain of custody is important to ensure the final results of the study are valid and usable, particularly for legal purposes.

A chain of custody record must be maintained. It must provide a comprehensive history of each sample. This includes how samples have been handled and transported (e.g. what temperatures samples were stored at) and who has been involved. Developing a reliable sample tracking system, using barcodes or unique sample IDs, is key to doing this effectively.

During experimental design, define the chain of custody, including where it starts, the data that should be recorded, and who is responsible. Throughout the study, record all steps from sample collection through to processing and analysis, including any variations away from the steps determined during experimental design.

Metadata and documentation

Collecting detailed metadata during sample collections will help to maintain a good chain of custody to support the analyses and interpretation of results. This is especially relevant when different teams collect and analyse data. The metadata scheme should be established before sample collection begins and be provided to collection teams ahead of fieldwork (Table 4). If possible, sampling kits can be prelabelled and templates provided for data collection.

Standardised metadata following the FAIR principles (Findable, Accessible, Interoperable, Reusable; Wilkinson et al. 2016) should be recorded across all projects. However, individual projects may have additional specific data that must be collected. The use of standardised ontologies (e.g. Darwin Core) and metadata schemes and repositories developed for use with molecular samples – such as GEOME – is highly recommended (Wieczorek et al. 2012, Deck et al. 2017, Riginos et al. 2020).

Table 4 Recommended metadata during sample collection

Metadata field	Notes	Example
Unique sample ID[1]	Clear code or designation of sample unique to the sample	eDNA-ProjCO6421, barcode
Name collector[1]	Ideally including contact details	Name Surname name.surname@email.com +61412345678
Sample type[1]	The type of sample that was collected	Water, scat, grain, bulk
Date + time[1]	Add estimated time of day if exact time is not possible	07/04/2022, 15:00
Location[1]	Site name and location	Battery Point, Hobart
GPS coordinates[1]	Latitude and longitude coordinates	−42.886201, 147.335827; 42° 53′ 10″ S, 147° 20′ 13″ E
Sample volume[1]	Volume of water filtered, weight of sediment or bulk sample collected	1 L, 10 g
Collection method[1]	Method used to collect the sample	Filtration method, sediment core, sweeping
Preservation method[1]	Method used to preserve sample	Freezing, drying, ethanol, Longmire's solution
Project name	Can be a code or formal designation	eDNA standards project, PR_eDNA_ST
Control	Is sample a negative control or not?	Negative control
Habitat type	Specific type of habitat	Coral reef, seamount, lake, stream, forest, desert
Environmental conditions	Environmental variables relevant to the project aims or which might influence DNA concentrations	Temperature (surface/at depth), weather conditions, currents
Other observations	Observations relevant to the project aims or which might influence DNA concentrations	Fishing vessels nearby, coral spawning, large storm prior to sampling

continues

Table 4 *continued*

Metadata field	Notes	Example
Problems	Issues that occurred during collecting that might compromise sample integrity	Half of sample spilled, gloves tore, filter clogged after 0.6 L
Filter type[1] (if used)	Type and pore size	Polyethersulfone, cellulose nitrate 0.2 µm, 1.2 µm
Collection depth	If sampling from water bodies	Surface, 10 m, 1200 m

[1] indicates minimum required metadata

3 Preserve samples for processing

Environmental DNA and RNA begins decaying immediately after it is shed by an organism. DNA and RNA continue to degrade after they are collected due to a range of factors, such as UV radiation, mechanical forces, microbial activity, and spontaneous chemical reactions (Goldberg et al. 2016). Repeated freeze–thaw cycles of samples during or after preservation can also cause significant DNA and RNA degradation. RNA is a less stable molecule than DNA and usually degrades quicker than DNA. However, recent studies have demonstrated that decay-rate constants for eRNA vary, but can be surprisingly similar to those of eDNA – environmental RNA can be detected in water samples for 13–72 hours after production, and eRNA concentrations can even be higher than corresponding eDNA (Yates et al. 2021).

Preservation of eDNA

To slow the degradation of samples, they should be preserved and processed using standardised protocols as part of the collection process (see Thomas et al. 2018), or as soon as possible after collection. Timeframes for preservation and processing should be specified in the experimental design. You may also need to consider whether long-term storage or archiving of samples will be required. Different preservation methods have different levels of handling involved, choosing a method that involves minimal handling will reduce the risk of cross contamination.

For preservation, samples can be kept in cold conditions until processing can occur (e.g. some samples can be kept in ice for up to 24 hours before preserving without compromising detection; Pilliod et al. 2013), although

this depends on the conditions affecting samples at the time of collection. In some cases, such as when collecting scat or soil samples, drying may be a better preservation option, especially when maintaining a consistent cold temperature is not possible. Drying can be achieved by using silica gel or calcium chloride moisture-absorbing bags (Nagy 2010, Guerrieri et al. 2020).

Filtration methods can also be used, with the advantage that large volumes of water or air can be rapidly filtered and samples preserved onsite using chemical preservation or dehydration (Thomas et al. 2019, Bruce et al. 2021). However, this may be difficult to perform within a suitable timeframe if appropriate sampling equipment is not available. If filtration is not practical, precipitation (Ficetola et al. 2008) can be used to preserve small samples by adding a salt (typically sodium acetate) and absolute ethanol in the field, followed by storing the sample at −20 °C. However, precipitation is unwieldy at large scale, and using ethanol and sodium acetate may enrich for large DNA at the expense of small, degraded DNA.

Establishing backup samples by splitting a single sample into several can help to mitigate against contamination but may reduce the chance of detection if the target is at very low concentration. For DNA aliquoting, it is a good idea to freeze multiple aliquots to avoid multiple freeze–thaw cycles during analysis.

Processing of eRNA

Extra care should be taken when targeting eRNA because RNA molecules are even more vulnerable to degradation than DNA. RNA stability depends strongly on the type of preservation in addition to sample collection, harvesting, handling, transportation, and the extraction protocol used. The gold standard for RNA preservation is immediate liquid nitrogen–based cryopreservation (−80 °C) after flash freezing (Auer et al. 2014) or filtration; however, this method may be problematic for many environmental sampling applications that are without access to liquid nitrogen or suitable freezing equipment. Alternatively, environmental samples can be submerged in aqueous, nontoxic stabilising solutions with a storage reagent that rapidly permeates samples to stabilise and protect cellular RNA (e.g. RNA*Later*, Invitrogen). The samples must be cooled to −80 °C as early as possible and kept there until processing can occur.

4 Extract and isolate DNA/RNA

Processes for extracting and isolating DNA/RNA should be based on validated commercial extraction kits and methods that are widely available and commonly used in eDNA-based research. Any novel extraction and isolation methods should be optimised and standardised for eDNA/RNA use. Methods must be chosen according to the needs of the project and the type of sample.

Extraction of eDNA

Commercial DNA extraction kits are available for different substrates, including soils, biofilms, tissue and water. Soil DNA extraction kits, for example, have been shown to be efficient at extracting DNA from many different substrates (Hermans et al. 2018). eDNA recovery rates vary with preservation and extraction methods (Hinlo et al. 2017). Furthermore, certain methods, such as precipitation methods, might not work well for small DNA fragments, so alternative methods should be considered when expecting heavily degraded DNA.

Extraction of eRNA

Commercial RNA extraction kits, used in conjunction with DNase kits to ensure no eDNA carryover, can be used to extract RNA from environmental samples. If DNase is not completed following RNA extraction, then one such step should be incorporated prior to reverse transcriptase of extracted RNA. The field of eRNA testing is still young and extraction and isolation methods are still being formally optimised and standardised for eRNA use. Methods must be chosen according to the needs of the project and the type of sample.

5 Ensure quality control and purification of extracts

All eDNA/eRNA extracts that do not produce positive results must be assessed for inhibitory substances (i.e. substances in a sample or extract that have a negative effect on the PCR, reducing assay sensitivity and increasing the risk of false negative results if they are not removed by the chosen extraction method). Processes should outline suitable methods to quantify total DNA/RNA yield and assess the presence of inhibiting factors. Inhibition can be assessed during qPCR in 2 ways – using a dilution series

of the eDNA extract with at least 2 dilution points (neat and 1:10) or an exogenous internal positive control.

In both cases, the presence of inhibitors is shown through evaluating the cycle threshold (Ct) values. Specifically, diluted eDNA extracts free of inhibition should behave quantitatively (i.e. 3.3 Ct shift between dilution points), while inhibited samples will show delayed amplification (Bylemans et al. 2017, Murray et al. 2015, White et al. 2020). In the same way, the exogenous internal positive control will also exhibit delayed amplification in the presence of inhibitors (Conte et al. 2018, Furlan et al. 2015). Some commercial DNA polymerase master mixes contain substances that can effectively counteract most qPCR inhibition from eDNA extracts (Jane et al. 2015), and commercial DNA extraction kits for complex substrates are able to remove common inhibitors – these should be used by accredited laboratories. When this is not possible, inhibitor removal steps can also be included post-extraction to avoid false negative detections (Schrader et al. 2012).

Table 5 Types of controls that can be implemented during different stages of environmental DNA workflows

When to use	Control type	Purpose	Method
Sample collection	Negative (= sampling blank)	Detect contamination during sample collection	During fieldwork, collect a sample known to not contain target DNA (e.g. filter 1 L of milliQ water)
	Positive exogenous control	Detect whether DNA collected in the field amplifies well (e.g. presence of inhibitors)	Add exogenous control to samples collected in the field. Single species (for qPCR), or a real or synthetic mock community for metabarcoding

continues

Table 5 *continued*

When to use	Control type	Purpose	Method
DNA extraction	Negative (= extraction blank)	Detect contamination during extraction	Extract a blank (no DNA) sample
	Positive exogenous control	Detect issues in extraction process	Add (non-target-species) DNA to samples collected in the field. Single species (for qPCR), or a real or synthetic mock community for metabarcoding
PCR	Negative	Detect contamination during PCR set up	Non-template technical replicates. These controls have all reaction components, except water replaces the target template
	Positive exogenous control	Detect inhibition of extracts in PCR process	Add (non-target-species) DNA to eDNA extracts. Single species (for qPCR), or a real or synthetic mock community for metabarcoding
		Determine method success across the whole workflow	Analyse environmental extracts using a generic primer assay designed to amplify abundant, non-target DNA that will be simultaneously sampled, captured, extracted and amplified (or only amplified) with the target species' DNA

Source: adapted from Furlan & Gleeson 2016.

The type of controls used depends on the project. Controls should be clearly defined by the sampling process and other methods, such as molecular biology and bioinformatics. Table 5 describes the range of possible controls.

6 Analyse extracts

There are several molecular methods being tested and explored for their suitability in testing environmental samples. These currently include the use of digital PCR (Doi et al. 2015), loop-mediated isothermal amplification (LAMP; Williams et al. 2017), and high-throughput sequencing methods coupled with metabarcoding techniques (Ruppert et al. 2019).

This section outlines the minimum requirements for authors, including:

- description of the molecular method used to analyse environmental samples
- explanation of what is measured by the method (e.g. absorbance, droplet ratio)
- description of the appearance and criteria for:
  - positive results
  - negative results
  - indeterminate results
  - unreliable results.

As an example, we provide minimum requirements for quantitative PCR assays used to test environmental samples.

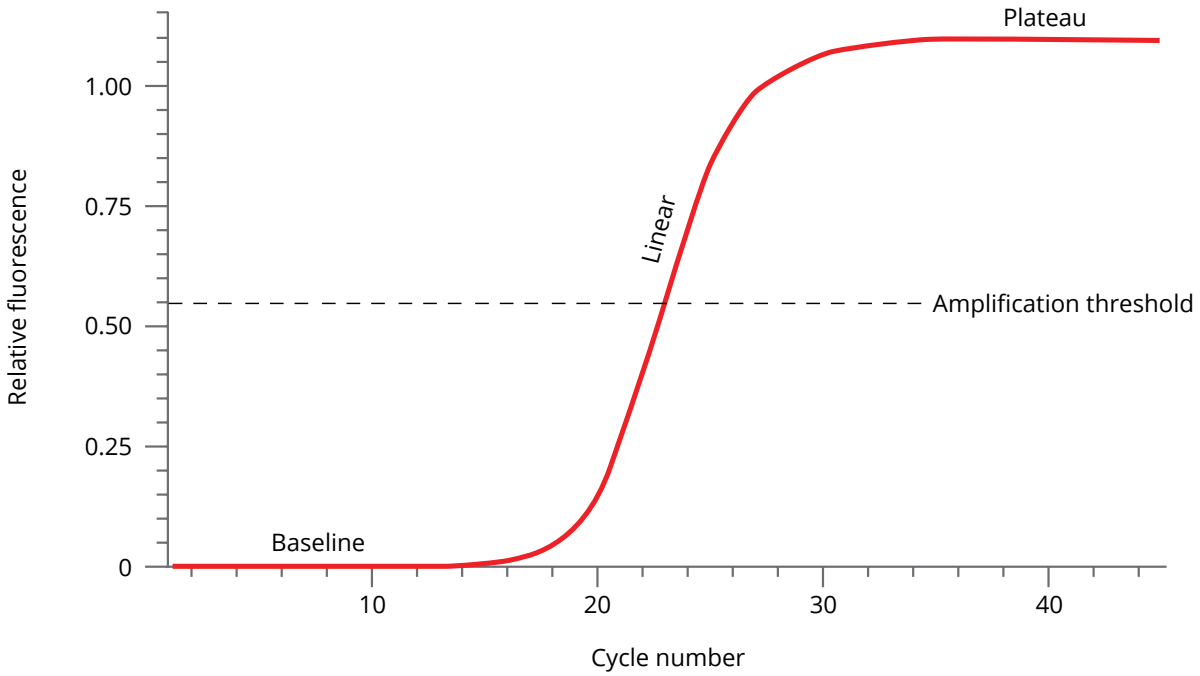
Quantitative PCR analysis

For species-specific assays, it is recommended that eDNA extracts are analysed and interpreted using probe-based quantitative PCR (qPCR) analysis because it is more sensitive and specific than conventional PCR or SYBR-based qPCR. Probe-based qPCR assays use specific probes that are labelled with a fluorescent dye (e.g. FAM, VIC, ABY or JUN) and a quencher (e.g. Minor Groove Binding quenchers, or Black Hole Quenchers) in conjunction with forward and reverse primers. During qPCR, samples containing the target eDNA bind the primer and probe and a fluorescent signal is measured in real time during PCR amplification.

A qPCR curve has 3 phases: the baseline, in which the reaction is only beginning to occur and no fluorescence can yet be captured; the exponential phase, in which the target DNA is ideally being doubled at every cycle; and a plateau phase, in which the reaction has exhausted amplification capacity and capture of absorbance reaches a non-incremental phase (Figure 2).

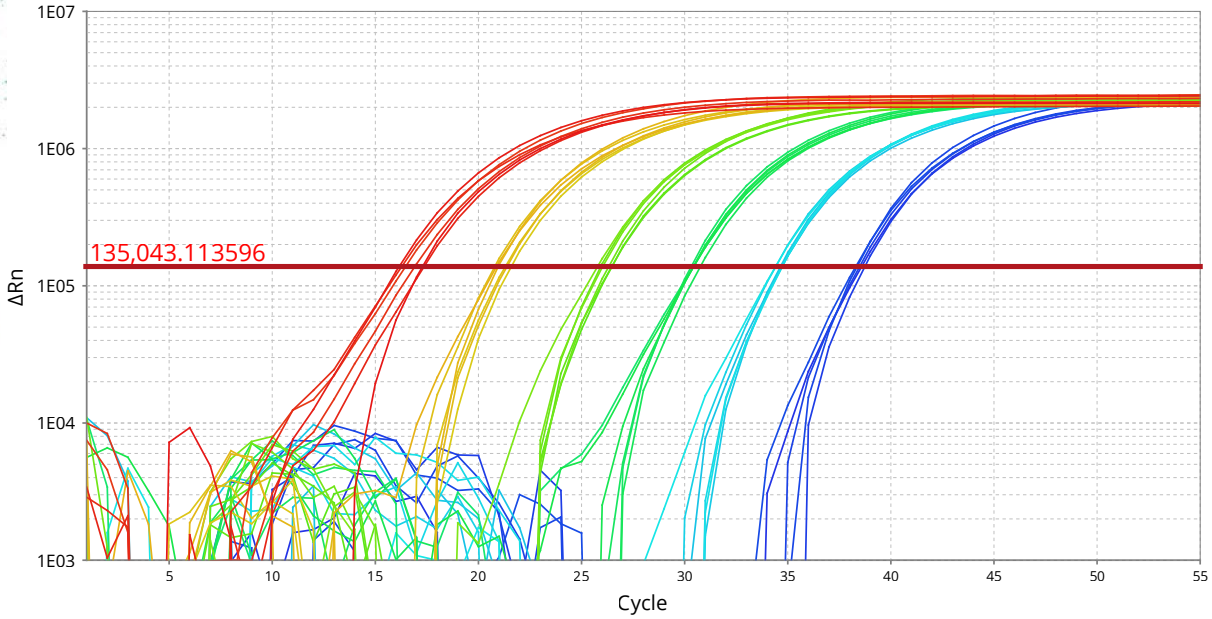
Amplification of targeted sequences results in the measurement of fluorescence that is directly proportional to the amount of double-stranded DNA present at each PCR cycle (Figure 2). Fluorescence in Applied Biosystems real-time PCR software presents results as a semi-log graph using the ΔRn, which is the magnitude of the signal generated by the given set of PCR conditions (Figure 3).

Variance in amplification among technical replicates can occur due to low template abundance in samples (Figure 4). Ideally, amplification of samples should cross the common threshold with little variation (Figure 5). Evidence of primer-dimer or early non-target amplification may be seen in the analysis (Figure 6).



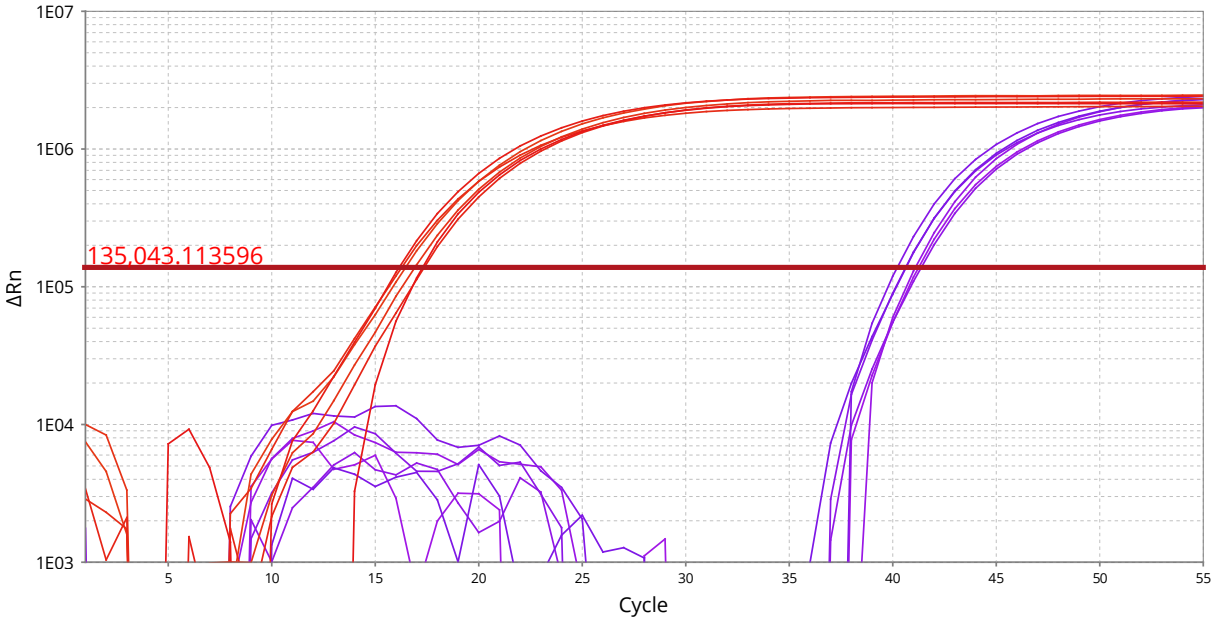
Source: modified from the SIGMA® Life Science qPCR technical Guide.

Figure 2 PCR amplification plots of relative fluorescence (standard X–Y plot)



Amplification plots obtained using a 10-fold serial dilution of the same target (5 replicates of each level). Samples with high concentrations of target template exhibit fluorescence in early cycles (e.g. red), while samples with low concentrations of target template fluoresce in late cycles (e.g. blue). The horizontal red line is the common fluorescence threshold of the analysis.

Figure 3 Probe-based quantitative PCR amplification plot



The horizontal red line is the common fluorescence threshold of the analysis.

Figure 4 Probe-based qPCR amplification plot of high (red) and low (purple) concentrations of target DNA

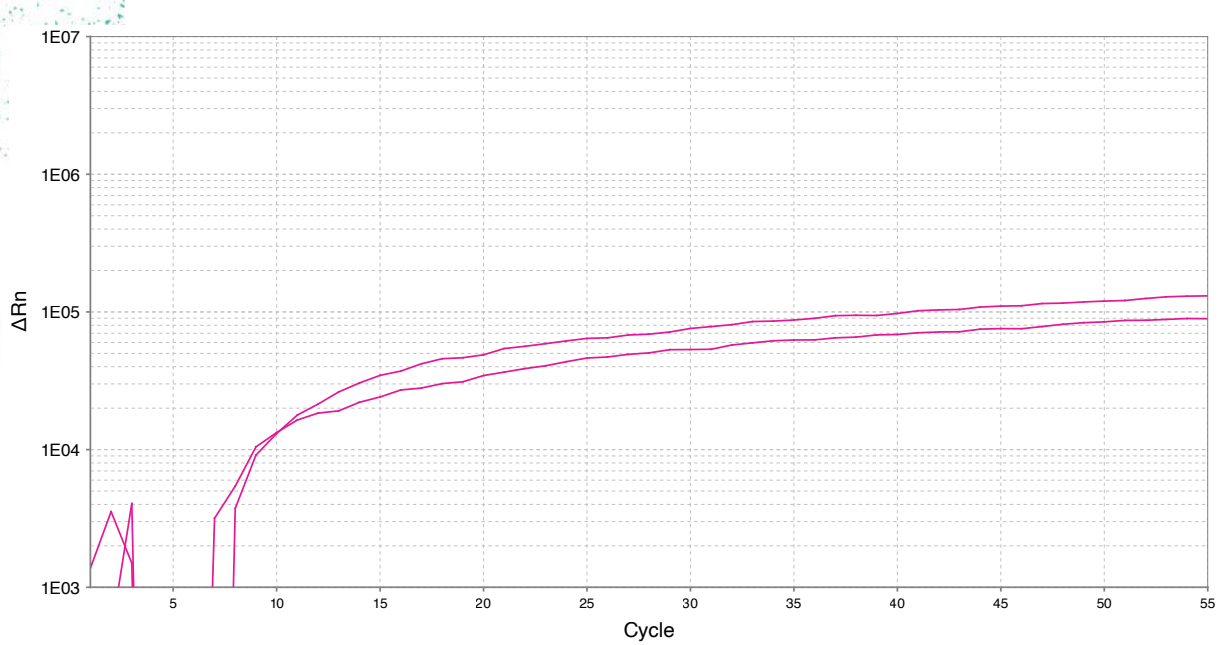


Figure 5 Primer-dimer or non-target amplification in probe-based quantitative PCR amplification plots

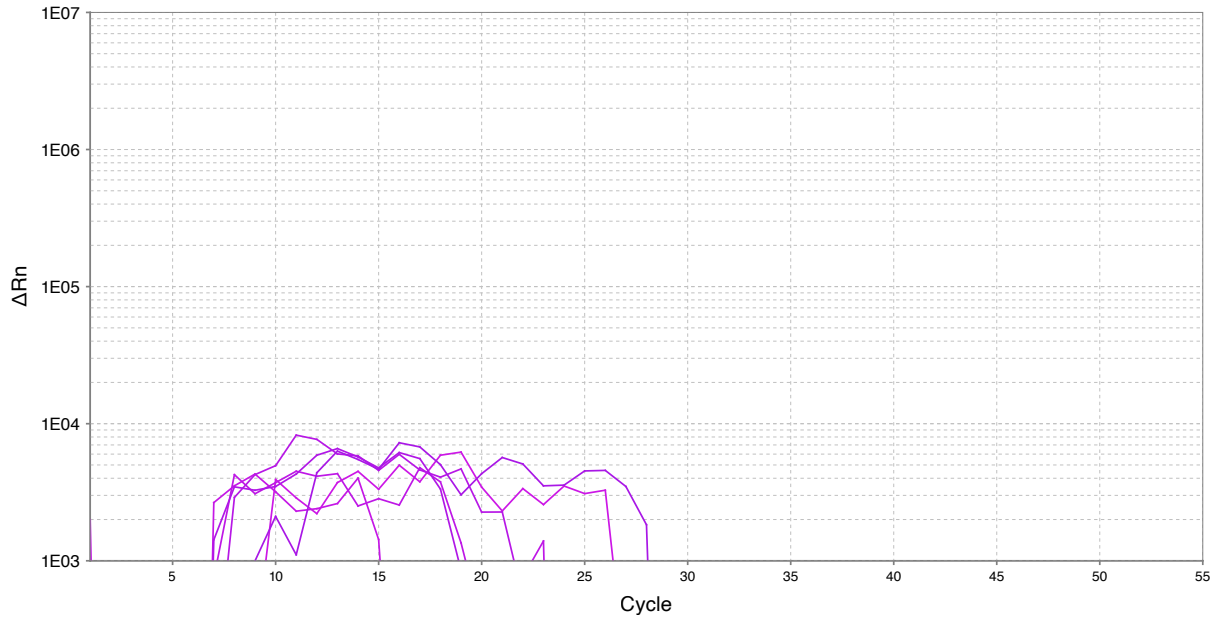


Figure 6 Negative detection signal in probe-based quantitative PCR amplification plots

Positive detection

For positive results to be accepted as reliable, the following criteria are recommended:

- All positive controls must exhibit positive amplification curves that cross the common fluorescence threshold at the expected cycle.
- All non-template controls, blank extraction and field controls should not show amplification.
- Test sample PCR replicates exhibit positive amplification curves that cross a common fluorescence threshold and are within Ct cut-off values that are based on the limit of quantification and detection for each assay.

If these conditions are met, the results are assumed to be positive. To confirm the positive result, amplicons must then be confirmed by Sanger sequencing and show 99–100% pairwise similarity to accepted accessions in online repositories.

Negative detection

For negative results to be accepted as reliable, the following criteria are recommended:

- All positive controls must exhibit positive amplification curves that cross the common fluorescence threshold at the expected cycle.
- All non-template controls, blank extraction and field controls should not show amplification.
- Test sample PCR replicates exhibit amplification curves that do not cross a common fluorescence threshold or fall within Ct cut-off values that are based on the limit of quantification and detection for each assay (Figure 6).

Indeterminate

PCR replicates are considered as indeterminate if:

- all positive controls cross the common fluorescence threshold.
- all non-template controls and blank extraction and field controls do not show amplification.
- test sample PCR replicates exhibit amplification curves that cross a common fluorescence threshold outside of Ct cut-off values based on the limit of quantification and detection for each assay.

If these conditions are met, the results are assumed to be indeterminate. To confirm the indeterminate result, amplicons must then be confirmed by Sanger sequencing and show 99–100% pairwise similarity to accepted accessions in online repositories.

Unreliable

Results are considered unreliable if they show any of the following 3 outcomes (Figure 7):

- No positive controls show amplification.
- Non-template or blank controls show amplification.
- Field or extraction negative controls show amplification.

If this occurs, samples must be reanalysed with the following inclusions in the plate:

- Reaction replicates must be completed without template DNA or eDNA to confirm reagent contamination. If any of these replicates crosses the common fluorescence threshold, then reagents used in the prior plate are contaminated and should be disposed of immediately. Fresh stocks of primer, probes, mastermix and water (and IPC, if used) should be used from that point forward.
- Reaction replicates with extract from the extraction and field blank samples. If any of these replicates crosses the common fluorescence threshold, then cross contamination may have occurred during sample collection (field controls) or during eDNA extraction processing (extraction controls), and all analysed samples must be re-extracted. Samples should be tested again.

If none of these replicates cross the common fluorescence threshold, then cross contamination may have occurred during the prior plate set up or loading of positive controls and standard dilutions. Results from this plate should be accepted instead of the previous results.

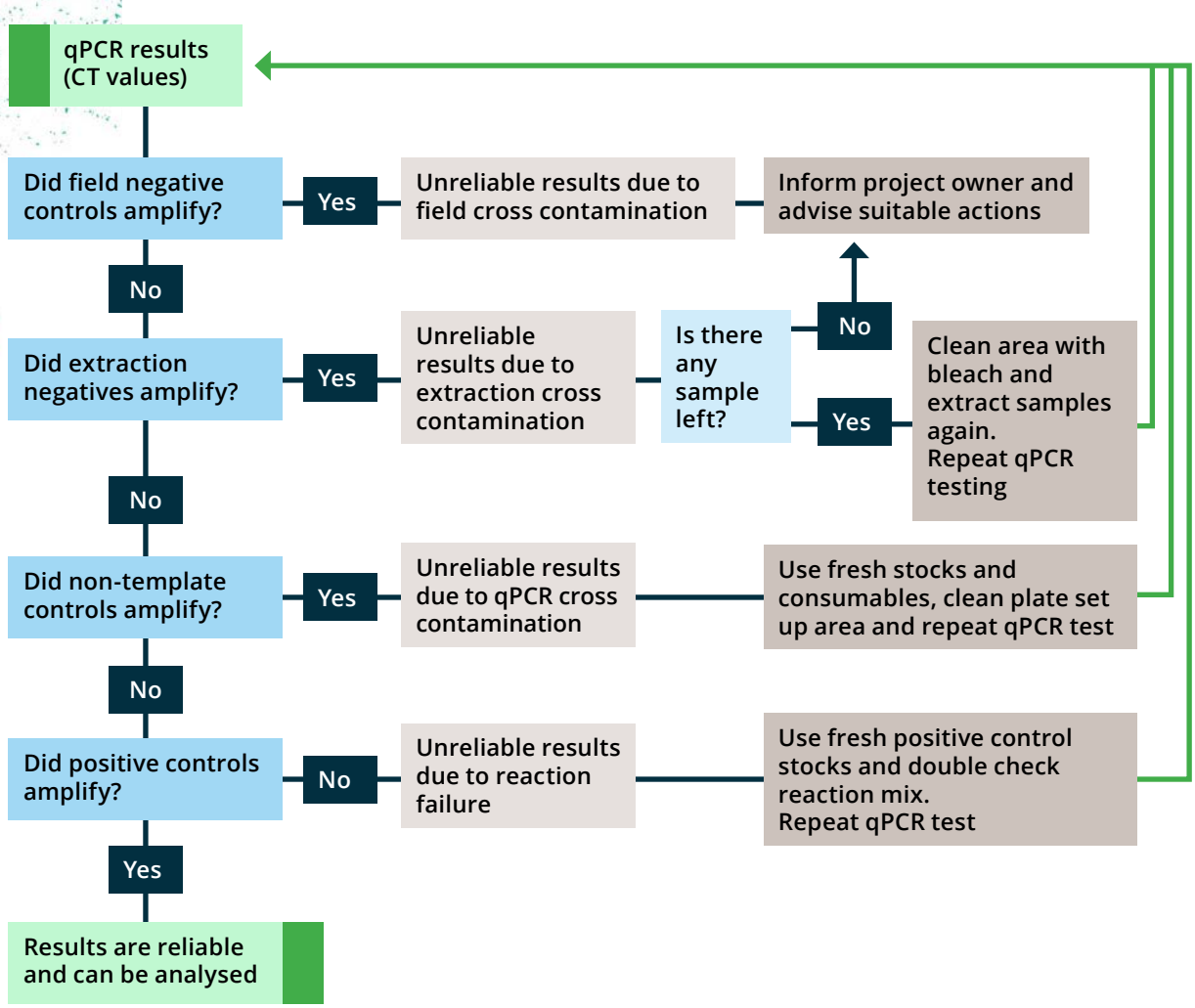
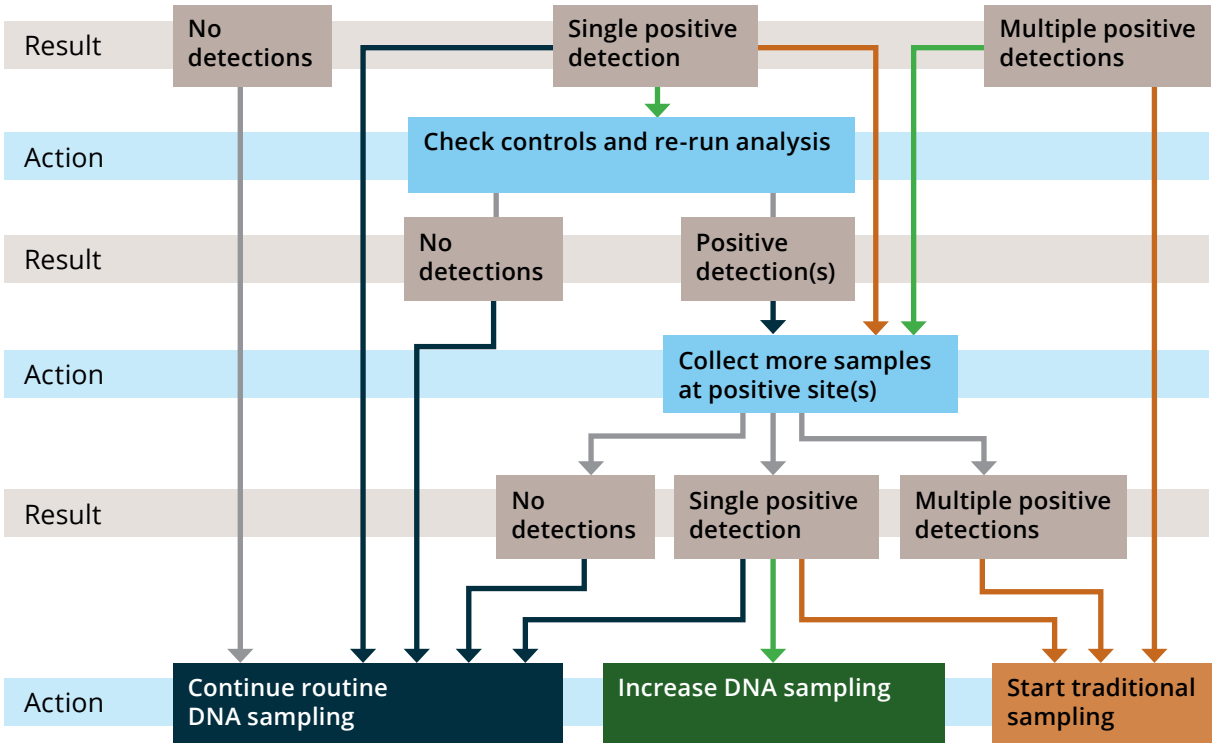


Figure 7 Identifying unreliable results in qPCR analyses

7 Interpret and communicate results

Results of eDNA surveys can require a different approach to interpretation from traditional surveys. Target species detected in eDNA surveys might not have been detected in previous or parallel surveys using traditional methods. Such differences have raised concerns in the past (Sepulveda et al. 2020) and might need to be addressed. Typical differences between eDNA metabarcoding methods and traditional methods investigating species assemblages include detecting more cryptic and/or transient species. This is a reflection of normal traditional and eDNA method biases, rather than errors in the metabarcoding process.

When high levels of stringency are required, as can be the case in biosecurity applications, addressing the level of certainty in positive or negative detections of a target species might be crucial to inform management (Figure 8; see also Ensure good communication). Likewise, the potential for false negative or positive results should be considered when interpreting results. This approach ties in with explicitly considering the limitations inherent to eDNA studies in general and those specific to the survey results.



Bold arrow colours indicate risk of significant impacts; dark blue: low risk; green: medium risk; orange: high risk.

Figure 8 Example of a management decision tree for high-stringency, species-specific eDNA sampling results, such as for biosecurity projects

When communicating results, explain the meaning of results clearly and accurately. Ensure any data visualisation does not distort meaning.

It is important to emphasise limitations and explain uncertainties (e.g. surrounding relative abundance data, taxonomic resolution, incomplete reference libraries) to avoid end users misinterpreting data. This would likely also include discussing the differences between false positives and negatives and how they might relate to the purpose of the project. A clear way to report negative results is describing them as 'below detection level' instead of 'zero'. Asking clients to voice their interpretation of results can help to check if they have correctly understood the meaning of important results.

The purpose of many eDNA applications is to inform management decisions. While these decisions are unlikely to be taken by the team executing an eDNA project, results of a study might require considering the options for follow-up actions to provide clearer results, confirm positive and negative detections, or address flaws in the study design.

Summary of key steps for environmental DNA/RNA test protocols

Step 1 Experimental design:

- Define purpose
- Decide level of replication, spatial extent, assay
- Decide on appropriate analysis methods
- Consider fieldwork logistics
- Account for realistic budget

Step 2 Minimum protocols for collecting samples:

- Wear personal protective equipment (PPE), at minimum gloves when collecting, take steps to avoid cross contamination
- Sterilise all equipment
- Include negative controls
- Record appropriate metadata

Step 3 Minimum protocols for preserving samples for processing:

- Specify the collection medium (e.g. filter mesh size, material, size)
- Specify the collection volume, container material
- Record the preservative details (e.g. concentration, hazardous details)

Step 4 Minimum protocols to extract and isolate DNA/RNA:

- Sterilise equipment and consumables (use single-use consumable where possible)
- Use a validated extraction kit or method
- Perform an extraction of negative controls
- Include an endogenous control

Step 5 Minimum protocols to ensure quality control and purification of extracts:

- Determine inhibition
- Include inhibitor removal steps and purification processes
- Determine final eDNA/eRNA extract yield and concentration

Step 6 Minimum protocols for analysis (this uses qPCR as an example, but should be tailored to the selected molecular technique):

- Include positive and negative controls, including field and extraction negatives
- Record reaction concentrations and thermal profiles
- Include standard serial dilutions curve preparation and quality
- Record details on technical replicates and assay limit of detection
- Determine false positive amplification and cross contamination
- Determine non-target amplification
- Confirm positive detection

Step 7 Minimum protocols for interpretation and communication:

- Ensure interpretation takes the study purpose and stringency needs into account
- Communicate the results precisely and accurately, including method limitations and levels of certainty

Resources

International best-practice guidelines:

- European Union: Bruce K, Blackman R, Bourlat SJ, Hellström AM, Bakker J, Bista I, Bohmann K, Bouchez A, Brys R, Clark K, Elbrecht V, Fazi S, Fonseca V, Hänfling B, Leese F, Mächler E, Mahon AR, Meissner K, Panksep K, Pawlowski J, Schmidt Yáñez P, Seymour M, Thalinger B, Valentini A, Woodcock P, Traugott M, Vasselon V & Deiner K (2021) A practical guide to DNA-based methods for biodiversity assessment. Advanced Books, doi:10.3897/ab.e68634.
- Japan: Minamoto T, Miya M, Sado T, Seino S, Doi H, Kondoh M, Nakamura K, Takahara T, Yamamoto S, Yamanaka H, Araki H, Iwasaki W, Kasai A, Masuda R & Uchii K (2021). An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols. *Environmental DNA* 3(1):8–13, doi:10.1002/edn3.121.
- Switzerland: Pawlowski J, Apothéloz-Perret-Gentil L, Mächler E & Altermatt F (2020). *Environmental DNA applications in biomonitoring and bioassessment of aquatic ecosystems: Guidelines.* Federal Office for the Environment, Bern, www.researchgate.net/publication/346511410_Environmental_DNA_applications_for_biomonitoring_and_bioassessment_in_aquatic_ecosystems.

Recent review papers:

- Beng KC & Corlett RT (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodivers Conserv* 29(7):2089–2121.
- Gaither MR, DiBattista JD, Leray M & von der Heyden S (2022). Metabarcoding the marine environment: from single species to biogeographic patterns. *Environmental DNA* 4(1):3–8.
- Pawlowski J, Bruce K, Panksep K, Aguirre FI, Amalfitano S, Apothloz-Perret-Gentil L, Baussant T, Bouchez A, Carogati L, Cermakova K, Cordier T, Corinaldesi C, Cost FO, Danovaro R, Dell'Anno A, Duarte S, Eisendle U, Ferrari BJD, Frontalini F, Fruhe L, Haegerbaeumer A, Kisand V, Krolika A, Lanzen A, Leese F, Lejzerowicz F, Lyautey E, Macek I, Sagova-Mareckova M, Pearman JK, Pochon X, Stoeck T, Vivien R, Weigand A & Fazi S (2022). Environmental DNA metabarcoding for benthic monitoring: A review of sediment sampling and DNA extraction methods. *Science of the Total Environment* 818:151783, doi:10.1016/j.scitotenv.2021.151783.
- Rourke ML, Fowler AM, Hughes JM, Broadhurst MK, DiBattista JD, Fielder S, Walburn JW & Furlan EM (2022). Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environmental DNA* 4(1):9–33.

- Yates MC, Derry AM & Cristescu ME (2021). Environmental RNA: a revolution in ecological resolution? *Trends Ecol Evol* 36(7):601–609, doi:10.1016/j.tree.2021.03.001.

Relevant methods papers:

- Calderón-Sanou I, Münkemüller T, Boyer F, Zinger L & Thuiller W (2020). From environmental DNA sequences to ecological conclusions: How strong is the influence of methodological choices? *J Biogeogr* 47(1):93–206.
- Furlan EM, Gleeson D, Hardy CM & Duncan RP (2016). A framework for estimating the sensitivity of eDNA surveys. *Mol Ecol Resour* 16(3):641–654, doi:10.1111/1755-0998.12483.
- Kumar G, Eble JE & Gaither MR (2020). A practical guide to sample preservation and pre-PCR processing of aquatic environmental DNA. *Mol Ecol Resour* 20(1):29–39.
- Wood SA, Pochon X, Ming W, von Ammon U, Woods C, Carter M, Smith M, Inglis G & Zaiko A (2019). Considerations for incorporating real-time PCR assays into routine marine biosecurity surveillance programmes: a case study targeting the Mediterranean fanworm (*Sabella spallanzanii*) and club tunicate (*Styela clava*). *Genome*, doi:10.1139/gen-2018-0021.
- Zaiko A, Greenfield P, Abbott C, von Ammon U, Bilewitch J, Bunce M, Cristescu ME, Chariton A, Dowle E, Geller J, Ardura Gutierrez A, Hajibabaei M, Haggard E, Inglis GJ, Lavery SD, Samuiloviene A, Simpson T, Stat M, Stephenson S, Sutherland J, Thakur V, Westfall K, Wood SA, Wright M, Zhang G & Pochon X (2021). Towards reproducible metabarcoding data: Lessons from an international cross-laboratory experiment. *Mol Ecol Resour* 22:519–538, doi:10.1111/1755-0998.13485
- Zhang S, Zhao J & Yao M (2020). A comprehensive and comparative evaluation of primers for metabarcoding eDNA from fish. *Methods Ecology Evol* 11(12):1609–1625.

Other useful resources:

- Atlas of Living Australia: Australia's largest repository of biodiversity occurrence data, including eDNA data
- Australian Microbiome Initiative: Characterising the diversity and ecosystem services of microorganisms, includes lab and bioinformatic workflows

References

Auer H, Mobley J, Ayers L, Bowen J, Chuaqui R, Johnson LA, Livolsi VA, Lubensky IA, McGarvey D, Monovich LC, Moskaluk CA, Rumpul CA, Sexton KC, Washington MK, Wiles KR, Grizzle WE & Ramirez NC (2014). The effects of frozen tissue storage conditions on the integrity of RNA and protein. *Biotech Histochem* 89, 518–528. doi:10.3109/10520295.2014.904927.

Barnes MA, Chadderton WL, Jerde CL, Mahon AR, Turner CR & Lodge DM (2021). Environmental conditions influence eDNA particle size distribution in aquatic systems. *Environmental DNA* 3:643– 653, doi:10.1002/edn3.160.

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL &Lodge DM (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environ Sci Technol* 48(3):1819–1827, doi:10.1021/es404734p.

Bruce K, Blackman R, Bourlat SJ, Hellström AM, Bakker J, Bista I, Bohmann K, Bouchez A, Brys R, Clark K, Elbrecht V, Fazi S, Fonseca V, Hänfling B, Leese F, Mächler E, Mahon AR, Meissner K, Panksep K, Pawlowski J, Schmidt Yáñez P, Seymour M, Thalinger B, Valentini A, Woodcock P, Traugott M, Vasselon V & Deiner K (2021). *A practical guide to DNA-based methods for biodiversity assessment*. Advanced Books, doi:10.3897/ab.e68634.

Coutant O, Cantera I, Cilleros K, Dejean T, Valentini A, Murienne J & Brosse S (2021). Detecting fish assemblages with environmental DNA: does protocol matter? Testing eDNA metabarcoding method robustness. *Environmental DNA* 3 (3):619–30.

DAWE (Australian Government Department of Agriculture, Water and the Environment) (2021). *Guide for authors writing an ANZSDP*, www.agriculture.gov.au/animal/health/laboratories/procedures/anzsdp-reviews/author-guidelines.

Deck J, Gaither MR, Ewing R, Bird CE, Davies N, Meyer C, Riginos C, Toonen RJ & Crandall ED (2017). The Genomic Observatories Metadatabase (GeOMe): A new repository for field and sampling event metadata associated with genetic samples. *PLoS Biology* 15(8):e2002925, doi:10.1371/journal.pbio.2002925.

Deiner K, Bik HM, Mächler E, Seymor M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I, Lodge DM, de Vere N, Pfrender ME & Bernatchez L (2015). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Mol Ecol* 26(21):5872–5895, doi:10.1111/mec.14350.

Deiner K, Lopez J, Bourne S, Holman L, Seymour M, Grey EK, Lacoursière A, Li Y, Renshaw MA, Pfrender ME, Rius M, Bernatchez L & Lodge DM (2018). Optimising the detection of marine taxonomic richness using environmental DNA metabarcoding: the effects of filter material, pore size and extraction method. *Metabarcoding and Metagenomics* 2 (February):e28963.

Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H & Minomoto T (2015), Use of droplet digital PCR for Estimation of fish abundance and biomass in environmental DNA survey. *PloS One* 10(3): e0122763, doi:10.1371/journal.pone.0122763.

Furlan EM & Gleeson D (2016) Improving reliability in environmental DNA detection surveys through enhanced quality control. *Marine and Freshwater Research* 68:388-395, doi:10.1071/MF15349.

Furlan EM, Gleeson D, Hardy CM & Duncan RP (2016). A framework for estimating the sensitivity of eDNA surveys. *Mol Ecol Resour* 16(3):641–654, doi:10.1111/1755-0998.12483.

Furlan EM, Gleeson D, Wisniewski C, Yick J, Duncan RP & Strecker A (2019). eDNA surveys to detect species at very low densities: A case study of European carp eradication in Tasmania, Australia. *The J Appl Ecol* 56(11):2505–2517, doi:10.1111/1365-2664.13485.

Guerrieri A, Bonin A, Münkemüller T, Gielly L, Thuiller W & Francesco Ficetola G (2021). Effects of soil preservation for biodiversity monitoring using environmental DNA. *Mol Ecol* 30: 3313–3325, doi:10.1111/mec.15674.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Strickler KM, Waits LP, Fremier AK, Takahara T, Herder JE & Taberlet P (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods Ecol Evol* 7(11):1299–1307, doi:10.1111/2041-210X.12595.

Handsley-Davis M, Kowal E, Russell L & Weyrish LS (2021). Researchers using environmental DNA must engage ethically with Indigenous communities. *Nature Ecology and Evolution* 5(2):146–148.

Harrison JB, Sunday JM & Rogers SM (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B* 286, 191519:20191409.

Hermans SM, Buckley HL & Lear G (2018). Optimal extraction methods for the simultaneous analysis of DNA from diverse organisms and sample type. *Mol Ecol Res* 18(3):557–569, doi:10.1111/1755-0998.12762.

Hinlo GD, Lintermans M & Furlan E (2017). Methods to maximise recovery of environmental DNA from water samples. *PloS One* 12(6), e0179251–e0179251, doi:10.1371/journal.pone.0179251.

IPPC (International Plant Protection Convention) (2016). *International Standard for Phytosanitary measures 27: Diagnostic protocols for regulated pests.* In: Food and Agriculture Organization of the United Nations: International Plant Protection Convention, accessed 18 June 2021, ippc.int/static/media/files/publication/en/2016/01/ISPM_27_2006_WithoutApp2_En_2015-12-22_PostCPM10_InkAmReformatted.pdf.

Jerde CL (2021). Can we manage fisheries with the inherent uncertainty from eDNA? *J Fish Biol* 98(2):341–353.

Jo T, Murakami H, Masuda R, Sakata MK, Yamamoto S & Minamoto T (2017). Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA. *Mol Ecol Resour* 17(6):e25–e33, doi:10.1111/1755-0998.12685.

Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC & Richter CA (2020). Reporting the limits of detection (LOD) and quantification (LOQ) for environmental DNA assays. *Environmental DNA* 2:271–282, doi:10.1002/edn3.29.

Langlois VS, Allison MJ, Bergman LC, To TA & Helbing CC (2021). The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. *Environmental DNA* 3*(*3):519–527, doi:10.1002/edn3.164.

Li J, Lawson Handley LJ, Read DS & Hänfling B (2018). the effect of filtration method on the efficiency of environmental DNA capture and quantification via metabarcoding. *Mol Ecol Resour* 18:1102–1114, doi:10.1111/1755-0998.12899.

Minamoto T, Miya M, Sado T, Seino S, Doi H, Kondoh M, Nakamura K, Takahara T, Yamamoto S, Yamanaka H, Araki H, Iwasaki W, Kasai A, Masuda R & Uchii K (2021). An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols. *Environmental DNA* 3(1):8–13, doi:10.1002/edn3.121.

Nagy ZT (2010). A hands-on overview of tissue preservation methods for molecular genetic analyses. *Org Divers Evol* 10:91–105, doi:10.1007/s13127-010-0012-4.

OIE (World Organisation for Animal Health) (2021). *Manual of diagnostic tests and vaccines for terrestrial animals 2021*, OIE, Paris, accessed 15 June 2021, oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access.

Pawlowski J, Apothéloz-Perret-Gentil L, Mächler E & Altermatt F (2020). *Environmental DNA applications in biomonitoring and bioassessment of aquatic ecosystems: Guidelines.* Federal Office for the Environment, Bern, researchgate.net/publication/346511410_Environmental_DNA_applications_for_biomonitoring_and_bioassessment_in_aquatic_ecosystems.

Pokon X, Ziako A, Fletcher Lm, Laroche O & Wood SA (2017). Wanted dead or alive? Using metabaroding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. *PloS One* 12(11):e0187636, doi:10.1371/journal.pone.0187636.

Riginos C, Crandall ED, Liggins L, Gaither MR, Ewing RB, Meyer C, Andrews KR, Euclide PT, Titus BM, Overgaard Therkildsen N, Salces-Castellano A, Stewart LC, Toonen RJ & Deck J (2020). Building a global genomics observatory: Using GEOME (the Genomic Observatories Metadatabase) to expedite and improve deposition and retrieval of genetic data and metadata for biodiversity research *Mol Ecol Resour* 20(6):1458–1469, doi:10.1111/1755-0998.13269.

Ruppert KM, Kline RJ & Rahman MS (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Glob Ecol Conserv* 17:e00547, doi:10.1016/j.gecco.2019.e00547.

Salomé Stauffer S, Jucker M, Keggin T, Marques V, Andrello M, Bessudo S, Cheutin M-C, Borrero-Pérez GH, Richards E, Dejean T, Hocdé R, Juhel J-B, Ladino F, Letessier TB, Loiseau N, Maire E, Mouillot D, Martinezguerra MM, Manel S, Fernández AP, Valentini A, Velez L, Albouy C, Pellissier L & Waldock C (2021). How many replicates to accurately estimate fish biodiversity using environmental DNA on coral reefs? *Ecology and Evolution* 11(21):14630–14643, doi:10.1002/ece3.8150.

SPHD (Subcommittee on Plant Health Diagnostics) (2019). Development of National Diagnostic Protocols – Procedures for Authors Reference Standard (SPHD RS No.2), National Biosecurity Diagnostic Network, Plant Health Australia, accessed 31 August 2021, plantbiosecuritydiagnostics.net.au/initiatives/national-diagnostic-protocols.

Stoeckle BC, Beggel, S, Cerwenka AF, Motivans E, Kuehn R & Geist J (2017). A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. *PloS One* 12(12):e0189119–e0189119, doi:10.1371/journal.pone.0189119.

Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS & Bruce K (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA* 3(4):823–836, doi:10.1002/edn3.189.

Thomas AC, Howard J, Nguyen PL, Seimon TA & Goldberg CS (2018). eDNA Sampler: A fully integrated environmental DNA sampling system. *Methods Ecol Evol* 9(6):1379–1385, doi:10.1111/2041-210X.12994.

Thomas AC, Nguyen PL, Howard J & Goldberg CS (2019) A self-preserving, partially biodegradable eDNA Filter. *Methods Ecol Evol* 10(8):1136–1141.

Waitangi Tribunal Report 2012. *The stage 1 report on the national freshwater and geothermal resources claim.* Lower Hutt, Legislation Direct. p258, https://forms.justice.govt.nz/search/Documents/WT/wt_DOC_59941926/Wai2358W.pdf.

Wieczorek J, Bloom D, Guralnick R, Blum S, Doring M, Giovanni R, Robertson T & Vieglais D (2012). Darwin Core: an evolving community-developed biodiversity data standard. *PloS One* 7(1):e29715, doi:10.1371/journal.pone.0029715.

Wilkinson MD, Dumontier M, Aalbersberg IJ, Appleton G, Axton M, Baak A, Blomberg N, Boiten JW, da Silva Santos LB, Bourne PE, Bouwman J, Brookes AJ, Clark T, Crosas M, Dillo I, Dumon O, Edmunds S, Evelo CT, Finkers R, Gonzalez-Beltran A, Gray AJG, Groth P, Goble C, Grethe JS, Heringa J, 't Hoen PAC, Hooft R, Kuhn T, Kok R, Kok J, Lusher SJ, Martone ME, Mons A, Packer AL, Persson B, Rocca-Serra P, Roos M, van Schaik R, Sansone S-A, Schultes E, Sengstag T, Slater T, Strawn G, Swertz MA, Thompson M, van der Lei J, van Mulligen E, Velterop J, Waagmeester A, Wittenburg P, Wolstencroft K, Zhao J & Mons B (2016). The FAIR Guiding Principles for scientific data management and stewardship. *Scientific Data* 3:160018, doi:10.1038/sdata.2016.18.

Williams MR, Stedtfeld RD, Engle C, Salach P, Fakher U, Stedtfeld T, Dreelin E, Stevenson RJ, Latimore J & Hashsham SA et al. (2017). Isothermal amplification of environmental DNA (eDNA) for direct field-based monitoring and laboratory confirmation of *Dreissena* sp. *PloS One* 12(10):e0186462, doi:10.1371/journal.pone.0186462.

Zaiko A, Greenfield P, Abbott C, von Ammon U, Bilewitch J, Bunce M, Cristescu ME, Chariton A, Dowle E, Geller J, Ardura Gutierrez A, Hajibabaei M, Haggard E, Inglis GJ, Lavery SD, Samuiloviene A, Simpson T, Stat M, Stephenson S, Sutherland J, Thakur V, Westfall K, Wood SA, Wright M, Zhang G & Pochon X (2021). Towards reproducible metabarcoding data: Lessons from an international cross-laboratory experiment. *Mol Ecol Resour* 22:519–538, doi:10.1111/1755-0998.13485.