A framework for assessing confidence in environmental DNA qPCR assays and results

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Keywords

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Further information

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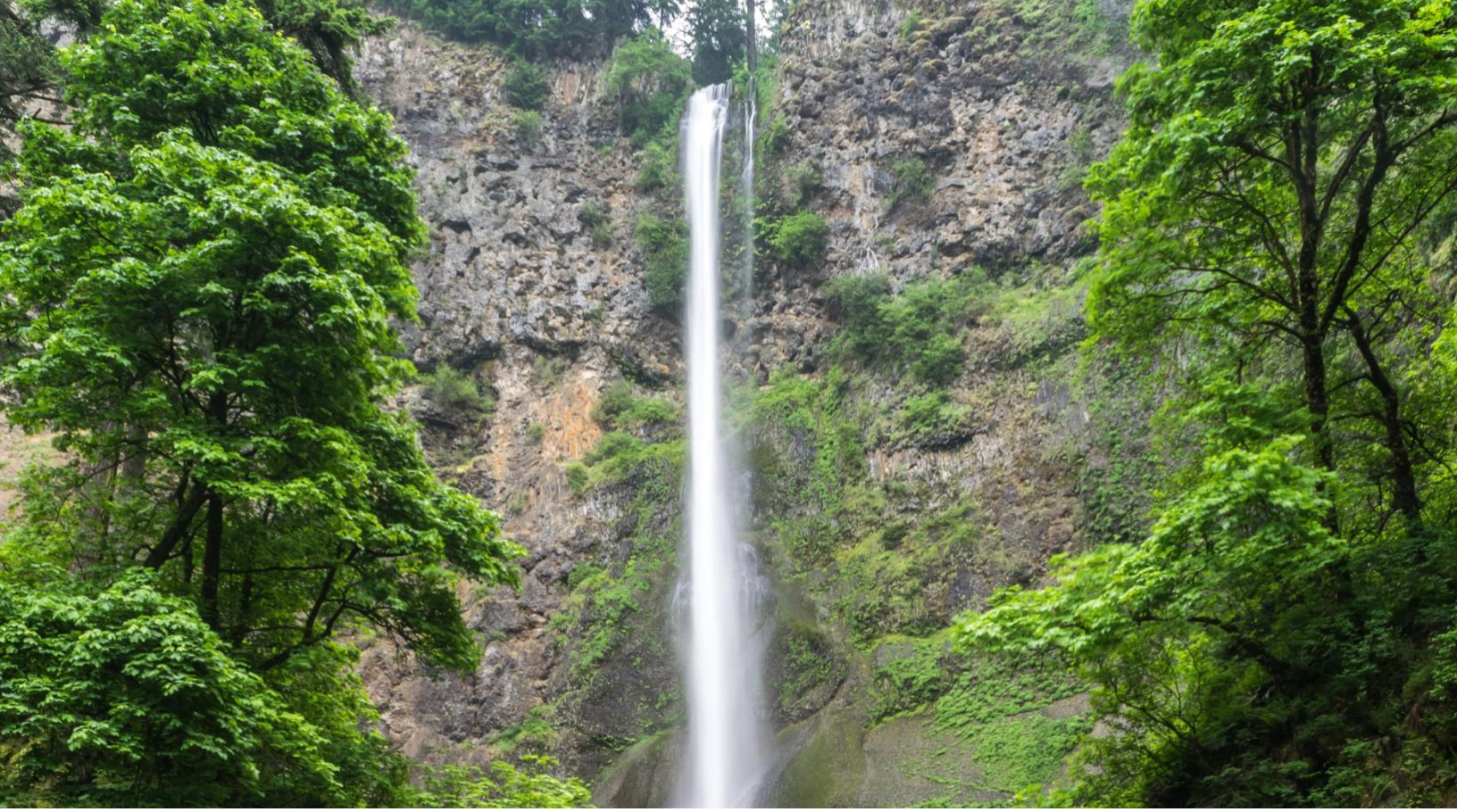
Forward

DNA – based methods offer a significant opportunity to change how we monitor and assess biodiversity. However, for most techniques, there is still much development required before they can be used in routine monitoring. Natural England has been exploring the further use of these methods for environmental monitoring for several years, delivering a series of reports which focus on the development of DNA-based methods with potential in a particular area.

One such area that is increasingly being used is the detection of ecologically important species in aquatic habitats using environmental DNA (eDNA). This is often conducted by employing species-specific quantitative polymerase chain reaction (qPCR) assays. There are many available qPCR assays and often more than one for a given species. Different assays may have been validated to varying degrees and this affects the interpretation of results. There are also numerous eDNA analysis providers, each providing results in a non-standard format. This situation has led to challenges for widely adopting eDNA qPCR approaches outside of the few highly validated assays.

The goal of this project was to address some of these challenges through the development of an eDNA qPCR assay and project validation framework, and this report describes the production of an online tool to assess confidence in results generated from eDNA qPCR assays.



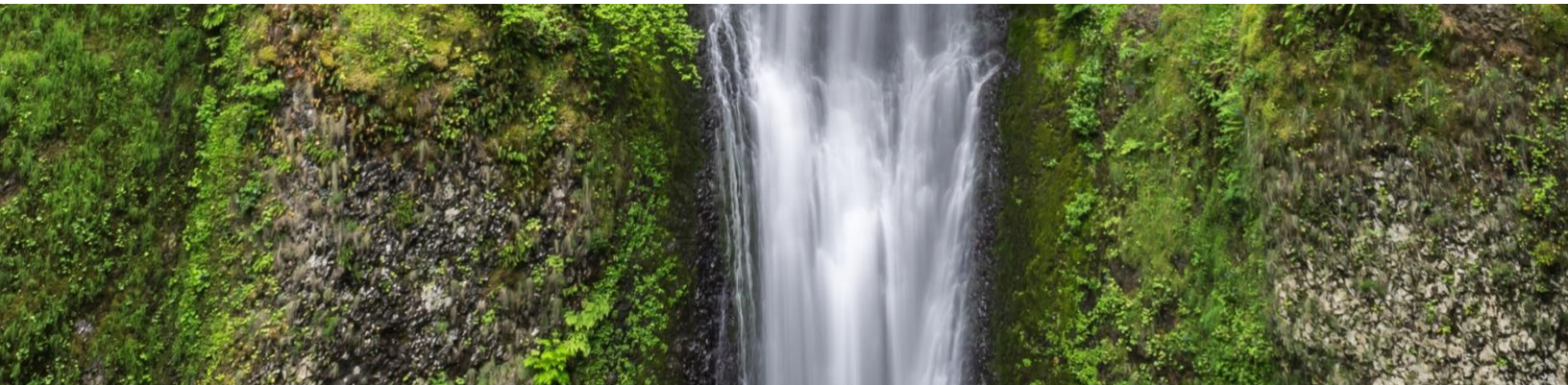
NATURE METRICS
DNA-BASED MONITORING

A FRAMEWORK FOR ASSESSING CONFIDENCE IN ENVIRONMENTAL DNA qPCR ASSAYS AND RESULTS

Submitted by NatureMetrics

April 2021

Kirsten J. Harper, Cuong Q. Tang, Kat Bruce, Andrea Ross-Gillespie, Vere Ross-Gillespie, Bastian Egeter



Executive Summary

Natural England is increasingly using environmental DNA (eDNA) detection methods for a range of applications. One of the most common applications is the detection of ecologically important species in aquatic habitats. This is often conducted by employing species-specific quantitative polymerase chain reaction (qPCR) assays. Quantitative PCR has been shown to outperform traditional sampling methods in a multitude of published studies, particularly when the study species is difficult or costly to survey for using traditional methods.

For some species, for example the great crested newt, there are well-specified, highly validated and quality assured qPCR assays. Validating assays to this high degree is a very expensive and quite exhaustive process involving landscape-scale experiments. The primary benefit of using a highly validated assay is the ability to interpret negative results as indicative of species absence from a site. However, this is not necessary for many surveys, where obtaining species presence information (and not species absence information) can be extremely valuable. Quantitative PCR assays are available in both the scientific literature and the commercial context. These have been validated to varying degrees and this affects the interpretation of results. There are many available assays and often more than one for a given species. There are also numerous eDNA analysis providers, each providing results in a non-standard format.

This situation has led to challenges for widely adopting eDNA qPCR approaches outside of the few highly validated assays. The goal of this project was to address some of these challenges through the development of an eDNA qPCR assay and project validation framework.

This report details the results of a literature review and consensus building workshop. It also provides the proposed eDNA qPCR assay and project validation framework, outlines tools developed to implement the framework, instructs the user how to apply the tools, and offers future recommendations associated with the continuing development of the framework.

Introduction

Background

Natural England is increasingly using environmental DNA (eDNA) detection methods for a range of applications. One of the most common applications is the detection of ecologically important species in aquatic habitats. This is often conducted by employing species-specific quantitative polymerase chain reaction (qPCR) assays. Quantitative PCR has been shown to outperform traditional sampling methods in a multitude of published studies, particularly when the study species is difficult or costly to survey for using traditional methods.

For some species, for example the great crested newt, there are well-specified, highly validated and quality assured qPCR assays. Validating assays to this high degree is a very expensive and quite exhaustive process involving landscape-scale experiments. The primary benefit of using a highly validated assay is the ability to interpret negative results as indicative of species absence from a site. However, this is not necessary for many surveys, where obtaining species presence information (and not species absence information) can be hugely valuable.

For other species, assays are available in both the scientific literature and the commercial context. These have been validated to varying degrees and this affects the interpretation of results. There are many available assays and often more than one for a given species. There are also numerous eDNA analysis providers, each providing results in a non-standard format.

What is qPCR?

Quantitative PCR (qPCR) is a method for measuring the amount of DNA through the addition of fluorescence. Thermal cyclers include a fluorometer to detect the fluorescence, which is emitted throughout the amplification process and produces cycle quantification (Cq) values, a threshold used to differentiate signal from noise. The Cq value, when combined with appropriate standards and reference values, translates fluorescence into absolute or relative quantities of target DNA. This amplification can be interpreted, simply, as the presence of that target.

There are two common methods of qPCR. The first method includes a non-specific fluorophore, such as SYBR Green, which binds to all double stranded DNA present including non-specific products such as primer dimer. This can interfere with, or even prevent, detection of the target product. The secondary analysis of the properties of the amplification (i.e., melt curve analysis) can be compared to known standards to potentially identify the source of the amplification. The second method includes the use of a sequence-specific DNA probe, such as a TaqMan probe, which has a fluorescent label attached. Since the probe only fluoresces in the presence of complementary DNA, specificity is increased and detection of non-specific products such as primer dimer are prevented.

This report and the associated framework and tools pertain only to probe-based qPCR assays (e.g., the great crested newt eDNA protocol). Natural England does not commonly use other types of qPCR assays for eDNA analysis.

The application of qPCR in the context of eDNA

Environmental DNA is DNA that has been extracted from environmental samples such as soil, sediment, or water (Rees et al. 2014). Organisms shed DNA traces into their habitat through a variety of processes, such as faeces, saliva, mucous, and gametes (Thomsen and Willerslev 2015). Environmental DNA detection methods provide a means of rapid detection of a target species without either visual or physical confirmation of the species presence (Kim, Yoon, and Shin 2020), and can increase the efficiency and reliability of species identification (Mauvisseau et al. 2019). Over the last decade there has been a shift from using eDNA to detect microbial taxa to using eDNA to detect macro-organisms for purposes such as protecting and preserving ecosystems from invasive species or for the conservation of endangered species (Roux et al. 2020; Thomsen and Willerslev 2015). Targeted qPCR assays applied to eDNA samples have the potential to overcome many of the limitations associated with traditional methodologies, particularly for early detection (Mauvisseau et al. 2019; Roux et al. 2020).

Quantitative PCR is most typically applied in the medical and research laboratories with clean and concentrated DNA sources for analyses such as gene expression or genotyping. In comparison, eDNA analysis is typically at the lower limit of qPCR capabilities. The increasing application of eDNA-based approaches to answer ecological questions continues to push the limits of qPCR-based detection, and its power has resulted in the proliferation of different assays, which has highlighted the need for highly optimised procedures, stringent analytical validation of a given assay (Roux et al. 2020) and special considerations for applying them to often highly dilute and complex environmental samples (Langlois et al. 2020).

Current qPCR assessment frameworks

There is a need to understand that not all published eDNA qPCR assays are equal in performance (Klymus et al. 2020). An assay's reliability to detect only the target DNA (specificity) and its ability to detect low quantities of target DNA (sensitivity) can vary greatly depending what criteria were used to design, select, optimise, and test the assay (Klymus et al. 2020). Additionally, the reporting of quantitative measures of an assay's performance has been generally lacking in literature (Klymus et al. 2020). For example, there is inconsistency among studies with regards to how Limit of Detection (LOD) and Limit of Quantification (LOQ) are calculated. These are both measures of assay sensitivity, where LOD is the concentration of DNA in a sample at which detection becomes unreliable and LOQ is the concentration of DNA in a sample at which accurate measurement of DNA concentration becomes unreliable. There is also inconsistency in reporting the number of replicates used, how data are interpreted and which metadata are reported (Loeza-Quintana et al. 2020; Roux et al. 2020).

However, there has been a recent move to improve consistency within the field (e.g. Klymus et al. 2019; Nicholson et al. 2020; Mauvisseau et al. 2019; Thalinger et al. 2021). Klymus et al. (2019) highlighted the need for improving accuracy for single-species qPCR assays when interpreting results and proposed standardised methods and reporting the LOD and LOQ, while Nicholson et al. (2020) stated that there is a need for improved metadata reporting before eDNA studies can become standard practice.

Outside the field of eDNA, there have been previous attempts to standardise reporting of qPCR metadata. For example, Bustin et al. (2009) created Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE), which includes a checklist of essential and desirable data for publishing and interpreting qPCR results.

However, although the MIQE guidelines provide a good reference for development of eDNA guidelines, in particular the testing and reporting of the assay efficiency, linear dynamic range and precision, there are some MIQE guideline criteria that are irrelevant for the application of eDNA (Klymus et al. 2019). Additionally, few eDNA studies utilise the MIQE guidelines as they stand (Mauvisseau et al. 2020; 2019). As the field of eDNA continues to move forward, there is a clear need to be able to analyse samples consistently across laboratories, which requires not only the standardisation of reporting on assay conditions but also confirming results are comparable across laboratories (Klymus et al. 2019).

Thalinger et al. (2021) proposed a scale to assess how ready an eDNA qPCR assay is to be implemented for routine species monitoring. The validation scale is composed of a list of 122 variables, which once assessed rate an assay on a scale of '1 – incomplete' to '5 – operational'. The goal of this validation scale is to standardise the way in which an existing or newly developed assay is evaluated for potential use. For this to work effectively the reporting of the qPCR analyses and metadata should also be standardised.

False positives and false negatives in eDNA qPCR

Results from eDNA approaches can be subject to false positives and false negatives (Rees et al. 2014). A false negative result stems from a target species being present but DNA not being detected by the assay (Mauvisseau et al. 2020). Conversely, a false positive result stems from a target species not being present but DNA being detected by the assay. False positive results are typically caused by cross-contamination during sampling or laboratory work (Gentile Francesco Ficetola, Taberlet, and Coissac 2016) but can also arise as a result of poorly designed assays. False negatives may occur because of PCR inhibition (Goldberg et al. 2016), low DNA shedding rates of the target species (Klymus et al. 2015; Mauvisseau et al. 2020), and low population sizes (Dougherty et al. 2016). The consequences of either a false negative or false positive can be ecological and/or economical in value. For example, a false positive result for an invasive species could lead to a conclusion that the target species is present in an area and resources may be wasted attempting to eradicate a non-existent species of concern, whereas a false negative for an endangered species may result in the loss of the target species habitat, and possibly the target species itself.

Interpreting qPCR amplification curves

Interpreting the results of a probe-based qPCR is based on the assessment of amplification curves, a graph depicting the amount of cumulative amplification (measured by fluorescence) over time (typically measured in amplification cycles). Fluorescence only happens when there is amplification, and amplification only happens when there is the target DNA. Briefly, a qPCR is made up of reagents that will enzymatically replicate (amplify) the target DNA. This is a buffered solution containing at least the following:

- Primers: short DNA sequences that are designed to flank a variable DNA region of interest
- Probe: a short DNA sequence that falls between the two primers. The probe is made up of a fluorophore and a quencher that counteract each other when in close proximity
- Polymerase: an enzyme, guided by the two primers, that amplifies the DNA sequence
- dNTPs: DNA building blocks used by the polymerase to amplify the DNA

In the presence of target DNA, the primers and probe will bind to a stretch of target DNA that they were designed to be complementary to, and the polymerase will use the dNTPs to replicate the stretch of DNA between the two primers. As the polymerase reaches the probe, the fluorophore is cleaved from the

quencher and this fluorescence is captured by the qPCR machine. The polymerase continues to amplify to the point where the target DNA is doubled. This is done in cycles of heating and cooling, which in turn act to separate DNAs from the PCR reagents and to promote their binding and amplification. After each cycle the target DNA is doubled, and the fluorescence increases exponentially until the reagents run out.

Typical qPCR curves are sigmoidal (Figure 1A), there is a baseline (aka initiation or ground) phase before any noticeable fluorescence is observed (phase 1), an exponential phase where there is true doubling of the DNA (phase 2), and lastly a plateau phase where the cumulative fluorescence declines with the diminishing reagents (phase 3).

Differentiating true amplification from background noise requires that a threshold (sometimes called a noise band) is set, which can be a manual or an automatic process. The background level of fluorescence can be caused by quenchers decaying as a result of the thermocycling or probe splitting. The cycle number after which a sample's amplification curve passes this threshold is called the quantification cycle (Cq; also known as the cycle threshold Ct, crossing point Cp, or take-off point TOP). The Cq value can be calibrated against a set of standards to quantify the target DNA. Importantly, the crossing of this threshold is the basis for calling a sample positive in a qualitative assay.

Amplification should only happen when you have target DNA in the sample, and early amplification is caused by higher concentrations of the target DNA – as exemplified by the standard dilution curves in Figure 1A. However, there are factors that can affect this typical amplification and curves sometimes require vetting and interpretation. It should be noted however that many of these factors can be resolved through optimization of an assay or can at least be acknowledged in the validation process.

Amplification curves with indistinguishable phases indicate that something is amiss with the assay. Indistinguishable phases can be caused by many factors. For instance, the qPCR conditions may be inefficient. If your melting temperature (Tm) is too high, the primers will not be able to bind down on priming sites as effectively (Langlois et al. 2020), which can reduce per-cycle efficiency and leads to a weak slope during phase 2, as can be seen for the right most amplification curves in Figure 1B. If the qPCR run includes a positive control, which shows a strong slope during phase 2 similar to amplification curves seen in Figure 1A, then it is likely that the sample showing the weak slope is inhibited to some degree. In this instance, it can be determined that inhibition is high enough to interfere with efficiency, but not high enough to result in an absolutely negative result. Amplification curves exhibiting the weak slope during phase 2 may also be caused by primers exhibiting poor specificity, which can also be caused by having a too low a melting temperature, and this can cause primers to bind indiscriminately to products. The addition of a probe reduces the risk of non-target amplification; however, the risk is not zero. In some instances, non-target products may exhibit enough sequence similarities to the probe, which can cause a signal to be detected. Therefore, even when using a probe-based qPCR assay caution should be used when interpreting results with an amplification curve exhibiting a weak slope. PCR efficiency can be measured by including standards of known concentration on a qPCR plate, which can monitor many of these issues.

Another unusual amplification curve that is commonly observed in qPCR involves the amplification curve ending up below the Cq instead of plateauing as expected during phase 3. Though this may appear like a negative result, it is in fact the result of an extremely high starting template DNA concentration. Since the signal is detected very early in the sample, the qPCR software is assuming that it is background noise, and the curve is "corrected" by an algorithm, which removes the real signal. The amplification curve may not cross the threshold at all, or it may cross and then recross the threshold at a later point. Samples exhibiting this amplification behaviour need to be diluted and retested in order to achieve the expected sigmoidal amplification curve.

There is a tendency to assume that late amplification of a sample is indicative of poor quality or highly dilute target DNA, however it can also be an artefact of carryover template during reaction set up. Amplification curves with lower-than-expected height could be caused by differences in probe concentration or differences in fluorescence intensity between dyes. Spikes early in the cycles can occur due to poor mixing of the reaction prior to thermocycling. These spikes may cross the threshold but should not be considered an indication of positive reaction.

Software will apply specific correction algorithms to the raw data to make sense of true signal from background. When dealing with many samples at once, a wider range of background across all samples may be observed, which can make it more difficult to identify true signal within the noise. This can result in inappropriate corrections being applied to some amplification curves. Automated baseline calling may be applied for some samples, but manual baseline calling may be required for others, such as when samples of very high and very low target DNA concentration are present on the same plate.

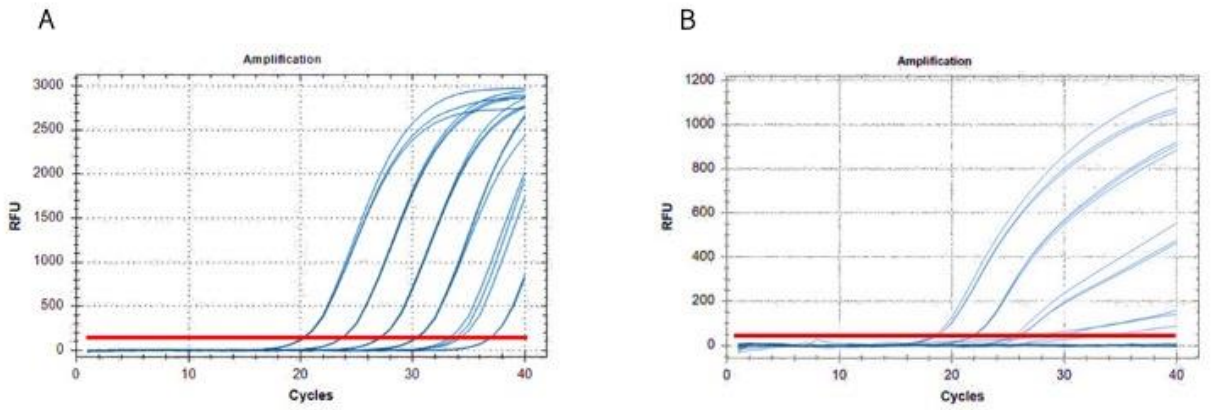


Figure 1. Examples of standard curves derived from three replicates each comprising of six dilutions (*n* = 18). Each dilution is a 10-fold dilution of the previous dilution, and the highest concentration is on the left with decreasing concentrations moving right. The horizontal line (red) represents the threshold set for the quantification cycle (Cq). The Cq is determined as the point where a sample crosses the threshold. A) An acceptable standard curve B) A poor standard curve. (Modified from Klymus et al. 2020).

Interpreting qPCR results

The number of technical replicates can directly influence the ability to detect DNA and to accurately calculate detection probabilities (Klymus et al. 2019). It is generally agreed that the number of qPCR replicates is important in minimising false negatives (Ficetola et al. 2015), with three qPCR replicates being the minimum limit to ensure reliable data while up to 12 qPCR replicates may be necessary when the probability of detection is low (Biggs et al. 2015; Coutant et al. 2020). This is especially true when dealing with very dilute sources of the target DNA, which is typical of eDNA (Taberlet et al. 1996). However, there is currently no guidance on how many of the qPCR replicates must be positive in order to classify a sample as positive for a target species (Friebertshauser et al. 2019; Harper et al. 2018), and thresholds for scoring samples as positive (or negative) for a target species varies from study to study (Qu and Stewart 2019). Positive samples have previously been described in eDNA literature by as little as one out of three, one out of eight (Jerde et al. 2013; 2011; Mahon et al. 2013; Piaggio et al. 2014) or one out of 12 (Biggs et al. 2015) qPCR replicates.

It may be necessary to use more conservative threshold to promote consistency in results (Darling and Mahon 2011), which would also mitigate false positive results. However, there is a case to be made that less stringent thresholds better protect target species where probability of detection is low despite the increased risk of false positives (Harper et al. 2018). Until a detection threshold is implemented, it may be necessary to re-analyse samples that yield one positive qPCR replicate to prevent false positives (Harper et al. 2018; Rees et al. 2014). If re-analysis is not possible, it may still be possible to claim a sample as positive if only one replicate exhibits a positive signal if the reasoning is scientifically sound and/or the original study from where the assay has been selected from had low detection thresholds. For example, it may be possible to use the LOD or LOQ to determine if a sample is a true positive with only one positive replicate. As previously mentioned, there is disparity in how eDNA studies report the LOD and LOQ. Some studies report the LOD and LOQ as a Cq value, while some studies report LOD and LOQ as more traditionally as DNA quantity. In both instances the LOQ Cq value is higher or more concentrated than the LOD, and both values have been determined through the production of a standard curve. Thus, if the one positive replicate's Cq value is within the maximum number of cycles, has an LOD or LOQ higher than that of the previously determined limits, and all controls perform as expected it can be designated as positive for the target organism.

Aims

The intricacies of eDNA qPCR assays have led to challenges for widely adopting eDNA qPCR approaches outside of the few highly validated assays. These include:

- Ascertaining a confidence level in an eDNA qPCR assay can be difficult, particularly without a framework for reference.
- Interpreting the results of an eDNA qPCR survey or project can be difficult, as the level of confidence in the eDNA qPCR assay will affect the interpretation.
- Results from eDNA analysis providers are provided to Natural England in different formats, making databasing of results difficult.
- Results from eDNA analysis providers sometimes do not contain the information that is required to make fully informed interpretations.

The goal of this project was to address these challenges through the development of an eDNA qPCR assay and project validation framework.

As part of this framework, the specific aims were to:

1. Design a tool for assessing confidence in a given eDNA qPCR assay
2. Design a tool to aid in the interpretation of eDNA qPCR results
3. Provide a template for standard reporting of eDNA qPCR results

It is expected that these tools will be utilised by the providers of eDNA qPCR analyses, so that outputs can be submitted to evaluators and/or project managers.

Framework Development

Background research and consultation

We first conducted a literature review of existing qPCR assessment frameworks including both scientific and grey literature across disciplines (e.g., ecology, microbiology, medicine) ensuring that any existing frameworks were duly considered. We concurrently conducted a consultation with members of the scientific community, practitioners, evaluators and other stakeholders. This was carried out through the organisation of a workshop hosted by Natural England and NatureMetrics that took place online on the 14th December 2020 *Developing a confidence assessment process for eDNA-qPCR assays*. Making the process inclusive allowed us to ensure that the resultant framework and tools could be user-friendly, easy to interpret and have an underlying scientific basis that was agreed upon across a range of experts.

Assessing confidence in eDNA qPCR assays

Confidence in how well a qPCR assay works is determined by a number of tests that are carried out in three stages, *in silico*, *in vitro*, and *in situ*. During the *in silico* stage the proposed primers and probe are optimised using software such as Primer-BLAST (Ye et al. 2012) or EcoPCR (Ficetola et al. 2010). The purpose of *in silico* testing is to test the efficiency and the specificity of the primers and probe. Efficiency testing involves assessing factors such as GC-content, and annealing and melting temperatures, while specificity testing involves assessing whether non-target products will be amplified in addition to the target species of interest. During the *in vitro* stage the primers and probe are optimised for application, assessed for sensitivity, and tested against tissue derived DNA from the target species. The primers and probe should also be tested against tissue derived DNA from closely related co-occurring species to ensure only the target species DNA amplifies. During the *in-situ* stage the optimised primers and probe are applied to environmental samples taken from sites where the target species is known to occur and known not to occur to ensure the assay works as expected. Assuming the basic validations have been met, it is the extent of *in situ* validation that determines the confidence of an assay. During the validation stages positive detections are also generally sequenced to confirm the positive signal is a result of target species DNA.

Thalinger et al. (2021) devised a validation scale for eDNA qPCR assays. Along with 35 experts in the field of targeted eDNA detection, an extensive list of 122 variables deemed important for validation was generated. This was developed into a scientific study entitled "A validation scale to determine the readiness of environmental DNA assays for routine species monitoring" that involved: 1) constructing of a comprehensive literature database of existing targeted qPCR assays; 2) scoring each variable for each eDNA qPCR assay; 3) assigning variables to thematic blocks and; 4) placing the thematic blocks on a 5-level scale ranging from "incomplete" to "operational". The result is a comprehensive tool to score eDNA qPCR assays. The downside is that it is somewhat complicated to implement, requiring time and a reasonably high level of eDNA qPCR knowledge. This, along with discussions during the stakeholder workshop, prompted the development of a simplified method to assess how confident a user can be that a positive or negative qPCR result is indicative of the target species presence or absence (Table 1 and 1). Note that this checklist is also implemented in the confidence assessment tool (see next section).

Information Box 1: Assay Validation Level checklist

To develop the checklist, key questions were identified and categorised into three levels of confidence: Low, Medium, or High. These levels are based on the risk of obtaining false negatives or positives.

Risk of false negatives	Risk of false positives	Confidence Level
High	High	Low
High	Low	Medium
Low	Low	High

To classify an assay the Assay validation checklist (**Table 1**) is completed. It is expected that the user of this checklist has a reasonable knowledge of eDNA qPCR. See the outcomes below to interpret the results.

Outcome	Confidence Level	Comments
Not all validation steps in the "Low" category have been completed	No confidence level	Users are advised not to proceed with applying the assay to any environmental samples until further validation has been conducted
All validation steps in the "Low" category have been completed	Low	The risk of false positives or false negatives is high. Weak positive qPCR results cannot be reliably interpreted.
All validation steps in the "Low" category have been completed **plus** **All** validation steps in the "Medium" category have been completed	Medium	A positive qPCR result is unlikely to be false, and any weak qPCR signals can more reliably be interpreted as a positive. However, false negatives are still possible - a negative qPCR result does not mean that the target species is absent.
All validation steps in the "Low" category have been completed **plus** **All** validation steps in the "Medium" category have been completed **plus** **All** validation steps in the "High" category have been completed	High	There is little risk of either a positive or negative qPCR result being false.

Table 1. Assay validation checklist to assess the level of confidence in an eDNA qPCR assay. See 1 for details on how to implement the checklist. Note that this checklist is also implemented in the confidence assessment tool (see next section).

Validation step	Confidence level category	Yes/No
Was in silico testing conducted and primers shown to amplify the target species?	Low	
Were the primers tested on tissue from the target species?	Low	
Was in silico testing conducted and potential cross-amplification of non-target species shown to be low?	Low	
Were primers tested on non-target tissue of closely related potentially co-occurring species?	Low	
Did the assay successfully detect the target species at a site of known presence?	Low	
Did the assay successfully detect the target species at multiple sites of known presence?	Medium	
Did the assay return negative results for the target species at multiple sites of known absence?	Medium	
Has assay sensitivity (Limit of Detection and/or Limit of Quantification) been assessed?	Medium	
Has site occupancy modelling (or equivalent) been conducted?	High	
Has the probability of detecting a target species at a site been calculated?	High	
Has the number of water samples needed to achieve reliable detection from a site been calculated?	High	
Has the number of water samples needed to estimate probability of species absence given negative results from a site been calculated?	High	
Has the number of qPCR replicates needed to achieve reliable detection in an eDNA sample been calculated?	High	

COASTER: Confidence assessment tool for eDNA qPCR assays and results

Development

The standardisation and reporting of qPCR performance metrics are key to enabling the assessment of eDNA across studies, and ultimately providing managers with a solid foundation for decision making (Loeza-Quintana et al. 2020). In order to move towards this goal, a web-based tool called *COASTER: Confidence Assessment Tool for eDNA qPCR Results* was developed. COASTER requires a standardised data input, as well as user-defined settings, and can be accessed through common web browsers. The tool operates in R under the Shiny framework.

We identified key variables for reporting associated with qPCR replicates (Table 2). In addition, experimental parameters were identified as being key to provide transparent reporting and apply confidence assessments (Table 3). COASTER incorporates the Assay Validation Level checklist (Table 1).

A decision tree to aid the interpretation of qPCR results was designed and implemented within COASTER (see Appendix 3 for a visual representation). The decision tree begins with the Cq values attained for the qPCR replicates. Based on the LOD and the performance of Positive and Negative controls, replicates are designated as either Positive, Negative, Tentative or Inconclusive. Briefly, Positive means that the Cq value for the replicate was lower than the LOD and all controls performed as expected, Negative means that there was no signal passing the baseline threshold and all controls performed as expected, Tentative means that the Cq value for the replicate was higher than the LOD and all controls performed as expected, and Inconclusive means that one or more of the controls did not perform as expected. As noted above, there are a number of ways LOD can be defined and specified, but however the LOD is defined, it should be clearly reported to allow a full interpretation. Other than contamination being detected or positive controls failing to amplify, an Inconclusive result is often caused by degradation or inhibition. Degradation is monitored by adding a spike of known DNA to the sample at the point of collection (or before) while inhibition is monitored by adding a spike at the PCR setup stage. These are both referred to as Internal Positive Controls (IPC). If a sample is degraded, a new sample is generally required, however if a sample is inhibited there are two courses of action. The inhibited sample can either be diluted, or the sample can be cleaned up using a specialist kit. Once inhibition has been accounted for the sample should be rerun and will likely produce either a Positive or Negative result.

Next, based on the outcomes of the qPCR Replicates, a DNA Sample can be assigned as Positive, Negative, Tentative or Inconclusive. This is based on the number of qPCR Replicates that were Positive (if any) and the number that were inhibited. These thresholds are usually set as part of an assay protocol.

Finally, the DNA Samples can be aggregated at the Sampling Location level, again as being Positive, Negative, Tentative or Inconclusive. The interpretation of these outcomes is dependent on the confidence level of the assay (see Table 1 and 1). If a Sampling Location returns a Negative result and the Assay Validation Level is Low, it is impossible to determine if target DNA was not present and since no assay sensitivity testing was conducted, target DNA may actually be present but be below detectable limits. If the Assay Validation Level is Medium it is still possible that target DNA may be present. However, Medium assays have conducted sensitivity testing, which enables greater confidence that a Negative result is not the product of low concentrations of target DNA. The only way to be confident that a Negative result represents a species absence is if an Assay Validation Level is High due to extensive testing and site occupancy modelling having been conducted (Table 1). For Positive Sampling Locations, only Medium and High assays are considered for interpretation purposes due to the high risk of false positives using Low assays (sequencing these can provide the required level of confidence in these cases). For Medium and High assays, the Positive result is likely a true positive, meaning that DNA of the target species is present in the sample. For Tentative results (e.g., where some qPCR replicates had weak amplification, with a Cq value higher than the LOD), sequencing is usually the best way to confirm species presence.

As with the other tools, there are certain underlying assumptions, for example, that sampling was performed at an appropriate time of year for the target species, that an appropriate amount of field and laboratory replication was applied, that eDNA equipment and laboratory best practices were followed.

User guide

The most up to date user guide is available through the COASTER user interface. The following is for reference and to provide a general overview of how to use COASTER.

Each parameter setting can be chosen by the user according to assay requirements. For example, if an assay has a detection threshold specified i.e., 4/12 replicates must return a positive signal for the sample to be considered positive, the user would enter "4" into the appropriate cell of the setting column. If there is no detection threshold specified for an assay, the tool currently has a default setting of "2", i.e., 2/12 replicates must return a positive signal for the sample to be considered positive. Similarly, if other parameters have a default setting, the default setting value is listed in the description. If the user does not enter a specified value in the setting column, the tool will analyse the data using the default values. For the Limit of Detection Cq (LOD_Cq), the user can either choose to allow the tool to calculate the LOD from the DNA standards (if standards were included) or can enter a known LOD_Cq value.

It is a **five-step process** to analyse and interpret the data using the tool guidelines, and generate a report for a project:

Step 1: A modifiable template (csv file) can be downloaded from the dashboard (Figure 2).

Step 2: After the template has been downloaded and filled out locally with the required information (see Appendix 1 for an example), it can be uploaded in the appropriate section of the homepage (Figure 2).

Step 3: Once the upload is complete the user should enter a project title. Additionally, the user may choose to select for the report to include an appendix with interpretation of the results at the qPCR Replicate Level, which will generate a table with the results inputted to the template file plus an additional column with an interpretation of each replicate result.

Step 4: The user must now set the appropriate parameters in the 'Set parameters' section of the tool homepage (Figure 2). To help indicate deviations from default settings in the report a justification must be made when default settings are changed.

Step 5: A full report can be generated. The generated report will give the date the report was generated, followed by four sections: summary of results, settings, results and interpretations, and appendix. An example of a generated report can be seen in Appendix 2, but a brief description of each section is as follows:

1. Summary of results includes the Assay Validation Level, if contamination was observed in any controls, if positive controls amplified as expected, and if standards were included in the uploaded data the associated R^2 and PCR efficiency values.
2. Settings includes a table with the parameters listed as set by the user.
3. Results and interpretations include a table summarising the results at 1) the Sampling Location Level 2) the DNA Sample Level and 3) [optional] the qPCR Replicate Level. Each of these tables can also be downloaded in csv format directly from the front page.
4. Samples will either be classified as Positive, Negative, Inconclusive, or Tentative. A Tentative outcome is generally the result of weak signal (Cq value below the limit of detection).

Table 2. Variables required by COASTER for each qPCR replicate, as presented in the downloadable template.

Variable	Description
Plate	The qPCR plate ID (number or name)
Well	Location of the qPCR replicate on the plate (e.g., A1, A2, …)
Sample_Type	Type of DNA sample. There are six sample types allowed: external negative control (extnc), PCR negative control (pcrnc), field negative control (fieldnc), positive control (pc), standard (std), and unknown (unkn)
DNA_Sample	DNA sample ID (number or name)
Replicate	qPCR replicate ID for a sample (number or name)
Target_Cq	Cq value obtained for the target taxon
IPC_Cq	Cq value obtained for the Internal Positive Control
Std_Conc	Concentration of the standard (any units)
Sampling_Location	Location ID (number or name)
Extraction_Batch	Extraction batch ID (number or name)
Volume_Water_Processed	Volume of water processed for a sample (mL)

Table 3. Overview of the parameters required by COASTER to analyse and interpret the qPCR results uploaded by the user. The default setting value listed in the description can be used or the user can enter an assay specific value.

Parameter	Parameter ID	Description
Number of qPCR Cycles	N_cycles	Total number of cycles used in qPCR run
Minimum Number of Positive Replicates	Pos_minreps	Minimum number of positive qPCR replicates for a DNA sample to be considered positive (default: 2).
Limit of Detection Cq Value	LOD_Cq	Cq value for the Limit of Detection (default: 0).
Internal Positive Control Cycle Delay	IPC_delay	Inhibition threshold is calculated relative to the PCR negative control (pcrnc) cycle number (default: 1).
Internal Positive Control Cq Value	IPC_Cq	Override inhibition threshold by entering the minimum cycle number that the IPC should have attained (default: 0).
Minimum Number of Internal Positive Control Replicates tested	IPC_minreps	Minimum number of replicates the must have been tested for inhibition for a DNA sample to be considered conclusively negative (default: 12).
Maximum Number of Internal Positive Control Replicates Exhibiting Inhibition	IPC_Inh_maxreps	Maximum number of replicates that exhibited inhibition for a DNA sample to be considered conclusively negative (default: 0).

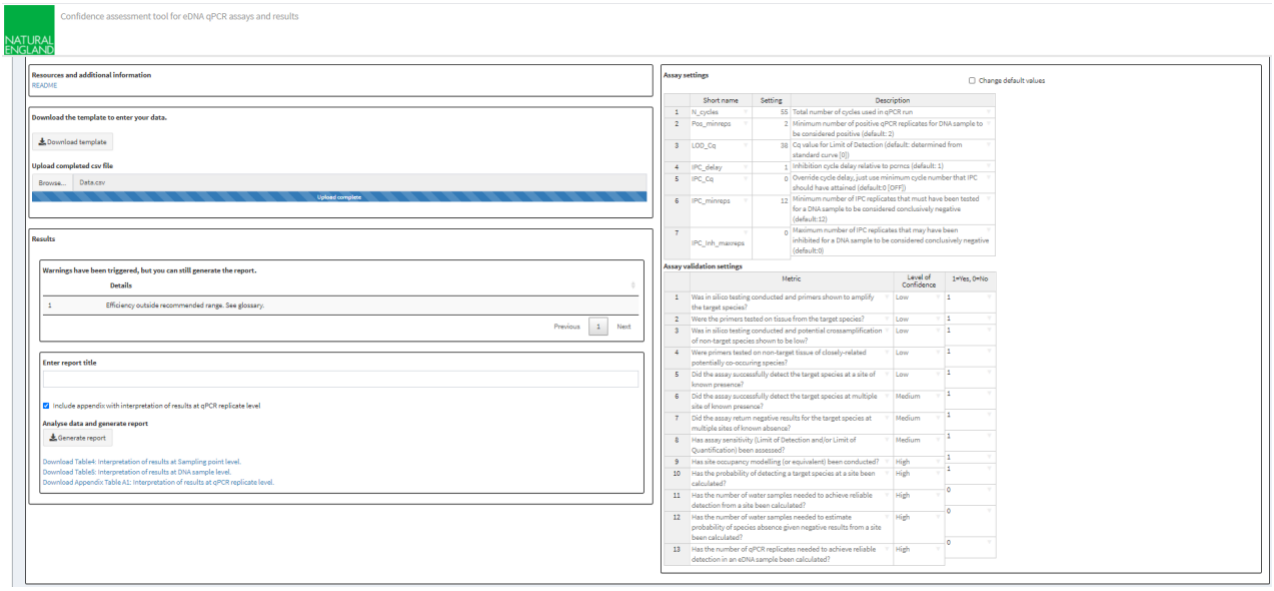


Figure 2. Example of the COASTER user interface

CAPA: Checklist for Assay and Project Adherence

An additional difficulty with assessing eDNA qPCR projects is ensuring that a project has adhered to the assay as it was originally specified. Although this is not built into COASTER at this point, we have summarised recent guidelines from the literature into a checklist (Appendix 4). This is developed from studies that have constructed best practice guidelines for eDNA (Goldberg et al. 2016; Klymus et al. 2020; Nicholson et al. 2020; Thalinger et al. 2021) and expands upon the original MIQE guidelines for qPCR produced by Bustin et al. (2009). To enable continuity between MIQE and CAPA, metrics were given an importance score of either Essential (must be reported) or Desirable (reported if available).

CAPA provides an additional resource for interpreting the results from COASTER. CAPA can also be used to determine how well developed an assay or project is before beginning a project. For example, if a sample is reported as Tentative CAPA can be consulted to determine if the reported assay meets all the Essential metrics. In particular, the user should consult the qPCR validation section and if all Essential metrics were met, the user would have increased confidence that a Tentative sample may actually be a true Positive. The ability to report that Essential CAPA metrics were met for an eDNA qPCR assay will provide the user with sound scientific reasoning for declaring a Sample or Sampling Location as Positive or Negative for a target species when reported in conjunction with the assay confidence level and the report generated by COASTER.

eDNA qPCR validation framework

The goal of this project was to develop an eDNA qPCR assay and project validation framework through the following aims:

1. Design a tool for assessing confidence in a given eDNA qPCR assay
2. Design a tool to aid in the interpretation of eDNA qPCR results
3. Provide a template for standard reporting of eDNA qPCR results

These tools were developed as part of this project and they form the basis of an overall eDNA qPCR assay and project validation framework (Figure 3). It is expected that the tools will be utilised by the providers of eDNA qPCR analyses, so that outputs can be submitted to evaluators and/or project managers. Managers can refer to the framework herein when designing projects or assessing project outcomes.

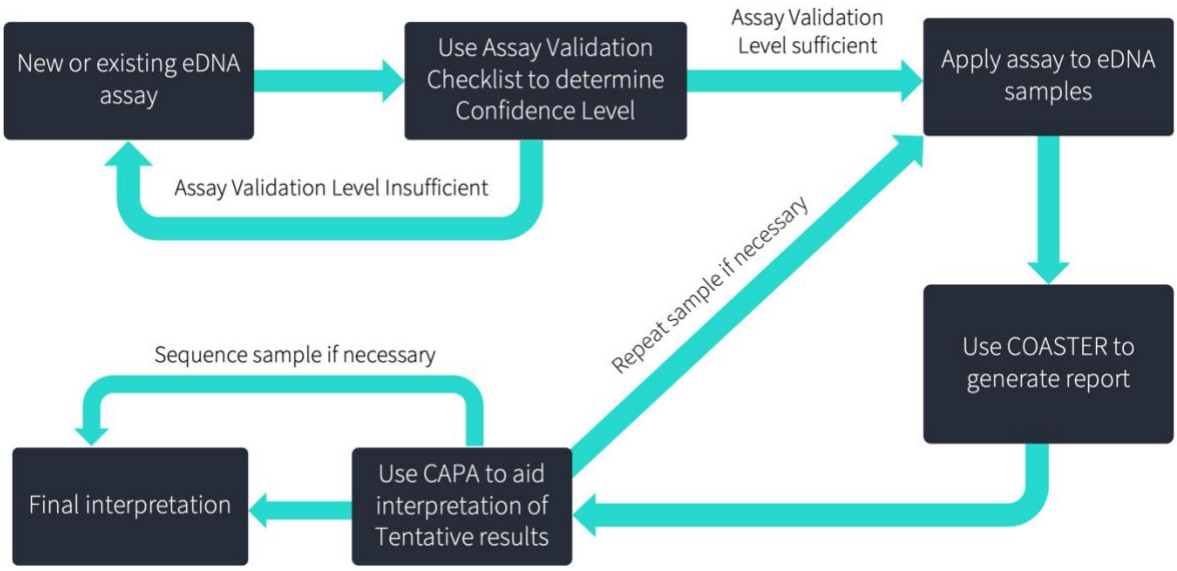


Figure 3. eDNA qPCR assay and project validation framework

Caveats and limitations

The developed tools make several assumptions that should be taken into account when interpreting results. These include: all controls (field and laboratory) perform as expected, inhibition is tested and controlled for, sampling is performed at an appropriate time for the target species, there is an appropriate amount of replication (field and laboratory), and that eDNA equipment and laboratory cleaning protocols are followed. If these assumptions are not met, the user should proceed with caution when using the tools for eDNA qPCR data interpretation.

It is important to note that while Positive results can be interpreted as “species DNA present in sample”, it is not always possible to infer species presence at a location. For example, the species may not actually be physically present at the site but instead the DNA could have been introduced by another means such as predation or downstream flow. This can generally be overcome by considered study design.

Finally, any change in conditions affecting an assays reported specificity or sensitivity, for example using different reagents (Klymus et al. 2020; 2019; Svec et al. 2015) or applying the assay to a different geographical region (Klymus et al. 2020), will change how the assay performs. As such, any reported protocol and interpretation should be revisited whenever new conditions are applied.

Conclusions

The features of the developed tools were designed to meet Natural England's need to understand and interpret the results of qPCR assays for different taxa dependent on their level of development, and to determine how confident they can be in the accuracy of these results, including the associated risk of a false positive or false negative. The assay validation checklist enables the user to assign a confidence level of Low, Medium, or High to an assay dependent on the level of development. Subject to the confidence level assigned, the user can determine how confident they can be that a target species DNA was present, or if not detected, that the target species DNA was truly absent. COASTER provides the user a standard recording data sheet for qPCR data and the ability to define parameters, including the Assay Validation Level, on an assay-by-assay basis. COASTER's report provides an interpretation of results on a qPCR Replicate Level, DNA Sample Level, and Sampling Location Level as either Positive, Negative, Inconclusive or Tentative. It also provides an easy to digest summary of the performance of all the relevant controls and. provides a transparent view of how data were treated through the reporting of settings applied. The visual representation in Appendix 3 and additional CAPA in Appendix 4 offer the ability to visually understand the results generated by COASTER, and aid the user in interpreting Tentative results as a true or false positive including whether other steps should be taken to confirm the result. The development of this eDNA qPCR validation framework contributes to a greater understanding of eDNA qPCR assays and results enables a more transparent process for reporting such results.

Recommendations for future work

Currently COASTER does not incorporate metadata, such as such as target species name, sample GPS co-ordinates, sampling method etc. Future recommendations would be to incorporate metadata into the tool and to interpret results with respect to sample metadata. Doing so would offer more breadth and depth to data evaluation and interpretation. It would be useful to incorporate CAPA into COASTER as a way to more comprehensively evaluate an assay and a project's ability to detect a target species based on how many essential reporting metrics are met. There is also opportunity to utilise the presence/absence results from COASTER and apply the data to other occupancy modelling analyses such as the "ednaoccupancy" (Dorazio and Erickson 2017).

An important future development would be the databasing of new information pertaining to a given assay when it becomes available. For example, a database of assays and their associated levels of validation would 1) allow labs to enter validation variables as they become available, growing the necessary body of information for each methodology, identify knowledge gaps / uncertainties for each methodology 3) act as a resource for practitioners to search for existing, well-validated methodologies 4) provide associated information of the methodology relevant to provide quality assurance of project outcomes 5) identify geographic areas that the assay has been validated in-situ, safeguarding against the application of the assay in regions that have not yet been thoroughly tested. A very recent substantial move in this direction has been made (Thalinger et al. 2021) and is now available online (https://edna-validation.com/).

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Appendix 1

Example of raw data entered into the downloadable template. This data was also used to generate the example report. Please see the COASTER user interface for more details

Plate	Well	Sample_Type	DNA_Sample	Replicate	Target_Cq	IPC_Cq	Std_Conc	Sampling_Location	Extraction_Batch	Volume_Water_Processed
1	A1	unkn	2136	1	25	32		abc	1	800
1	A2	unkn	2136	2	40.43	33.03		abc	1	800
1	A3	unkn	2136	3	0	32.84		abc	1	800
1	A4	unkn	2136	4	42.79	0		abc	1	800
1	A5	unkn	2136	5	0	33.04		abc	1	800
1	A6	unkn	2136	6	0	32.87		abc	1	800
1	A7	unkn	2136	7	43.79	33.4		abc	1	800
1	A8	unkn	2136	8	0	33.62		abc	1	800
1	A9	unkn	2136	9	0	33.26		abc	1	800
1	A10	unkn	2136	10	0	33.36		abc	1	800
1	A11	unkn	2136	11	41.87	33.06		abc	1	800
1	A12	unkn	2136	12	41.76	33.5		abc	1	800
1	F13	unkn	2137	1	0	33.35		abb	2	1000
1	F14	unkn	2137	2	0	33.44		abb	2	1000
1	F15	unkn	2137	3	0	33.27		abb	2	1000
1	F16	unkn	2137	4	0	33.82		abb	2	1000
1	F17	unkn	2137	5	0	33.34		abb	2	1000
1	F18	unkn	2137	6	0	33.26		abb	2	1000
1	F19	unkn	2137	7	0	33.24		abb	2	1000
1	F20	unkn	2137	8	0	33.34		abb	2	1000
1	F21	unkn	2137	9	0	33.09		abb	2	1000
1	F22	unkn	2137	10	0	33.04		abb	2	1000
1	F23	unkn	2137	11	0	32.98		abb	2	1000
1	F24	unkn	2137	12	0	33.29		abb	2	1000

Plate	Well	Sample_Type	DNA_Sample	Replicate	Target_Cq	IPC_Cq	Std_Conc	Sampling_Location	Extraction_Batch	Volume_Water_Processed
1	D1	unkn	2142	1	0	33.17		xyz	2	1200
1	D2	unkn	2142	2	0	33		xyz	2	1200
1	D3	unkn	2142	3	0	33.11		xyz	2	1200
1	D4	unkn	2142	4	0	33.21		xyz	2	1200
1	D5	unkn	2142	5	0	33.19		xyz	2	1200
1	D6	unkn	2142	6	0	33.16		xyz	2	1200
1	D7	unkn	2142	7	0	33.05		xyz	2	1200
1	D8	unkn	2142	8	0	33.28		xyz	2	1200
1	D9	unkn	2142	9	0	33.39		xyz	2	1200
1	D10	unkn	2142	10	0	33.16		xyz	2	1200
1	D11	unkn	2142	11	0	33.43		xyz	2	1200
1	D12	unkn	2142	12	0	33.4		xyz	2	1200
1	G1	unkn	2143	1	0	32.75		xyz	2	200
1	G2	unkn	2143	2	0	32.97		xyz	2	200
1	G3	unkn	2143	3	0	32.86		xyz	2	200
1	G4	unkn	2143	4	0	33.24		xyz	2	200
1	G5	unkn	2143	5	0	32.86		xyz	2	200
1	G6	unkn	2143	6	0	32.67		xyz	2	200
1	G7	unkn	2143	7	0	32.95		xyz	2	200
1	G8	unkn	2143	8	0	32.89		xyz	2	200
1	G9	unkn	2143	9	0	32.86		xyz	2	200
1	G10	unkn	2143	10	0	32.82		xyz	2	200
1	G11	unkn	2143	11	0	33.05		xyz	2	200
1	G12	unkn	2143	12	0	33.02		xyz	2	200
1	N13	extnc	EB	1	25	-1		none	1	
1	N14	extnc	EB	2	0	-1		none	1	
1	N15	extnc	EB	3	0	-1		none	1	
1	N16	extnc	EB	4	0	-1		none	1	
1	N17	extnc	EB	5	0	-1		none	1	
1	N18	extnc	EB	6	0	41.62		none	1	

Plate	Well	Sample_Type	DNA_Sample	Replicate	Target_Cq	IPC_Cq	Std_Conc	Sampling_Location	Extraction_Batch	Volume_Water_Processed
1	N19	extnc	EB	7	0	-1		none	1	
1	N20	extnc	EB	8	0	-1		none	1	
1	N21	extnc	EB	9	0	-1		none	1	
1	N22	extnc	EB	10	0	-1		none	1	
1	N23	extnc	EB	11	0	-1		none	1	
1	N24	extnc	EB	12	0	-1		none	1	
1	P21	std	S1	9	24.15	-1	0.1	none		
1	P22	std	S2	10	28.26	-1	0.01	none		
1	P23	std	S3	11	31.99	-1	0.001	none		
1	P24	std	S4	12	36.2	-1	0.0001	none		
1	P21	std	S1	1	24.15	-1	0.1	none		
1	P22	std	S2	2	28.26	-1	0.01	none		
1	P23	std	S3	3	31.99	-1	0.001	none		
1	P24	std	S4	4	0	-1	0.0001	none		
1	P21	std	S1	5	24.15	-1	0.1	none		
1	P22	std	S2	6	28.26	-1	0.01	none		
1	P23	std	S3	7	31.99	-1	0.001	none		
1	P24	std	S4	8	0	-1	0.0001	none		
1	P14	pcrnc	TNC	2	0	35		none		
1	P15	pcrnc	TNC	3	0	35		none		
1	P16	pcrnc	TNC	4	0	35		none		
1	P17	pcrnc	TNC	5	0	35		none		
1	P18	pcrnc	TNC	6	0	35		none		
1	P19	pcrnc	TNC	7	0	35		none		

Appendix 2

Example of the generated report from COASTER.

A report generated by Natural England's eDNA qPCR assay and project validation tool on 12 February 2021. The software has accepted qPCR results and user/defined settings as input and assigned interpretations to the results at three levels: 1) the qPCR replicate level (see Appendix) 2) the DNA sample level and 3) the Sampling Point level.

Project title:

Warnings table. Please note any warnings carefully.

warning
Efficiency outside recommended range. See glossary.

Summary of results

Assay validation level: Medium
Did any qPCR negative controls (pcrnc) indicate contamination: No
Did any extraction negative controls (extnc) indicate contamination: No
Did any field negative controls (fieldnc) indicate contamination: No
Did all positive controls (pc) amplify: Yes
Were standards included on the plate: Yes
R2 = 0.98
PCR efficiency = 102%

Settings

Table 1: Assay settings supplied by user

shortname	Setting	Description
N_cycles	55	Total number of cycles used in qPCR run
Pos_minreps	1	Minimum number of positive qPCR replicates for DNA sample to be considered positive (default: 2)
LOD_Cq	38	Cq value for Limit of Detection (default: determined from standard curve [0])
IPC_delay	5	Inhibition cycle delay relative to pcrncs (default: 1)
IPC_Cq	40	Override cycle delay, just use minimum cycle number that IPC should have attained (default:0 [OFF])
IPC_minreps	1	Minimum number of IPC replicates that must have been tested for a DNA sample to be considered conclusively negative (default:12)
IPC_Inh_maxreps	1	Maximum number of IPC replicates that may have been inhibited for a DNA sample to be considered conclusively negative (default:0)

Table 2: Assay validation answers supplied by user

Metric	Level of Confidence	Yes/No
Was in silico testing conducted and primers shown to amplify the target species?	Low	Yes
Were the primers tested on tissue from the target species?	Low	Yes
Was in silico testing conducted and potential crossamplification of non-target species shown to be low?	Low	Yes
Were primers tested on non-target tissue of closely-related potentially co-occuring species?	Low	Yes
Did the assay successfully detect the target species at a site of known presence?	Low	Yes
Did the assay successfully detect the target species at multiple site of known presence?	Medium	Yes
Did the assay return negative results for the target species at multiple sites of known absence?	Medium	Yes
Has assay sensitivity (Limit of Detection and/or Limit of Quantification) been assessed?	Medium	Yes
Has site occupancy modelling (or equivalent) been conducted?	High	Yes
Has the probability of detecting a target species at a site been calculated?	High	Yes
Has the number of water samples needed to achieve reliable detection from a site been calculated?	High	No
Has the number of water samples needed to estimate probability of species absence given negative results from a site been calculated?	High	No
Has the number of qPCR replicates needed to achieve reliable detection in an eDNA sample been calculated?	High	No

Table 3: Interpretation of results at Sampling point level

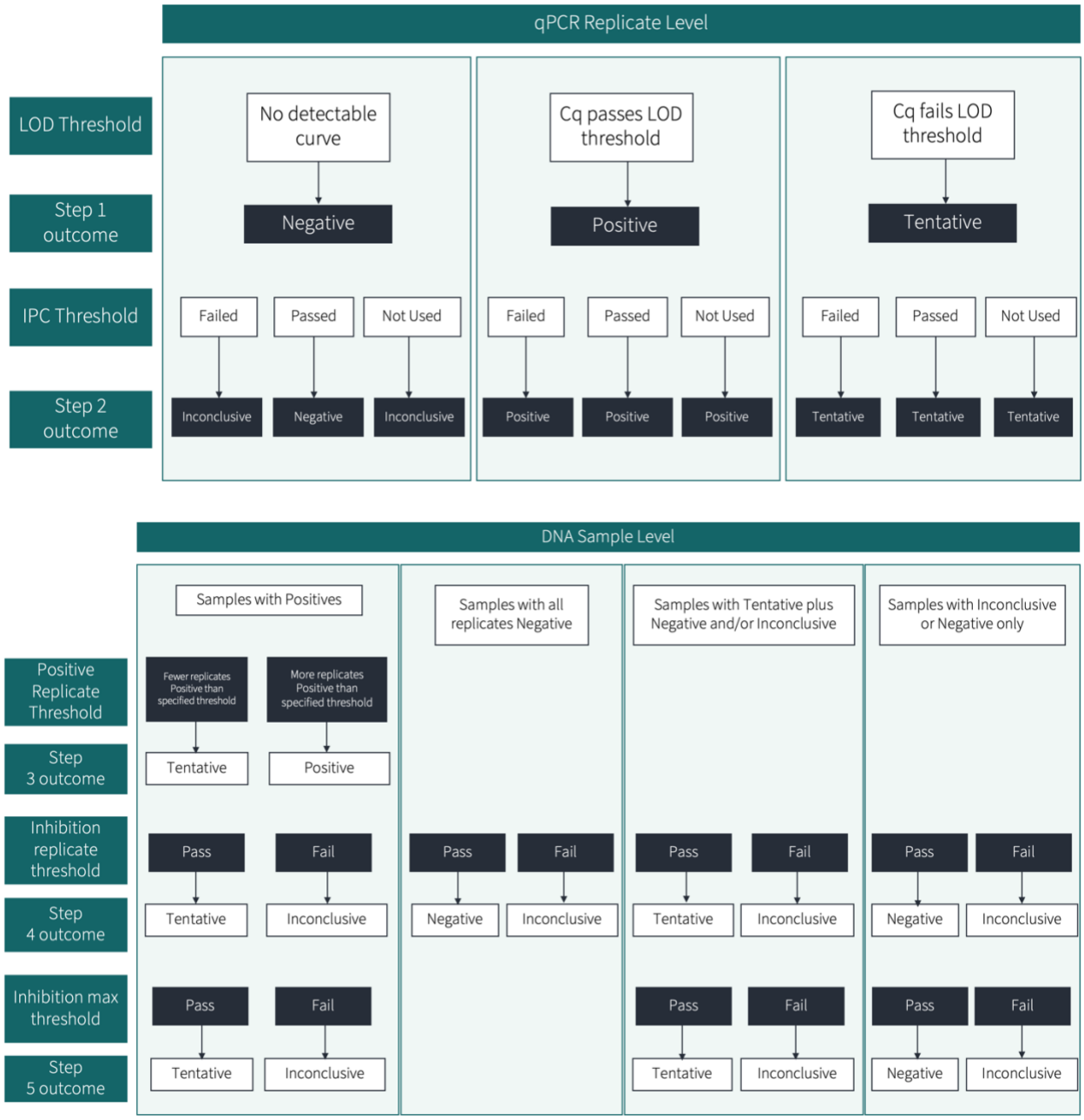
	Inconclusive	negative	positive	Tentative	Interpretation	Notes
abc	0	1	1	0	Positive	Species DNA is present
none	1	1	4	0	Positive	Species DNA is present
ttt	0	2	0	4	Tentative	Suggest sequencing or further assay validation
xxx	0	4	0	1	Tentative	Suggest sequencing or further assay validation
xyz	0	0	0	3	Tentative	Suggest sequencing or further assay validation
yyy	5	4	0	4	Tentative	Suggest sequencing or further assay validation

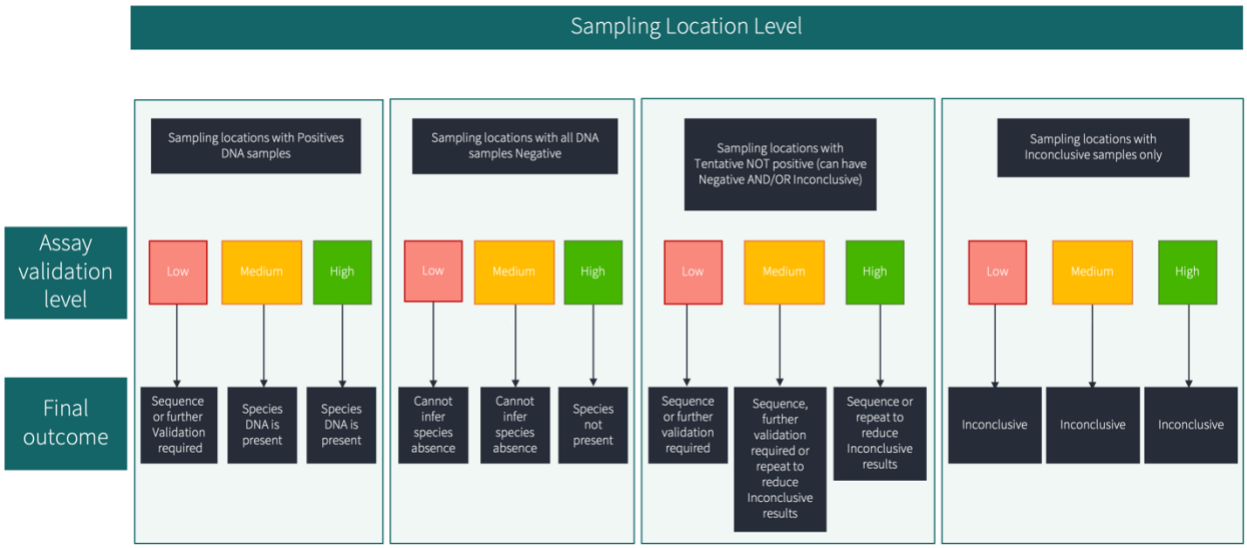
Table 4: Interpretation of results at DNA sample level

DNA Sample	Sample Type	nReps	nPos	nIPC	nFailedIPC	Interpretation	Notes
2136	unkn	12	1	12	1	positive	1 of 12 replicates positive and passing all controls (min=1)
2136 1:2	unkn	12	0	8	0	Tentative	weak signal in 2 replicates
2136 1:4	unkn	12	0	11	0	Tentative	weak signal in 6 replicates
2137	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2137 1:2	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2137 1:4	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2142	unkn	12	0	12	0	Tentative	weak signal in 5 replicates
2142 1:2	unkn	12	0	8	0	Tentative	weak signal in 4 replicates
2142 1:4	unkn	12	0	11	0	Tentative	weak signal in 1 replicate
2143	unkn	12	0	12	0	Tentative	weak signal in 6 replicates
2143 1:2	unkn	12	0	12	0	Tentative	weak signal in 5 replicates
2143 1:4	unkn	12	0	10	0	Inconclusive	not detected but inhibition was only tested in 10 replicates (min=1)
2144	unkn	12	0	12	1	Tentative	weak signal in 5 replicates
2144 1:2	unkn	12	0	9	1	Tentative	weak signal in 2 replicates
2144 1:4	unkn	12	0	11	0	Tentative	weak signal in 3 replicates
2145	unkn	12	0	0	0	negative	0 replicates were negative, 0 showed inhibition max=(1) and 12 were not tested for inhibition (min=1)
2145 1:2	unkn	12	0	11	0	Inconclusive	not detected but inhibition was only tested in 11 replicates (min=1)
2145 1:4	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2167	unkn	12	0	12	0	Tentative	weak signal in 12 replicates
2167 1:2	unkn	12	0	12	0	Tentative	weak signal in 12 replicates
2172	unkn	12	0	12	1	negative	11 replicates were negative, 1 showed inhibition max=(1) and 0 were not tested for inhibition (min=1)
2172 1:2	unkn	12	0	8	0	Inconclusive	not detected but inhibition was only tested in 8 replicates (min=1)
2172 1:4	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2173	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2173 1:2	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2173 1:4	unkn	12	0	12	0	negative	all replicates negative and without inhibition
2174	unkn	12	0	0	0	negative	0 replicates were negative, 0 showed inhibition max=(1) and 12 were not tested for inhibition (min=1)
2174 1:2	unkn	12	0	1	0	Inconclusive	not detected but inhibition was only tested in 1 replicate (min=1)
2174 1:4	unkn	12	2	2	0	Inconclusive	extraction batch contaminated
EB	extnc	12	0	1	1	Inconclusive	extraction batch contaminated
GCN1	std	3	3	0	0	positive	3 of 3 replicates positive and passing all controls (min=1)
GCN2	std	3	3	0	0	positive	3 of 3 replicates positive and passing all controls (min=1)
GCN3	std	3	3	0	0	positive	3 of 3 replicates positive and passing all controls (min=1)
GCN4	std	3	1	0	0	positive	1 of 3 replicates positive and passing all controls (min=1)
TNC	pcrnc	6	0	6	0	negative	all replicates negative and without inhibition

Appendix 3

Visual representation of the primary workflow undertaken in COASTER. To make the illustration more easily readable, steps taken to check positive and negative controls (other than the Internal Positive Control) are not shown but these are implemented in COASTER. Explanations of each step and of each possible outcome can be found in section entitled COASTER: Confidence assessment tool for eDNA qPCR assays and results, as well as in Table 3.




Sampling Location Level
Assay validation level
Final outcome
Sampling locations with Positives DNA samples
Low
Medium
High
Sequence or further Validation required
Species DNA is present
Species DNA is present
Sampling locations with all DNA samples Negative
Low
Medium
High
Cannot infer species absence
Cannot infer species absence
Species not present
Sampling locations with Tentative NOT positive (can have Negative AND/OR Inconclusive)
Low
Medium
High
Sequence or further validation required
Sequence, further validation required or repeat to reduce Inconclusive results
Sequence or repeat to reduce Inconclusive results
Sampling locations with Inconclusive samples only
Low
Medium
High
Inconclusive
Inconclusive
Inconclusive

Appendix 4

Checklist of Assay and Project Adherence (CAPA)

METRIC	IMPORTANCE	REFERENCE	YES/NO/NA
EXPERIMENTAL DESIGN			
Definition of experimental and control groups/goal of study	E	Bustin et al. 2009; Goldberg et al. 2016	
Number within each group	E	Bustin et al. 2009	
Indicator species	D	Nicholson *et al.* 2020; Thalinger et al. 2021	
Species at risk	D	Nicholson et al. 2020; Thalinger et al. 2021	
Invasive alien species	D	Nicholson et al. 2020; Thalinger et al. 2021	
Method development	D	Nicholson et al. 2020	
Method comparison	D	Nicholson et al. 2020	
Transport of eDNA	D	Nicholson et al. 2020; Thalinger et al. 2021	
Fate of eDNA	D	Nicholson et al. 2020; Thalinger et al. 2021	
State of eDNA	D	Nicholson et al. 2020; Thalinger et al. 2021	
Origin of eDNA	D	Nicholson et al. 2020; Thalinger et al. 2021	
Assay carried out by core lab or investigators lab	D	Bustin et al. 2009	
Acknowledgement of authors contributions	D	Bustin et al. 2009	
SAMPLE			
Description	E	Bustin et al. 2009; Thalinger et al. 2021	
Volume/mass of sample processed or collected	E	Bustin et al. 2009; Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Method of water collection (filtration or precipitation)	E	Nicholson et al. 2020; Thalinger el al. 2021	
Laboratory or field	E	Nicholson et al. 2020; Thalinger et al. 2021	
Type of system (freshwater or marine)	E	Thalinger et al. 2021	
Wind conditions	D	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Precipitation	D	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
UV exposure	D	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Time of Day	D	Goldberg et al. 2016; Nicholson et al. 2020	
pH	D	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Flow rate	D	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	

METRIC	IMPORTANCE	REFERENCE	YES/NO/NA
Water temperature	D	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Distance from shore	D	Goldberg et al. 2016; Nicholson et al. 2020	
Distance between samples	D	Goldberg et al. 2016; Nicholson et al. 2020	
Date	D	Nicholson et al. 2020	
Container type	D	Goldberg et al. 2016; Nicholson et al. 2020	
Water depth	E	Goldberg et al. 2016; Nicholson et al. 2020	
Replication	E	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Field decontamination	E	Goldberg et al. 2016; Nicholson et al. 2020	
Month	D	Nicholson et al. 2020	
GPS coordinates/map	D	Nicholson et al. 2020	
Year	D	Nicholson et al. 2020	
Negative control	E	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Province/state	D	Nicholson et al. 2020	
Country	D	Nicholson et al. 2020; Thalinger et al. 2021	
Processing procedure	E	Bustin et al. 2009	
Sample preservation (Frozen or fixed)	E	Bustin et al. 2009; Goldberg et al. 2016; Nicholson et al. 2020	
Filter preservation (Frozen or fixed)	E	Bustin et al. 2009; Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Filter pore size	E	Nicholson et al. 2020; Thalinger et al. 2021	
Duration	D	Goldberg et al. 2016	
Filter Type	E	Goldberg et al. 2016; Thalinger et al. 2021	
Filtering location	E	Goldberg et al. 2016	
Sample storage conditions and duration (especially for FFPE samples)	E	Bustin et al. 2009	
NUCLEIC ACID EXTRACTION			
Procedure and/or instrumentation	E	Bustin et al. 2009	
Extraction location (field of laboratory)	D	Nicholson et al. 2020	
Clean lab room	D	Nicholson et al. 2020; Thalinger et al. 2021	
Lab decontamination	E	Goldberg et al. 2016; Nicholson et al. 2020	
Extraction Method (Inc. kit protocol adjustments)	E	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Negative control	E	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Name of kit and details of any modifications	E	Bustin et al. 2009; Thalinger et al. 2021	

METRIC	IMPORTANCE	REFERENCE	YES/NO/NA
Source of additional reagents used	D	Bustin et al. 2009	
Details of DNase or RNase treatment	E	Bustin et al. 2009	
Contamination assessment (DNA or RNA)	E	Bustin et al. 2009	
Nucleic acid quantification	E	Bustin et al. 2009; Thalinger et al. 2021	
Instrument and method	E	Bustin et al. 2009	
Purity (A260/A280)	D	Bustin et al. 2009	
Yield	D	Bustin et al. 2009	
RNA integrity method or instrument	E	Bustin et al. 2009	
RIN/RQI or Cq of 3' and 5' transcripts	E	Bustin et al. 2009	
Electrophoresis traces	D	Bustin et al. 2009; Thalinger et al. 2021	
Inhibition testing (Cq dilutions, spike or other)	E	Bustin et al. 2009; Thalinger et al. 2021	
qPCR TARGET INFORMATION			
Design and Validation methods	E	Goldberg et al. 2016; Thalinger et al. 2021	
If multiplex, efficiency and LOD of each assay	E	Bustin et al. 2009	
Sequence accession number	E	Bustin et al. 2009	
Location of amplicon	D	Bustin et al. 2009; Thalinger et al. 2021	
Amplicon Length	E	Bustin et al. 2009	
In silico specificity screen (BLAST, etc)	E	Bustin et al. 2009; Thalinger et al. 2021	
Pseudogenes, retropseudogenes or other homologs?	D	Bustin et al. 2009	
Sequence alignment	D	Bustin et al. 2009	
Secondary structure analysis of amplicon	D	Bustin et al. 2009	
Location of each primer by exon or intron (if applicable)	E	Bustin et al. 2009	
What splice variants are targeted?	E	Bustin et al. 2009	
qPCR OLOGONUCLEOTIDES			
Primer sequences	E	Bustin et al. 2009; Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Primer database	E	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
RTPrimerDB Identification Number	D	Bustin et al. 2009	
Probe sequences	E	Bustin et al. 2009; Thalinger et al. 2021	

METRIC	IMPORTANCE	REFERENCE	YES/NO/NA
Location and identity of any modifications	E	Bustin et al. 2009; Thalinger et al. 2021	
Manufacturer of oligonucleotides	D	Bustin et al. 2009	
Purification method	D	Bustin et al. 2009	
qPCR PROTOCOL			
Complete reaction conditions	E	Bustin et al. 2009; Thalinger et al. 2021	
Reaction concentrations	E	Goldberg et al. 2016; Thalinger et al. 2021	
Reaction Volume and amount of cDNA/DNA	E	Bustin et al. 2009; Thalinger et al. 2021	
Primer, (probe), Mg++ and dNTP concentrations	E	Bustin et al. 2009; Thalinger et al. 2021	
Inhibition detection and handling	E	Goldberg et al. 2016; Thalinger et al. 2021	
Positive control	E	Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Polymerase identity and concentration	E	Bustin et al. 2009; Thalinger et al. 2021	
Buffer/kit identity and manufacturer	E	Bustin et al. 2009; Thalinger et al. 2021	
Exact chemical constitution of the buffer	D	Bustin et al. 2009	
Additives (SYBR Green I, DMSO, etc)	E	Bustin et al. 2009; Thalinger et al. 2021	
Manufacturer of plates/tubes and catalogue number	D	Bustin et al. 2009	
Complete thermocycling parameters (including number of cycles and cycle time)	E	Bustin et al. 2009; Goldberg et al. 2016; Nicholson et al. 2020; Thalinger et al. 2021	
Reaction setup (manual/robotic)	D	Bustin et al. 2009	
Manufacturer of qPCR instrument	E	Bustin et al. 2009; Thalinger et al. 2021	
qPCR VALIDATION			
Evidence of optimisation (from gradients)	D	Bustin et al. 2009; Thalinger et al. 2021	
Specificity (gel, sequence, melt or digest)	E	Bustin et al. 2009; Thalinger et al. 2021	
For SYBR Green I, Cq of the NTC	E	Bustin et al. 2009	
Standard curves with slope and y-intercept	E	Bustin et al. 2009; Goldberg et al. 2016	
PCR efficiency calculated from slope	E	Bustin et al. 2009	
Confidence interval for PCR efficiency or standard error	D	Bustin et al. 2009	
r2 of standard curve	E	Bustin et al. 2009	
Linear dynamic range	E	Bustin et al. 2009	

METRIC	IMPORTANCE	REFERENCE	YES/NO/NA
Cq variation at lower limit	E	Bustin et al. 2009	
Confidence intervals throughout range	D	Bustin et al. 2009	
Evidence for limit of detection	E	Bustin et al. 2009; Thalinger et al. 2021	
Concentration range and number of replicate standards per concentration used for calculating LOD and LOQ	E	Klymus et al. 2019; Thalinger et al. 2021	
Determination approach used (i.e., discrete threshold of curve fitting modelling method)	E	Klymus et al. 2019; Thalinger et al. 2021	
Specific criteria for LOD probability detection (e.g., 95%) and LOQ precision (e.g., 35% CV) applied	E	Klymus et al. 2019; Thalinger et al. 2021	
If multiplex, efficiency and LOD of each assay	E	Bustin et al. 2009	
DATA ANALYSIS			
qPCR analysis program (source, version)	E	Bustin et al. 2009	
Cq method determination	E	Bustin et al. 2009	
Outlier identification and disposition	E	Bustin et al. 2009	
Results of NTC	E	Bustin et al. 2009	
Justification of number and choice of reference genes	E	Bustin et al. 2009	
Description of normalisation method	E	Bustin et al. 2009	
Number and concordance of biological replicates	D	Bustin et al. 2009; Thalinger et al. 2021	
Number and stage (RT or qPCR) of technical replicates	E	Bustin et al. 2009; Goldberg et al. 2016; Thalinger et al. 2021	
Repeatability (intra-assay variation)	E	Bustin et al. 2009	
Reproducibility (inter-assay variation, %CV)	D	Bustin et al. 2009	
Power analysis	D	Bustin et al. 2009	
Statistical methods for results significance	E	Bustin et al. 2009; Nicholson et al. 2020	
Software (source, version)	E	Bustin et al. 2009; Nicholson et al. 2020	
Cq or raw data submission using RDML	D	Bustin et al. 2009	

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