Environmental DNA test validation guidelines

Citation

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Contents

Glossary

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

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

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

Haplotype: A group of alleles that are inherited together from a single parent – for example, mitochondrial haplotypes. A haplogroup consists of haplotypes that shared a common ancestor with a single nucleotide polymorphism mutation.

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

HTS library: Amplicons amplified for the purpose of HTS, cleaned and pooled following indexing.

Inhibitory substances: Substances in a sample or extract that have a negative effect on PCR, reducing assay sensitivity and increasing the risk of false negative results.

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

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

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

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

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

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

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

Reference library: Database with DNA barcodes of specific species.

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

Introduction

The use of molecular assays to assess the presence of species using environmental DNA (eDNA) and RNA (eRNA) as analytes has diversified as the field of environmental surveillance advances. These methods can be used, for example, to screen for pest species as part of biosecurity measures and risk management, to screen for threatened species as part of development requirements, or for biodiversity monitoring purposes. To ensure that testing results are reliable, it is imperative to develop and validate molecular assays using stringent quality standards that can minimise the potential for false negative and false positive results.

What is the aim of these guidelines?

These *Environmental DNA test validation guidelines* provide harmonised quality control and minimum standard considerations for developing or validating eDNA/eRNA assays for the purpose of single-species or multi-species detection.

This document is a comprehensive guide for the development and use of eDNA/eRNA tests, recommended and curated by eDNA experts, stakeholders and end users in Australia and New Zealand. The guidelines are designed to support a consistent and best-practice approach to eDNA/eRNA testing to help detect species of interest. This approach ensures that surveillance and resource managers are provided with robust scientific evidence to support decision making.

Environmental DNA–based methods exist for a broad variety of organisms; however, assay performance has only been superficially tested for most available assays, and few assays are validated to ensure reproducibility (Thalinger et al. 2021). This document outlines minimal quality requirements to develop and validate eDNA/eRNA molecular assays and standard protocols for operational use in Australia and New Zealand.

Who are they for?

The *Environmental DNA test validation guidelines* provide minimum quality requirements and assurances to design and validate eDNA assays for biosecurity and surveillance applications. Although these guidelines will help improve the accuracy and reliability of eDNA assays, they are not explicitly designed to provide results for use in compliance and legal situations.

For researchers

The *Environmental DNA test validation guidelines* detail key steps to be used in assay development and validation for species-specific testing and metabarcoding.

For clients

The *Environmental DNA test validation guidelines* provide quality assurance for any contracted eDNA work. They tell end users what services and standards can be expected, and may also be used to inform staff collecting samples or involved in other areas of the project.

How have they been developed?

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

Guidelines for single-species detection assays use a framework based on the current principles and methods of validation for diagnostic assays as approved by the World Organisation for Animal Health and the International Standard for Phytosanitary Measures from the International Plant Protection Convention. These principles and methods define the minimum requirements needed to determine the fitness of an assay, including analytical sensitivity, assay specificity, limit of detection, limit of quantification, and eDNA-based ex situ testing.

Guidelines for multi-species detection assays use a framework based on current recommendations from existing eDNA metabarcoding guidelines, including the *Environmental DNA sampling and experiment manual* from the Japanese eDNA Society (Minamoto et al. 2021), *A practical guide to DNA-based methods for biodiversity assessment* (Bruce et al. 2021, Zaiko et al. 2021), and existing protocols from experts in Australia and New Zealand.

Updates

It is anticipated that the *Environmental DNA test validation guidelines* will be updated and expanded over time, with review and update as required.

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

Assay purpose and selection

It is important that the intended purpose of an assay is defined at the outset. This will help to determine whether a species-specific, metabarcoding or combined approach is appropriate. The characteristics of the assay can also be checked against its purpose throughout the development and validation process, to ensure that the assay is fit for purpose. This ensures that eDNA and eRNA assays and related procedures are appropriate and results are relevant to management.

Environmental DNA assay performance is affected by environmental factors that must be considered for assay optimisation. After initial optimisation for an intended purpose, characteristics of the performance of the assay must be tested and checked against the intended purpose and overall project principles.

The most common purposes of species-specific eDNA/eRNA assays (see Species-specific assay development and validation) are to:

- contribute to the detection of a targeted species in
  - an environment
  - waste products of a host organism
  - a compartment or storage area
- assess the presence of metabolically active species in
  - an environment
  - waste products of a host organism
  - a compartment or storage area
- contribute to the detection and eradication of invasive species and pests in defined areas.

The most common purposes of metabarcoding eDNA/eRNA assays (see Metabarcoding assay development and validation) are to:

- characterise and survey biodiversity in
  - an environment
  - waste products of a host organism
  - stomach contents of an organism
- contribute to the monitoring of species assemblages
- contribute to the detection and eradication of multiple invasive species and pests in defined areas.

For both species-specific assays and metabarcoding, there may be many more specific purposes for which assays can be developed. Such specific applications and their unique purposes need to be clearly defined within the context of a fully validated assay.

In addition, consider whether a combined approach may be useful. For example, you may decide to include metabarcoding as your first step in a screening tool to determine the range of species at the test site, to then guide the use of more targeted, species-specific assays.

Species-specific assay development and validation

Species-specific eDNA assays are currently PCR-based assays that involve amplification of a target DNA sequence present in environmental matrices, and can be validated and designed within a diagnostic framework. However, the availability of suitable eDNA samples and experimental set-ups to formally assess diagnostic sensitivity and specificity is rare. Validating eDNA/eRNA assays for the purpose of diagnostic assessments requires a quantitative analysis of amplification data with minimal variation to confirm diagnostic capability. Therefore, the robustness of eDNA/eRNA assays must be considered and explored extensively to determine how inhibiting factors and environmental factors affect the amplification of known eDNA/eRNA traces in controlled matrices.

Steps for species-specific assay development and validation are:

1 Define the intended purpose of the assay
2 Design and test the assay
3 Validate and optimise the assay using reference samples
4 Check analytical specificity
5 Check analytical sensitivity
6 Check repeatability
7 Check reproducibility
8 Determine thresholds (cut-offs)

1 Define the intended purpose of the assay

The purpose of the assay must be defined at the outset, to act as a benchmark for testing and validation (see Assay purpose and selection).

The purpose should be defined in terms of:

- the high-level purpose (e.g. to detect the target species in a certain area, to contribute to managing invasive species in an area)
- the target organism
- what aspect of the target organism will be measured (e.g. presence of live organisms, presence of excreta, presence of cells)
- the sampling matrix.

2 Design and test the assay

The robustness of eDNA detection relies heavily on the design quality of primers and probes (Langlois et al. 2021). Species-specific assays are recommended to be designed using probe-based chemistries for species-specific detection.

Assays must be designed and tested in accordance with the assay's intended purpose.

Assay choice or design

The first step is to ascertain whether tests have already been developed for the intended purpose or are available for testing. Suitable molecular assays may have been designed and validated for purposes outside the scope of eDNA testing. In the specific case of biosecurity applications, national diagnostic protocols may be available for targeted species with approved molecular assays that could be suitable for eDNA-based testing.

When designing or testing an eDNA/eRNA assay, DNA degradation and inhibition must be considered as important factors that affect assay performance. Environmental DNA/RNA assays are designed considering environmental factors that affect DNA integrity and the success of detection. Such factors include temperature, moisture, bacterial activity and UV radiation, which affect DNA degradation rates. Natural inhibitors such as algae, humic substances and suspended sediment particles can also affect detection success (Stoeckle et al. 2017).

Therefore, primers and probes are commonly designed to target short (80–280 base pairs) rather than large (>300 base pairs) fragments, because short fragments remain available for detection for longer (Jo et al. 2017). Targeted regions should be selected following currently accepted barcode regions, and considering the availability of reference DNA data for the species.

In silico testing

If relevant sequence data for the target species (and related non-target species) exist in online repositories, selected assays should undergo in silico testing for specificity using sequence alignment tools (e.g. BLAST). Common repositories include the National Center for Biotechnology Information, the Barcode of Life Data System, the European Molecular Biology Laboratory online repositories, and the CSIRO National Biodiversity DNA Library.

If such data do not exist, reference material will need to be obtained and sequenced to facilitate assay design. The level of specificity depends on the purpose of the assay; however, assays should assess the specificity of the target species, considering potential intraspecific variation in the study area (i.e. inclusivity), as well as non-amplification of co-occurring and closely related species (i.e. exclusivity). The biology and ecology of the target species should be considered – genetically diverse species will require greater reference resources to achieve a similar level of assay robustness to that of less diverse species.

Species-specific primers should be designed to display 0 base pair mismatches with the target species. Probes are recommended to be designed to display >2 mismatches with closely related species (Klymus et al. 2020a). If target species are part of a species complex or if the target has minimal genetic differences from closely related species, the use of amplification-refractory mutation system primers and locked nucleic acid probes is recommended to increase assay sensitivity and specificity of annealing temperatures (Stewart et al. 2016). Testing for specificity should comprise closely related non-target species, as well as commonly co-habiting species that users could encounter in the environment. Suitable species and samples for assay evaluation should be selected considering:

- taxonomy – include closest relatives (e.g. all members of the same genus or family, and members of all suspected subspecies/genetically distinct populations of the target species)

- geographic scope of intended use – include representatives of all taxa within the broader group (e.g. mammals) that might be encountered in the study area.

Inhibition testing

The robustness of eDNA assays must also be tested against inhibitory factors that interfere with assay performance. Assessment of robustness against inhibition should begin during the assay development and optimisation stages. The factors most likely to affect assay robustness include pH, temperature and organic matrix factors (Goldberg et al. 2016).

If you have access to suitable environmental reference samples (see Step 3), testing for inhibitory factors should be performed to confirm which factors are present and in what concentrations following extraction, and whether selected extraction methods are sufficient to yield high-quality DNA/RNA. Inhibition tests can then be undertaken using serial dilutions of confirmed inhibitory matrices spiked with known concentrations of synthetic oligonucleotides that reproduce the sequence of the qPCR assay target sequence. Synthetic oligonucleotides can include nucleotide inversions to allow for additional plate preparation controls to determine potential cross-contamination of synthetic oligonucleotides (Trujillo-González et al. 2021). Assays would be considered robust if 10fold serial dilutions of the synthetic oligonucleotides amplify within 3.3 cycles ± standard deviation (95% confidence intervals) of each other within serial dilutions of inhibitory matrices. Instances where amplification falls outside this acceptable range would indicate lack of robustness in the assessed inhibitory matrix concentration.

3 Validate and optimise the assay using reference samples

Optimisation aims to evaluate and adjust the most important physical, chemical and biological parameters of an assay to ensure that the performance characteristics of the assay are best suited to the intended application (OIE 2021). For analysing environmental samples, it is important to select reference samples that are representative of the target species and analyte. These reference samples may be fluids, tissues, excreta and environmental samples that contain the analyte of interest and are usually harvested from the target species as well as their environments. Assays should be optimised using both sets of reference samples (i.e. samples derived from the target species and its environment).

Environmental reference samples are considered to be samples known to contain the analyte of interest in varying concentrations (modified from OIE 2021, Chapter 2.2.6: Selection and use of reference samples and panels). They can ideally be collected from ex situ settings used to maintain the target species; however, samples collected from natural environments or urban locations with confirmed records of the target species may also serve as suitable confirmed environmental samples. Sequences could be obtained from the study area to account for potential genetic variation; however, this may only be possible in locations where the target species is already present in the environment. It is not a viable option for biosecurity applications where target species can be considered exotic or their presence is unknown. The suitability of environmental samples for assay optimisation must be assessed based on the size of the habitat, the abundance of the target species (Goldberg et al. 2016) and the availability of samples to be appropriately standardised.

The availability of confirmed environmental samples may be limited, depending on the target species. Reference environmental samples spiked using extracted DNA/RNA may be prepared in the laboratory from an original starting material (e.g. serial dilution of a highly concentrated tissue-derived extract) by spiking a suitable environmental matrix. The matrix into which the analyte is placed or diluted should be identical to, or resemble as closely as possible, the samples that ultimately will be tested in the assay. Wherever possible, large quantities of these reference samples should be collected or prepared and preserved for long-term use.

4 Check analytical specificity

Analytical specificity is the ability of the assay to distinguish the target analyte (i.e. the nucleic acid sequence of the target species) from non-target analytes (OIE 2021). The assessment is qualitative, and the choice and sources of sample types, organisms and sequences for evaluation of analytical specificity should reflect test purpose and assay type.

Specificity tests should assess cross-reactivity of the assay against co-occurring species and species that are closely related to the target organism.

5 Check analytical sensitivity

The limit of detection (LOD) is a measure of the analytical sensitivity of an assay. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified percentage of the time. The LOD is based on detection/non-detection criteria and describes an assay's ability to detect the target sequence at low levels (Klymus et al. 2020b).

The LOD should be assessed using 10-fold dilutions of the target analyte in a suitable matrix. Dilutions can be completed using different matrices and reference samples to compare the LOD of the assay between tissue-derived and environmental DNA; however, the LOD of the eDNA assay should be ultimately estimated using suitable environmental reference samples. The LOD of an eDNA assay should be assessed as the last dilution showing 100% positive amplification across all technical replicates. A more accurate estimate may be obtained by a second-stage experiment using narrower intervals in the dilution scheme, focusing on the region between 100% and 0%.

6 Check repeatability

Repeatability is the level of agreement between assay results, which can be quantified within and between relevant hierarchical levels, such as machine runs and operators, when applying the same test method within the same laboratory (OIE 2021). Repeatability can be used to define the expected precision of an assay in detecting a range of analyte concentrations under normal operating conditions.

Repeatability is estimated by evaluating variation in results from a minimum of 3 confirmed eDNA-positive independent samples (samples collected during different sampling events or at different locations, using cleaned or sterile equipment) within the operating range of the assay – samples should range from well-detectable concentrations of target DNA to concentrations close to the LOD. Initial tests for repeatability should assess assay sensitivity with DNA reference samples using 6 technical replicates in at least 5 separate runs completed on multiple days. The variation in results should be explored within technical replicates of each sample within and between runs, expressed as standard deviations or coefficients of variation (standard deviation ÷ mean of replicates).

For control samples to provide valid inferences about assay precision, they should be treated in exactly the same way as test samples in each run of the assay, including the use of blank controls, extraction-negative controls, positive controls and standard dilutions to assess analyte concentration. It is not acceptable to prepare a final working dilution of a sample in a single tube from which diluted aliquots are pipetted into reaction vessels, or to create replicates from one extraction of nucleic acid rather than preparing new dilution standards for each run. Such 'samples' do not constitute valid replicates for repeatability studies (OIE 2021).

7 Check reproducibility

Reproducibility is the ability of a test method to provide consistent results, as determined by estimates of precision, when applied to aliquots of the same samples (OIE 2021). Assay reproducibility is required for assay recognition and implementation where assays have been designed for the purposes of biosecurity and diagnostics – these assays should be tested in different laboratories using the identical assay (protocol, reagents and controls). For routine surveillance outside the scope of biosecurity, assays can be tested for reproducibility by individual laboratories, although inter-laboratory testing is highly recommended.

To assess the reproducibility of an assay, each of at least 3 laboratories should test the same panel of samples (blinded) containing a suggested minimum of 20 samples, with identical aliquots going to each laboratory. Measurements of precision can be estimated for both the reproducibility and repeatability data.

The reproducibility process should be designed to suit the intended purpose of the assay. For example, if the results are intended to be used in biosecurity compliance applications, more laboratories should be used and/or more samples should be tested to increase confidence in the results. If tests are designed to be used by a single laboratory, establishing repeatability across operators, relevant equipment, batches of consumables and time may be sufficient.

8 Determine thresholds (cut-offs)

Selection of the assay cut-off values should reflect the intended purpose of the assay and its application. Options and descriptive methods for the best way to determine the cut-off values of eDNA qPCR assays are available (Caraguel et al. 2011). The main difficulty in establishing assay cut-off values based on environmental reference samples is the lack of diverse environmental matrices that are representative of the target species and its habitat. Moreover, eDNA extracts may contain amplifiable traces of target DNA that fall outside the defined cut-off values of the assay. Despite the utility of cut-off values in molecular assays, any amplification considered positive, regardless of whether it falls within or outside assay cut-off values, must be confirmed by sequencing before confirming results.

Summary of key steps in species-specific qPCR assay development and validation

Step 1 At the outset of the project, define the intended purpose of the assay

Step 2 Design and test the assay:

- Identify whether an assay exists for the intended purpose.
- Identify whether an assay exists for similar purposes or species that could be adapted for the intended purpose.
- Identify environmental factors that may affect DNA integrity and detection success (e.g. temperature, moisture, bacterial activity, UV radiation).
- Identify natural/human-made inhibitors that may affect detection success (e.g. algae, humic substances, suspended sediment, chemical contaminants).
- Design probes and primers to target short DNA fragments (80–280 base pairs) following currently accepted barcode regions, if no suitable assay exists.
- Test the specificity of the designed or identified assays in silico using sequence tools against online repositories.
- Test the robustness of the designed or identified assays in inhibition testing.

Step 3 Validate and optimise the assay:

- Collect reference samples containing both the analyte and the environmental matrix.
  - Collect a sample that contains the target species and analyte (e.g. sera, fluids, tissues, excreta).
  - If no known sample exists, make a reference sample by creating a matrix that resembles the natural environmental setting of the target species, and spiking it with the analyte.
- Test assay robustness using known concentrations of reference eDNA samples spiked into representative environmental matrices.
- Test the assay sensitivity using serial dilutions of reference samples to determine the level of analyte that can be successfully detected.

Step 4 Check analytical specificity:

- Assess cross-reactivity of the assay against co-occurring species and species that are closely related to the target organism.

Step 5 Check analytical sensitivity:

- Assess the LOD using 10-fold dilutions of the target analyte in a suitable matrix.

Step 6 Check repeatability:

- Evaluate variation in results of independent replicates from a minimum of 3 confirmed eDNA-positive samples within the operating range of the assay.

Step 7 Check reproducibility:

- Arrange for at least 3 laboratories to test the same panel of samples (blinded) containing at least 20 samples, with identical aliquots going to each laboratory.

Step 8 Determine thresholds (cut-offs):

- Use your preferred method to determine thresholds, with the understanding that amplification must be confirmed by sequencing.

Resources

- eDNA Validation Scale: https://edna-validation.com/
- Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS & Bruce K (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA* 3(4):823–836, doi:10.1002/edn3.189.
- Furlan EM &Gleeson D (2016). Improving reliability in environmental DNA detection surveys through enhanced quality control. *Marine and Freshwater Research* 68(2):388–395, doi:10.1071/MF15349.
- Furlan EM, Gleeson D, Hardy CM & Duncan RP (2016). A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources* 16(3):641–654, doi:10.1111/1755-0998.12483.
- Wilkes Walburn J, Rourke ML, Furlan E, DiBattista JD, Broadhurst MK, Fowler AM, Hughes JM & Fielder S (2022). Robust environmental DNA assay development and validation: A case study with two vulnerable Australian fish. *Aquatic Conservation* 32(7):1225–1231, doi:10.1002/aqc.3809.

Metabarcoding assay development and validation

Metabarcoding is the parallel sequencing of complex bulk samples through the analysis of short, conserved gene regions. Metabarcoding assays have the capacity to inform users about the presence of multiple species present in multiple samples simultaneously, providing valuable information for community-level species assemblages.

Environmental DNA assays designed for metabarcoding and high-throughput sequencing (HTS) can be validated and designed within experimental set-ups. Experimental set-ups to formally assess the performance of metabarcoding assays can comprise mesocosm studies representative of the target environment and species. Given the complexity of metabarcoding assays and their corresponding data analysis requirements, developing metabarcoding assays requires validation not only of the molecular assay itself but also of the analytical pipeline used to curate and analyse the resulting high-throughput data.

Steps for metabarcoding assay development and validation are

1. Define the intended purpose of the assay (define the targeted taxa and outline reference database)
2. Design and test the assay (develop and test the associated bioinformatic pipeline for data analysis)
3. Validate and optimise the assay using reference samples
4. Check reproducibility.

1 Define the intended purpose of the assay

The purpose of the assay must be defined at the outset, to act as a benchmark for testing and validation (see Assay purpose and selection).

The purpose should be defined in terms of:

- the high-level purpose (e.g. to detect the range of a target species in a certain area, to contribute to managing invasive species in an area)
- the target organisms
- what aspect of the target organisms will be measured (e.g. presence of live organisms, presence of excreta, presence of cells)
- the sampling matrix.

2 Design and test the assay

The robustness of metabarcoding assays relies heavily on the design quality of primers and the availability of reference DNA sequence databases at suitable resolution to address the question. Assays must be designed and tested in accordance with the assay's intended purpose.

Assay choice or design

The first step is to ascertain whether assays have already been developed for the intended purpose or are available for testing. The length of the targeted amplicons and gene regions (i.e. barcode) must be considered when designing PCR primers for metabarcoding. The barcode cannot be too short because it must be taxonomically well resolved and should span sufficient genetic variation to distinguish closely related species. However, it also cannot be too long, because it otherwise does not fit technical features of current sequencing technologies.

Currently, most of the barcodes used in metabarcoding studies range between 200 and 500 base pairs (Pawlowski et al. 2020). Shorter barcodes (less than 120 base pairs) are sometimes used, especially for microbial species detection, but such short gene fragments persist longer in the environment and therefore may provide information that is less well resolved in time and space, and has lower taxonomic resolution (Pawlowski et al. 2020). Fusion-tagged primers (i.e. primers designed to include unique tag identifiers and sequencing adapters) can simplify library preparation steps, and improve control of tag-jumping events and chimeric sequences.

Sequencing errors during metabarcoding workflows are common during PCR amplification (Berney et al. 2004, Aird et al. 2011) and sequencing (Yoshitake et al. 2021). These technical errors include nucleotide substitutions and insertions introduced by the polymerase enzyme (Eckert & Kunkel 1991, McInerney et al. 2014, Lee et al. 2016), nucleotide substitutions induced by the DNA damage caused by temperature cycling during PCR (Potapov & Ong 2017), and formation of chimeras (Fonseca et al. 2012). Chimeric PCR products are generated when small DNA fragments that did not finish the elongation during one step are carried over in subsequent amplification steps. The final amplicon will be a chimeric sequence that does not exist in any living organism and is composed of 2 or more different DNA fragments that originate from 2 or more different organisms.

Moreover, it is important to be aware that metabarcoding primers will not amplify all DNA equally in a sample. They are likely to favour sequences already predominant in the environment, leading to a biased abundance ratio between DNA from different species (Elbrecht & Leese 2015, Piñol et al. 2015). One of the major factors driving PCR bias is the number of primer mismatches for each species in the community – species with fewer, or no, mismatches will amplify more efficiently during PCR (Clarke et al. 2014). Gene copy number (e.g. the number of mitochondria per cell), differences between species in ease of DNA extraction, and the type and position of primer mismatches also influence PCR efficiency and bias.

PCR biases can be minimised by using quality measures and controls that include suitable assay optimisation, using fusion-tagged primers to minimise PCR steps and identify chimeric sequences, using high-fidelity polymerase within reactions to improve replication sensitivity, and using multiple PCR replicates per sample. Use of unique molecular identifiers when designing fusion-tagged primers or when using two-step PCR protocols (MacConaill et al. 2018) can also increase analytical resolution, and allow greater confidence in identifying sequencing and PCR biases (Yoshitake et al. 2021).

Assay testing and mesocosm studies

Assays must be tested using appropriate environmental samples from areas with confirmed presence of targeted species, or from appropriate mesocosm experimental set-ups (Zaiko et al. 2021) with known species abundances. Alternatively, artificial samples or mock communities can be designed following protocols in Zaiko et al. (2021).

Library preparation

Amplicons produced using metabarcoding assays must be cleaned and pooled to create a 'library' for HTS technologies (i.e. an HTS library). Several methods are available for HTS library preparation and the steps needed to prepare amplicons for sequencing. Metabarcoding assays must clearly outline what library preparation strategy is to be used.

The following steps must be clearly outlined:

- PCR amplification regime and replication. Describe how many PCR technical replicates per sample and per control will be used during the selected amplification steps. Indicate the reaction mix and temperature regime to be completed during amplification.
- Indexing strategy. Describe the method to be used for the addition of sample-specific identifiers (e.g. 1-step PCR with fusion primers, 2-step PCR using untagged primers with sequence overhangs, 2-step PCR using primers with tags and sequence overhangs, tagged PCR and library build on amplicon pool; see Bohmann et al. 2021).
- Amplicon pooling for library preparation. Describe how amplicons are to be pooled for library preparation, indicating required concentrations and how to select suitable PCR technical replicates for pooling.
- Troubleshooting. HTS and the pooling of PCR replicates is highly complex, and entails costs associated with preventing, detecting and eliminating errors and biases. Each metabarcoding approach has advantages and disadvantages that end users must understand to better troubleshoot and analyse HTS data. This section should provide assay-suitable information on what complications are to be expected using the selected method, addressing issues relating to cross-contamination risk, PCR amplification efficiency, chimera formation, tag jumping and index misassignment (see Bohmann et al. 2021).

Sequencing

HTS libraries can be sequenced using several methods, including:

- sequencing by Synthesis (Illumina)
- single-molecule real-time sequencing (Pacific Biosciences)
- Ion Torrent sequencing (ThermoFisher Scientific)
- 454 pyrosequencing technology
- oligonucleotide ligation and detection (SOLiD) sequencing (Life Technologies)
- nanopore sequencing (Oxford Nanopore Technologies)
- small genome sequencing (GenapSys).

These different methods achieve different read lengths and reads per run, and have advantages and disadvantages associated with sequencing accuracy, time per run, costs and reagents. Assays must describe which method is selected in accordance with the purpose of the assay. Sequencing should be undertaken as per the manufacturer's instructions. Deviations from the standard method must be described clearly, supported by published research.

Data analysis

Data analysis of sequenced outputs requires standardised bioinformatic scripts that include data quality control, sample demultiplexing and taxonomic identification against a reliable reference database (Figure 1; Zaiko et al. 2021). Scripts should be written with enough guidance for end users to reliably run each script and understand what each step achieves in curating the data.

There are important aspects that bioinformatic scripts should consider and include for final analysis and communicating results:

- Quality control measures. Outline how data will be curated by the script, providing an indication of how many reads are being filtered by each step from each sample. Quality control measures should consider sequence quality of reads, pairing of paired end reads (if applicable), tag and primer congruence, and filtering of chimeric or singleton sequences. It is essential that the metabarcoding assay includes an indication of the accepted minimal quality requirements, considering the application and context of the proposed assay.
- Sequence taxonomic identification. The taxonomic assignment of curated sequences must be completed against suitable reference databases, either available in online repositories or prepared by users before the analysis. Reference databases can be generated with a series of scripts (see Arranz et al. 2020), but should always aim to include sequences that correspond to the targeted genomic region (e.g. 16S gene region, cytochrome oxidase I), correspond to the appropriate targeted taxonomic group and taxonomic level, and include sequences with suitable metadata associated with either museum accessions or peer-reviewed publications.

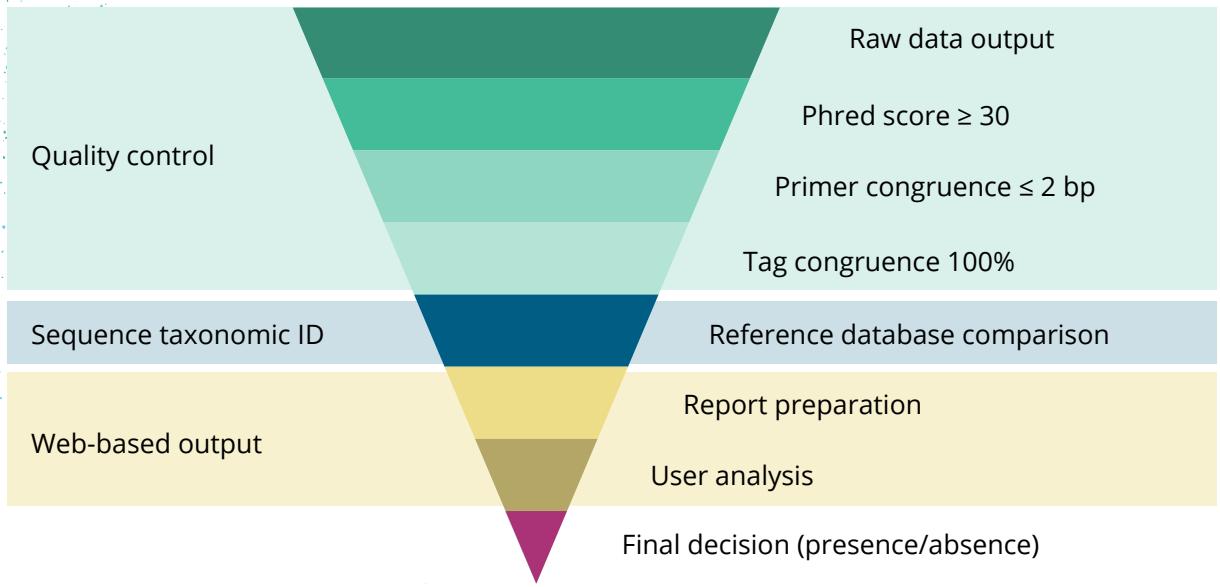

Figure 1 Example of a bioinformatic pipeline for analysing metabarcoding assay results

3 Validate and optimise the assay using reference samples

Optimisation aims to evaluate and adjust the most important physical, chemical and biological computational parameters of an assay to ensure that the performance characteristics of the assay are best suited to the intended application (OIE 2021). To optimise metabarcoding assays, it is important to select reference samples that are representative of the target environment. These may be environmental samples that contain a variety of analytes of interest and are usually harvested from environments with known occurrence of targeted organisms. Environmental samples may be spiked using extracted DNA from target organisms in the environment in serial dilutions of highly concentrated tissue-derived extracts (Coghlan et al. 2021). Mock mesocosm experiments can also be used to collect environmental samples containing DNA from known species assemblages and abundances under controlled conditions (Kelly et al. 2014, Evans et al. 2015).

4 Check reproducibility

Metabarcoding assays can be assessed for reproducibility by processing homogenised samples using standardised DNA extraction protocols, primers and bioinformatic analyses (Zaiko et al. 2021). In this context, metabarcoding assays should consistently provide comparable results between independent laboratories, although these may show variation in raw results associated with the use of different amplification regimes, instruments and user-associated error (Zaiko et al. 2021). Verifying assays through an independent laboratory is preferred.

Summary of key steps in metabarcoding assay development and validation

Step 1 At the outset of the project, define the intended purpose of the assay

Step 2 Design and test the assay:

- Identify whether an assay exists for the intended purpose.
- Identify whether an assay exists for similar purposes or species that could be adapted for the intended purpose.
- Design primers to target longer DNA fragments (200–500 base pairs) following currently accepted barcode regions.
- Address potential PCR biases (e.g. nucleotide substitutions and insertions) during analyses.
- Test the assays using environmental samples from areas with confirmed presence of targeted species, a mock community or mesocosm experiments.
- Outline a library preparation strategy and create a library for HTS.
- Sequence the HTS library.
- Filter and curate sequenced data before taxonomic assignment, and use a curated reference database suitable for the targeted gene region using curated sequences from available molecular repositories.

Step 3 Validate and optimise the assay:

- Evaluate and adjust the physical, chemical and biological parameters of the assay to suit the intended application, using reference samples representative of the target environment.

Step 4 Check reproducibility:

- Process homogenised samples using standardised DNA extraction protocols, primers and bioinformatic analyses.

Resources

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