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Laboratory Analysis Guidelines and Best Practices for Environmental Genomics Applications Relevant to the Energy Sector

This Guidance is publicly available as a draft version for readers for review and trial its use. Any comments that improve the content or workability of the draft guidance are welcomed by the JIP.

Please send comments to **info@iogp-edna.org**.

We will endeavour to address comments, if received by Feb 28th 2025, in the Final (edited and formatted) version of the guidance which will be available early in 2025. Any comments received after this date or any comments that cannot be addressed within this timeframe will be considered for future updates to the guidance. Updates are planned every 1-2 years to capture the rapid advances in this field.

Prepared by CEGA and eDNAtec for the International Oil and Gas Producers' Association Joint Industry Program on Environmental Genomics.

REVISION HISTORY

RELEASE	NAME	COMPANY	DATE OF ISSUE	COMMENTS
R1	Nicole Fahner	eDNAtec	2024-Jan-26	First release.
R2	Nicole Fahner	eDNAtec	2024-Aug-19	Revisions throughout to address IOGP and external reviewer comments. Reordered sections to lead with QMS requirement as the foundation for the rest of the guidance.
R3	Nicole Fahner	eDNAtec	2024-Oct-07	Revisions throughout to address more reviewer comments, fix formatting inconsistencies, and integrate text from RFP2. Update to questionnaire template in Section 11.2 to also include lab competency and services.
R4	Nicolas Tsesmetzis	Shell	2024-Oct-11	Addition of Revision History and Disclaimer
R5	Michael Marnane	Chevron	2024-Nov-21	Addition of Instructions to readers

Contents

1 Scope of Document and Regulatory Context

This document specifies guidelines and best practices for environmental genomics laboratory analysis and offers service providers direction and minimum expectations when implementing environmental genomics programs for industry applications with a focus on the oil and gas (and more broadly, energy) clients. It covers physical, operational, and technical recommendations from environmental sample receipt, storage, and nucleic acid extraction to molecular assay setup and testing, including both sequencing and qPCR/dPCR approaches.

In addition, this document serves as a comprehensive guide to assist industry clients in selecting a qualified service provider for environmental genomic laboratory analysis, by outlining the essential requirements, operational practices, and best practice methodologies.

The focus of this document is on currently accepted approaches with briefs on upcoming technical advances. It is not meant to be a how-to or training manual, but rather it instructs on how to improve repeatability of results and confidence in environmental genomics data through the design of appropriate systems and procedures.

1.1 Status of Regulatory Requirements and Oversight

It is important to understand the status of regulatory requirements and oversight in environmental genomics (EG), as this provides important context for standards and quality control within the field. There is an evolving acceptance and application of EG approaches by regulatory agencies in various jurisdictions, although usage remains largely exploratory. For example, EG has started to be accepted by certain agencies in the United States in a regulatory context, particularly under the Endangered Species Act and National Environmental Policy Act (Laschever et al., n.d.). Evidence from eDNA-based studies has been used in management and research based on the Endangered Species Act for several species since 2018 (Laschever et al., n.d.). In this context, peer-reviewed studies and data were used (e.g., for critical habitat designation in bull trout (McKelvey et al., 2016; Wilcox et al., 2013)) and no standardized field or laboratory methods were required to use eDNA data as evidence. In all cases, eDNA data was used alongside other data sources and not as a standalone method (Laschever et al., n.d.). eDNA has also been included as a monitoring method in an environmental impact statement prepared by the Bureau of Ocean Energy Management for an offshore wind energy development (Bureau of Ocean Energy Management Office of Renewable Energy Programs, 2023). eDNA sampling was noted as a survey method for marine mammal monitoring; however, no methodological information is included (Bureau of Ocean Energy Management Office of Renewable Energy Programs, 2023). The environmental impact statement does not represent rule-making or a court decision but it is subject to development review under the National Environmental Policy Act (Laschever et al., n.d.). Overall, despite increasing acceptance as a data stream in regulatory contexts in the US, there are currently no standards or protocols on laboratory analysis of eDNA for regulatory purposes.

The United Kingdom is one of a few countries where EG approaches have been accepted for regulatory enforcement. Targeted qPCR eDNA surveys are a nationally recognized method to detect the presence/absence of the endangered great crested newt in ecological impact assessments for the planning and development sector (The Use of Environmental DNA Test for Great Crested Newt Licensing Purposes, 2022) and only results from laboratories passing the standardized proficiency test are accepted.

While the uptake into regulatory contexts has been slow, many agencies have shown their commitment to including or accepting EG into regulations through published strategies and roadmaps as well as several useful resources to guide laboratory analysis (see **Table 11-1**). The guidelines vary in scope (e.g., sample types, environments included, analyses) and prescriptiveness. Many of these resources focus on targeted eDNA approaches, as these approaches are well-established, and methods are less complex. As uptake of EG continues to increase among agencies, the guidance and oversight from these agencies is likely to evolve to reflect technological advances and adoption of new approaches. An overview of these resources is provided in (Appendix Section 11.3). The government-issued documents make clear the intention to move eDNA to regulator application, but they do not provide standards for laboratory analysis.

The lack of broadly accepted standards is a gap often cited by regulators, which has led to several non-governmental standardization bodies working on EG approaches. Most recently, the International eDNA Standardization Task Force (iESTF) was formed by members of the international eDNA community with a mandate to accelerate the development of standards by acting as a hub for multiple working groups and national eDNA bodies, and lead engagement with stakeholders across user groups. The Canadian Standards Association (CSA), European Standards Organization (Comité Européen de Normalisation, CEN), the eDNA Society (in Japan), and International Standards Organization (ISO) have work in various stages on EG laboratory analysis standards (see Appendix Section 11.3). The development of standards from independent bodies is relatively recent and has not yet been formally integrated into regulations.

A second commonly cited gap limiting regulatory implementation of eDNA is the need for accredited EG laboratories to support the use of eDNA in enforcement or forensic applications. This was demonstrated in Canada during the 2021 response to invasive zebra mussels being important on moss ball products (Cowell et al., 2021). Government labs were not accredited, and staff did not have the training to provide expert witness testimonies, nor were there accredited EG service providers with this capability at the time. As a result, eDNA was only used as a screening tool to recommend follow up with other methods and could not be used as the only evidence used to support charges. Regulatory recognition of a set of standards and accreditation of labs to these standards would support increased regulatory uses of eDNA in future.

Information from all the published protocols, guidelines, and standards referenced in Appendix Section 11.3 and **Table 11-1** were used in the drafting of this document.

2 Abbreviations and Acronyms

Abbreviation	Description
BAC	Benzalkonium chloride
bp	Base pair
COI	Cytochrome c oxidase subunit 1
Cq or Ct	Quantification Cycle or Cycle Threshold
Cytb	Cytochrome b
DNA	Deoxyribonucleic acid
cDNA	Copy or complementary DNA
cpDNA	Chloroplast DNA
eDNA	Environmental DNA
mtDNA	Mitochondrial DNA
rDNA	Ribosomal DNA
DNase or RNase	Deoxyribonuclease or ribonuclease
DRS	DNA removal solution
EDTA	Ethylenediaminetetraacetic acid
EG	Environmental Genomics
HEPA	High efficiency particle absorbing [filter]
HRMA	High resolution melting curve analysis
ID	Identification
IAC	Internal amplification control
IPC	Internal positive control
ISO	International Organization for Standardization
ITS	Internal transcribed spacer
LIMS	Laboratory information management system
LOD	Limit of detection
LOQ	Limit of quantification
MLA	Machine learning algorithms
NaCl	Sodium chloride
NGS	Next generation sequencing
NRT	No reverse transcription control
NTC	No template control
PCR	Polymerase chain reaction
dPCR	Digital PCR
qPCR	Quantitative PCR
PPE	Personal protective equipment
QC	Quality control
QMS	Quality management system
rbcL	Ribulose bisphosphate carboxylase large subunit
RNA	Ribonucleic acid
eRNA	Environmental RNA
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
SDS	Sodium dodecyl sulphate
SOP	Standard operating procedure
Tris-HCl	Trisaminomethane hydrochloride
UV	Ultraviolet

3 Introduction

3.1 Working with Environmental DNA or RNA

Environmental DNA (eDNA) or RNA (eRNA) samples are distinct from conventional molecular or genomic samples due to their unique sources and the inherent challenges this presents. eDNA and eRNA are collected from environmental substrates like water, soil, sediments, and air, where they are present in minute quantities amidst a complex mixture of organic and inorganic matter, unlike DNA and RNA from pure cultures or well-characterized tissue samples. The complexity of these samples requires specific methods that are highly sensitive to discern the target sequences from a backdrop of genetic material from myriad other organisms. Moreover, environmental factors may partially degrade eDNA and eRNA, so specialized molecular assays and preservation techniques are required.

These features of eDNA and eRNA make samples very sensitive to contamination from the laboratory environment and cross-contamination from other samples, so working with eDNA and eRNA requires stringent laboratory controls to test for contamination and careful processes to prevent it. Accurate and reliable detection of diverse species requires highly sensitive and precise molecular laboratory instruments as well as stringent assays validation.

3.2 Environmental Genomics Laboratories

An EG laboratory analyzes environmental DNA (eDNA) and RNA (eRNA) samples, adhering to validated protocols for sample handling, nucleic acid extraction, molecular assays, and analysis to provide reliable and reproducible results. The features of eDNA and eRNA make samples very sensitive to contamination from the laboratory environment and cross-contamination from other samples, so stringent laboratory controls are applied to test for contamination and careful process design minimizes risks.

The EG laboratory needs technical expertise and specialized infrastructure to conduct these analyses. EG laboratory expertise includes standardized processes throughout operations and highly competent personnel having formal and site-specific standardized training in all relevant areas. Infrastructure requirements include dedicated equipment and machinery (such as pipettors, thermocyclers, analytical instruments, cold storage units) and lab spaces designed for effective project execution while minimizing contamination risks. All components of the operation are controlled under a Quality Management System (QMS).

3.3 Quality Management Systems – A Layered Approach

To serve industry clients, a QMS shall be in place and should be certified.

A well-built, robust QMS is essential to producing reliable, traceable EG data (Centre for Environmental Genomics Applications, 2023). There is currently no international standard specific to EG laboratories, however, a working group has recently (in 2024) been appointed to develop an ISO standard for environmental DNA and RNA methods (ISO - International Organization for Standardization TC 147/SC5 Working Group 13 “eDNA, DNA and RNA methods”). In the interim, the International Organization for Standardization (ISO) offers multiple globally recognized quality management standards that relate to EG laboratories including, but not limited to, ISO 9001:2015 (Quality Management System), ISO 17025:2017 (Testing and Calibration Laboratories), ISO 11063:2020 (Soil Quality- Direct Extraction of Soil DNA) and ISO 20387:2018 (General Requirements for Biobanking). These existing standards allow EG laboratories to adopt a base layer of internal standardization through a formal QMS and then additional elements may be layered as programs advance through further levels of standardization, outlined below.

The first layer for EG laboratories is the implementation of an internal QMS to ensure consistency within the laboratory. The basic QMS framework encompasses everything from general documentation, reporting procedures, adherence to Good Laboratory Practices, training protocols, device validation, and other aspects of ISO 9001:2015 like risk management (Leese, 2023). The QMS establishes standardized procedures and protocols, enhancing the accuracy and reproducibility of results and maintains consistency within the laboratory. Furthermore, certification to a QMS standard includes external auditor review of the full system. A quality management system in good standing with all relevant EG services in its scope and certified under ISO 9001:2015 replaces the need for industry clients to individually vet these

elements of reliability and reproducibility of a prospective service provider. It is the recommended minimum entry point for providing EG laboratory analysis services to industry.

Looking forward in the standards development pipeline, proficiency testing is the next layer in the lab selection process by facilitating comparisons among laboratories. Proficiency testing not only ensures the reliability of analytical results but also aids in identifying areas for improvement. It contributes to the overall quality assurance by promoting competitiveness and transparency within the laboratory landscape and can help clients confidently choose an EG laboratory to carry out their projects. Proficiency testing is guided by sample type, and specific certification of analysts and laboratories according to existing ISO standards, such as ISO 13528:2015 (Statistical methods for use in proficiency testing by interlaboratory comparison)(Leese, 2023).

The third layer introduces technical minimal specifications, outlining specific parameters that must be met for service providers to conduct a particular test, such as emphasizing sample type-specific technical standards (Leese, 2023). This layer is pivotal in guaranteeing the validity and precision of test outcomes across different laboratories and includes considerations for negative and positive controls, sequencing depths, purity standards, and data management in alignment with the FAIR principles (Findable, Accessible, Interoperable, and Reusable)(Leese, 2023; "(PDF) The FAIR Guiding Principles for Scientific Data Management and Stewardship," 2024). These requirements act to safeguard data reliability and accuracy, fostering a more robust and standardized testing environment.

The fourth layer and ultimate goal for standardization of EG laboratory analyses, is achieved through establishment of reference laboratories. A reference laboratory not only sets benchmarks but also establishes expected results across laboratories for a particular test and contributes to the creation of technical standards. This structured trajectory reflects a gradual ascent in performance expectations. Reference laboratories oversee testing of EG service providers by quantifying accuracy and precision using a defined "blind sample" specifically a DNA sample with a known composition(Leese, 2023). This process necessitates engagement with an accredited reference lab, reinforcing the importance of collaboration and adherence to stringent quality control (QC) measures. The involvement of reference labs contributes to the overall reliability, traceability, and comparability of results across different laboratory settings. The ISO 17025:2017 (Testing and Calibration Laboratories) Standard includes this level of QC using external reference standards and is what the future ISO standard for eDNA, DNA and RNA methods aims to achieve.

Rigorous QC measures are essential for maintaining the credibility and scientific validity of eDNA data.

3.4 Choosing a Service Provider

Selecting an appropriate laboratory for EG work is a critical decision for industry members, as it directly impacts the reliability and credibility of data generated. Commercial laboratories for eDNA analysis are becoming established and, as a business, are expected to have standardized practices for sample handling, analysis, reporting, and turn-around-time for completion of services. Commercial laboratories will also provide a list of available services, a pricing structure, and a mechanism to issue quotations to establish an agreement for scope of services.

This standard provides the basis for evaluating laboratory service providers to determine if a candidate meets minimum expectations. Selection of a qualified laboratory is a critical first step to ensure the data generated will meet the stringent requirements of potential applications, including decision-making, legal contexts, conservation efforts, regulatory adherence, and research.

See Section 11.2 in the Appendix for a draft questionnaire to determine if a service provider adheres to this standard.

4 Risk Management and Best Practices in the EG Laboratory Environment

A laboratory-derived false positive detection is defined as an erroneous detection of a molecular target within an environmental sample while a laboratory-derived false negative is defined as the erroneous non-detection of a molecular target that is truly present within an environmental sample. A core responsibility of EG laboratories is identification, tracking, and mitigation of sources of these errors through deliberate facility design (Section 4.1.1), operational practices (Sections 4.1.2 and 4.2), and assay design and implementation (Sections 5.2, 6, 8, 9, and 10)

4.1 Risk Assessment

EG laboratories shall identify risks specific to working with eDNA and eRNA and maintain a record of the mitigating efforts for each risk as part of their QMS. The risks and mitigations shall be reviewed annually and as part of planning for change processes.

4.1.1 Facility Design Risks and Mitigations

The suitability of new or existing spaces for carrying out EG laboratory analyses is evaluated in the risk assessment and considers all aspects of the physical working environment including workspace layout, storage, ventilation, plumbing, and materials. Three types of EG risks linked to the laboratory environment and examples of suitable mitigations are outlined in **Table 4-1**. These suggested mitigations may be replaced by alternative measures that achieve a similar outcome as needed. For example, when physical space is at a minimum and separate dedicated work areas are not available, labs may plan SOPs to use operational practices like secondary containment (e.g., double bagging) to isolate eDNA samples and their derivatives in shared storage spaces and use separation in time for shared workspaces with thorough decontamination of surfaces, equipment, and PPE between activities.

Table 4-1 Facilities Risk Assessment

Risk	Suggested Facility Mitigations

1. Sample cross-contamination from high concentration samples	• Establish dedicated eDNA work areas and equipment, physically separated by contamination risk. The minimum contamination risk categories to be considered are:
	  ○ Reagent only handling and storage
	  ○ Environmental sample handling and storage (pre-amplification)
	  ○ Post-amplification handling and storage
	  ○ Tissue handling and storage
	• The layout of workspaces should support a unidirectional workflow from no DNA (reagent handling), then low DNA concentration (e.g., environmental samples), and high DNA concentration (post-amplification). Allow sufficient storage space for materials in each work area to facilitate this workflow and minimize contamination risks. Install separate sinks for pre- and post-amplification areas.
	• Identify cold storage needs of each work area to accommodate the unidirectional workflow, contamination risk, and volume of samples at each stage.

2. Sample contamination from the laboratory environment	• Use non-absorbent materials for work surfaces, seating, floors, and cabinetry resistant to the chemicals used for routine decontamination. • Design layout and ventilation (e.g., positive pressure system) to prevent direct air flow from the outdoors or from the post-amplification workspace into the reagent

	handling and pre-amplification areas unless the air is HEPA-filtered. • Complete work within secondary containment (e.g., laminar flow hoods). • Block routes that pests may use to enter workspaces (e.g., floor drains should have one-way valves). • Design floor plan to facilitate controlled access to pre-amplification areas to reduce unnecessary foot traffic.
3. Loss of sample and reagent integrity due to improper environmental conditions	• Integrate climate control systems that can maintain temperature and humidity within the optimal range for cold storage units and sample handling equipment. • Integrate remote monitoring systems and integrated back-up power generators on cold storage circuits. Ensure cold storage capacity includes redundancy in case of equipment failure or maintenance.

4.1.2 Operational Risks & Mitigations

As part of the QMS risk assessment, EG laboratories shall systematically identify risks related to handling and analysis of eDNA/eRNA and the associated mitigating operational practices. Seven types of risks linked to EG operational practices and examples of possible mitigations are outlined in **Table 4-2**. The EG laboratory shall ensure the specific needs of working with eDNA/eRNA are addressed throughout the QMS, including in SOPs, equipment maintenance and calibration program, training program, and other areas identified in the risk assessment that affect the ability to produce high quality, reliable eDNA data.

Table 4-2 Operational Risk Assessment

Risk	Suggested Operational Practice Mitigations
1. Sample contamination from the laboratory environment and personnel	• Require PPE that eliminates direct physical contact between them and samples (e.g., gloves, lab coat, mask, hairnet). • Train staff to change gloves after touching a potentially contaminated surface. • Require indoor shoes or shoe covers in the laboratory. • Routine workspace and equipment decontamination. • Do not perform DNA extractions after working with amplified DNA on the same day, especially where physical separation is not ideal. • Use certified sterile/DNA-free/molecular biology grade supplies. • Control access to pre-amplification laboratory areas to only trained staff.
2. Sample to sample contamination	• Should change gloves between environmental samples • Designate separate PPE for pre- and post-PCR activities . • Maintain PPE (e.g., scheduled lab coat cleaning). • Ensure properly fitted PPE. • Routine decontamination of equipment and tools.

	• Introduce pipetting practices that reduce risk of sample carry over from aerosols including using filter tips and pipetting sample into liquid.
3. Contamination is undetected	• Use negative controls in laboratory SOPs
4. Samples or reagents degrade due to improper shipping, storage, or handling.	• Use comprehensive sample and reagent receiving SOPs. • Include sample and reagent storage requirements in all laboratory SOPs. • Implement equipment maintenance program to increase the life expectancy of cold storage units and reduce the risk of malfunctions. (e.g. scheduling regular defrosting). • Prepare emergency response procedures for equipment failure or power outages. • Require DNA storage plasticware to be free of DNase and RNase, and material that demonstrates low DNA adsorption.
5. EG analysis fails or data quality is negatively impacted due to analytical equipment failure or malfunction.	• Implement an equipment maintenance and calibration program to monitor performance of key equipment • Require SOPs to specify controls or standards to run with assays to assess equipment performance and how to interpret the results. Suggest corrective actions.
6. EG analysis fails or has quality impacted because operational practices are not followed correctly or consistently	• Implement a quality management system, including staff training. Keep training records. Use standard operating procedures. Keep track of non-conformances and corrective actions.
7. Information is lost or incorrect	• Develop a system of record keeping, such as a Laboratory information management system (LIMS) • Provide LIMS training to all relevant staff. • Include LIMS requirements in SOPs.

4.2 EG Laboratory Best Practices

Best practices are the collective, specific actions that support quality assurance and are derived from the risk assessment. EG laboratories shall create and implement a best practices guide for EG laboratory staff that describes the specific set of general practices applied throughout EG operations to mitigate risks, including personal protective equipment (PPE), decontamination, tissue handling, and environmental sample handling. The following subsections provide background information and widely accepted best practices that may be implemented.

4.2.1 Personal Protective Equipment

In addition to ensuring the health and safety of laboratory personnel, PPE serves as a physical barrier that protects eDNA samples from contamination. The standard PPE for eDNA work is disposable gloves and separate pre-PCR and post-PCR lab coats. Other PPE is added based on risk, such as surgical masks and hairnets.

4.2.2 Decontamination

Decontamination removes unwanted DNA that might interfere with the processing of samples and is separate from general cleaning practices that remove dirt, debris, or dust. Contaminant DNA can result in false positives downstream; therefore, decontaminating surfaces and equipment at the beginning and end of every work task prevents cross-contamination between projects, sample batches, and controls. Thorough decontamination and cleaning also removes nuclease enzymes that would break down DNA or RNA if spread to samples.

There are three recognized and reliable methods used for decontamination: sodium hypochlorite (bleach), commercial DNA removal solutions, and Ultraviolet (UV) radiation.

Sodium hypochlorite: Freshly made 1-10% sodium hypochlorite solution (Bockrath et al., 2023; Jerney et al., 2023) with an appropriate contact time based on the application method, concentration, and surface can efficiently decontaminate surfaces and equipment (Fischer et al., 2016; Nilsson et al., 2022). It is cheap and readily available, but caution is needed as it reacts with other chemicals used in eDNA processing (such as guanidine thiocyanate) and creates toxic fumes.

Commercial DNA removal solutions: Commercially available DNA Removal Solutions (DRS) such as ELIMINase™ (Decon Laboratories, Inc., PA, USA), DNAOUT™ (G-Biosciences, MO, USA), and others can be applied to instantly remove DNA from surfaces, labware, pipettes, and other major equipment, unless the equipment's user manual recommends otherwise. Some products, such as ELIMINase™, also remove DNase and RNase enzymes to further protect samples. It is important to review product specifications before selecting a DRS.

All decontaminant solutions require a rinse step with deionized or distilled water before sample handling to avoid removing the eDNA in samples.

Radiation: Decontamination is possible with short-wave UV radiation of 254 nanometers. It will only work on surfaces directly exposed to UV light, so best practice is to combine UV radiation with other methods to ensure complete decontamination. It should not be used as the sole method (Gefrides et al., 2010).

Novel decontamination methods by an EG laboratory must be justified and verified by tests showing that the decontamination method works, and these tests must be documented and available for review.

Decontaminating all the molecular biology supplies before or after each use in an EG laboratory is difficult and introduces additional processes and risks. Single-use consumables are the norm and discarded after use to prevent cross-contamination. Products that make direct contact with samples, such as primary storage containers, pipette tips, or laboratory water, should be certified molecular biology grade or sterile, DNA-free, and nuclease-free where possible. On receipt of these products, the protective packaging may be decontaminated before being placed in the lab for use.

4.2.3 Reference Tissue Specimen Handling

Reference tissue (i.e., a whole organism or a subsample from one) has multiple purpose in the EG laboratory: to generate sequences from a known specimen to add to reference databases for taxonomic assignment, to identify new marker gene regions to aid in developing new molecular assays, and to act as positive controls to validate molecular assay performance. Tissue specimens have much higher concentrations of DNA compared to environmental samples and likely belong to species that the laboratory is trying to detect in the environmental samples. Storage and handling practices should maximize separation between the reference tissues, their DNA extracts, and environmental samples.

Tissue samples shall be extracted, stored and handled separately from environmental samples, and procedures that include reference tissue or DNA must directly address how cross-contamination risks will be mitigated.

4.2.4 Environmental Sample Receiving

EG laboratories shall retain clear records for chain of custody during receipt of samples, whether the samples are handed off in-person or shipped. To support this, samples sent to EG laboratories should include a sample submission list provided by the client. The sample receiver shall cross-reference this list with the samples received. The sample receiver should know the intended storage and transportation conditions and shall record the status (e.g., temperature, integrity of packaging) on arrival at the laboratory. The condition and list of samples received shall be documented in a sample submission receipt for the client and internal records.

Samples should be individually packaged in some sort of sterile primary containment and then biological replicates may be grouped together for ease of receiving in a secondary container or bag. The eDNA samples shall be physically isolated from any tissue samples in the shipment and never transported in containers previously used for tissue samples without

thorough decontamination. If sample packaging for transport is not done correctly, it shall be reported by the EG laboratory receiving log as well.

Environmental metadata should be included as part of the sample submission process because it will inform sample processing and analysis steps and help determine expectations for normal QC test results. Recommended metadata fields for data analysis are covered in [RFP 2 and 4] but fields of particular importance to the lab are listed in **Table 4-3**. All relevant information should be updated in a laboratory information management system (LIMS) used to track samples (see 4.3. below).

Table 4-3 Metadata fields important for EG laboratories

Metadata Fields	Importance
Sample type (e.g., water, sediment) Submission type (e.g., extract, filter) Storage condition Preservation method Preservative solution composition Organism (tissue)	Sample and Analysis Descriptors: Different laboratory handling, storage, and extraction methods are applied depending on these categories.
Sample size Volume filtered Soil or sediment type and quantity (g)	eDNA Quantity Variables: Dilution factor and inhibitor content may vary depending on these categories.
Site Transect Sediment layer depth (cm) Sample water depth (m)	Troubleshooting Variables: Unexpected QC results can be compared with replicates from the same sampling groups.
Genetic material (DNA, RNA, extract, etc.) DNA volume DNA storage DNA extraction method DNA concentration Storage buffer Plate ID Well ID Primers (Forward & Reverse) Indices (Forward & Reverse) Library prep kit Average fragment length Spike-in	Genomic Variables: Additional information for submission of DNA or RNA extracts or derivatives (e.g., amplicons, sequencing libraries).
List of target species or taxonomic groups Geographic location (GPS coordinates)	Marker Selection: Additional information used to select or confirm molecular assay type, DNA markers, and primer sets.

4.2.5 Sample Stability

Environmental samples, eDNA, and eRNA, need to be stored appropriately to benefit from the archival opportunities these samples present. There are different storage requirements of different sample types and commercially available preservatives which affect sample handling to ensure sample integrity is maintained. Laboratory storage conditions shall

be logged and should be the same for all samples within a project. If storage procedures change, this shall be reported along with the results of the laboratory analysis.

An EG laboratory should have different cold storage equipment to meet the storage recommendations of samples at different stages in the workflow. Ultra-low freezers, that can be set to -80°C, are optimal for long-term storage of received samples and derived DNA materials (Corrales & Astrin, 2022). Manual-defrost -20°C freezers are another cold storage equipment that is essential to an EG lab for temporary storage of samples and permanent storage of some reagents. Auto-defrost freezers should not be used to store samples or reagents because this feature introduces temperature fluctuations and increases evaporation. Fridges are also necessary for short-term storage of amplicon and indexed plates (i.e., stable amplified DNA and tagged DNA) and long-term storage of some reagents.

To avoid cross-contamination, samples should be stored in multiple layers of containment (e.g., plastic bags, plastic containers). It is essential that eDNA samples are physically isolated from any tissue samples or amplified DNA and not placed in storage units previously used for these high DNA content samples if they have not been thoroughly decontaminated. Likewise, there should be dedicated freezers and fridges for pre-PCR and post-PCR work areas.

4.2.5.1 Environmental Samples

eDNA in environmental samples will degrade relatively quickly when stored at ambient conditions. The temperature at which to store environmental samples depends on the type of the sample and whether a preservative (**Table 4-4**) is being used.

Liquid, solid, and mixed samples that are neither desiccated nor chemically preserved should be frozen at -80°C until they are ready for processing (i.e., filtration and/or extraction). Likewise, environmental samples that are received frozen, regardless of preservation method, should be stored frozen to avoid additional freeze-thaw cycles.

Desiccated filter membrane samples and samples preserved in ethanol may be stored at room temperature for short-term storage (up to 1 month), but fridge storage is preferable because it provides a consistent, controlled environment. For long term storage of these samples, they should be transferred to a -20°C freezer or colder.

Filter membrane samples and sediment/soil samples preserved in stabilizing buffers like Longmire's may be held at 4°C until DNA extraction can be performed.

eRNA in environmental samples is more prone to degradation and more sensitive to contamination than eDNA. Therefore, for RNA-focused projects, filter membrane samples should be preserved with an RNA stabilizing solution (such as RNAlater) and stored at -80˚C as soon as possible to prevent enzymatic degradation of the RNA molecules in the sample. Freezing and thawing cycles should be minimized as much as possible to protect the integrity of the eRNA sample. Due to the uncertainty surrounding RNA preservation in soil and sediment samples, the EG laboratory should optimize and validate the preservation method fit for the purpose of the study.

RNA or DNA stabilizing solutions should not be used to preserve soil or sediment samples as they tend to react with humic acids in the samples, causing DNA/RNA degradation and potential biodiversity signal loss (Bruce et al., 2021; Corrales & Astrin, 2022; Wort et al., n.d.). Unless logistically impossible or the preservation method has been directly verified as suitable for the intended analysis, soil and sediment samples should be preserved by freezing.

EG laboratories should keep a record of preservatives verified to be compatible with nucleic acid isolation SOPs and communicate to clients which preservation methods have been verified before any analysis is conducted (and preferably prior to collection). The EG laboratory should develop guidance or a decision tree for choosing whether to reject samples with an incompatible preservative, perform verification testing for a new preservative, or proceed with the analysis without verification at the client's risk.

Table 4-4 Common Chemical Preservatives for Environmental Samples (adapted from (Bruce et al., 2021)).

Preservative	Lyses cells?	Kills microbes?	Preserves RNA?	Recommended sample type(s)	Composition, Preservation Notes, and Practical Considerations
Ethanol	No	Yes	No	Filters (water or air), bulk tissue	Ethanol (non-denatured) and water. Final concentration should be >80% ethanol.
					Can be stored for at least 6 days at room temperature without a significant decrease in DNA (Minamoto et al., 2016)
					Ethanol is readily available, relatively inexpensive, and can be used to preserve samples at room temperature which makes it practical for use in the field. Water drawn out from bulk tissue or damp sediment samples can dilute the ethanol, and thus a high ratio of ethanol to sample may be required to maintain a suitable final concentration, or the ethanol may need to be replaced once the moisture has been drawn out from the sample. It is, however, (1) flammable, posing dangerous goods risk for certain modes of transport (air), (2) incompatible with reagents of many common extraction kits and must be fully removed before beginning extraction processes, and (3) import to certain countries is strictly regulated which can cause shipping delays.
RNAlater™	No	Yes	Yes	Filters (water or air), bulk tissue	Commercial product, proprietary formula
					Stable for a day at 37°C, a week at room temperature, a month at 4°C or indefinitely at -20°C to -80°C*. eRNA preserved ≥ 1 week at -20°C
					Can pose challenges with nucleic acid extractions, requires optimization. May not be appropriate for soil preservation. Relatively expensive preservative solution.
DNA/RNA Shield™	Yes	Yes	Yes	Filters (water or air), bulk tissue	Commercial product, proprietary formula
					DNA Stability: Ambient temperature (4 to 25 °C) >2 years. RNA Stability: Ambient temperature (4 to 25 °C) >1 month. DNA and RNA: Frozen (<−20 °C): Indefinitely*
					May precipitate at low temperatures but will return to solution following vortexing and heating to 37°C for 5 minutes
Longmire's buffer	Yes	No [Yes]	No	Filters (water or air)	Distilled water, 1M Tris-HCL, 5M NaCl, 20% SDS, 0.5M EDTA [Optional: 5% (w/v) sodium azide]

					Can store at room temperature for 150 days without a significant loss in DNA (Wegleitner et al., 2015). Other sources suggest 8 months at room temperature (Mauvisseau et al., 2021)
					Hazardous when it contains sodium azide. May precipitate at low temperatures but will return to solution following vortexing/warming
Sarkosyl buffer	Yes	Yes	No	Filters (from water or air samples)	100 mM Tris, 100 mM EDTA, 10 mM NaCl, 1% Sodium N-lauroylsarcosinate
					8 months at room temperature (Mauvisseau et al., 2021)
					Will not precipitate at low temperatures, making it an attractive alternative to Longmire's
0.01% benzalkonium chloride (BAC)	No	Yes	TBD	Water	0.01% BAC
					Water samples with BAC can be preserved at room temperature for 8 hours (with ~92% retention of eDNA). After filtering, samples should be stored at -20℃ (Yamanaka et al., 2017). Room-temperature incubation for 10 days retained 50% of eDNA in the water samples (Yamanaka et al., 2017).
					This can be an affordable way to preserve water samples for short-term transportation at ambient temperature so that filtration equipment is not required on-site in the field. However, it might interfere with extraction (Tsuji, Nakao, et al., 2022) and/or inhibit PCR (T. Jo et al., 2021), depending on the sampling environment and the target. BAC must be validated for the study purpose before being used as a preservative.

*As displayed on website, not specific to environmental samples.

4.2.5.2 DNA, RNA, and Derivatives

Storage conditions vary in molecular workflows due to differences in stability of DNA, RNA, and their derivatives and recommendations are summarized in **Table 4-5**. Note that storage recommendations might differ depending on various factors including:

1) reagents used during DNA/RNA extraction

2) material of plasticware used to store the samples

3) concentration of DNA/RNA in the sample.

EG labs should test and validate that the storage temperature used to preserve the sample is retaining the integrity of the DNA/RNA in the sample. Several lyophilization methods have been used for long-term storage of DNA at room temperatures for biobanking purposes. However, the freeze-drying process may alter the integrity of the DNA extract by shearing the molecules during the freezing process, and the drying process may concentrate the buffer salts. (Corrales & Astrin, 2022; X. Tan et al., 2021) Therefore, the decision to lyophilize eDNA extracts for long-term storage should be taken with caution and considering these disadvantages.

When establishing storage conditions for an EG workflow, it is also important to minimize the freeze-thaw cycles to avoid shearing and degradation of the molecules and consider how long each sample or derivative will be retained. In EG laboratory analysis, best practice is to aliquot the samples into working volumes to

1) prevent contamination of the original sample

2) reduce the freeze-thaw cycles

3) ensure some volume is reserved for archiving.

Archiving may be important for EG laboratories with industry clients because it facilitates repeat testing to confirm results, cross-lab validation, calibration of time series, and re-analysis with new assays or molecular tools in the future. DNA archives offer physical backups in case of data loss and an efficient use of space compared to the original environmental samples.

Table 4-5 Recommended storage conditions for DNA and RNA samples and derivatives.

Molecule	Material	Recommended Storage
DNA	Environmental sample	Varies with sample type and preservation method. See section 4.2.5.1
	Extract in storage buffer with EDTA	DNA extracts can be stored at 4°C for ≤ 1-3 months; recommend aliquoting working volumes and storing stock extracts at -80°C long-term.
	Extract in water or storage buffer without EDTA	DNA extracts can be stored at 4°C for ≤ 2 weeks; recommend aliquoting working volumes stored at -20°C and storing stock extracts at -80°C long-term. Less common methods of preserving extracts include flash freezing in liquid nitrogen, encapsulation, and lyophilization (freeze-drying)(Corrales & Astrin, 2022).
	Amplicon	Amplicons, purified after PCR to remove leftover reagents, are stable at 4°C for 1-3 months; should be stored at -20°C (preferably -80°C) for long-term.
RNA	Environmental Sample	eRNA samples should be stored at -80°C until extraction.
	Extract	RNA extracts should be frozen at -20°C immediately after extraction unless cDNA synthesis proceeds right after. RNA in water or EDTA-free buffer may be stored at -80°C for up to a year. For longer-term, RNA may be stored as a precipitate at -80°C (*Oligonucleotide Handling & Stability*, n.d.).
	cDNA	Unamplified single stranded or double stranded cDNA should be stored ≤-20°C for short-term, -80°C for long term.
	Amplicon	Amplified cDNA (such as qPCR products) is stable at 4°C for 1-3 months; should be stored at -20°C (preferably -80°C) for long-term.

4.3 Record Keeping and Document Management

A centralized, shared, digital record-keeping system or LIMS ensures sample traceability from receipt through analysis as well as supporting quality audits.

4.4 Client Review of EG Laboratories

Industry clients may choose to verify that an EG laboratory has a well-maintained QMS by requesting proof of a third-party certification or accreditation. Third-party audits from a reputable certification body save both the lab and the client time by alleviating the need for repeating the detailed review of operations and documentation.

Secondly, an EG laboratory shall provide a completed Quality Assurance and Laboratory Competence Questionnaire (see Section 11.2 in the Appendix).

5 Overview of Analysis Workflows and Procedures

EG analysis can be applied to eDNA or eRNA. Ultimately, the decision to use eRNA or eDNA is based on the project goals and needs to be made prior to sample collection during the study design and project planning phase. Following from this decision, the selection of nucleic acid extraction method must be tailored to the sample type, considering factors such as the source material (water, soil, sediment, etc.), expected level of contaminants, and potential inhibitors. The quality of extracted DNA or RNA influences the success of subsequent molecular testing.

eDNA analysis can leverage a variety of molecular detection techniques. Central to many of these methods is Next Generation Sequencing (NGS), where DNA molecules from multiple samples, genes, and/or organisms are read (sequenced) simultaneously, with or without enrichment for specific DNA molecules of interest. Utilizing NGS, metagenomics analyzes all genetic material recovered from environmental samples. In its simplest implementation, no enrichment is involved and metagenomics is a quantitative approach, allowing the identification and relative quantification of organisms within communities. Metabarcoding, another NGS-based method, combines the specificity of polymerase chain reaction (PCR) with sequencing to amplify (enrich for) DNA from specific taxonomic groups at a single genetic locus, enabling the sensitive, qualitative detection of multiple species within groups of interest. In cases where the assay is designed for a single species, it is referred to as targeted sequencing. Hybridization capture enrichment, also known as target enrichment, uses DNA baits (short DNA probes) in conjunction with NGS to enrich and sequence specific DNA fragments from multiple genetic loci, spanning a pre-selected list of target species from diverse taxonomic groups (Günther et al., 2022; Li et al., 2023; Wilcox et al., 2018).

PCR-based detection methods can also be quantitative, allowing estimation of the relative biomass of specific target species. Real-time PCR, also known as quantitative PCR (qPCR), is a well-established approach for detection and quantification of specific DNA molecules from target species using fluorescing DNA-binding dyes or probes. Digital PCR (dPCR) is a newer, more precise variant of qPCR that is less prone to inhibitors, providing absolute quantification of DNA sequences for highly accurate and sensitive analysis (S. C. Taylor et al., 2017; Zhao et al., 2016).

Each of these eDNA analysis methods offers unique advantages, from broad ecosystem surveys to the focused detection of specific species.

Likewise, eRNA analysis can involve a similar variety of molecular detection techniques with modifications to methods based on the different characteristics of these molecules compared to eDNA. Metagenomics sequencing of all RNA recovered from environmental samples is referred to metatranscriptomics. It is largely the same as DNA metagenomics from the laboratory perspective, except that RNA extractions are lengthier and involve more toxic reagents. The distinctions and challenges in this workflow fall on the data processing side. Likewise, hybridization capture enrichment, qPCR, dPCR, and metabarcoding can all be applied to eRNA samples with modifications to the molecular probe or primer design process to bind to the types of RNA of interest (messenger or mRNA, ribosomal or rRNA, or transfer or tRNA). eRNA offers distinct application opportunities compared to eDNA including a view of what genes and metabolic pathways were active in the community at the time of sampling as well as detection of RNA-based viruses.

5.1 Workflow Selection

The molecular detection method should be established together with the client during the study design phase (see RFP1 and RFP2), guided by the project's objectives and resource availability. EG laboratories offering EG services are expected to provide expert advice to ensure the chosen molecular detection method aligns with each project's needs.

Key Considerations for Selecting a Molecular Detection Method:

Number of Species Targets

For projects requiring the detection of multiple species simultaneously, opt for sequencing. NGS excels in parallel detection, making it ideal for comprehensive biodiversity studies or when dealing with complex samples. In contrast, limit the use of qPCR and dPCR to projects with fewer species targets due to their multiplexing constraints.

Validated Assay Options

With limited budgets, prioritize methodologies with validated (see Section 5.2) assays for the target species. Choosing a validated method enhances reliability and accuracy, streamlining the project workflow and reducing the risk of errors. EG laboratories may opt to develop a new assay specific to a project, but the time and cost involved with assay validation will need to be considered.

Sensitivity

Select dPCR or NGS when high sensitivity is paramount, particularly for detecting rare targets or analyzing samples from challenging locations. These methods, especially dPCR, are well-suited for low abundance targets. It is important to compare the limit of detection (LOD) of assays because sensitivity can vary with specific methods used. For example, there could be multiple qPCR assay options for the same target, and these could differ by several orders of magnitude depending on the methods, and the same is true for dPCR and NGS.

Taxonomic and Genomic Scope of Molecular Analysis

Choose NGS for projects that require analysis of community biodiversity, broad taxonomic groups, and/or multiple genetic regions. NGS is beneficial for validating species identities, calculating biotic indices, conducting functional analyses, and population-level studies.

Quantification

For projects demanding a quantitative measure of target species eDNA or estimate of relative abundance, use dPCR or qPCR. The added sensitivity of dPCR is particularly suitable for low-concentration templates. Consider metagenome sequencing if quantification of species at the community-level is of interest and budget allows. Consider targeted sequencing or metabarcoding only if quantification is a secondary objective, as this area is still developing (see Section 11.4) and standard workflows do not accurately capture biomass.

Cost-effectiveness

Evaluate the financial aspects of a project thoroughly. Projects with single targets, limited budgets, or low sample sizes favor qPCR or dPCR, as they typically involve lower costs and are faster. Consider sequencing for mid- to large-scale projects.

Throughput

Match the chosen molecular method with the project's scale and timeframe. Sample processing capacity should align with the project's timeline.

Tolerance to False Positives and False Negatives

For projects where accuracy is critical, such as those with regulatory implications, evaluate the relative likelihood of false positives or negatives. Where available, known error rates should be discussed. The importance of false positives or negatives depends on the context or application of a project so communication between client and EG laboratory is key.

Future Analyses

For future re-analysis of data without additional lab work, choose NGS; data can be reanalyzed as reference databases and bioinformatic techniques evolve, potentially offering additional value over time.

Data Volume

Consider the data management needs and capacity. If handling large volumes of data is not feasible or desired, qPCR or dPCR is the appropriate choice. If this is not a concern, NGS offers added information on the specificity of each detection by retaining sequence records, ability to reassess detections in the future as reference databases grow, and statistical power to detect community level ecosystem impacts by generating data on hundreds or thousands of taxa simultaneously.

Reference Sequence Availability

Choose metabarcoding or metagenomics when there are limited reference DNA data for target species or ecological communities; these approaches can provide information on previously undescribed or unknown species in absence of records in the reference library. Metabarcoding assays recover DNA sequences within higher-order taxonomic groups of interest (e.g. eukaryote assay or vertebrate assay) whereas metagenomics provides a broad overview of biodiversity, albeit with low sensitivity to detect rare species and greater emphasis on microbial diversity. The sequences recovered from the samples can be stored for future reanalysis if there is no exact match and higher-order taxonomy can be estimated based on the closest matches. For species or ecosystems with good reference DNA database coverage, capture enrichment, qPCR, or targeted sequencing are all viable options for precise species detection because these assays rely on existing sequence knowledge for assay design and validation.

Regional Biodiversity Knowledge

Choose metabarcoding or metagenomics when there are gaps in knowledge of the local biota or if the information may be out of date; these approaches have the potential to recover unexpected biodiversity (i.e., lack of historical records, recent range extensions, or previously unreported invasive species) unlike capture enrichment, targeted sequencing, qPCR, or dPCR assays.

5.2 Workflow Implementation, Optimization, & Change Management

EG laboratory analysis workflows, made up of connecting SOPs from the initial sample to the data output, require optimization and validation. This applies to implementing novel workflows (e.g., eRNA workflows), new assays within an existing workflow (e.g., a new DNA metabarcoding added to an existing workflow) or making substantial changes to an existing procedure (e.g., replacing one commercial kit for DNA extraction with another).

New assays are further divided into different categories of optimization and validation depending on whether it's an entirely new design, an existing assay from another lab that is being introduced for the first time, or an adaptation of an existing design to a broader scope than previously tested.

Optimization refers to fine-tuning processes to make them as efficient and effective as possible. For example, implementing a new DNA extraction procedure will require optimization to maximize yield and species detections. This could encompass testing various lysis protocols to find the one which can lyse a variety of cell types of interest (see section 7.2 below). Optimized procedures based on the same kit or chemistry will vary between labs due to differences in available lab equipment, supplies, and automation.

Validating a method involves rigorous testing and documentation to demonstrate an assay's fitness-for-purpose. Assay validation parameters include sensitivity, specificity, precision, accuracy, linear range, and robustness (Abbott et al., 2021;

De Brauwer et al., 2022a; DNAqua-Net, n.d.). For example, for an EG lab to make its own DNA or RNA extraction procedure rather than using a commercial kit, it must validate its kit can extract from the desired of sample types, from samples with low nucleic acid concentrations, that results are repeatable, and to establish assay controls. Some of the validation could be substituted with commercially available kits that are fit for the application in question, but some form of in-lab testing demonstrating that the procedure performs as intended is always necessary.

Consistency is imperative within projects, and between sample sets where direct comparisons are to be made. EG laboratories shall maintain "versioning" of specific procedures used in the workflow, and maintain documentation on the details of these procedures. EG laboratories shall notify clients when changes are made within a project that may directly affect the results (e.g., commercial kit change, volume or number of subsamples, sequencing depth, etc.). The laboratory shall maintain documentation outlining the justification for the change (including results of optimization testing), expected outcomes and provide relevant information to clients as needed.

5.3 Positive and Negative Controls

The implementation of controls and their regular monitoring is crucial for environmental DNA (eDNA) analysis, due to the low quantities of eDNA in samples, even smaller proportion of DNA from genes and species of interest, and high sensitivity of methodologies like PCR to trace contaminants. Controls are critical for the accuracy and reliability of the results, help ensure transparency in assay results, increase confidence in the lab results, and assist in root cause analysis for corrective actions.

5.3.1 Positive Controls

Positive controls produce an expected result or signal when a process is completed successfully. Inclusion of positive controls allows EG laboratories to identify false-negative results (e.g., PCR inhibition) and prepare corrective actions. There are three types: internal positive controls (IPCs), where known material is added to the environmental samples, and external positive controls that are separate samples of known composition (i.e. mock samples, calibration standards).

Internal Positive Controls, IPCs: Commercially available IPCs like synthetic DNA fragments may be spiked into samples at the start of analysis to verify successful execution of the full workflow or specific steps or added to preservation buffer to assess preservation. Internal amplification controls (IAC) are a type of IPC used to normalize between reactions and test for inhibition or reaction failure in PCR. IACs co-amplify along with the target under the same reaction conditions but with different primers.

External Positive Controls: Commercially available microbial mock communities made up of known mixtures of microbial DNA or cells (e.g. Zymo Research, (*ZymoBIOMICS Microbial Community Standards*, n.d.)) may be used as an external positive control to verify the efficacy of nucleic acid isolation as well as downstream processing steps in some applications. There are no commercially available eukaryotic mock samples. EG laboratories may also choose to make their own mock communities or select environmental samples from past successful analyses to use as positive controls. For example, the positive control may be a tissue extract of the targeted species or a synthetic DNA molecule that matches the sequence of the target species. A calibration standard or quantification calibrator is a type of external positive control used to interpret measurements from an assay by creating a standard curve. For example, serial dilution of known DNA concentrations generates a standard curve used to infer DNA quantity.

EG laboratories shall include positive controls in all species-specific EG workflows and may include positive controls in community assessment assays. EG laboratories shall characterize positive controls to determine the expected range for measurements in each workflow. The number and type of positive controls may vary depending on the biodiversity targets and assay, but where used, there should be at least one positive control per sample batch for each procedure or test.

5.3.2 Negative Controls

Negative controls are expected to produce null results. They can be divided into different categories including no template controls, negative sample controls, and no reagent controls.

No template controls (NTCs) are when all components and conditions are equal to the samples being analyzed, except no environmental material or DNA is added. NTCs are used to detect cross-contamination, reagent contamination, and establish thresholds for background noise in the assay.

Negative sample controls are reactions that contain all the same components as a sample reaction but it is known that the template DNA does not contain the target. This may be DNA from the tissue of a non-target species or eDNA from a site where the target is known not to exist. Negative sample controls are used to test for specificity by identifying false positives.

No reagent controls are reactions where a key reagent is left out. For example, no-reverse transcription controls (NRTs) lack the reverse transcriptase enzyme and serve to detect presence of (unwanted) DNA in RNA analysis.

EG laboratories shall include negative controls in all EG workflows. The number and type of negative controls included will depend on the procedure but there should be multiple negative controls for each batch of samples, distributed between technicians, automation equipment, or analytical instruments that process the samples, and at each step of the analysis for thorough monitoring of when and where potential contamination occurs.

5.4 Commercial Assays and Kits

The field of environmental DNA (eDNA) analysis is rapidly evolving, with commercial laboratory assays emerging as a pivotal development. These assays, leveraging proprietary methods, offer a suite of advantages for accurate and efficient DNA analysis. EG laboratories can also benefit from a range of existing molecular biology kits for DNA/RNA extraction, quantification, and purification. This section outlines the unique benefits commercial chemistries provide, underscoring their impact on eDNA analysis and role in advancing environmental studies.

Reproducibility and Quality Control: Standardized protocols are a benefit of commercial assays. This uniformity helps reduce variation between different laboratories, providing consistent results. Additionally, incorporated QCs maintain the integrity and reliability of results in environmental DNA analysis. In other words, commercial kits are a common way to offset certain risks by outsourcing aspects like primer design or chemical formulation to specialized third parties.

Time Efficiency: Utilizing commercial products saves time and resources that would otherwise be spent on designing and optimizing custom methods. This efficiency allows users to concentrate on their primary client service objectives.

Ease of Use and Support: Designed for user-friendliness, commercial assays are accompanied by detailed manuals, reports, and dedicated customer support that enables users to implement them as intended without excessive training. Additionally, ongoing support and updates ensure users have access to the latest advancements in data analysis.

Designed with Large-Scale Applications in Mind: One of the standout features of commercial assays is their inherent scalability. These assays are designed and rigorously tested to perform consistently across extensive projects. This scalability ensures that they can accommodate large-scale environmental studies, providing reliable and uniform results even when the project scope expands significantly. This feature is particularly valuable for large environmental impact assessments or widespread biodiversity monitoring programs, where the ability to scale up without compromising accuracy or efficiency is crucial.

When choosing to implement a commercial assay, consider the reputation of the manufacturer, availability of technical support, production scale (bespoke vs. large scale operations), product lifecycle (long term availability), product stability or shelf life, compatibility with automation, acceptance by other commercial laboratories, and technical specifications. If a commercial assay is implemented within a core workflow, EG laboratories must maintain records of batch or lot numbers and any quality issues (e.g., non-conformances in the QMS). Since the exact chemical composition of most

commercial assays is proprietary, EG laboratories must also have a procedure in place to manage the transition from one kit to another and verify performance of a new kit and maintain a record of this.

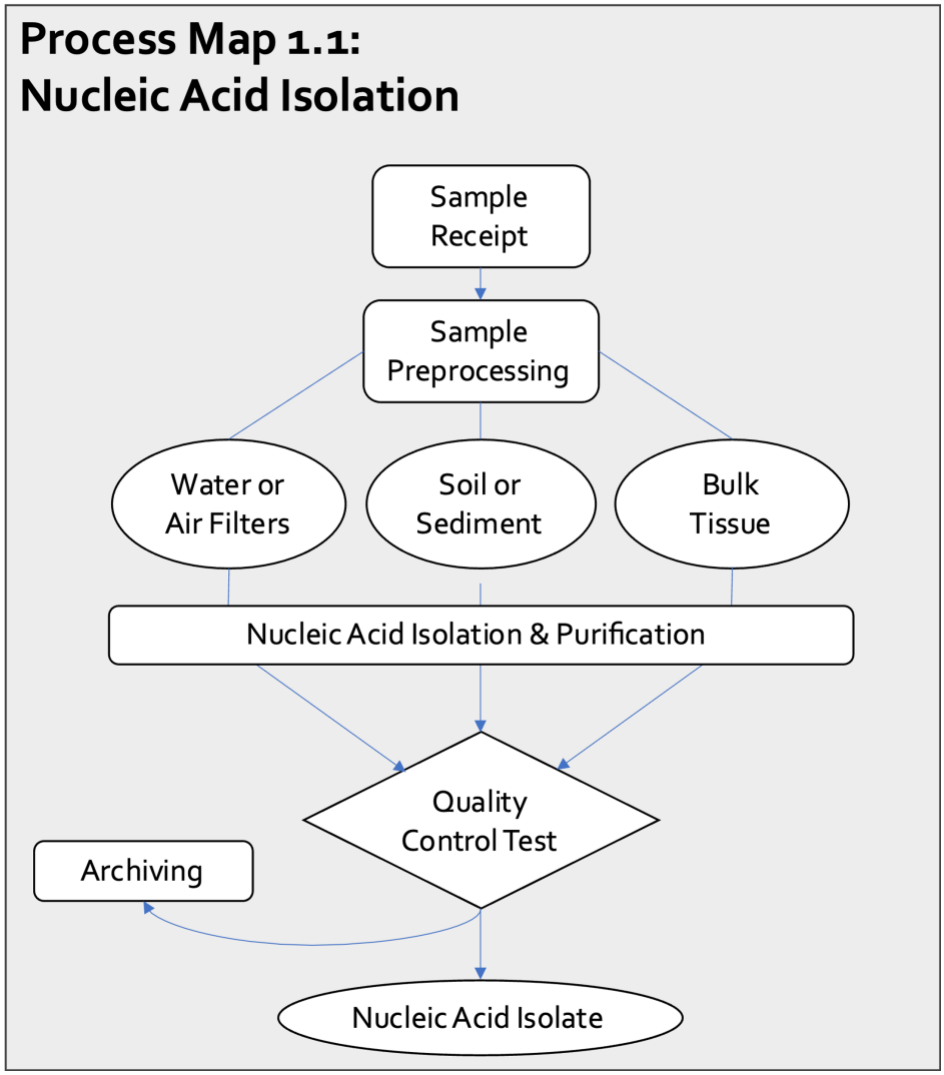
5.5 Sample Batching

When analyzing large sets of samples, it is important to incorporate strategies to account for batch effects, which are technical variations, often small but unrelated to study factors of interest, that can significantly impact results and confound the planned environmental comparisons. For example, in time series analyses spanning multiple years, batch effects such as changes in reagent batches over time or variations in storage conditions could affect the consistency of results. A notable instance might be a change in reagent formulation over several years leading to subtle but impactful shifts in chemical reactions that obscure or make it harder to separate ecological changes occurring at the sites over that time. To mitigate these effects, sample sets may be randomized for analysis so that any batch-related biases are not systematically associated with any particular group. Alternatively, a linker set of reference samples may be repeated in each batch, serving as a consistent reference point and enabling more accurate assessment of batch-to-batch variations. Likewise, it is necessary to keep detailed workflow versions and documents on the potential impact of any changes on results as noted in Section 5.2.

5.6 Transitioning between EG Laboratories

Transitioning between EG laboratories during a project is possible, and there are ways to verify transferability of a method to an alternate laboratory. Inter-laboratory calibration experiments can verify comparability among multiple laboratories for results of a given procedure. Commercially produced positive controls or reanalysis of some previous samples by the second lab can aid with these transitions. These considerations also apply if planning to compare a new data set to a historical data set from another laboratory that may have used earlier methods or older instruments.

6 Sample Pre-Processing & Nucleic Acid Isolation



This section focuses on key factors in nucleic acid (DNA and RNA) extraction from environmental samples. The early stages of eDNA or eRNA analysis can be broken down into these steps: sample pre-processing; homogenization (for some sample types); isolation and purification of DNA or RNA; and stabilization of extracted material for storage. Adherence to standard operating procedures (SOPs) and rigorous QC measures ensure the consistency and reproducibility of results while detailed record-keeping and risk analysis mitigate potential challenges (see Sections 3.3 and 4).

EG laboratories should inform clients when the pre-processing or nucleic acid isolation steps will limit potential for future downstream analyses from the DNA or RNA isolate (e.g., DNA extraction chemistry preferentially extracts from animal cells and not walled cells like bacteria, plants or fungi, or lysis method fragments DNA so long read sequencing chemistry not suitable, etc.).

6.1 Sample pre-processing

In sample pre-processing, EG laboratories shall ensure any subsample taken is representative of the original sample.

6.1.1 Water and Air eDNA Samples

Sample filtration should take place in the field so eDNA or eRNA samples from water or air enter the lab as material bound to a filter membrane but these sample types may be submitted as frozen or liquid volumes[1] due to field constraints. Samples on filter membranes are preserved by freezing, desiccation, or the addition of a chemical preservative (See **Table 4-4**).

If in situ filtration is not possible and eDNA samples are submitted as frozen or liquid water, filtration or centrifugation shall be used to separate the water from solid material. Frozen samples stored at -80°C should be transferred to -20°C for ≥12 hours and then thawed at 4°C; once thawed, they should be processed immediately. If not immediately processed, samples can be compromised after delays on the order of hours. There should be a record of the sample storage temperatures and durations.

For larger volumes (>50mL), the standard method for processing water samples is filtration. Multiple filters may be used for each sample and multiple samples may be filtered on the same filter membrane, depending on project specifications for replication. There are a variety of filter membranes commercially available, and the method of filtration depends on the type of filter chosen, membrane composition, pore size, and water volume to be filtered. Filters may be open or enclosed in permanent or removable housing. After filtration, filters should be dry and stored frozen or in lysis buffer (Majaneva et al., 2018), unless immediately proceeding through nucleic acid isolation.

Centrifugation may be used as an alternative option for eDNA capture however, it is only suitable for small volumes and is less effective than filtration. Precipitation may be used in conjunction with centrifugation to increase nucleic acid yield (Eichmiller et al., 2016; Xing et al., 2022) but it is only suited for even smaller volumes (approximately 30 mL). The method targets large (intact) DNA fragments, which can be useful for certain applications such as metagenomics.(De Brauwer et al., 2022b; Sahu et al., 2023; Tsuji et al., 2019) It is important to note that some large-volume filter types (e.g. Waterra eDNA Filters; Waterra, Mississauga, ON) call for a centrifugation step after filtering. This is distinct from centrifugation-only methods and does not include the same shortcomings. After the suspended cellular material has been separated from the water samples, this concentrated sample may be temporarily stored at –20°C (or –80°C for long-term storage) until proceeding to the next stage.

6.1.2 Soil and Sediment eDNA Samples

The heterogeneity of sediment and soil samples presents a challenge, as samples can often contain large chunks of tissue, or the substrate might be rocky and too large for the lysis tubes, making it difficult to obtain a representative subsample for extraction. Soil and sediment samples also have high risks of contamination, due to dusty or wet field sampling conditions, particulates collecting around the opening of tubes and caps, and the higher yields of DNA present compared to water and air samples. Decontamination is more demanding for soil and sediment samples, with increased diligence needed to maintain a clean work environment and prevent cross contamination.

Nucleic acids should be isolated from sediment and soil samples frozen immediately (ideally flash frozen in liquid nitrogen) and stored at -20°C (ideally -80°C, especially if RNA is targeted) unless processing is within a few hours of sampling.

If freezing cannot be maintained through collection and transport, keep samples at ~ 4°C for up to two weeks or desiccate samples at ambient temperature (~ 20°C) (Clasen et al., 2020). 4.2.5.1 Liquid preservation agents such as RNA*later* (Thermo Fisher Scientific), and ethanol should be used with caution for soil samples because some studies have shown a bias towards gram positive bacteria (Iturbe-Espinoza et al., 2021; Rissanen et al., 2010) and lower yields of DNA (Delavaux et al., 2020; Rissanen et al., 2010). Refer to Section 4.2.5.1 for more information about logistical considerations when using ethanol.

[1] Air sampling protocols may collect particulates into traps filled with a liquid or onto a plate that is then rinsed into a collection vile.

Strategies for sample homogenization depend on the project goals and the sample collection method used. If distinguishing between layers is relevant to the project, appropriate sampling equipment such as tube cores should be used to collect the samples, and layers should be isolated before any homogenization. In some cases, surface layers may be avoided to reduce DNA input from sources outside the intended sampling area. Otherwise, samples and subsamples shall be thoroughly mixed to ensure homogeneity. Visible pieces of materials other than the substrate sampled (e.g., leaves, roots, shells) should be removed. Composite field samples may be created by mixing multiple field replicates to produce a representative sample for a larger area (Dickie et al., 2018; Hestetun, Lanzén, & Dahlgren, 2021; Taberlet et al., 2012).

After mixing, it is common to take one or multiple subsamples from a sediment or soil sample (Fahner et al., 2016; Pearman et al., 2021; Sakata et al., 2020). With multiple subsamples, replicates may be combined either before or after the final DNA purification or elution to produce a single DNA extract. Alternatively, these subsamples can be kept as technical replicates and carried through the whole analysis. The selection of the subsampling technique and number of replicates to carry through downstream analyses depends on factors such as the research question, site-specific characteristics, and practical considerations like cost and time constraints.

The volume of sediment or soil to be subsampled is specified by the chosen extraction kit to ensure efficient removal of humic acids (PCR inhibitors). Small amounts of input material (0.5 g) are suitable for characterizing prokaryote diversity, but larger quantities of input material (≥ 10 g) are preferred for characterizing metazoan communities (Brandt et al., 2021; Bruce et al., 2021; Dopheide et al., 2019). However, as large sample volumes are difficult to automate, they represent a considerable hindrance on throughput for EG laboratories. It has been shown that several small subsamples pooled after extraction can effectively be used to describe eukaryotic communities, allowing implementation on extraction robots (Hestetun, Lanzén, Skaar, et al., 2021). In general, less input material and/or fewer replicates are required to characterize microbial and meiofaunal richness, whereas macrofauna richness requires more sampling effort either through increased sample volume or more replicates to detect differences in richness across sites. The weight of the sediment or soil (sub)sample should be measured to maintain consistency between samples and to follow the specifications of the kit.

Soil and sediment samples are typically collected in higher volumes than what is needed for extraction, and the remaining samples can be stored long-term at -80°C following subsampling. This makes it possible to extract from the same sample multiple times, so additional single-use subsamples of the sediment or soil sample may be stored where repeated extraction procedures are likely.

Further research is required to develop comprehensive guidelines on appropriate sample sizes, replication levels, subsample depths, and DNA extraction input quantities needed to generate ecologically relevant data from various environment types (Pawlowski et al., 2022). It is currently accepted that there are multiple ways to subsample sediment and soil samples prior to DNA extraction with no single best or correct method. EG laboratories shall record subsampling information in the project records.

6.1.3 Tissue Samples

Tissue samples either represent individual organisms (e.g., fin clips, muscle tissue, leaf punch, etc.), or mixtures of tissue from many organisms (e.g., insect traps, plankton tows, macrofauna retained on 1 mm sieve, faeces, stomach contents, biofilms, microbiome, etc.), often referred to as bulk tissue samples. The presence of highly concentrated DNA represents a major contamination risk, therefore, tissue samples must be processed and extracted in a separate, contained area from where eDNA samples are processed, and meticulous decontamination efforts are required to mitigate cross-contamination risks. Like soil and sediment samples, tissue samples can undergo multiple subsampling and extraction rounds from the same sample, provided there is enough material.

Tissue samples may be transported to an EG facility frozen, dried, or in preservative solutions such as ethanol or propylene glycol (see section 4.2.5.1) (Robinson et al., 2021). Frozen samples may be thawed at room temperature for subsampling.

Subsampling in single species analysis depends on multiple factors including type of organism and tissue. For example, the best practice for fish species is to subsample from muscle tissue. To minimize the risk of contaminating the sample with exogenous DNA, subsampling should avoid the specimen's surface and areas of the digestive system (stomach, intestines, etc.) (Rimet et al., 2021). Commercial extraction kits provide recommendations for volume or mass, extraction method, and type of tissue to use. In-depth information about subsampling and extracting from various tissue types can be found in *Biodiversity Biobanking - A Handbook on Protocols and Practice* (Corrales & Astrin, 2022).

In the case of bulk tissue samples, the EG laboratory shall ensure subsamples are representative of the overall sample. Where the sample constituents are microscopic, like plankton tows, samples may be shaken or inverted multiple times to mix. For small volume samples, a device such as a beadmill may be used to homogenize the sample, and for larger volume samples of macroorganisms (e.g., insect traps, sediment macrofauna), a blender or similar may be used. Centrifuging may be used to separate and remove excess liquid from the tissue samples before or after homogenization. If sample volume is large, it is possible to serially centrifuge the entire sample or only a subsample. If the resulting pellet is sizable, additional subsampling into replicates is also possible (Yuan et al., 2015).

In summary, there are different approaches used to subsample tissue and bulk tissue samples prior to DNA extraction depending on the type of organisms and material provided by the client. EG laboratories shall ensure the subsampling approach is appropriate to the type of tissue or organism(s) based on *Biodiversity Biobanking* (Corrales & Astrin, 2022) and that subsampling information is included in the project records.

6.2 Cell Disruption & Lysis

Another critical aspect of environmental sample processing is the lysis process which breaks open cell walls and membranes to release DNA and RNA into solution for purification and recovery. Lysis procedures are typically incorporated into commercial extraction kits with recommended modifications to accommodate various sample types. Lysis methodology can limit the recovery of intact, high molecular weight DNA, which is necessary for some types of genome sequencing, versus smaller, more fragmented molecules that can be used for metabarcoding or qPCR tests. If chemical preservation was used for subsamples, compatibility of the lysis process shall be verified or modified as necessary by the EG laboratory. For example, ethanol interferes with Qiagen's DNeasy PowerWater kit (Hinlo et al., 2017) so the EG laboratory must add steps to completely remove the ethanol from the sample prior to further processing. Storage preservatives that lyse cells release DNA into solution and EG laboratories may improve yields by extracting from the preservative as well (Bruce et al., 2021).

With all environmental sample types, the lysis process is a combination of usually 2 or more of: physical disruption (bead beating), sonic disruption, heat disruption, cold disruption (cryogrinding), chemical disruption (detergents), and/or enzymatic disruption (e.g., Proteinase K). For water filters, release of cell material and DNA from the filter membranes is accomplished by either removing the filter from its enclosure for treatment or by conducting the lysis within the filter capsule directly. For sediment samples, bead beating is the standard method, followed by a thorough strategy for inhibitor removal. Bead beating may be performed with high-throughput cell lysers (Hestetun, Lanzén, Skaar, et al., 2021) or benchtop vortexes. In general biodiversity analysis, or where biodiversity scope of a project may expand, a versatile method should be applied to lyse all cell types, including plant, fungi, algae, and bacteria cell walls. If the biodiversity analysis is focused on a particular taxonomic group, the lysis method must be suitable for this cell or tissue type. For example, a study focused on fish only can use a lysis method for cells without cell walls such as that found in the Qiagen Blood & Tissue kit.

The EG laboratory shall document evidence of effective lysis of cells or otherwise demonstrate suitability of the chosen method to the service being provided, whether that is general biodiversity or for specific taxonomic groups. EG laboratories shall keep a record of verification tests and maintain a list of compatible preservatives to provide to clients prior to field sample collection (see Section 4.2.5.1). For each sample set, the EG laboratory must keep records of the

lysis procedure including kit with expiry date, version of lysis procedure, and any values for any project specific parameters.

6.3 DNA Isolation

The choice of DNA extraction methods and their underlying chemistry is pivotal. The ideal protocol will be robust towards diverse chemical and physical backgrounds found in environmental samples. It should also ensure a high yield of DNA, crucial for samples with limited starting material and for detection of rare targets. Furthermore, the purity of the extracted DNA is essential as it should be free from contaminants that could interfere with PCR and other downstream processes, hereby referred to as inhibitors. As with lysis, choice of isolation method may impact the integrity of DNA molecules which can be important for certain genomic sequencing applications [see section 9].

EG laboratories shall perform extractions using commercially available kits that have been validated for environmental substrates due to the multiple benefits outlined in Section 5.4**.** Qiagen extraction kits are frequently utilized for eDNA samples, notably the DNeasy Blood & Tissue Kit for (bulk) tissue extraction, the DNeasy PowerWater Kit for water filters, and the PowerSoil kits for soil, sediment, bulk tissue, and faeces (Hermans et al., 2018; Pawlowski et al., 2022; Sahu et al., 2023; van der Loos & Nijland, 2021). Commercial DNA extraction kits specific to other forms of eDNA, such as the DNeasy PowerBiofilm, or higher throughput formats that allow automated extraction of batches of 96 samples, like DNeasy PowerSoil Pro Qiacube HT kit. Users are still advised to verify and optimize the extraction procedure for any new sample types. Consider yield, inhibitor control, type of sample, ease of use, throughput, cost, and reliability. There are several inhibitors common to environmental samples. Humic acids in soils and decaying biomass, polysaccharides in marine organisms and plants, and calcium ions in environmental samples are some commonly encountered inhibitors (Schrader et al., 2012). For example, water samples from turbid environments with high suspended sediment loads may benefit from using a soil DNA extraction kit which is optimized to reduce inhibitors from sediment. DNA extraction from soils laden with heavy oil require additional modifications to remove heavy metals and organic inhibitors (Basim et al., 2020; Puentes et al., 2019).

Not all extraction methods will release DNA bound to particles such as clay. If it is suspected that much of the DNA in a sample is particle bound (e.g., in highly turbid waters), then using kits optimized for soil extraction (e.g., Qiagen PowerSoil) or lysis buffers containing trisodium phosphate are needed to release adsorbed DNA (Kirtane et al., 2023; Sellers et al., 2018). If not, then adsorbed DNA will not be extracted.

For long read sequencing, high molecular weight DNA is required and has additional considerations. Specialized commercial kits or methods are required to isolate high molecular weight DNA without shearing it. Also, high molecular weight DNA may require heat treatment or shearing to homogenize any clumps prior to purity and concentration assessments (‘Giron’ Koetsier & Cantor, 2021).

The EG laboratory must keep records for each sample of extraction kit used, lot number and/or the expiry date of reagents used, and version of the internal SOP followed.

6.4 DNA Stabilization & Archiving

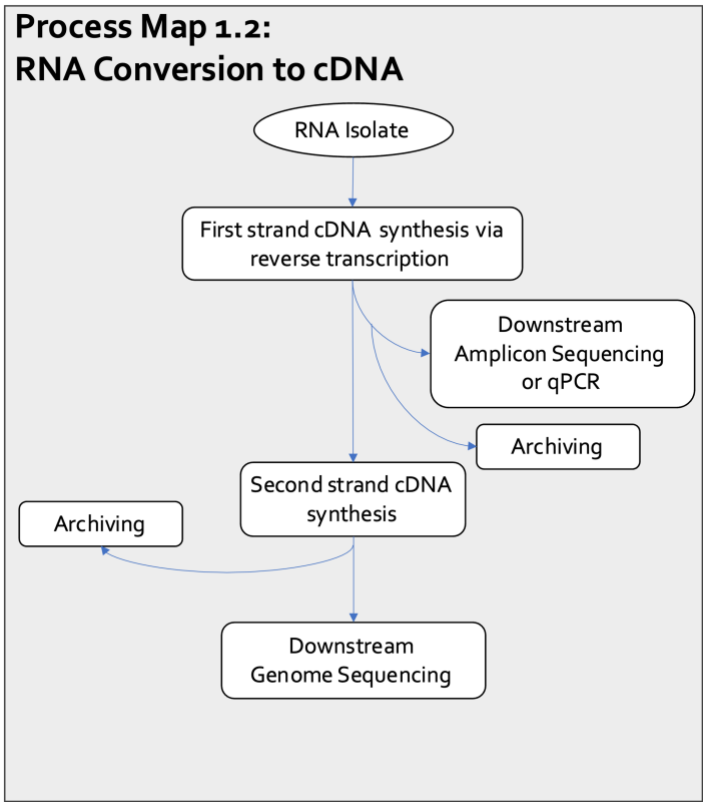
General storage considerations (Section 4.2.5.2) and operational practices (Section 4.1.2) apply here. EG laboratories shall make an aliquot of the DNA for the planned analyses which may be preserved at 4°C or -20°C if in water (referred to here as “working” DNA). The remaining archival DNA shall be transferred to long term storage immediately.

6.5 RNA Isolation

The inherent instability of eRNA presents unique challenges compared to isolation of eDNA. A particular challenge in RNA isolation is the ubiquitous presence of RNases, enzymes that degrade RNA molecules. Found almost everywhere—including in the environment, in the air, on human skin, and on laboratory surfaces—they can rapidly degrade RNA samples, such that even a minute amount of RNase contamination poses a significant risk and requires specialized mitigations throughout operations (see Section 4).

The receiving laboratory for environmental RNA (eRNA) projects should engage in a preliminary consultation with clients to ensure that their field collection and preservation methods are compatible with the laboratory procedures. For example, samples must be submitted in RNA stabilization reagents that are compatible with the sample type and extraction chemistry (See Section 4.2.5.1). Alternatively, samples may be frozen.

Different sample types (water, soil, air) require specific RNA extraction methods for optimal results; there are several methods available with varying efficiencies and specificities. RNA extractions need a lysis buffer proven to be compatible with RNA preservation, such as commercially available RNA lysis buffers, and an RNase inhibitor to protect RNA during the extraction process (e.g., β-mercaptoethanol). Lysis conditions must efficiently release RNA while minimizing DNA contamination, so DNase digestion may be applied at the end of the extraction workflow to obtain DNA-free RNA. EG laboratories shall perform RNA extractions using commercially available kits that have been validated for environmental substrates due to the multiple benefits outlined in **Section 5.4.** Qiagen's RNeasy PowerSoil Total RNA kit is one common example. Additionally, RNA samples can optionally be enriched or depleted using specialized commercial kits to focus on specific types of RNA, such as rRNA depletion, mRNA enrichment, pathogen RNA depletion, or targeted RNA capture.



eRNA samples require one additional step prior to downstream analysis where the RNA molecules are converted to complementary DNA (cDNA) using reverse transcriptase enzymes (see Process Map 1.2). There are both standalone cDNA kit and kits where the cDNA synthesis is included as a step within the downstream analysis. The additional step requires its own set of controls to ensure the conversion is successful.

In summary, it is especially important to obtain detailed information from the client about sample collection, sample type and conditions, storage conditions, and preservation, as these can impact the quality and yield of recovered RNA. The EG laboratory must keep records of the kit used, lot number and/or the expiry date of reagents used, and version of the internal SOP followed.

6.6 QC and Performance Verification

Negative Controls: Separate NTCs shall be included for in-lab filtration and nucleic acid isolation and may be included for specific subsampling or pre-processing procedures as needed. For a filtration NTC, water from the same supply used to clean filtration equipment shall be filtered following the same procedure as the samples and carried through nucleic acid isolation as if it was a sample. No-reverse transcription controls (NRT) shall be included to identify genomic DNA contamination and success of DNase digestion in RNA isolates. The number of NRT controls will depend on the specific objective of each study. Once the sample has been shown to be free of genomic DNA contamination, the continued inclusion of NRT controls is desirable but not mandatory.

Positive Controls: There is no universal positive control for sample pre-processing and nucleic acid isolation in EG so labs may develop and implement controls specific to applications as required.

Procedures: Specific quality assurance checkpoints, outlined in **Table 6-1**, are necessary to confirm the successful completion of sample pre-processing and nucleic acid isolation procedures by ascertaining the quality of the resulting isolates. EG laboratories shall include a summary of QC procedures and results in reports to clients.

Table 6-1 QC Measures for Nucleic Acid Isolation

Assessment	Method, Outcomes, and Corrective Actions
Quantification of DNA or RNA Yield	***Standard Procedure:*** Sensitive fluorescent dyes that specifically bind DNA or RNA are used for quantification (e.g., Picogreen assay). Spectrometry methods (i.e., Nanodrop) are not sensitive enough to quantify eDNA in most cases. EG laboratories shall measure all samples and control samples.
	Normal Result: Negative controls (NTCs and NRTs) read below the detection limit. Positive controls fall within the expected range indicating high extraction efficiency. Sample concentrations vary but align with the expected values for given sample types and outliers are few. The quantity of DNA or RNA required will depend on the subsequent assay requirements (e.g., sequencing platform and library preparation kit input amounts).
	Other Outcomes and Corrective Actions: • If negative extraction controls have quantifiable DNA or RNA, (i) Review and revise the extraction protocol, (ii) Eliminate contamination sources, (iii) Re-extract samples, if possible, otherwise carry through all planned analyses and carefully evaluate impact on results. • If positive controls are not within the expected range, (i) perform root cause analysis on the extraction including reagents and extraction equipment, (ii) re-extract samples, if possible, otherwise (iii) carefully evaluate impact on results. • If sample eDNA yield is lower than required or highly variable, and the negative and positive controls are normal, (i) re-evaluate the extraction method and sample handling processes, if possible, or (ii) inform client and make a decision regarding the likelihood of success if the analysis is continued.
Evaluation of DNA and cDNA Integrity	***Standard Procedure:*** Confirm successful amplification of target DNA or cDNA using final detection method, or at minimum use gel or capillary electrophoresis to confirm PCR products have expected size. In cases where gel electrophoresis does not have enough sensitivity, use qPCR or sequencing. A representative subset of samples can be used for this QC.
	Normal Result: Observe detection of assay targets using qPCR or sequencing; or at a minimum confirm that DNA or cDNA is amplifiable and PCR products are of expected size.

	Positive controls fall within expected range and NTC and NRT negative controls do not amplify.
	Other Outcomes and Corrective Actions: • If no amplification is observed in positive controls or fragments are not the expected size, (i) Review extraction method applicability to this sample type, cDNA conversion (RNA), and PCR reaction setup and cycling conditions including how inhibitors were handled. • If positive control amplifies but samples do not amplify or fragments are not the expected size, (i) Repeat test with additional samples, (ii) conduct inhibitor testing. • If NRT controls for RNA samples show amplification, (i) Apply or repeat DNase treatment on samples.
Evaluation of PCR Inhibitors	***Standard Procedure:*** Test for inhibition by running PCRs on a serial dilution of samples or using a qPCR-based assay that can measure PCR inhibition. Another common approach is to spike a known amount of positive control DNA into eDNA samples and compare its amplification in samples to its amplification alone. For large sample sets it may be possible to check a representative subset of samples (5-10%).
	Normal Result: Amplification is successful at one or more of the tested dilutions. Negative controls have no amplification and positive controls give expected result.
	Other Outcomes and Corrective Actions: • If control samples are normal but inhibitors are detected in samples, (i) perform additional purification and/or PCR optimization measures. Consider commercial kits such as the Zymo OneStep PCR Inhibitor Removal Kits or Qiagen PowerClean kits, use of chemical enhancers such as betaine, bovine serum albumin (BSA) or dimethyl sulphoxide (DMSO) in the PCR reaction, or dilution of the DNA extract*. Repeat the inhibitor test. Or (ii) review extraction method applicability to this sample type. * Dilution reduces false negatives from inhibition but dilution may also dilute the target DNA below the assay LOD. EG laboratories may compensate for this effect by increasing the number of PCR replicates performed in the assay or increasing the volume of extracted DNA added to each reaction.

6.7 Client Perspective and Verification

The EG laboratory should recommend one or more options for sub-sampling and nucleic acid isolation based on client-provided project specifications (e.g., compatibility with chosen field methods or preservatives, biodiversity testing objectives, comparability with previous sample sets, etc.) and shall make available data to support this recommendation on request. The EG laboratory should also clearly identify any limitations of the chosen analysis that are relevant to the project.

7 Molecular Assay Techniques and Associated Parameters

Within each assay workflow, multiple molecular techniques are applied. This is a short overview of parameters involved in these techniques that influence outcomes and are optimized during workflow implementation.

7.1 Polymerase Chain Reaction (PCR)

The specific conditions for PCR must be established for each primer set.

PCR thermocycling conditions are optimized for each specific primer set. Determining optimal reaction efficiency is important because less than optimal thermocycling conditions can lead to under amplification of the target, and lower output after sequencing. Reaction efficiency should ideally fall between 90-110%, and low efficiency can lead to low output where low-abundance targets can be missed (Smith, 2021).

The choice of **DNA polymerase** is critical, as it directly affects the performance, efficiency, and fidelity of PCR. Taq polymerase is typically used; however, other polymerases such as high fidelity or proofreading polymerases may be better suited to application that require higher accuracy or complex targets.

PCR volumes, including amount of DNA sample (ng and µL) added to each reaction, can range based on the overall goals of the assay. Suitable volumes should be established during performance testing of an assay based on sensitivity requirements.

Technical replicates are essential in eDNA amplicon sequencing due to the heterogeneity of the template DNA where biodiversity components are often rare or low abundance. Multiple reactions help mitigate some PCR biases and stochastic effects of working with small subsamples of DNA. These replicates must contain the same amounts of reagents, DNA input, and undergo the same thermocycling conditions. Replicates can be pooled together after PCR amplification or treated as individual samples and sequenced independently to assess the reproducibility, consistency, and reliability of the overall amplicon sequencing workflow. The number of replicates will depend on the overall goals of the study, sample type, and rarity of target taxa, and should be established during the assay performance verification process. Increasing the number of replicates improves the likelihood of detecting species with low DNA copy numbers (Bruce et al., 2021). Power analysis may be performed to help determine the appropriate number of replicates needed to achieve a desired level of statistical power.

Post-PCR clean up procedures remove materials left from PCR that may inhibit subsequent reactions when PCR is an intermediate step in the workflow. Commercial kits (e.g., silica columns, magnetic beads) and other chemistries (e.g., ethanol precipitation) will differ in DNA yield, fragment size selection, and purity.

7.1.1 Quantitative PCR

qPCR uses the same process of PCR, however with the addition of real-time fluorescence detection.

Fluorescent markers are added to the reaction mixture and emit light when activated. There are many different kinds of fluorescent markers used in qPCR analysis, however the most recommended is probe-based qPCR due to its increased sensitivity. Species-specific probes are added into a PCR reaction and bind to the target DNA. During the amplification step, the DNA polymerase enzyme cleaves off the probe, resulting in fluorescence activation. Therefore, the fluorescence signal increases as more DNA is copied.

7.2 DNA Sequencing

Sequencing platforms differ in total capacity, read length, run time, accuracy or error rates, computational resources, and cost (Jennings et al., 2017). Illumina platforms are the standard in EG analysis due to their low error rates and scalability. The weaknesses of Illumina technology are higher DNA/RNA input requirements, shorter read lengths, and high cost of reagents. Oxford Nanopore sequencing can sequence long-read lengths and is portable (pocket-sized), which is desirable for real-time sequencing in the field (Marx, 2023; Satam et al., 2023). Long-read sequencing can offer better assembly and characterization of complex genomic regions or transcriptomes within environmental samples; however,

with these advantages come at the significant limitation of higher sequencing error rates when compared to other short-read sequencing instruments. The accuracy of nanopore sequencing has been improving over the years (Bock et al., 2023; Marx, 2023; Satam et al., 2023). Other sequencers like ThermoFisher's Ion Torrent platform and PacBio's Single-Molecule Real-Time (SMRT) platform are less commonly seen in EG Laboratories.

Sequencing depth, the number of sequences generated per sample, is optimized based on the application. For the detection of rare species in a community analysis, deeper sequencing (more reads per sample) increases assay sensitivity and accuracy, whereas general biodiversity assessments focused on common taxa require less depth (Singer et al., 2019). Broadly speaking, metagenomics and metatranscriptomics samples require deeper sequencing than metabarcoding or targeted sequencing. Optimal sequencing depth may also be empirically evaluated during assay performance testing, especially if a lower sequencing depth is going to be used.

Library preparation method or kit is chosen based on the sequencing platform and type of sequencing assay (e.g., amplicon sequencing, metagenomics, metatranscriptomics, etc.). Protocol steps such as amplification, fragmentation, size selection, or enrichment may bias the final library composition or introduce sequence errors and these factors are assessed during workflow validation. Fragmentation is applied in target enrichment, metagenomic, metatranscriptomic, and genome skimming workflows but not commonly applied in amplicon sequencing. Common fragmentation methods include enzymatic digestion (e.g. Illumina Tagmentation protocol), or mechanical shearing (e.g., sonication). Size selection procedures using magnetic beads (e.g., Beckman Coulter AMPure XP beads) or electrophoresis (e.g., Sage Science Pippin Prep) help to isolate DNA fragments within the desired size range to ensure an appropriate size for sequencing. DNA fragmentation may not be necessary for long-read sequencing platforms.

Indexing is the process of attaching unique short, synthetic sequences to DNA from different samples. This allows multiple samples to be sequenced simultaneously because the unique index sequence is then used to correctly reassign the resulting sequence reads to the original samples during the bioinformatic analysis stage. There are multiple indexing strategies (single, dual, unique dual, combinatorial), commercial providers, and lengths of indices available. Index hopping (or tag jumping) is where the wrong index becomes attached to a sequence from a different sample resulting in misassignment during the data analysis. Index hopping and cross-contamination of indices are two important sources of false positive detections in sequencing analysis that EG laboratories need to manage.

Sample **normalization** is the process of equalizing DNA concentration across multiple samples to prevent sequencing biases from uneven DNA concentrations. There are various approaches to sample normalization. For example, fluorescent-based quantification is used to create an equimolar pool so that each sample and amplicon has equal chance of contributing to the final sequencing data. Alternatively, there are commercial kits for magnetic bead-based normalization. The normalization process shall be validated to ensure that the strategy used does not affect the representation of target in the downstream analyses**.**

7.3 Technical And Practical Considerations For Combining qPCR and Amplicon Sequencing

Targeted qPCR has the capacity to analyze thousands of samples per day, and it boasts a short turnaround time, often ranging from a few hours to a single day. A qPCR instruments are common in molecular labs, making it an easy option. High sensitivity, specificity, and accuracy are notable attributes, though sequencing positive detections may sometimes be necessary to ensure accuracy. Compared to metabarcoding, qPCR data analysis is relatively simple and offers a quantitative measure of relative eDNA abundance.

In contrast, amplicon sequencing employs high-throughput technologies for sequencing specific genomic regions providing information on taxa identity and provide qualitative detection results. Unlike qPCR, metabarcoding is well-suited for detecting many species simultaneously, allowing comprehensive biodiversity surveys. Like qPCR, metabarcoding provides high sensitivity, specificity, and accuracy. While it also can analyze thousands of samples per day, sequencing tends to have longer turnaround times due to sample preparation and data analysis but provides more information than qPCR in the resulting data. Analysis of this sequencing data can be complex and requires specialized expertise.

Combining targeted qPCR and amplicon sequencing is possible and may be recommended in some cases. For example, sequencing positive detections in a new qPCR assay may be used to confirm detection of the target and rule out false positives. Moreover, qPCR can be used as a targeted approach to verify detections obtained through metabarcoding and quantify specific target species. Several studies have successfully combined metabarcoding and qPCR to approximate absolute abundance of various aquatic species (Evans & Lamberti, 2018; Ficetola et al., 2008; Klymus et al., 2015; Pont et al., 2023; Yates et al., 2019). Similarly, qPCR can be used to screen for known targets (e.g. indicator species) and combined with metabarcoding to get an overview of biodiversity, including rare or not well-described species.
The choice to combine both approaches depends on the specific study question, taxa of interest, and available resources. An effective strategy might focus sequencing on screening samples and then apply qPCR for confirmation and/or quantification. **Table 7-1** below outlines the differences and similarities between amplicon sequencing and qPCR and **Table 7-2** outlines a comparison of validations used for each method.

Table 7-1. Similarities and differences between amplicon sequencing and qPCR.

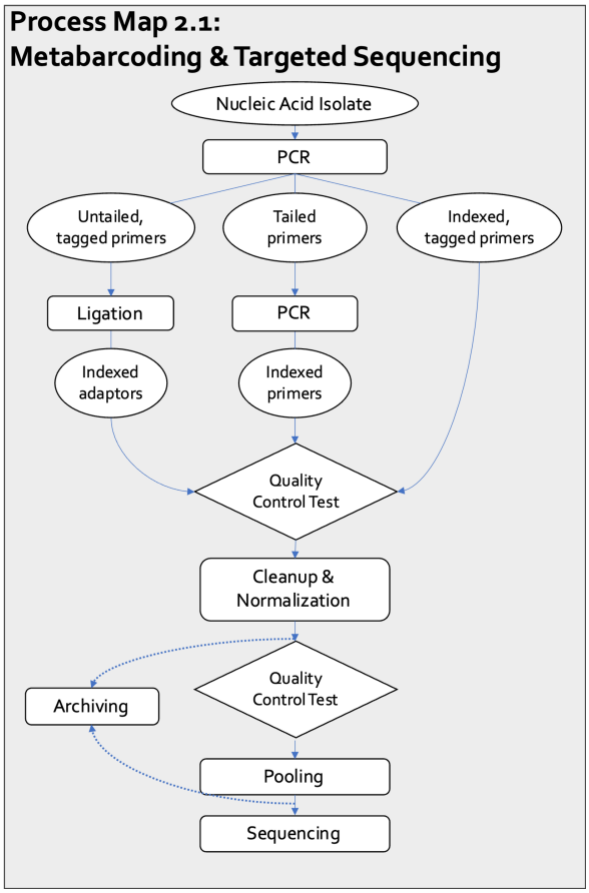
	Amplicon Sequencing	qPCR
Principle	High throughput sequencing of select genomic regions for taxonomic identification of single species or whole communities	Amplification and quantification of genomic regions using specific primers and probes to quantify target species
Target Multiplexing	Well-suited for highly multiplexed assays with many target species (beyond 6). Description of whole communities.	Can examine multiple target species (up to 6) within the same reaction, but typically single species
Sample multiplexing / Throughput	Can analyze thousands of samples per day	Can analyze thousands of samples per day
Turnaround Time	Longer turnaround time due to sample preparation and data analysis	Shorter turnaround time, typically a few hours to a day, simplified data analysis
Sensitivity	High	High
Specificity	Verifiable from sequences obtained	Relies on validation data
Accuracy	High	High. Sequencing of positive detections may be required.
Data Analysis	Can be complex and requires bioinformatics expertise	Relatively simple
Data Type	Qualitative, detect/non-detect (Quantitative or semi-quantitative possible for unicellular organisms or with modification of assays)	Quantitative, copy numbers

Table 7-2. Summary of types of validations in qPCR and NGS

Validation type	NGS	qPCR

Specificity validations - *in silico*	• Primers match target taxa • Marker can resolve target species from close relatives	• Primers and probe closely match target haplotypes • No predicted amplification of close relatives • No predicted non-specific amplification
Specificity validations - *in vitro*	• Amplification of DNA from target species from eDNA • No off-target amplification	• Amplification of DNA from target species from eDNA and tissue • No amplification of DNA from close relatives • No non-specific amplification from eDNA
Sensitivity validations	• PCR efficiency • Limit of detection (LOD)	• PCR efficiency • Limit of detection (LOD)
Fit for purpose	• Review applicability of validations • Test samples for PCR inhibitors	

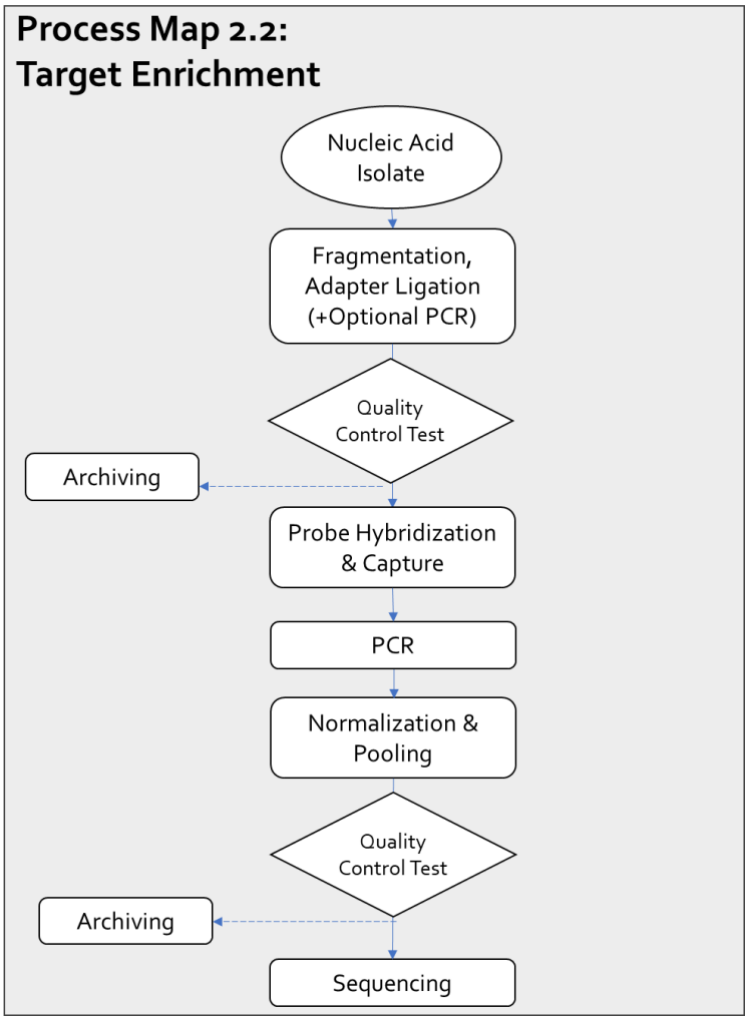
8 Metabarcoding & Targeted Sequencing



Metabarcoding and targeted sequencing (Process Map 2.1) refers to any workflow featuring PCR amplification of specific genetic fragments (markers, DNA barcodes) belonging to one or more species from an environmental sample and sequenced with a high-throughput sequencing platform.

Metabarcoding allows simultaneous detection of multiple species in a single PCR reaction by amplifying DNA markers which are common across species but have enough variability to differentiate them, including the standardized DNA barcode regions. The identification of multiple species within a single PCR reaction is an efficient approach to gain a comprehensive overview of community composition in eDNA samples. This selective amplification reduces the amount of non-relevant DNA taking up limited sequencing space, enabling the detection of low-abundance species.

Targeted sequencing uses species-specific primers designed to bind and amplify genome regions that distinguish the species of interest from closely related taxa. Compared to general metabarcoding, the species-specificity of targeted sequencing improves sensitivity for efficient detection of the species of interest.



Target enrichment (Process Map 2.2) is a special case where precisely defined genetic fragments of interest are selectively captured from an eDNA sample using probes (short single stranded DNA, complementary to regions of interest) ahead of PCR amplification (Jensen et al., 2021; Ribière et al., 2016; Seeber et al., 2019; Wilcox et al., 2018). In other words, assay specificity in a target enrichment workflow is based on probes rather than PCR primers, and target enrichment assays may be designed to capture as many different genes and species as necessary for the intended application. Capture based molecular assays are an area of active development (Günther et al., 2022; Li et al., 2023; Seeber et al., 2019; Wilcox et al., 2018). This approach reduces the complexity of the eDNA sample and increases the relative abundance of captured target DNA, making it possible to cost-effectively detect the species of interest. Probes

are added to eDNA samples, DNA from target species binds to the probes, and unbound non-specific DNA is washed away, leaving only the target-enriched DNA fragments. These enriched fragments can then be amplified through PCR to boost the amount of target DNA for subsequent analysis or sequenced directly if concentration allows.

8.1 Molecular Design Guidelines

Design and optimization of an amplicon sequencing assay shall consider Sections 5.1 and 5.2 and the following key parameters to ensure assays are fit for purpose.

Client Assay Requirements: Requirements for a molecular assay shall be defined at the start of the design process or prior to implementing a published assay. This includes establishing the biodiversity targets, expectations for taxonomic resolution, geographic scope, data output (quantitative vs presence-absence), and any additional thresholds for sensitivity or accuracy that need to be met.

Technical Assay Requirements: Sequencing platform specifications (e.g., sequencing read lengths, error rates, etc.) and type of assay (e.g., metabarcoding or target enrichment, multiplex or single assay, etc.) should be decided at the start of the design or assay review process to identify additional assay requirements.

Reference Sequences: Taxonomic and geographic coverage of reference databases are the primary limiting factor in assay design, but these databases are constantly being updated. Accession numbers are unique IDs that are assigned to DNA sequences in databases such as GenBank. A record should be kept of individual accession numbers used for assay design for reproducibility of the design process by providing unambiguous identification of the target sequences used and facilitating future reviews of the assay.

The target gene region(s) shall be selected based on assay requirements. In metabarcoding of general biodiversity, certain genetic regions are widely accepted for common taxonomic groups because reference database coverage and taxonomic resolution have been established for these groups at large. For example, the 16S ribosomal DNA (rDNA) gene region for bacterial diversity, the 18S rDNA gene for eukaryotic diversity, the cytochrome b (Cytb) for vertebrates, the cytochrome c oxidase subunit 1 (COI) for metazoans, the internal transcribed spacer (ITS) 1 or 2 for fungi and plants, the mitochondrial 12S rDNA gene for fish, the mitochondrial 16S rDNA for vertebrates (See **Table 8-2**). Other regions are used as secondary markers, such as the D-loop mitochondrial region targeted in cases where Cytb and COI do not provide enough specificity(Tsuji et al., 2019). For targeted sequencing, database coverage for the species of interest and their sister species can be assessed individually to determine potential gene regions for the assay.

***In Silico* Specificity, Resolution, and Molecular Design:** Primers and probes used to recover the target DNA fragments from environmental samples must be (1) specific to the gene regions of interest, (2) specific to the biodiversity target(s) while avoiding similar non-target sequences, and (3) compatible with the intended sequencing platform. The DNA fragment that will be sequenced must provide sufficient information to or resolve taxonomy to the taxonomic level in the project specifications, while minimizing risks for false positive or false negative detections.

To meet the requirements in Sections 5.2, EG laboratories must perform a minimum set of checks and verifications on each newly designed, or newly implemented, assay:

Specificity validation: New primer or probe sequences should first be reviewed against known haplotypes (genetic variants) of the target taxa at the binding sites, to measure their capacity to detect all biodiversity targets. This assessment requires multiple reference sequences for the target species and examines the conservation of primer binding sites within the taxa to estimate likelihood of PCR amplification. This cannot be evaluated if only one reference sequence is available for the gene region of a target species or if all reference sequences are identical. Geographic origin of reference sequences should be reviewed. For new designs, the placement of metabarcoding primers or hybridization capture probes should be adjusted based on findings to maximize selective amplification and reduce the chances of false negatives. Secondly, assay specificity in targeted sequencing assays should be assessed by aligning primers or probes to reference sequences from closely related non-target species to predict what may be co-amplified in the assay and change nucleotide sequences if needed. This test requires at least one reference sequence from each related species. It

is recommended to perform an *in silico* PCR, i.e. simulate a PCR on an appropriate database, in order to verify if primers or probes target only taxa of interest.

Taxonomic resolution: Close relatives of the target species may share high sequence similarity, so it is critical that the DNA marker chosen provides enough taxonomic resolution for the project aims. If a marker lacks enough resolution, it may lead to misidentifications or incomplete biodiversity inventories, as its DNA sequence will be identical across many taxa. The DNA marker or probe should cover enough nucleotide mismatches between the targets and close relatives to unambiguously identify the target. The number of mismatches required will depend on the error-rates of the library prep and sequencing technology, as well as the effectiveness of any bioinformatic methods used to mitigate these errors. Testing this requires at least one reference sequence from each related species and EG laboratories should maintain in their records a list of species used in marker evaluation as well as key species missing from the assessment.

For general biodiversity studies, published, verified and widely used PCR assays are preferred over new, untested designs, as this maximizes taxonomic assignment success. For each assay designed or newly implemented at an EG laboratory, internal records shall include the (1) design requirements the assay was evaluated against, (2) lists of species, database accession numbers, search terms, and/or library versioning of reference information used in the design or review process, (3) sequencing platform and length of amplicon that was targeted, (4) primer or probe sequences, and (5) outcomes of checks and verifications along with any expected limitations of the design that could not be avoided. This review process shall be repeated if an assay will be applied in a new context, new platform, or new reference information is available.

Assay production: The choice of primer or probe purification method is based on assay requirements. Desalting is a common method that works well for routine applications, more advanced purification methods such as High-Performance Liquid Chromatography and Polyacrylamide Gel Electrophoresis provide higher purity and may be combined for both high purity and high-resolution and are generally recommended for NGS. Cartridge-based purification (chemistries can be silica-based or magnetic bead-based) is best for large volumes. Manufacturer and purification method for primer or probe production should be included with assay records since synthesis methods and QC procedures may vary.

Performance Testing: Newly designed or newly implemented sequencing assays shall undergo laboratory testing to verify that they perform as intended and optimize the reaction conditions.

Sensitivity validation: EG assays need to accurately detect low abundance target sequences. It is important to evaluate efficiency of amplicon sequencing assays to minimize false negatives and/or variability in detection limits which are assumed constant. To validate efficiency of an amplicon sequencing assay, PCR conditions are first optimized. Factors like annealing temperature and time, primer concentrations or number of PCR cycles (~30) are modified to achieve maximal amplification. Following optimization, a dilution series PCR test is used to evaluate amplification efficiency and influence of inhibitors. These tests require positive control eDNA samples that mimic the expected DNA concentrations, compositions, and chemical profiles of the intended application.

Every molecular assay has a LOD. It is a probabilistic measurement, typically the lowest concentration at which there is target detection 95% of the time among replicate runs. Determining the LOD provides a measurement of sensitivity threshold for meaningful interpretation of negative results or non-detects and facilitates comparisons of different molecular assays for the same target. EG laboratories should determine LOD for targeted sequencing assays, matching qPCR practices, through serial dilutions of target tissue DNA or dilution of a synthetic gene. For metabarcoding, more work is needed to establish a standard practice for LOD calculation. However, it is not feasible to validate every target of a multiplex assay to the same extent as an individual species of interest but a representative subset may be reviewed.

Specificity validation: EG laboratories must perform tests with eDNA samples to confirm the efficient and specific recovery of DNA marker sequences from target taxa. Positive control eDNA samples are brought through to sequencing to confirm successful detection of the targets and measure proportion of target versus off-target detection.

For each assay designed or implemented at an EG laboratory, internal records shall include performance testing procedure and outcomes, including any potential limitations of the assay.

Table 8-1 Overview of validation stages, influencing parameters, and critical applicability considerations to review for any new study.

Validation stage	Review applicability of prior validation to current study	Key influencing factors to optimize
Assay inclusivity of haplotypes *in silico*	• Are the reference haplotypes from the study area? • How conserved is the primer or probe binding region among haplotypes?	• Target gene region • Primer sequences • Amplicon size
Marker taxonomic resolution *in silico*	• Are there reference sequences available for all close relatives in the study area? • Are there reference sequences available for all close relatives?	• Target gene region • Primer and probe sequences • Amplicon size
Amplification of DNA from targets	• Were the eDNA samples used for validation relevant to the study area?	• Primer design • Assay optimization: Annealing temperature, primer concentrations, etc.
Amplification of DNA from close relatives	• Was the specificity validated for the closest relatives relevant to the study area?	• Primer design • Assay optimization: Annealing temperature, primer concentrations, etc.
PCR efficiency	• Comparable sample matrix to that used in validation? • Are the same PCR reagents used?	• Thermocycling protocol (times and degrees) • PCR volume • PCR cycles • Primer concentrations • Technical replication • Sample dilution
Limit of detection (LOD)	• Comparable sample matrix to that used in validation? • Are the same PCR reagents used?	• Technical replication • PCR volume • Sample dilution • Sequencing depth

8.2 Core Metabarcoding Markers

The best DNA markers for metabarcoding demonstrate successful recovery, taxonomic resolution, and annotation across many species within broad taxonomic groups, and once designed, these DNA markers are widely applicable for different monitoring projects and geographic areas (Fahner et al., 2016). There are several well-established DNA metabarcoding markers, **Table 8-2** , as the result of global research efforts and many more can be found in published documents (e.g., for more narrow taxonomic groups or to address particular challenges) that could be implemented at EG laboratories with completion of further performance testing.

Table 8-2 Examples of commonly used DNA markers for metabarcoding (not exhaustive)

Gene region	Target Taxa	Example Marker	Mean amplicon length (bp) including primers	Taxonomic Resolution
12S	Fish	MiFishU (Miya et al., 2015) F: NNNNNNGTCGGTAAAACTCGTGCCAGC R: NNNNNNCATAGTGGGGTATCTAATCCCAGTTTG	234	Medium
	Vertebrates	12SV05 (Riaz et al., 2011) F: TTAGATACCCCACTATGC R: TAGAACAGGCTCCTCTAG	133	Low
16S	Bacteria	515F–806R (V4 region) (Apprill et al., 2015; Parada et al., 2016) F: GTGYCAGCMGCCGCGGTAA R: GGACTACNVGGGTWTCTAAT	390	Medium
	Mammals	16Smam (P. Taylor, 1996) F: CGGTTGGGGTGACCTCGGA R: GCTGTTATCCCTAGGGTAACT	135	Low
18S	Eukaryotes	18SV4M1 (V4 region) (Stat et al., 2017) F: GCAGTTAAAAAGCTCGTAG R: TCCAAGAATTRCACCTCT	346	Low
COI	Eukaryotes	F230 (Gibson et al., 2015) F: GGTCAACAAATCATAAAGATATTGG R: CTTATRTTRTTTATNCGNGGRAANGC	281.5	High
	Metazoans, Macroalgae	Leray XT (Wangensteen et al., 2018) F: GGWACWRGWTGRACWITITAYCCYCC R: TAIACYTCIGGRTGICCRAARAAYCA	368	High
	Insects	BF3BR2 (Roger et al., 2022) F: CCHGAYATRGCHTTYCCHCG R: TCDGGRTGNCCRAARAAYCA	458	High
ITS	Fungi	ITS3ITS4 (ITS2 region) (White et al., 1990) F: GCATCGATGAAGAACGCAGC R: TCCTCCGCTTATTGATATGC	306	Medium
	Plants	ITS2S2FITS4 (ITS2 region) (Fahner et al., 2016) F: ATGCGATACTTGGTGTGAAT R: TCCTCCGCTTATTGATATGC	420	High
rbcL	Diatoms	[Diat_rbcL_708F_1 + Diat_rbcL_708F_2 + Diat_rbcL_708F_3] + [R3_1 + R3_2] (Bruce et al., 2021) F: AGGTGAAGTAAAAGGTTCWTACTTAAA + AGGTGAAACTAAAGGTTCWTACTTAAA + AGGTGAAGTTAAAGGTTCWTAYTTAAA R: CCTTCTAATTTACCWACWACTG + CCTTCTAATTTACCWACAACAG	312	Medium
	Plants	rbcLa (Fahner et al., 2016) F: ATGTCACCACAAACAGAGACTAAAGC R: GTAAAATCAAGTCCACCRCG	596	Medium

8.3 Library Preparation and Sequencing Guidelines

Illumina sequencing is the standard for metabarcoding and targeted sequencing assays in EG and thus standard library preparation is based on Illumina's "16S metagenomic sequencing library preparation" procedure (Illumina, n.d.): amplification of the DNA marker with tailed primers, post PCR clean-up, addition of unique sample indices in a second PCR, second post PCR clean-up, quantification, normalization, pooling, and sequencing. First and second PCR may be combined into a single reaction by using tagged-primers such as outlined in (Esling et al., 2015; Mauffrey et al., 2021) followed by sequencing adapter ligation, and there are also new kits to streamline the quantification, normalization, and pooling steps into a single procedure.

The workflow for target enrichment is distinct from amplicon-based DNA metabarcoding or targeted sequencing workflows and more closely resembles metagenomic library preparation in the initial steps. As a newer method, EG laboratories should use commercial kits such as the Illumina DNA Prep with Enrichment kit and adhere to the associated procedures and manufacturer's guidance.

For amplicon sequencing workflows, all steps of library preparation including PCR, PCR clean up, indexing, normalizing, and pooling shall be evaluated during initial implementation to identify and minimize taxonomic biases (metabarcoding) or maximize sensitivity (targeted sequencing, target enrichment) using positive controls or representative samples. PCR thermocycling conditions, PCR volumes, selection of DNA polymerase, number of technical replicates, and post-PCR clean up parameters are established for each primer set or DNA marker as part of the optimization testing. Index hopping rates for the library preparation and sequencing workflow should be assessed during workflow development. Alignment of sequencing platform, read length, DNA marker size, PCR clean-up, and size selection protocols prevents loss of sensitivity and specificity. Sequencing depth shall be evaluated against assay requirements during workflow development because a minimum depth is required to accurately examine diversity from eDNA samples (García-Machado et al., 2023).

Specific Requirements:

- A minimum of three PCR replicates per sample shall be used for each metabarcoding assay.
- To mitigate the risk of index hopping, EG laboratories shall carry negative controls through sequencing, use a unique dual indexing strategy, and perform a post-indexing PCR clean up to remove excess indices.
- Metabarcoding, targeted sequencing, and target enrichment shall be performed on Illumina sequencing platforms unless performance of an alternative platform is first validated against Illumina sequencing.
- Metabarcoding sequencing assays shall aim for a minimum depth between 100,000 and 1,000,000 reads per sample per marker unless a lower depth is validated against the higher depth for a specific application or taxonomic group of interest.
- Higher depth may be used for detection of the rare taxa with general metabarcoding markers.
- Target sequencing assays may use a lower sequencing depth per sample per species target.

8.4 Standard QC Procedures

Metabarcoding assays shall include NTCs and may include positive controls where specific applications require. Targeted sequencing assays shall include NTCs and positive controls. Target enrichment assays shall include NTCs and shall include at least one positive control.

At minimum, one NTC for the assay shall be included in each reaction plate. These NTCs shall be in addition to negative controls from previous steps like field or extraction NTCs to allow tracking of separate contamination sources. The control samples can be screen using an electrophoresis type procedure following the logic outlined in **Table 8-3**.

Table 8-3 QC Measures for Metabarcoding and Targeted Sequencing

Assessment	Method, Outcomes, and Corrective Actions
Screening of assay controls	***Standard Procedure:*** Electrophoresis (gel or capillary) to assess presence and size of DNA fragments following amplification or enrichment steps.
	Normal Result: NTCs present without DNA fragments of the expected size. Positive controls have DNA fragments of the expect size. Regardless of a normal result, controls must still be brought through the final sequencing stage to monitor for contamination because NGS is more sensitive than most electrophoresis-based QC testing options.
	Other Outcomes and Corrective Actions: • If electrophoresis indicates target DNA is present in one or more of the assay NTCs, then the assay shall be repeated. (i) Review and revise the assay protocol, (ii) eliminate contamination sources, and (iii) re-run the assay. • If electrophoresis indicates amplification of NTCs from prior stages of the workflow, but not the assay NTCs, then (i) Review the procedure associated with the failed NTC, (ii) eliminate contamination sources and repeat prior steps if possible, otherwise (iii) carry the NTC through all planned analyses to evaluate impact on results. • If positive controls do not produce the expected DNA fragments, then (i) Review the assay protocol including reagents, control samples, and equipment, and revise as needed, and then (ii) re-run the assay.
Evaluating assay controls	For interpretation of controls post-sequencing, please see bioinformatics section.

8.5 Laboratory Outputs & Reporting

The EG laboratory shall provide raw sequencer output files to the bioinformatics service provider along with a mapping file of sample IDs and indices.

The EG laboratory shall provide at minimum the information listed in **Table 8-4** to clients.

Table 8-4 Minimum reporting for assay parameters in NGS amplicon sequencing

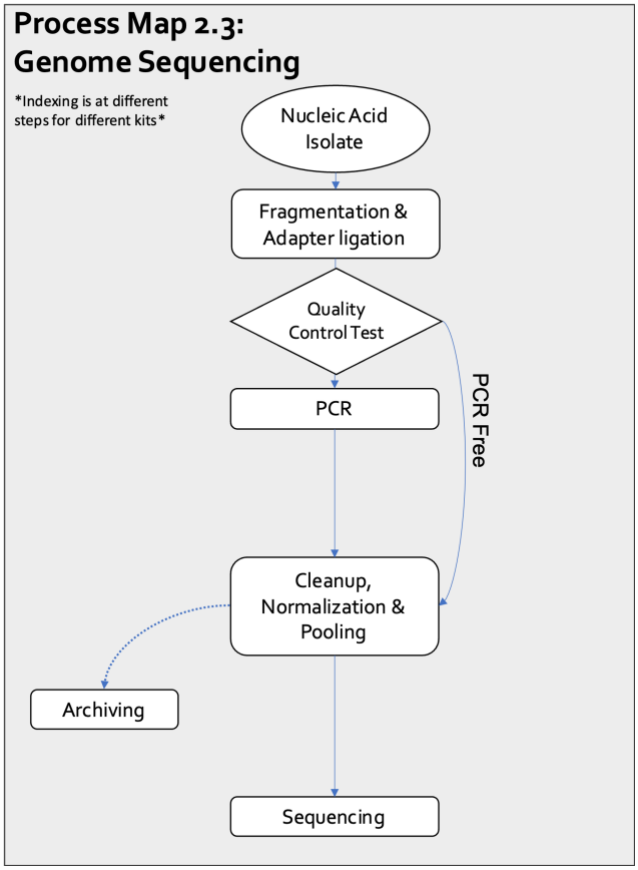
Component	Reporting Parameters
Amplicon sequencing primer or probe selection	• Overview of assay design specifications and performance testing • LOD if measured • Known limitations of assay
Sequencing library preparation	• Library preparation workflow name and version • Number of technical replicates and when/if pooling occurs • Estimated rate of index hopping if a data correction is not applied
NGS	• Sequencing depth • Sequence read length
Data analytics	• Sample normalization or how read counts should be interpreted • Summary of negative and positive control sample results
Deviations from standard workflow	• Application notes on any major deviations from the standard • Application notes justifying use of non-Illumina sequencing platform, alternative library preparation methodology, or lower sequencing depth

8.6 Client Perspective

Before starting the analysis, clients can expect the EG laboratory to recommend the molecular analysis approach based on client project specifications. The lab should select the DNA marker(s) or target enrichment panel and make available information supporting this decision if requested, including assay limitations, design specifications, suitability of the assay to detect the species or taxonomic groups of interest, and validation or performance testing that was conducted. Clients can also expect the lab to outline number and where technical replicates, positive controls, and negative controls will be used in the workflow and if a non-standard library preparation and/or sequencing method will be used.

After completion of the analysis, clients can expect the EG laboratory to provide the necessary information to the bioinformatics service provider for demultiplexing the data by sample and denoising sequence reads. Clients should also receive a report that includes a summary of the work performed and the reporting parameters outlined in **Table 8-4**.

9 Metagenomic, Metatranscriptomic, and Genome Sequencing



Metagenomic, metatranscriptomic, and genome sequencing are all sequencing-based molecular analysis techniques with limited or no enrichment towards particular genes or species and instead provide a broader view of genetic information from eDNA, eRNA, or tissue samples, respectively.

9.1 Metagenomics and Metatranscriptomics

Metagenomics refers to the random sequencing of fragments of total DNA of an environmental sample, to look at genomic content across organisms in the environment. The random nature of the sequencing means the distribution of read counts will reflect the prevalence of that sequence in the original DNA extract. This method will best reflect the taxon abundances in samples, aside from any biases or enrichment for certain taxa or cell types that occur during collection, pre-processing, or extraction stages. It is reasonable to expect that prokaryote sequences and genes that occur in high copy number will be the most prevalent, which is why this approach is mainly applicable to prokaryotes at current sequencing depths.

Metagenomic sequencing offers a comprehensive and unbiased view of the microbial community, especially in complex or poorly characterized environments. A hybrid approach, applying both metagenomic and amplicon sequencing methods to the same samples, can leverage their respective strengths. Metagenomics can help enhance resolution, provide quantitative and functional information, cross-verify results obtained from amplicon sequencing, and help overcome database limitations of poorly characterized species of interest because it is not constrained by choice of DNA marker.

Metagenomics requires library preparation from a high-quality DNA extract. DNA is fragmented and then prepared for sequencing with end-repair, adenosine nucleotide tailing, and ligation of adapters. In the case of the Illumina Tagmentation protocol, enzymatic fragmentation and adapter ligation are done as a single reaction. Sequencing adapters

are ligated to the fragments to prepare for sequencing using a PCR-free approach. Alternatively, adapters are ligated to the fragments and subsequently sequencing adapters are added during PCR amplification. Size selection procedures are applied to isolate fragments of the appropriate size for sequencing. Short read sequencing (150 bp) is often used for deep sequencing, and long read sequencing (1000 bp) can help provide longer fragments to help with assembly (Liu et al., 2022) since average length of a prokaryotic gene transcript is roughly 320 amino acids or 900 bp and longer for eukaryotes. It is also possible to prep samples for both long- and short-read sequencing to benefit from the advantages of each (Priest et al., 2021).

Nanopore sequencing can sequence long-read lengths and is portable (pocket-sized), which is desirable for real-time sequencing in the field(Marx, 2023; Satam et al., 2023). Long-read sequencing can offer better assembly and characterization of complex genomic regions or transcriptomes within environmental samples; however, with these advantages come at the significant limitation of higher sequencing error rates when compared to other short-read sequencing instruments. The accuracy of nanopore sequencing has been improving over the years (Bock et al., 2023; Marx, 2023; Satam et al., 2023).

Similarly, metatranscriptomics is the random sequencing of fragments of RNA or cDNA. By sequencing RNA rather than DNA, metatranscriptomics focuses on expressed genes only, providing information on the active metabolic processes and other cellular functions within the environment at the time of sampling. Specific types of RNA can be enriched or depleted in samples in response to project specification (see Section 6.5). For example, RNA samples are typically dominated by rRNA, which is of limited use for evaluating functional diversity so EG laboratories may apply a strategy to increase the mRNA content of samples prior to library preparation. Metatranscriptomics is also used for monitoring RNA viruses in the environment which cannot be detected using DNA-based surveys.

In short read sequencing, RNA samples are fragmented to the desired size range by incubation with divalent cations (e.g. Mg^{+2}) or enzymatically (e.g. RNase II) prior to cDNA conversion. Subsequent library preparation procedures follow the workflow for metagenomics.

Recently, Oxford Nanopore released a sequencing platform for direct sequencing of RNA. This procedure begins with total RNA or enriched/depleted RNA and does not require fragmentation. While cDNA is synthesized from the RNA during library preparation, only the RNA is sequenced.

9.1.1 Library Preparation and Sequencing Guidelines

For metagenomic and metatranscriptomic workflows, all steps of library preparation including fragmentation, enrichment, amplification, indexing, normalizing, and pooling shall be evaluated during initial implementation using positive controls or repeated measures of representative samples to identify and minimize biases. Index hopping rates for the library preparation and sequencing workflow should be assessed during workflow development. Alignment of sequencing platform, read length, fragment size, PCR clean-up, normalization, and size selection protocols prevents loss of sensitivity and specificity.

Sequencing depth shall be evaluated during initial implementation for a specific application and preferably should be re-evaluated for each new project based on diversity estimates. Diversity, and thus depth requirements, can vary widely from 200 Gb (670 million reads with 150 bp paired-end sequencing) for highly diverse soil samples (Rodriguez-R & Konstantinidis, 2014) to 1.2 Gb (4 million reads) for Antarctic rock samples (Tremblay et al., 2022). In some cases, sequencing depth may be assessed for the total pool of samples rather than individual samples, depending on project goals (Tremblay et al., 2022). EG laboratories should optimize sequencing depth by deep sequencing metagenomic libraries from a few samples and statistically estimating impact of depth on biodiversity metrics. Alternatively, 16S metabarcoding can provide estimates of relative abundance for microbial taxa which can be used to predict metagenomic coverage at different sequencing depths (*Metagenomics - Calculate Sampling Depth*, n.d.).

If long read sequencing is selected, nucleic acid isolation methods and sample storage and handling procedures should be reviewed and optimized for recovery of high molecular weight DNA or RNA as outlined in Sections 4.2.5.2 and 6.

Specific Requirements:

- EG laboratories should use commercial kits for library preparation, such as the Illumina DNA Prep kit or Nextera XT DNA Library Preparation Kit.
- To mitigate the risk of index hopping, EG laboratories shall carry negative controls through sequencing, use a unique dual indexing strategy, and perform a post-indexing PCR clean up to remove excess indices.
- Short read sequencing shall be performed on Illumina sequencing platforms with at least 150 bp paired-end read length unless performance of an alternative platform or shorter read length is first validated against this.
- Metagenomic and metatranscriptomic sequencing assays shall aim for a minimum depth between 3-32 Gb (10-100 million reads per sample with 150 bp paired-end reads) unless a lower depth is validated as "fit-for-purpose" for a specific application or taxonomic group of interest.
- EG laboratories shall maintain records of any modifications to kit protocols during library preparation and justification for reproducibility and troubleshooting purposes based on deep sequencing or estimated from 16S metabarcoding.

9.1.2 Standard QC Procedures

At minimum, one NTC for library preparation shall be included in each sample plate, in addition to field and laboratory negative controls from previous steps. In metatranscriptomics, a NRT control is added where cDNA synthesis occurs (see Section 6.6) which may be during library preparation. One or more positive controls may be included where specific applications require to confirm that the library preparation and sequencing are working as expected. The controls are used to assess library preparation and determine if steps should be repeated prior to sequencing (**Table 9-1**).

Table 9-1 QC Measures for Metagenomics and Metatranscriptomic Library Preparation

Assessment	Method, Outcomes, and Corrective Actions
Screening of assay controls	***Standard Procedure:*** Electrophoresis (capillary) to assess size distribution of DNA fragments. Measurement of DNA concentration using qPCR or fluorometric method. Both of these are monitored at multiple points in the library preparation.
	Normal Result: Negative controls from library preparation stages present null results in both size and quantification assays. Library concentration for samples falls within the optimal range for sequencing and size distribution is within the analysis parameters. Positive controls have DNA fragments of the expected size and concentration. Regardless of a normal result, controls should still be brought through the final sequencing stage to monitor for low levels of cross-contamination and index hopping which may not be detected in QC testing.
	Other Outcomes and Corrective Actions: • If electrophoresis indicates DNA contamination is present in one or more of the library preparation negative controls, then the library preparation shall be repeated. (i) Review and revise the assay protocol, (ii) eliminate contamination sources, and (iii) re-run the assay. • If electrophoresis indicates amplification of NTCs from prior stages of the workflow, but not the library preparation NTCs, then (i) Review the procedure associated with the failed NTC, (ii) eliminate contamination sources and repeat prior steps if possible, otherwise (iii) carry the NTC through all planned analyses to evaluate impact on results. • If positive controls do not produce the expected DNA library size and concentration, then (i) Review the assay protocol including reagents, control samples, and equipment, and revise as needed, and then (ii) re-run the assay.
Evaluating assay controls	For interpretation of controls post-sequencing, please see bioinformatics section.

9.1.3 Laboratory Outputs and Reporting

The EG laboratory shall provide raw sequencer output files to the bioinformatics service provider along with a mapping file of sample IDs and indices.

The EG laboratory shall provide at minimum the information listed in **Table 9-2** to clients.

Table 9-2 Minimum reporting for assay parameters in metagenomics and metatranscriptomics

Component	Reporting Parameters
Sequencing library preparation	• Library preparation workflow name and version • Number of technical replicates and when/if pooling occurs • Estimated rate of index hopping if a data correction is not applied
NGS	• Sequencing depth • Sequence read length
Data analytics	• Sample normalization or how read counts should be interpreted • Summary of negative and positive control sample results
Deviations from standard workflow	• Application notes on any major deviations from the standard • Application notes justifying use of non-Illumina sequencing platform for short-read sequencing, laboratory-derived library preparation methodology (i.e., non-commercial kit), or lower sequencing depth

9.2 Genome Skimming

Genome skimming is the random (shotgun) sequencing of single-species tissue DNA extracts at a much lower sequencing depth than what is used for complete genome assembly. This "shallow" sequencing is biased towards regions of the genome in high copy number including mitochondrial DNA (mtDNA), chloroplast DNA (cpDNA), and ribosomal DNA (rDNA). Likewise, these same genomic regions are used in EG because they're more likely to be detected in the environment. Compared to conventional amplicon sequencing that generates reference sequences for one marker region at a time, genome skimming efficiently generates reference sequences for all potential metabarcoding markers in one sequencing preparation (Trevisan et al., 2019). Additionally, this technique generates sequence information for primer binding sites, which is important for evaluating existing assay suitability and designing novel assays.

9.2.1 Library Preparation and Sequencing Guidelines

The genome skimming laboratory workflow is identical to the metagenomic workflow outlined in Section 9.1 and Illumina sequencing is also the standard for short-read shotgun sequencing. As with metagenomics, EG laboratories shall use commercial kits for library preparation but the high yield of DNA from tissue samples provides greater flexibility in terms of kit selection. Key considerations for library preparation and sequencing include those listed above in Section 9.1 for metagenomics except for sequencing depth. For genome skimming, sequencing depth should be optimized to provide adequate coverage of the high copy number genomic regions and depends on genome size of the organism including mitochondrial and chloroplast genomes. EG laboratories should optimize sequencing depth for groups of related organisms by starting with higher depth than necessary for a few test specimens and calibrating based on the data.

9.2.2 Standard QC Procedures

EG laboratories shall include negative and positive controls for library preparation as outlined for metagenomics in Section 9.1.2 to monitor for contamination and successful execution of library preparation prior to sequencing. EG

laboratories may choose to carry these controls through sequencing but it is less important for genome skimming because contamination is simpler to detect in single-source DNA samples during bioinformatics.

9.2.3 Laboratory Outputs and Reporting

The EG laboratory shall provide raw sequencer output files to the bioinformatics service provider along with a mapping file of sample and taxonomic IDs and indices.

The EG laboratory shall provide at minimum the information listed in **Table 9-3** to clients. Reference sequences, verification of species identities, and any associated metrics (e.g., fold coverage) are determined from bioinformatics.

Table 9-3 Minimum reporting for assay parameters in genome skimming

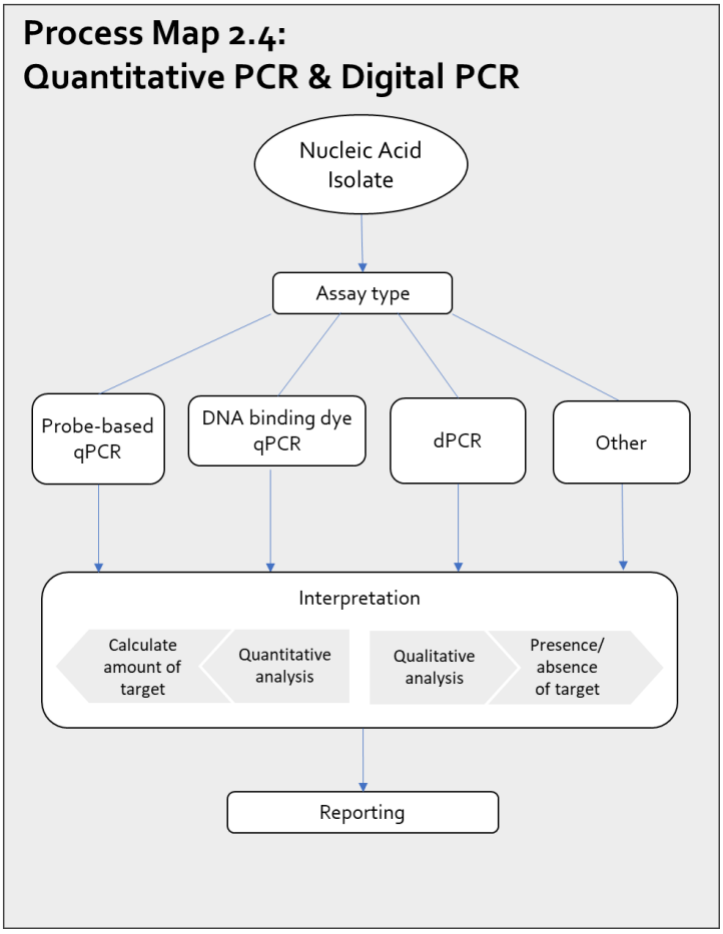
Component	Reporting Parameters
Sequencing library preparation	• Library preparation workflow name and version
NGS	• Sequencing platform • Sequencing depth • Sequence read length
Data analytics	• Summary of negative and positive control sample results

9.3 Client Perspective

Not all EG laboratories may offer metagenomics, metatranscriptomics, or genome skimming. If there is a possibility that these approaches will be relevant to a project, it is important to find an EG laboratory that offers these services. Clients can expect an experienced EG laboratory to recommend when and where it is suitable to apply these techniques over more focused or targeted approaches like metabarcoding or qPCR, or if a combined approach is best. Metagenomic approaches are commonly deployed for prokaryote community investigations. Eukaryotic metagenomic and metatranscriptomic studies are less common and are still in the early stages of application. The EG laboratory will also help identify reference database gaps where genome skimming could be beneficial, so that clients may plan to collect and preserve key tissue samples. These techniques are more computationally intensive so the decision should also be based on a discussion with the bioinformatics service provider if separate from the laboratory.

Clients can expect the laboratory to provide information on why the selected workflow is fit for purpose and an overview of the validation testing that was performed. The laboratory should outline number and where technical replicates, positive controls, and negative controls will be used in the workflow and planned sequencing method. After completion of the analysis, clients can expect the EG laboratory to provide the necessary information to the bioinformatics service provider for demultiplexing the data by sample. Clients should also receive a report that includes a summary of the work performed and the reporting parameters outlined in **Table 9-2** or **Table 9-3**.

10 Quantitative PCR and Digital PCR



Quantitative Polymerase Chain Reaction (qPCR) and digital PCR (dPCR) are PCR-based techniques that enable users to both detect and quantify DNA of a target species in a sample, with applications such as invasive species monitoring or indicator species detection for ecosystem health assessment. These methods are highly sensitive, specific, and allow users to estimate the relative abundance[2] of a target species in environmental samples, providing a rapid and cost-effective solution to infer concentration of target species. Contrary to NGS-based methods, dPCR and qPCR only target one or a few species at a time (lower throughput) but once an assay is available for a species, the implementation is much faster due to simplicity of the workflow.

The inherent nature of eDNA samples leads to more inhibitors and if inhibitors cannot be addressed with other measures (see **Table 6-1**), EG laboratories should use dPCR over qPCR or have plans to test for and mitigate inhibitors that must be done for each new sample set.

10.1 qPCR Assay Chemistries and Oligos

Hydrolysis probes are the most prevalent qPCR assay type used in eDNA analysis due to high specificity and ease of design (Pilliod et al., 2013; Tajadini et al., 2014; Wilcox et al., 2013). Hydrolysis probes are oligonucleotide probes equipped with a fluorescent dye and a quencher molecule at either end. When the probe is whole, the quencher's proximity to the fluorescent dye suppresses the fluorescence. These probes, during PCR, anneal to the target sequence between the primer sites. DNA polymerase activity degrades the bound probe during DNA replication in PCR, separating

[2] It is important to understand that, like in all eDNA approaches, quantification is dependent on the amount of DNA released by the organisms, its environmental persistence, the suitability of the chosen gene region, among other factors (sampling, storage, etc.). As such, these quantifications are best used as a measure of relative abundance.

the fluorescent dye from the quencher and enabling a detectable fluorescence signal. Hydrolysis probes can contain modifications to increase specificity, like a 3' minor groove binding moiety or locked nucleic acids.

Another type of qPCR assay uses DNA binding dyes (e.g., SYBR Green I) that indiscriminately binds to double-stranded DNA, and the fluorescent signal increases after each cycle as long as the species-specific primers are amplifying DNA in the sample. Assays based on SYBR Green I are less frequently employed for eDNA analysis due to the lower specificity than assays using hydrolysis probes. Due to SYBR green's nature of binding to any double-stranded DNA, false positives can be triggered by non-specific amplification, necessitating carefully designed primers and secondary melt-curve analysis. Since no probe is required, costs and design time are potentially reduced. However, hydrolysis probe specificity is higher than SYBR green chemistry because the target sequence-specific probe avoids non-specific signals from primer-dimers or off-target amplification.

Apart from hydrolysis probes and SYBR Green chemistries, alternative qPCR chemistries like Molecular Beacons, Dual-labelled probes (e.g., FRET probes), and Scorpion Primers also provide certain advantages. Each of these chemistries have unique strengths and limitations, with the choice depending on the experiment's specific requirements. This may encompass factors such as the need for high specificity, multiplexing capabilities, ease of design, or cost considerations.

EG laboratories shall use hydrolysis probes for qPCR-based target species detection or quantification from eDNA.

10.2 Digital PCR (dPCR)

Digital PCR, or dPCR, is a variant of qPCR (Langlois et al., 2021) and used for precise quantification of nucleic acid molecules. It is particularly useful in applications where quantification of rare targets is important, such as invasive or endangered species monitoring. The digital aspect of dPCR comes from the fact that the reaction is partitioned into numerous discrete units, allowing for more accurate and precise quantification compared to traditional single-volume qPCR. Types of dPCR include droplet digital PCR (ddPCR), where the partitions are a suspension of oil droplets, and chip-based digital PCR, where the partitioning occurs on the assay plate.

Though the reaction format and instrumentation may vary, dPCR can use the same primers and probes as qPCR and offers several advantages:

- **Precision**: dPCR is highly precise, especially at low target concentrations. Each partition essentially represents a binary "yes" or "no" answer regarding the presence of the target, allowing much more precise quantification..
- **Sensitivity**: It is particularly sensitive and can detect low-abundance targets that might be challenging to quantify accurately using other methods.
- **Robustness**: dPCR is less influenced by PCR inhibitors and is more suitable for challenging sample types, common in eDNA studies.

EG laboratories should consider the differences, **Table 10-1,** between qPCR and dPCR when choosing techniques. qPCR should be chosen if relative quantification or qualitative results (e.g., presence/absence of targets) is sufficient to meet project goals. dPCR should be chosen if precise and accurate absolute quantification is required, especially for low-abundance targets or when dealing with complex sample matrices, such as eDNA from sediments. It should be noted that qPCR can be modified by scaling the reaction volume or increasing the sample input to increase assay sensitivity for lower abundance targets (Quan et al., 2018). Consider the specific features and limitations of each method based on the goals of the project.

Table 10-1. Comparison of qPCR and dPCR for use in EG applications.

	qPCR	dPCR
Target Abundance	Well-suited for moderately abundant targets but can be	Ideal for precise quantification of low-abundance targets

	adapted for low-abundance targets	
Precision and Sensitivity	High sensitivity; suitable for detecting small changes in gene abundance	Higher precision for absolute quantification, especially at low target concentrations
Absolute vs. Relative Quantification	Primarily used for relative quantification; can perform absolute quantification but may be less accurate, especially at low concentrations	Well-suited for absolute quantification, providing accurate measurement of target copy number
Cost and Throughput	Usually more cost-effective; higher throughput	May be more expensive per sample; suitable for projects with a smaller number of samples requiring high precision
Ease of Use	Widely used and more straightforward; commonly available equipment	Requires specialized equipment and training; user-friendly once trained
Multiplexing	More amenable to multiplexing, allowing simultaneous detection of multiple targets	Multiplexing can be more challenging and may require additional optimization
Applications	Suitable for routine screening and large-scale studies where relative quantification is sufficient	Ideal for applications demanding high precision in quantifying low-abundance targets, e.g., rare species detection in eDNA
Sample Complexity	Suitable for a wide range of sample types and concentrations	More robust in the presence of inhibitors, making it suitable for complex sample matrices
Technology Familiarity	Well-established and widely adopted technology	A newer technology with a growing user base; familiarity may vary

10.3 Optimization and Validation

EG laboratories may implement published assays or design new assays for specific targets. Primers and probes designed for qPCR should be *species-specific* and only bind to the intended target DNA sequences, not to any non-target sequences.

There are several specific requirements in set up of every qPCR or dPCR assay, including optimizing efficiency of the assay, implementation of suitable controls and quantification calibrators, testing and controlling for inhibitors, and validating specificity/sensitivity of the assays. All the design guidelines outlined for Amplicon Sequencing in Section 8.1 apply to these assays but with some additional validation requirements outlined in the sections to follow. The DNAqua-Net program, focused on developing DNA-based approaches for aquatic habitat assessment and monitoring, created a targeted assay validation scale (Thalinger et al., 2021). The scale, shown in **Figure 10-1**, provides a common, user-friendly

method for assessing published targeted assays to determine their suitability for monitoring applications and can be a useful tool for tracking and communicating progress during validation steps. An eDNA validation website is also available that introduces the validation scale and provides a library of validated assays (in development)(DNAqua-Net, n.d.).

Since qPCR is still more common than dPCR, it will be discussed hereafter; however, most of these measures equally apply to dPCR.

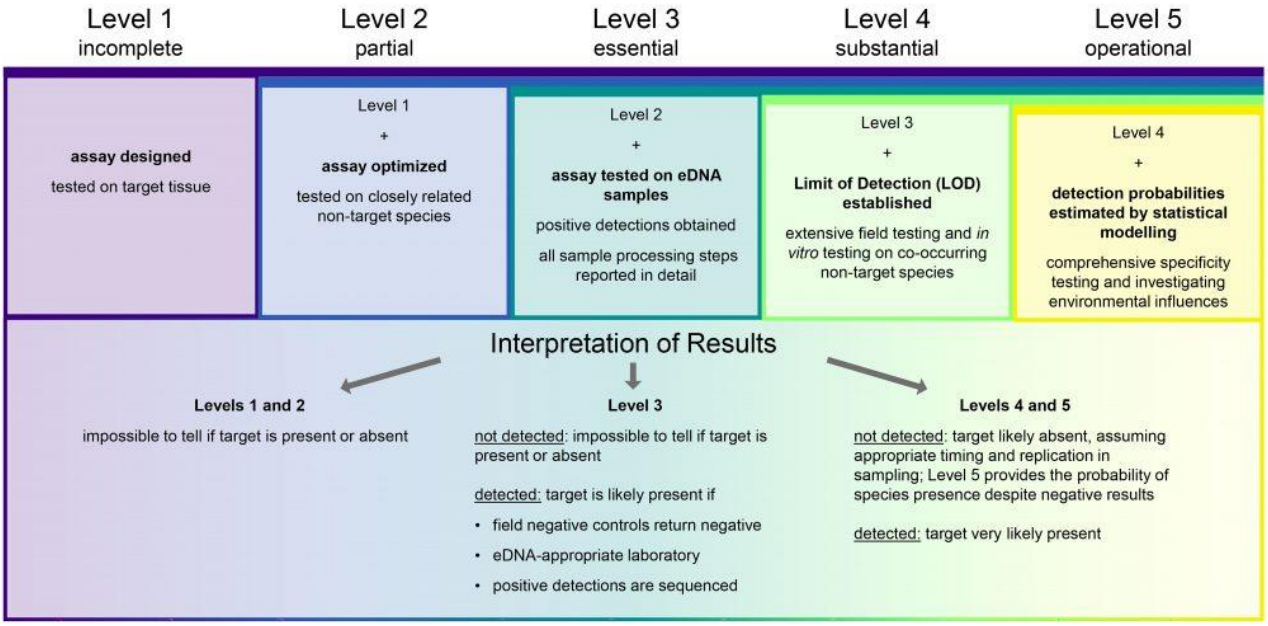


Figure 10-1 Overview of the DNAqua-Net qPCR assay validation scale from (DNAqua-Net, n.d.)

10.3.1 Validate Specificity *in silico*

The first step in specificity validation is the *in silico* (computational) analysis of target haplotypes. The primers must be designed to amplify a unique and conserved region of the target species' DNA. If there's a genetic variant in the target species that the primers do not bind to, amplification may fail leading to false negatives. This step requires multiple reference sequences covering the target species' haplotypes and an effective primer will demonstrate a match to all the target sequences. The assessment should be considered incomplete or inconclusive if a species is only represented by one or two sequences in the database or if all the reference sequences are from a limited geographic range where gene flow with the population being monitored is unlikely.

The next step is the *in silico* analysis of potential cross-reactivity among close relatives of the target species, especially those who co-occur in the study area. False positives occur when primers and probes bind to DNA from non-target species to produce the fluorescent signal. This analysis requires reference sequences from all close relatives (best case), but in practice reference sequences from a subset of relatives are reviewed based on available information, and primers and probes should have 2 or more mismatches with each of these non-target taxa. It is also prudent use *in silico* PCR to check whether the primers are likely to amplify anything else in the nucleotide database, known as non-specific amplification. A mismatch analysis can be used to predict the likelihood of binding target sequences and off-target sequences.

Proper primer design and specificity validation minimizes the risk of false positives and cross-reactivity with non-target DNA. The name of the target species, gene region, length of amplicon and accession number should be checked for accuracy and reported (Bustin et al., 2009; The dMIQE Group et al., 2020). Consideration for these decisions is outlined in Section 8.1.

10.3.2 Performance Testing

10.3.2.1 Optimizing Efficiency

Optimizing efficiency of a qPCR assay is crucial, as it directly impacts the accuracy and reliability of results. EG laboratories should follow the standard optimization parameters for qPCR(Svec et al., 2015).

Reaction efficiency should be reported to clients, to enhance confidence in the assay results. In general, reaction efficiencies should fall between 90-110% (Svec et al., 2015). An efficiency of 100% represents a perfect doubling of DNA during the amplification cycles. Low values indicate a non-optimal reaction and can lead to incorrect quantification, false negatives, difficulty interpreting results. Numerous technical and eDNA standards have discussed evaluating reaction efficiency in qPCR (Abbott et al., 2021; Bustin et al., 2009; CSA W219:23, Performance Criteria for the Analyses of Environmental DNA by Targeted Quantitative Polymerase Chain Reaction, 2023; De Brauwer et al., 2022b; Pabinger et al., 2014; Svec et al., 2015; The dMIQE Group et al., 2020).

To optimize efficiency, several factors must be considered. A meticulous approach will aid in maintaining a high-quality, efficient, and reliable qPCR:

- Primer design is crucial, considering factors like melting temperature, GC content, and sequence specificity to avoid off-target amplification.
- Master mix chemistries can be evaluated by testing different commercial products.
- The concentration of template DNA and primers must be adjusted to achieve the highest efficiency without saturation or inhibition. This can be achieved by performing a gradient PCR on these parameters.
- Annealing temperature and time should also be optimized to find the values at which the highest yield of the target product is produced without non-specific amplification. This can be achieved by performing a gradient PCR on these parameters.
- A standard curve to determine the efficiency and linearity of the reaction (not typically required for dPCR).
- Lastly, monitor the specificity of the reaction by performing a high resolution melting curve analysis (HRMA) post-amplification, or another independent method such as sequencing of positive detections.

Consistent PCR efficiency across experiments is essential for ensuring the repeatability of results. EG laboratories shall verify PCR efficiency for each new sample set or whenever changes are made to a validated protocol, to demonstrate equivalency (or enhancement) of results before and after the change.

10.3.2.2 Sensitivity Validation

LOD and if applicable, limit of quantification (LOQ) are critical parameters to understand the sensitivity of the qPCR assay, especially when dealing with low-abundance targets in eDNA samples. Determining an LOD establishes the lowest concentration at which the assay can reliably detect a target. Targets present in lower quantities might not be detected below this limit, so LOD provides meaningful interpretation of negative results or non-detects. LOD also allows comparison of sensitivity of different qPCR assays and helps ensure reproducibility between labs. For EG studies focusing on presence/absence or other qualitative results, determining LOD is required but LOQ might not be necessary.

LOQ is a crucial assay parameter to obtain *quantitative* results. It represents the lowest concentration at which targets can accurately be quantified in the assay. It is at a higher point than the LOD, and it is possible to have detections of target that are above the LOD but below the LOQ. Numerous studies have detailed procedures on determining LOQ and LOD in qPCR and dPCR assays (Forootan et al., 2017; Hunter et al., 2017; Klymus et al., 2020; Pabinger et al., 2014), however the value of these limits is always calculated using multiple PCR replicates (5-12) for each concentration of the DNA standards.

Following recent standardization effort, EG laboratories shall define LOD in qPCR as the lowest concentration at which 95% of the technical replicates of the standard amplify, while LOQ is the lowest concentration for which the coefficient of variation (CV) value is < 35% for the used standard DNA (Klymus et al., 2020); definitions also adopted by (Thalinger et al., 2021). Based on these definitions, it is still possible (and common) to detect target DNA at concentrations below the

theoretical LOD, especially when multiple technical replicates are run for each environmental sample. Detections below the LOD may be considered as lower confidence level in interpretation of results but should not be ignored.

For dPCR, the lowest concentration that can theoretically be detected and measured is one target molecule per reaction (Deprez et al., 2016; Hunter et al., 2017). The same practical definition of LOD as used in qPCR shall be applied to dPCR (95% positive detections of standard DNA) meaning that it is still possible to obtain valid detections below the LOD. Calculation of the mean target concentration of samples should include non-amplifications as zero-estimates, droplets beyond the fluorescence as positive events, and those below threshold as negative events.

EG laboratories should verify the LOD during implementation of all qPCR assays. Determining at least the LOD ensures the assay has been optimized, is efficient, can detect low amounts of target, and will address uncertainties surrounding false negatives.

10.3.2.3 Validation Specificity in vitro

The steps for a successful *in vitro* specificity validation with eDNA samples are as follows:

Verify amplification of DNA from tissue of target haplotypes. It is essential to validate that the assay efficiently amplifies the target using a tissue extract of target haplotypes. Alternatively, a synthetic gene can serve this purpose if no tissue extract is available; however, this requires knowledge of local haplotypes and synthetic DNA should be spiked into a representative eDNA sample to reproduce matrix effects.

Test amplification of DNA from tissue of close relatives. If available, cross-testing amplification results of the assay using tissue from non-target close relatives in the area offers more robust information on the specificity of the assay. This cannot always be done as it may not be possible to obtain tissue from the related species in the area. A direct test provides sound information about the reliability of the assay and confidence in the results, so collecting samples from related species should be part of the sampling plan.

Verify amplification of targets from eDNA samples. It is essential to confirm the assay detects the target species from eDNA samples. Detections can be confirmed by sequencing the amplicons from positive signals, or another method (such as visual confirmation of the target, or PCR and sequencing another diagnostic region). Alternatively, melt-curve analysis can be performed to assess false positives from non-specific amplification. This can be done as a pilot study, or on a subset of samples from the study area prior to a large-scale analysis.

10.3.3 Review Applicability of Validations

It is critical to determine for pre-existing assays whether the available validation data are applicable for each new sample set and/or project, as parameters for validation may change across all eDNA sample types. **Table 10-2** contains a list of considerations for reviewing validations.

Table 10-2. Considerations to review applicability of validations for new projects and sample sets.

Parameter to review	Questions to determine applicability
Specificity testing in silico	Was the previous validation relevant to the study area? Has the reference database changed since the previous validation? If so, determine whether the primer and probe designs are still relevant to the current available information.
Specificity testing in vitro	Were the eDNA samples used for validation relevant to the study area?

	Was the specificity validated for all the closest relatives relevant to the study area? Did the eDNA samples tested have similar species composition and chemical matrix to the study area? If not, consider repeating some specificity testing steps.
Sensitivity testing	Did the samples tested have similar species composition and share chemical/physical properties to the study area? If not, the LOD may need to be re-evaluated on the current sample set.

10.4 Standard QC Procedures

There are several different types of controls required in qPCR and dPCR:

NTCs for the assay must be included in each qPCR or dPCR reaction plate, in the same number as the number of technical replicates for the assay (Bustin et al., 2009; CSA W219:23, Performance Criteria for the Analyses of Environmental DNA by Targeted Quantitative Polymerase Chain Reaction, 2023, p. 219; The dMIQE Group et al., 2020). A negative sample control, where the target species is known to be absent, should also be included with the assay. A NRT should also be included in RNA-based assays.

An external positive control shall be included in each assay of either quantified tissue DNA extract of the targeted species, purified amplicons (e.g., Currier et al., 2018), or a synthetic standard (e.g., gBlocks™)(Langlois et al., 2021) with identical primer and probe binding sites to that of the target species being tested. Where quantitative results are required, a quantification calibrator (standard curve made from external positive control) shall also be included to convert the fluorescence measurements from the assay into quantities (copy numbers) of the target. Additionally, EG laboratories may include an IAC to co-amplify with the target for direct inhibitor testing.

Inhibitor testing is crucial in qPCR because inhibitors can impair the activity of DNA polymerases, interfere with primer binding, quench the fluorescence of fluorophores, decrease reaction efficiency, increase reaction variability, or lead to false negatives (Sidstedt et al., 2020). Inhibitor testing shall be carried out on each new sample set, since inhibitors vary depending on environmental conditions (Kumar, Girish et al., 2021).

Table 10-3 Interpretation of Assay Controls for qPCR and dPCR

Assessment	Method, Outcomes, and Corrective Actions
Inhibitor testing	***Standard Procedure:*** Inhibition is revealed with either an IAC, by performing a dilution series on a subset of samples from the sample set (if the target is known to be present in the samples), or by spiking in tissue of a known concentration into the eDNA samples. A minimum subset of samples (5-10%) should be evaluated for inhibitors in qPCR and dPCR assays, since inhibitors have a pronounced affect on quantitative measurements. Ensure a representative set of samples are examined (e.g., range of sites and DNA concentrations).
	Normal Result: Amplification is successful at one or more of the tested dilutions. Negative controls have no amplification and positive controls give expected result.
	Other Outcomes and Corrective Actions: • If control samples are normal but inhibitors are detected in samples,

	(i) perform additional purification, dilution, and/or PCR optimization measures. Consider testing PCR additives such as betaine or bovine serum albumin to counter inhibitor effects. Or (ii) review extraction method applicability to this sample type.
Evaluating assay controls	***Standard Procedure:*** The laboratory includes assay NTCs on each assay plate. Each assay has an external positive control and may also include negative sample controls, NRT controls, standard curve, and internal positive controls.
	Normal Result: Negative controls have no amplification. Positive controls amplify and have the expected quantitative result.
	Other Outcomes and Corrective Actions: • Any positive detections in negative would result in rejecting the results of the plate and repeating the assay to eliminate the source contamination. Negative sample controls should not amplify but may reveal non-target amplification if they do. NRT amplification indicates contamination of RNA samples with genomic DNA. (i) Review and revise the assay protocol, (ii) eliminate contamination sources, and (iii) re-run the assay. • If NTCs from prior stages of the workflow amplify (e.g., extraction), but not the assay NTCs, then (i) Review the procedure associated with the failed NTC, (ii) eliminate contamination sources and repeat prior steps if possible, otherwise (iii) reject the results for the associated samples. • If positive controls do not produce the expected amplification, then (i) Review the assay protocol including reagents, control samples, and equipment, and revise as needed, and then (ii) re-run the assay.

10.5 Interpretation of qPCR and dPCR

qPCR assays are interpreted using the Quantification Cycle or Cycle Threshold (Cq or Ct) values. As shown in **Figure 10-2**, Cq is the cycle number at which a sample's amplification curve intersects the fluorescence threshold. A lower Cq value means a higher starting quantity of DNA. Most modern qPCR software can determine this value automatically. Cq thresholds can be used to ensure detections are above the LOD, and above the LOQ when making quantitative measurements. For quantification, Cq values are calibrated against a standard curve of positive controls with known copy numbers of the target (see **Figure 10-3**), allowing for a report of copy numbers by unit of volume. Relating this back to a quantification of the number of organisms present in the environment is dependent on many variables which are difficult to model. For this reason, quantification is usually relative, and reporting the Cq or copy number value is sufficient.

For dPCR, results will be obtained in gene copies/µL instead of Cq values without the need to calibrate to a standard curve. The number of positive partitions which contain the target will be given, along with the total number of partitions. This allows the user to calculate the concentration of the target DNA in the original sample in copies/µL. The interpretation of either dPCR or qPCR results will depend on the overall goal of the study.

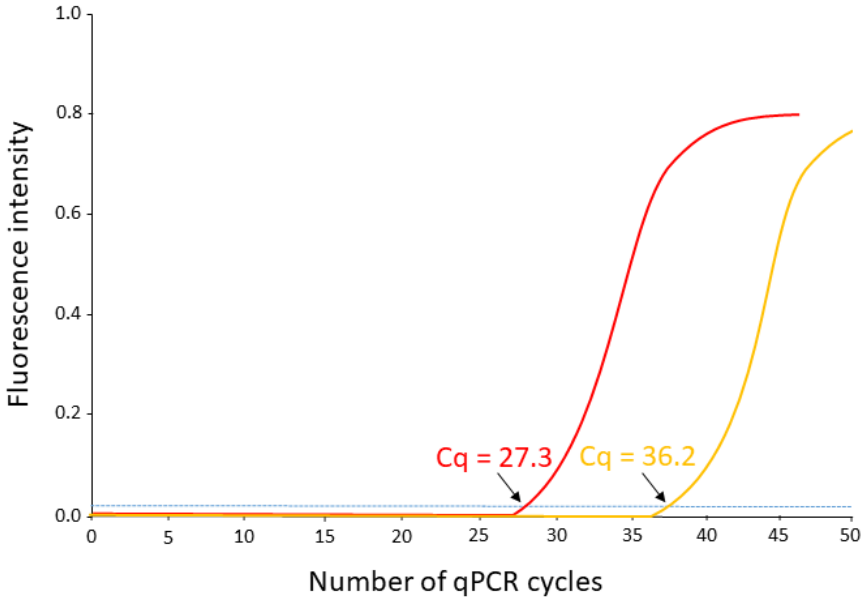


Figure 10-2 Schematic diagram of a qPCR curve from (Abbott et al., 2021) where the red line represents a sample with more target DNA than the sample represented by the yellow line.

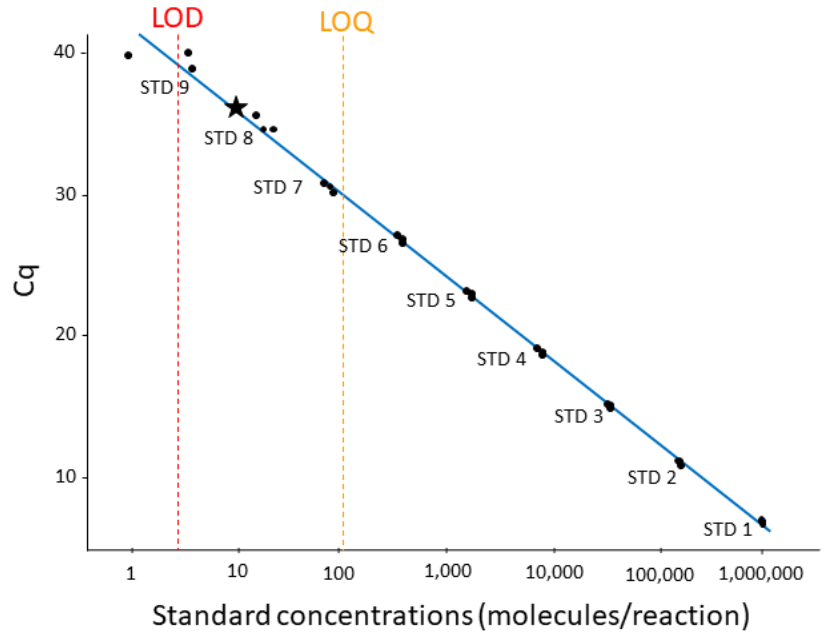


Figure 10-3 A qPCR assay standard curve showing results of solutions with known (circles) and unknown (star) DNA concentrations and limits of detection (LOD, red) and quantification (LOQ, yellow) from (Abbott et al., 2021).

10.6 Laboratory Outputs & Reporting

The EG laboratory report to the client shall include, at minimum, the level of validation of each qPCR or dPCR assay within the DNAqua-Net framework shown in **Figure 10-1** and a summary of specificity and sensitivity testing. The specificity testing summary shall include the geographic region considered in the validation, a list of species and number of distinct specimens included in specificity testing, whether each species was evaluated *in silico* from reference

sequences or *in vitro* using DNA, and outcomes and/or limitations identified during this testing. The sensitivity testing summary shall provide the LOD and LOQ for each assay, if determined.

The report shall include a list of negative and positive controls for each assay, number of technical replicates, decision criteria used for determining a positive or negative technical replicate, and the results (detect/non-detect, Ct, or copy number) for each technical replicate of all controls and samples. The standard curve for estimating the target eDNA concentration in the environmental media shall be provided when appropriate.

10.7 Client Perspectives

Before starting the analysis, clients can expect the EG laboratory to recommend the molecular analysis approach based on client project specifications. The lab should select the target species assay and make available information supporting this decision if requested, including assay limitations, design specifications, suitability of the assay to detect the species or taxonomic groups of interest, and validation or performance testing that was conducted. Clients can also expect the lab to outline number of technical replicates, positive controls, and negative controls that will be used in the workflow.

After completion of the analysis, clients can expect to receive a report that includes a summary of the work performed, the reporting parameters outlined in Section 10.6, and interpretation of the results based on initial specification for detection and/or quantification.

11 Appendices

11.1 References

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11.2 Quality Assurance and Laboratory Competency Questionnaire

The following form may be used as a template to collect information on the quality assurance practices of the EG laboratory or to guide EG Labs on the practices that should be in place for their operations.

Lab Information

1. Lab Name	
2. Lab Location	
3. Lab Director	
4. Lab Phone	

Part 1: Quality Management System

Does your facility adhere to an existing Quality standard (e.g., ISO9001:2015)? Is it certified or accredited by a third-party? If so, please provide a copy of the most recent certification document.

5. Standard	
6. Scope of certification	
7. Certification type	
8. Certificate number	
9. Date of last audit	
10. Result of last audit	
11. Copy of most recent certification document attached? (yes/no)	
12. Copy of most recent audit report attached? (yes/no)	

Describe, in brief, the following components of your Quality Management System as they relate to your Environmental Genomics service offerings.

Quality Assurance

13. Quality policy and quality objectives	
14. Who is responsible for the QMS?	
15. How frequently are internal and external audits performed?	
16. How is upper management involved in your Quality program?	

Training & Competency

17. How is training conducted and competency established for new staff?	
18. How is competency monitored for existing staff?	

Equipment Maintenance & Calibration

19. Who is responsible for tracking equipment maintenance and calibration?	
20. List of calibration service providers for EG lab equipment	
21. What equipment maintenance and calibration records are available for review, if requested?	

Procedures

22. Are SOPs in place for all laboratory analyses conducted?	
23. When SOPs are revised, how is versioning tracked and what is communicated to clients?	

Data Management

24. What are your laboratory data governance policies?	
25. How is the sample chain of custody traceable and secure?	

Continuous Improvement

26. Does the laboratory have a formal process for identifying, implementing, and documenting non-conformances and improvements?	
27. How is client feedback collected and integrated?	

Part 2: Environmental Genomics Services and Experience

Nucleic Acid Extraction

28. What substrates and preservation methods are accepted for DNA extraction?	
29. Is eRNA extraction available and if so, what substrates and preservation methods are accepted?	
30. Are commercial extraction kits used?	
31. How is quality of nucleic acid extracts assessed?	☐ Negative controls ☐ IPC ☐ Positive controls ☐ Inhibitor testing ☐ Other
32. What material is archived?	
33. Turnaround time?	

Metabarcoding & Targeted Sequencing

34. List of taxonomic groups, species, and/or published assays available.	
35. Number of technical replicates included per sample per DNA marker assay in the default service offering.	
36. Sequencing platform, read length, and sequencing depth per sample per DNA marker included in the default service offering.	
37. How is the quality of the library preparation and sequencing output assessed?	☐ Negative controls ☐ IPC ☐ Positive controls ☐ Inhibitor testing ☐ Other
38. What data is provided to the client? And does the laboratory offer bioinformatics services? Is a reporting standard followed and is an example report available?	
39. What material and data is archived?	
40. Turnaround time?	
41. Are new assay design services available?	

qPCR & dPCR

42. List of species assays available with level of validation testing completed.	
43. Number of technical replicates included per sample per assay in the default service offering.	
44. How is assay performance verified for each sample batch?	☐ Negative controls ☐ IPC ☐ Positive controls ☐ Inhibitor testing ☐ Other
45. What is included in the client report? Is a reporting standard followed and is an example report available?	
46. Turnaround time?	
47. Are new assay design services available?	

Metagenomics & Metatranscriptomics

48. Are commercial library preparation kits used?	
49. What enrichment or depletion options are available?	

50. Sequencing platform, read length, and sequencing depth per sample included in the default service offering.	
51. How is the quality of the library preparation and sequencing output assessed?	☐ Negative controls ☐ IPC ☐ Positive controls ☐ Inhibitor testing ☐ Other
52. What data is provided to the client? And does the laboratory offer bioinformatics services? Is a reporting standard followed and is an example report available?	
53. What material and data is archived?	
54. Turnaround time?	

Reference Sequence Generation

55. Does the lab offer DNA barcoding or genome skimming services to generate reference sequences from tissue samples?	
56. What data is provided to the client and what is archived?	

Laboratory Service Credentials

What is the laboratory history and qualifications for EG analysis?

57. Years of business providing EG services	
58. Years of experience for key staff	
59. Geographic areas served	
60. List of published scientific articles using data produced by the laboratory	
61. References from clients	
62. Number of projects completed	
63. Number of samples analyzed	
64. Other	

11.3 EG Guidance Documents from Regulators

Fisheries and Oceans Canada released guidance for the use of targeted qPCR assays for endangered and invasive species monitoring (Abbott et al., 2021). This document provides guidance on designing, conducting, reporting, and interpreting targeted eDNA studies in aquatic environments using qPCR. Guidance is not limited to a particular sample type or species and the document provides few specific recommendations, but rather indicates what information must be considered and reported for accurate, reproducible, and repeatable results. At the provincial level in Canada, an eDNA protocol for freshwater ecosystems was developed for the British Columbia Ministry of Environment for single-species qPCR analysis and reporting(Hobbs & Goldberg, 2017), although the standard has yet to be formally adopted by the government's internal standards committee. These are detailed protocols providing lists of materials required and step-by-step protocols for reference tissue sample collection, water sample collection, water sample filtration, sample preservation, and reporting along with recommendations for study design and reporting. This protocol is tailored to the specific environment, conditions, and species of interest for the BC government so the protocols may not be widely applicable.

Two documents for eDNA biomonitoring in Australia and New Zealand were released as an initiative of the Australian Government Department of Agriculture, Fisheries and Forestry (De Brauwer et al., 2023). The first document focuses on protocol development (i.e., how to develop protocols for a given project/program), sampling design, and operational practices for eDNA laboratories (De Brauwer et al., 2022a). The protocol development section is focused on analysis using qPCR. The second document focuses on eDNA test validation and provides guidance on the development and validation of both species-specific and general assays (De Brauwer et al., 2022b). Both sets of guidelines are not prescriptive and set minimum standards to support consistency in eDNA use. The National eDNA Reference Centre in Australia, a collaboration between the University of Canberra and the Department of Agriculture, Fisheries and Forestry, coordinates proficiency testing for facilities involved their network of collaborating eDNA centers (*eDNA Proficiency Testing Scheme*, 2023).

Two sets of guidelines that cover the metabarcoding workflow from sample collection to data analysis and reporting have been published. The first comes from the Nordic Council of Ministers for monitoring phytoplankton using DNA metabarcoding (Jerney et al., 2023). The guidelines provide an overview of methodological options available at each step of the workflow along with recommendations for each step. Recommendations are very specific for some stages (e.g., two specific primer sets recommended) and other stages included minimum standards (e.g., iinhibition testing should be done when a high load of humic substances is expected, and commercial extraction kits optimized for inhibitor removal should be used). More prescriptive recommendations were made since the guidelines focus on a particular target group of organisms (i.e., phytoplankton) in a particular environment (i.e., marine and brackish). The second set of guidelines that includes the whole metabarcoding workflow comes from the Swiss Federal Office for the Environment (Pawlowski et al., 2020). Their guidelines for eDNA biomonitoring in aquatic ecosystems are less prescriptive as they are designed for a broader range of applications. For example, the Nordic phytoplankton monitoring guidelines provide recommendations on two specific primers sets to use and the Swiss guidelines for aquatic ecosystem biomonitoring describe how to determine what primer sets to use. The Swiss guidelines also provide guidance on setting up a molecular lab for eDNA work and include targeted eDNA approaches as well as metabarcoding.

In addition to the resources described above, several regulatory agencies have released roadmaps or strategies for integrating eDNA into regulatory regimes including CSIRO Australia's roadmap to integrate eDNA for marine parks (De Brauwer et al., 2023), the United Kingdom's 25-year Environment Plan using eDNA for a range of applications (Handley et al., 2023), Scotland's development of habitat scale biodiversity monitoring (Bakker et al., 2023), Finland's roadmap for implementing molecular monitoring methods (Norros et al., n.d.), Europe's strategy for integrating eDNA-based methods into aquatic monitoring resulting from COST Action DNAqua-Net program(Bruce et al., 2021; Lefrançois et al., 2021), and the United States national eDNA strategy (Kelly et al., 2023) and National Oceanic and Atmospheric Administration genomics strategy (*NOAA 'Omics Strategy*, 2020). While these documents make clear the intention to move eDNA to regulator application, they do not provide specific guidance for laboratory analysis or service providers.

Independent standardization bodies are also helping to close this gap in laboratory standards. The Canadian Standards Association (CSA) has released two standards for EG: eDNA reporting requirements and terminology(CSA W214:21, Environmental DNA (eDNA) Reporting Requirements and Terminology, 2021) and performance criteria for eDNA analysis with targeted qPCR(CSA W219:23, Performance Criteria for the Analyses of Environmental DNA by Targeted Quantitative Polymerase Chain Reaction, 2023). The targeted qPCR standard focuses on the laboratory processing steps and QC for qPCR while the reporting standards cover information to be reported for both qPCR and metabarcoding workflows from study design to results. Both documents set minimum requirements for particular methods or stages in the workflow and are relevant across applications and environments. In Europe, the European Standards Organization (Comité Européen de Normalisation, CEN) has convened a working group on eDNA and eDNA methods for water analysis, which has released one standard EN 17809:2023 on sampling, capture and preservation of eDNA from water(*SIST EN 17805:2023 Water Quality - Sampling, Capture and Preservation of Environmental DNA from Water*, 2023). This standard is focused only on sample collection steps in the field primarily for the purpose of water quality monitoring, as such it is limited in scope to a certain environment, sample type, and application. The preservation and field metadata recording are pertinent to laboratory processing as this information must be passed on to the lab to inform subsequent steps (see 5.4 Environmental Sample Receiving). Finally, the International Standards Organization has recently convened a working group TC 147/SC5/WG13 on eDNA, DNA and RNA methods, which will be working towards the development of international standards for EG approaches(International Standards Organization, 2023). The development of standards from independent bodies is relatively recent and has not been formally integrated into regulations yet.

Table 11-1 Summary of resources created by regulatory agencies or relevant in a regulatory context listed by country with the scope of environment(s), target organism(s), and workflow stage(s) covered.

Country/Region	Resource Name	Environment(s)	Target Organism(s)	Workflow Stage(s)
Australia	Integrating Environmental DNA Science into Australia's Marine Parks: A Roadmap	Marine	*Not specified*	*Not specified*
Australia & New Zealand	Environmental DNA Protocol Development Guide for Biomonitoring	*Not specified*	*Not specified*	Experimental Design; Sample Collection & Preservation; DNA Extraction; Sample QA/QC; qPCR; Reporting
Australia & New Zealand	Environmental DNA Test Validation Guidelines	*Not specified*	*Not specified*	qPCR; Assay Validation
Canada	Guidance on the Use of Targeted Environmental DNA (eDNA) Analysis for the Management of Aquatic Invasive Species and Species at Risk	*Not specified*	*Not specified*	Sample Collection & Preservation; DNA Extraction; qPCR; Reporting
Canada	Environmental DNA Protocol for Freshwater Aquatic Ecosystems	Freshwater	*Not specified*	Sample Collection & Preservation; qPCR; Reporting
Canada	CSA W214:21, Environmental DNA (eDNA) Reporting Requirements and Terminology	*Not specified*	*Not specified*	Reporting
Canada	CSA W219:23, Performance Criteria for the Analyses of Environmental DNA by Targeted Quantitative Polymerase Chain Reaction	*Not specified*	*Not specified*	qPCR; Assay Validation; Reporting

Country/Region	Resource Name	Environment(s)	Target Organism(s)	Workflow Stage(s)
Europe	A Validation Scale to Determine the Readiness of Environmental DNA Assays for Routine Species Monitoring.	*Not specified*	*Not specified*	qPCR; Assay Validation
Europe	A Practical Guide to DNA-Based Methods for Biodiversity Assessment	Aquatic	*Not specified*	Sample Collection & Preservation; DNA Extraction; Sample QA/QC; qPCR; Metabarcoding
Europe	Strategy for Successful Integration of eDNA-Based Methods in Aquatic Monitoring	*Not specified*	*Not specified*	*Not specified*
Europe	SIST EN 17805:2023 Water Quality - Sampling, Capture and Preservation of Environmental DNA from Water	Aquatic	*Not specified*	Sample Collection & Preservation
Finland	Roadmap for Implementing Environmental DNA (eDNA) and Other Molecular Monitoring Methods in Finland – Vision and Action Plan for 2022–2025	*Not specified*	*Not specified*	*Not specified*
International	ISO/TC 147/SC 5 Biological Methods	*Not specified*	*Not specified*	*Not specified*
Japan	Environmental DNA Sampling and Experiment Manual, Version 2.1	Aquatic	Fish	Experimental Design; Sample Collection & Preservation; DNA Extraction; Sample QA/QC; qPCR; Metabarcoding
Nordic Countries (LIST)	Guidelines to Monitor Phytoplankton Diversity and Distribution in Marine and Brackish Waters	Marine; Brackish	Phytoplankton	Sample Collection & Preservation; DNA Extraction; Sample QA/QC; Metabarcoding; Bioinformatics; Data Management
Scotland	Phase 2 Main Report - Developing Habitat Scale DNA Monitoring in Support of Post 2020 Biodiversity Reporting Requirements	Marine; Freshwater; Woodland; Peatland	*Not specified*	Experimental Design; Sample Collection & Preservation
Switzerland	Environmental DNA Applications for Biomonitoring and Bioassessment in Aquatic Ecosystems	Freshwater	*Not specified*	Sample collection; DNA Extraction; qPCR; Metabarcoding; Bioinformatics; Data Management
United Kingdom	Analytical and Methodological Development for Improved Surveillance of the Great Crested Newt	Freshwater	Great Crested Newt (*Triturus cristatus*)	*Not specified*

Country/Region	Resource Name	Environment(s)	Target Organism(s)	Workflow Stage(s)
United Kingdom	Analytical and Methodological Development for Improved Surveillance of the Great Crested Newt. Appendix 5. Technical Advice Note for Field and Laboratory Sampling of Great Crested Newt (Triturus cristatus) Environmental DNA	Freshwater	Great Crested Newt (*Triturus cristatus*)	Sample Collection & Preservation; DNA Extraction; qPCR; Reporting
United Kingdom	An Evidence Review for Great Crested Newt eDNA Monitoring Protocols	Freshwater	Great Crested Newt (*Triturus cristatus*)	Sample Collection & Preservation; DNA Extraction; qPCR; Reporting
United Kingdom	A Green Future: Our 25 Year Plan to Improve the Environment	*Not specified*	*Not specified*	*Not specified*
United States	Toward a national eDNA strategy for the United States	*Not specified*	*Not specified*	*Not specified*
United States	NOAA 'Omics Strategic Plan	Marine	*Not specified*	*Not specified*

11.4 Technical Advances

In the ever-evolving field of environmental DNA (eDNA) analysis, keeping abreast of technical advancements can maximize the data obtained from each sample. While not exhaustive, the following advancements represent key innovations that are enhancing EG laboratory service offerings for biodiversity monitoring and ecological assessment.

11.4.1 Quantitative Approaches in eDNA Amplicon Sequencing

Advances in quantitative NGS approaches in environmental DNA (eDNA) analysis mark a significant step forward in multi-species monitoring (Porter & Hajibabaei, 2018). While qPCR has long been the gold standard for eDNA quantification, its application is generally limited to single-species analysis. Quantitative NGS methods, on the other hand, enable the parallel quantitative analysis of multiple species. This is achieved by incorporating internal controls, such as synthetic genes or DNA from different sources, during library preparation for normalization of read counts from target species (Hoshino et al., 2021; Tsuji, Inui, et al., 2022; Ushio et al., 2017). This approach accounts for sample to sample variation including PCR inhibitors and eDNA background. Recovering quantitative sequence data is a key step for meeting certain biodiversity monitoring criteria by providing data on relative abundance changes over time and space, thus enabling more effective management and protection strategies.

11.4.2 Population Level Studies in eDNA Amplicon Sequencing

Recent advances in bioinformatic processing enable to recover haplotype information from amplicon sequence data, thus allowing to resolve intraspecific diversity and conduct analyses in “metaphylogeography” (Adams et al., 2023; Andres et al., 2023; Antich et al., 2023; Dugal et al., 2022; Turon et al., 2020). This minimally invasive method can allow for detailed population genetic studies from eDNA without the need for catch-based sampling, reducing environmental disturbance and costs. By providing a clearer understanding of genetic diversity and population structure, these analyses can guide targeted conservation efforts and resource management strategies, helping to achieve ecological sustainability goals.

11.4.3 Alternate PCR Methods for Rapid Field Detection

Palm-sized, portable qPCR instruments that run on external 12V power supplies have been demonstrated to be capable of detecting a single DNA copy of Influenza A virus subtype H7N9 (Ahrberg et al., 2016). Researchers have demonstrated the use of portable PCR technology for targeted detection in eDNA studies with high detectability and a short 30 minute turnaround time (Doi et al., 2021). Portable instruments like this could be implemented in field applications to facilitate remote studies where results in the short-term will allow for dynamic site selection for further sampling or other urgent decisions.

Isothermal DNA amplification techniques like LAMP (Loop-Mediated Isothermal Amplification) and RPA (Recombinase Polymerase Amplification) have emerged as alternatives to traditional PCR methods. Their adaptability to various conditions and tolerance to inhibitors make them ideal for on-site monitoring (Wei et al., 2023; Williams et al., 2017). Though not yet applied to whole community monitoring, these methods are particularly advantageous for rapid and portable monitoring of individual or groups of species like harmful algal blooms (HABs), invasive species, or other groups of interest in remote or inaccessible locations (Blin et al., 2023; Kageyama et al., 2022). This aligns with the industry's need for efficient and effective environmental monitoring systems, ensuring timely responses to potential ecological threats.

11.4.4 Advances in Sequencing Technologies

Recent advances in sequencing technologies present new opportunities for eDNA analysis. For example, Oxford Nanopore Technologies offers a new sequencer that is more field-compatible, capable of reading longer DNA fragments and detecting methylation patterns, which could enable in-situ, real-time environmental monitoring (Urban et al., 2023). Illumina, on the other hand, continues to enhance the throughput of its instruments, while maintaining high read quality and reliability – essential features for comprehensive and accurate biodiversity assessments. A new sequencing company, Element Biosciences, is also making strides by offering more cost-effective solutions for deep sequencing, and this could make extensive eDNA studies more accessible (Carroll et al., 2023).

Additionally, there have been advances in long read technologies. Long read technologies, such as PacBio circular consensus sequencing, and can provide more information to improve taxonomic classification, especially as reference databases grow to include greater coverage beyond barcode regions (Bj et al., 2019; Portik et al., 2022). The accuracy of nanopore sequencing has been improving over the years so it is likely that this technology will become more widely used in EG studies in the future (Marx, 2023; Satam et al., 2023).

The growth of other competitors in the sequencing market is promising for the industry, indicating a trend towards more diverse, advanced, and cost-efficient sequencing options. These technological advancements collectively will enable more precise, efficient, and versatile eDNA analyses, supporting rigorous environmental monitoring and sustainable resource management.

11.4.5 Advances in Metatranscriptomics

A major challenge in metatranscriptomics is RNA degradation during sample processing. Considerable effort is devoted to developing methods that minimize this degradation, ensuring that the RNA data collected is as intact and representative of the in-situ conditions as possible (T. S. Jo, 2023).

Commercial kits for direct sequencing of RNA molecules without a cDNA conversion step have been developed for nanopore sequencers (Oxford Nanopore Technologies); the current kit offered can take poly(A)-tailed RNA or total RNA as input. Direct sequencing of RNA can help circumvent biases that may be introduced by reverse transcriptase or during PCR amplification. This could open many new possibilities for eRNA analysis.

11.4.6 Development of Integrated eDNA and eRNA Analyses

There are efforts to combine DNA and RNA data to gain a more holistic understanding of ecosystems (Bunholi et al., 2023; Giroux et al., 2023; Scriver et al., 2023). This integration would provide both genome and transcriptome signals, informing genomic capabilities and actual metabolic activities in the environment, providing insights into functions and interactions. Metagenomic data also improves taxonomic classification and metatranscriptomic data enable detection of RNA viruses. Finally, the differential persistence of RNA and DNA also provides multiple signals to infer the presence of species, potentially providing a clearer picture of the ecosystems.

11.4.7 Advances in Genome Skimming

In genome skimming, a balance between sequencing depth and coverage is essential. The goal is to maximize the information gain from sequencing while minimizing costs. This optimization is crucial for efficiently understanding the genetic makeup of ecosystems without unnecessary expenditure. There have been continual improvements in extracting information from low coverage datasets (Bohmann et al., 2020; Pouchon & Boluda, 2023; M. H. Tan et al., 2021).

A key advancement in genome skimming is the increased effort to build more extensive reference databases (Bohmann et al., 2020; Westfall et al., 2023). Sequencing of genomes or full organelles genomes (mitochondria and chloroplasts) via genome skimming provides a wealth of data, enabling more detailed analyses than is possible with only amplicon sequences.

11.4.8 Novel Methods for Multiplexing

Multiplex qPCR or dPCR is an extension of single target assays, facilitating simultaneous amplification and quantification of multiple target DNA sequences in one reaction. This method employs specific primers and fluorescent probes that emit at different wavelengths during PCR amplification. Multiplexing is efficient and cost-effective, since the ability to analyze multiple targets in a single reaction saves substantial hands-on time and reduces the use of sample DNA and reagents. Consequently, this also reduces the cost per sample, making multiplexed assays an attractive option for laboratories seeking to maximize their throughput. The number of targets has previously been limited to four and in a few cases, up to six at a time.

A novel qPCR multiplexing technique first described by (Rajagopal et al., 2019) demonstrates a way to increase the number of multiplexing targets beyond six, without loss in specificity. By varying the probe concentrations amongst targets in a single reaction, multiple targets can be measured in a single fluorometric channel. The advantage of this

discovery is that it can be used with existing, commercially available qPCR platforms and chemistry. The study measured nine different targets in a single reaction. This approach has been mainly used in healthcare applications but could significantly improve target multiplexing in eDNA monitoring programs as well.

Another new approach in qPCR multiplexing within the past decade involves target discrimination based on multiple detection temperatures (MuDT). This technique can detect two targets simultaneously within a single fluorescence channel, using only the amplification signal, and without the need for melt curve analysis (Lee et al., 2014). A change in fluorescence signals between different detection temperatures allows for individual detection of targets (Kreitmann et al., 2023; Lee et al., 2014). This next-generation molecular diagnostic approach is mainly used for human pathogen detection in the medical field but could be extended to pathogen detection studies for EG projects using eDNA and beyond.

11.4.9 DOTS (Droplet-On-Thermocouple Silhouette) qPCR

DOTS was first described as a novel technology in 2015 (Harshman et al., 2015). Amplification is detected by measuring reductions in droplet volume. Assay run-time is drastically reduced over traditional methods, providing results as early as the fourth cycle (or just under 4 minutes), due to thermocycling speeds of up to 28 seconds per cycle. It has been demonstrated to have a LOD below a picogram of DNA, making it an interesting option for eDNA studies where target abundance tends to be low. Successful implementation of this technology in eDNA studies could reduce turnaround time and increase laboratory throughput.

11.4.10 Advances in Laboratory Data Analysis

Recently machine learning algorithms (MLA) have been applied to interpret amplification and melt curves (Kreitmann et al., 2023). These MLA could combine information obtained from PCR and assay chemistries to improve multiplexing capabilities.