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Best Practice Guidelines for Targeted Environmental DNA-Based Proficiency Testing in Non-Regulatory Contexts

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ABSTRACT

The effective use of environmental DNA (eDNA) tools is contingent on strict adherence to established and validated methods. Differences in eDNA methods and quality assurance protocols may contribute to variability in results. However, quality assurance measures such as proficiency testing can provide independent evaluation of laboratory performance against pre-established test criteria. With this commentary, we discuss how broad implementation of recurring proficiency testing in eDNA laboratories can build decision-maker confidence in eDNA results. It can also create a culture of continuous evaluation and improvement that minimizes error and meets performance requirements to inform the sustainable use or monitoring of natural resources. We provide an overview of proficiency testing across molecular disciplines, review the state of proficiency testing in eDNA applications, and draft a roadmap for the expanded application of proficiency testing informed by best practices for targeted eDNA detection. We suggest that best practice proficiency testing can be conducted by an independent, third-party sample provider. By demonstrating that laboratories are competent and capable of producing reliable results, implementation of proficiency testing best practices should foster confidence in eDNA measurements and its use in decision-making processes. Increased confidence in eDNA methods and a clear expectation of what is considered satisfactory performance are also likely to create more favorable conditions for investments in eDNA-based monitoring.

1 | Introduction

Environmental DNA (eDNA) is a powerful, non-invasive tool for detecting species in the environment and is one of the few

monitoring approaches that can be applied at the vast scales needed to address the sustainable use of natural resources (Goodwin et al. 2024). The eDNA field has broadly diverse applications, spanning from the detection of bacteria and imperiled

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or invasive species to the evaluation of community responses to disturbance (Bass et al. 2023; Takahashi et al. 2023). There are multiple steps in the eDNA sampling and analytical pipeline that can be impacted by instrumental and human biases and error, including eDNA sample collection, extraction, amplification, and data interpretation. Therefore, it is critical to assess the repeatability and reproducibility of the data output, especially when results are being used to inform management decision-making (Sepulveda et al. 2019; Theroux et al. 2025). The rate of method advancement has surpassed the establishment of appropriate quality control and quality assurance measures, and largely unknown variability exists in the application of eDNA methodologies within and across laboratories. Broad implementation of quality assurance measures that reduce error and variability in eDNA results can improve natural resource decision-making and advance eDNA method implementation and uptake (Darling 2020; Stein et al. 2024).

Proficiency testing (PT) is an integral component of quality assurance in many laboratory-rooted disciplines, from forensics to human health and water quality. The purpose of PT is to demonstrate the validity and performance of a laboratory's protocol. When scored as satisfactory by an external reviewing body, PT provides independent verification that a laboratory's operations associated with a protocol are effective and the quality of work is being maintained (e.g., Mejia et al. 2020). A principal benefit of PT is that proficient laboratories have demonstrated their ability to produce data within the accepted limits—this can save money and time and support informed decisions.

Even with the benefits of PT, it has yet to be broadly implemented in eDNA laboratories despite calls by management and research communities for increased rigor (Darling et al. 2021; Trujillo-González et al. 2021; Kelly et al. 2023). This lack of eDNA-based PT is not due to unwillingness or disinterest, but rather to the challenges inherent in a rapidly evolving discipline across diverse applications. Additionally, standardized eDNA protocols or formal standard methods are a precursor to developing a PT scheme, but few eDNA formal standard methods are currently available (Theroux et al. 2025). Proficiency testing allows for the evaluation of service providers, the certification of network labs, and the internal assessment of technical staff. An eDNA-based PT schema can assist with efficient and confident uptake of eDNA data in management as well as aid in the development of eDNA formal standards and best practice guidelines (as defined by Theroux et al. 2025).

Here, we develop a roadmap for eDNA PT by providing an overview of PT across molecular disciplines, a review of the state of PT in eDNA applications and research, and propose an initial framework for expanded application of PT to eDNA sample analysis for targeted detection of rare, cryptic, or small taxa. Based on the precedent of other targeted eDNA PTs, we limit the scope to targeted eDNA approaches here (Langlois et al. 2021; Trujillo-González et al. 2021; Abbott et al. 2023; Rees et al. 2023), although PT efforts for eDNA metabarcoding are underway (e.g., Vasselon et al. 2025). The goal of this commentary is to identify eDNA best practice guidelines for PT that can be broadly applied across the high diversity of eDNA

laboratories and methods. These best practice guidelines may provide a common denominator of quality assurance that can be attained by any eDNA laboratory. Through participation in PT schemas that align with these best practices, an eDNA laboratory can provide additional evidence to end-users that their reported eDNA test results are reproducible and can be more confidently used to inform decision-making. Our best practice guidelines are not intended to compete with or supplant those associated with standards organizations (e.g., International Organization for Standardization [ISO], International Electrotechnical Commission [IEC]), but rather help bridge the gap until there are formal PT eDNA methods. These guidelines also provide a means to demonstrate credibility for laboratories that are unable to pursue formal accreditation (e.g., due to cost, capacity, or policy). This framework does not restrict the research activities focused on basic eDNA detection and methods development.

2 | Proficiency Testing Overview

2.1 | What Is Proficiency Testing?

Proficiency testing is the external evaluation of laboratory performance of a specific test or suite of tests against pre-established criteria. Results generated from PT are used to evaluate a participant laboratory's competency in performing a standard method, establish laboratory performance in comparison to other laboratories, and assess performance among analytical staff. Additionally, PT helps to identify areas for improvement, sources of bias or error, and helps to ensure confidence, reliability, and reproducibility of a laboratory's efforts (Horowitz 2013; Fleischman et al. 2019). Proficiency testing is a hallmark of numerous diagnostic procedures because it indicates that appropriate methods are being used, that these methods are being carried out properly, and that testing results from a specific laboratory can be trusted for high-stakes decision-making.

2.2 | Proficiency Testing Roles

There are three roles involved in PT: (1) A participant laboratory whose performance is being evaluated, (2) an independent provider to coordinate and oversee the testing, and (3) a reference laboratory to establish results used to determine participant laboratory performance (Figure 1). The provider and reference laboratory are often one and the same and are jointly referred to as “provider” hereafter.

Related, but different from PT, are inter-laboratory comparisons (ISO/IEC 17025 2017) that lack an independent, third-party provider and do not compare results to pre-established criteria. These can take multiple forms; in round-robin tests, they include multiple laboratories performing the same tests on the same samples generated by one or more participating laboratories (e.g., Amberg et al. 2015; Sepulveda et al. 2019). Inter-laboratory comparisons can be used in the development of a PT scheme and offer opportunities for laboratories to verify the competency of technicians and identify workflow areas that need improvement.

2.3 | Proficiency Testing Process

In a basic PT program, a third-party provider sends participant laboratories a blind panel of samples, either of known-composition reference material (RM) or ideally certified reference material (CRM; Figure 1). The participant laboratory conducts the analysis of the PT samples and submits the results to the provider. Test results are compared with the expected sample values, using qualitative and/or quantitative metrics. The provider then communicates to the participant laboratory an evaluation that includes the level of concordance with expected values and whether this level of concordance meets a predetermined threshold to demonstrate proficiency.

In some cases, there are rules on retesting when a participant laboratory fails to meet a desired threshold of concordance. In the United States, human clinical laboratories are regulated under

the Clinical Laboratory Improvement Amendments (CLIA 1988), with the Centers for Medical Medicaid Services (CMS) overseeing approval of PT programs. A variety of third-party, CMS-approved PT providers serve clinical laboratories (participants) across the country and report to CMS. The clinical laboratory must achieve a passing score for each regulated test that it intends to offer. If PT performance is determined to be unsatisfactory, the clinical lab must determine the cause of the error, document the actions to remedy this error, review and address any past clinical results that may have been affected by this error, and re-test.

Proficiency testing is most frequently performance-based, where the analysis protocol is not strictly defined, and participant labs use their local routine methods. The only required criterion is that test result interpretation is concordant with known sample composition. For example, the College of American Pathologist Proficiency Testing program allows participants to use their laboratory's routine procedures for several histocompatibility and immunogenetic PT assessments, such as antibody screening, where multiple testing methodologies exist (Sullivan et al. 2023). Participants receive a grade based on the accuracy of the results. Less frequently, PT is prescriptive where all participants use a similar analytical protocol or a standardized protocol with a defined list of acceptable variations. For example, the Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL) PT program requires use of a detailed protocol, but also includes a living list of “planned deviations,” including alternative DNA extraction equipment and probe fluorescent chemistries, which, once assessed for performance, are then added to a library of acceptable variants (Smart et al. 2021).

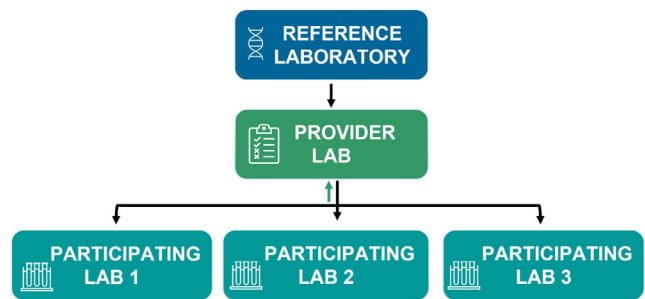


FIGURE 1 | Example of a simple proficiency testing (PT) schema that involves a reference laboratory, a provider, and participating labs. Testing reference material is prepared by the reference laboratory and sent to the provider for distribution to the participating labs (black arrows), who then analyze the test material and return results to the provider (green arrow). Refer to Trujillo-González et al. (2021) for an alternative schematic describing formal eDNA PT through ISO/IEC 17025 and ISO 17034 accreditation.

2.4 | Proficiency Testing Type: Formal Versus Best Practice Proficiency Testing

Formal PT is typically associated with the ISO/IEC 17025 (2017) testing and calibration accreditation, which requires

Formality	Category	Participating Labs	Reference Lab	Potential PT Outcome	eDNA Application	Testing intensity
	Regulatory	ISO/IEC 17025 accredited	ISO/IEC 17043 accredited	Accredited	Licensure to test for biosecurity	
	Regulatory/ non-regulatory	ISO/IEC 17025 accredited	No program available	Accredited or compliant	Commercial service lab	
	Non-regulatory	Not accredited	No program available	Compliant or evidence of competency	Research lab	
Best practice guidelines	Non-regulatory	Not accredited	Programs available, but not accredited	Evidence of competency	Assess lab staff competency	

FIGURE 2 | Examples of a range of proficiency testing (PT) schemes that might exist for environmental DNA (eDNA) applications. These schemes use reference material (RM) or certified reference material (CRM), typically produced by ISO 17034 accredited laboratories. ISO/IEC 17025 outlines general requirements for the competence of testing and calibration laboratories and ISO/IEC 17043 specifies general requirements for the competence and impartiality of PT providers.

that laboratories seeking accreditation demonstrate the validity of results through the implementation of techniques to record, monitor, and review data (Figure 2). Performance monitoring can be conducted through formal PT administered by a provider accredited under ISO/IEC 17043 (2023). The RM used in this process must be sufficiently homogeneous and stable to serve as a reliable benchmark for measurement and validation. More specifically, CRM is a type of RM that has known property values (i.e., metrologically valid), measurement uncertainty, and is accompanied by a certificate that provides the values of specified properties (e.g., microbial pathogen DNA standard 8376.pdf). The CRM is often produced by a laboratory accredited under ISO 17034 (2016). A RM may be used when a CRM is not an option (Holden et al. 2011), depending on accreditation needs. Some providers may not be formally accredited to the ISO/IEC 17043 or ISO 17034 standards but operate in accordance with them. While participation in formal PTs administered by ISO/IEC 17043-accredited providers is not mandatory, adequate justification for pursuing an alternative approach to formal PT must be provided by the participating laboratory. For instance, when a formal PT scheme is not available, inter-laboratory comparisons are an accepted alternate mechanism to demonstrate competence and validity of test results (ILAC 2024). Participation in formal, standardized PT schemes may be restricted to specific laboratories, such as those in a professional society. Figure 2 summarizes the different scenarios of PT testing and accreditation.

In comparison, best practice guidelines for proficiency testing (hereafter best practice PT) are those developed outside of a formal standards organization and are derived from professional consensus. A benefit of a best practice PT schema is that there is additional flexibility, making the tool available for a broader suite of laboratories. The reference, or provider, laboratory and participating laboratories can use RM or CRM and may or may not be accredited. Provider laboratories distribute the RM to participating laboratories (Figure 1).

The accreditation requirements for PT provider laboratories vary across industries, with human clinical laboratories subject to the most stringent regulations. For example, in the United States, PT providers for clinical laboratories operating under the CLIA (1988) are commonly accredited to ISO/IEC 17043 and use RM from sources accredited under ISO 17034 (General requirements for the competence of reference material producers). In contrast, the U.S. Department of Agriculture Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL), which develops and operates PT for the National Plant Protection Laboratory Accreditation Program in the United States, follows practices consistent with ISO/IEC 17043 and ISO 17034, but is not formally accredited under these standards due to cost considerations and the lack of regulatory requirements. Instead, it is considered ISO compliant as it adheres to the standards, without holding formal certification from a recognized accrediting body.

Proficiency testing can have regulatory and non-regulatory applications. For some sectors, such as human clinical laboratories, formal PT is mandated by U.S. federal and state laws (e.g., CLIA) to ensure quality laboratory testing. Laboratory licensure is contingent on enrollment in an approved PT

program and repeated, unsuccessful PT performance can result in a laboratory no longer being allowed to perform the respective test. Non-regulatory uses of PT are widely regarded as a best practice because results are used to identify workflow issues and training deficiencies, improve staff competency, and foster a culture of evaluation and continuous improvement (Earley et al. 2017). During the COVID-19 pandemic, non-regulatory PT proved to be an effective tool for enhancing the quality of SARS-CoV-2 detection. This was especially valuable amid the rapid expansion of methods and involvement by laboratories with a range of experience and technical capacity (Edson et al. 2020; Lau et al. 2022).

3 | Environmental DNA Proficiency Testing

There are a wide variety of legitimate analytical methods and protocol variations for eDNA analysis, but few formal standards or best practice guidelines have been developed to ensure data quality, rigor, and reliability across these methodological variations. Environmental DNA analysis relies on detecting trace genetic material from environmental samples, which presents distinct challenges that are not necessarily encountered in traditional diagnostics. For instance, eDNA concentrations of targeted organisms are often low and heterogeneous in the environment (e.g., Augustine et al. 2024). Analytical methods that are sufficiently sensitive to detect eDNA at low concentrations are also susceptible to contamination (Hutchins et al. 2022) and inhibition (Hunter, Ferrante, et al. 2019; Hunter, Meigs-Friend, et al. 2019). Errors from false negative and false positive data can be consequential, especially since it can be difficult to corroborate eDNA results for rare species using less sensitive, conventional approaches (Darling et al. 2021; Sepulveda et al. 2023). For example, false negative detections could impact at-risk species protections, while false positive results could lead to costly mitigation measures for invasive species.

Some impactful standards and PT schemes have been developed in recent years. Considerations for the development of formal PTs for eDNA studies were discussed by Trujillo-González et al. (2021). Examples include: a standard method (WC1067) guiding Great Crested Newt (*Triturus cristatus*) eDNA sampling and analysis in the United Kingdom, approved by Natural England (Biggs et al. 2014); standard pipelines adopted by industry and government in Japan as specified by The eDNA Society (Yamanaka et al. 2023); standards for quantitative PCR (qPCR) assay validation for eDNA analysis in Canada (CSA W219:23; Abbott et al. 2023); and best practice guidelines for eDNA assay validation and bio-monitoring applications in Australia and New Zealand (De Brauwier et al. 2023). However, many standards are limited in scope to specific geographies and certain species and may not be readily adaptable to other applications. The difficulty in applying the current standards and best practices more broadly is an important consideration since the eDNA field is rapidly evolving and often requires customized study designs and novel, case-specific methodologies to effectively capture target eDNA collected from dynamic environments (e.g., Hunter, Meigs-Friend, et al. 2019; Pochon et al. 2024). The limited best practices for eDNA study design and sampling

standards and the challenges of quality management systems keeping pace with ever-changing methods are an impediment to eDNA sampling implementation and uptake (Darling 2020; Stein et al. 2024; Theroux et al. 2025). However, we propose that best practice PT can play a key role in improving end-user confidence in eDNA methods now while eDNA standards and best practices are under development. Proficiency testing will also play a critical role in ensuring that eDNA methods are properly executed and broadly applicable, thereby facilitating more rapid and reliable transition of eDNA tools from research to management practice.

3.1 | Examples of Environmental DNA Proficiency Testing in Practice

Here, we highlight two successful schemes in practice, the Great Crested Newt PT and the Australian National eDNA Reference Centre PT. These schemes take different approaches (prescriptive vs. performance-based), which underscore that PT is not a one-size-fits-all process and is most impactful when it is designed in a fit-for-purpose framework. We also summarize recent research and inter-laboratory comparison studies in support of future PT efforts for the field.

3.1.1 | Great Crested Newt Proficiency Testing

Natural England (the United Kingdom Government advisor for the natural environment in England) has required annual PT of commercial laboratories that are testing water samples for Great Crested Newt (GCN) eDNA for the last 9 years (2017–2025). Surveying for GCN is required in the UK in relation to construction and development near their habitat. This was the first known PT scheme for eDNA sampling.

The current PT provider is Fapas Proficiency Testing (York, UK), and PT takes place once a year in January or February prior to the start of the GCN eDNA testing season 15th April to 30th June. Results from eDNA sampling are used to support GCN mitigation license applications (to carry out work that may affect GCN). To ensure that the population is safeguarded, mitigation to compensate for impacts is often a requirement of a license. Mitigation may involve changes in the timing of operations; capturing and excluding GCN; setting aside land for GCN; purchase of additional land; habitat creation; and post-development monitoring commitments (often through eDNA testing by laboratories participating in the PT scheme).

The GCN PT scheme uses a prescriptive laboratory analysis pipeline (Biggs et al. 2014). Participating laboratories supply their own eDNA sampling kits to the PT provider who adds simulated pond water. The sampling kit sent to the PT provider consists of 6 × 50-mL tubes each containing 33.5 mL of 100% molecular biology grade ethanol and 1.5 mL of 3M sodium acetate pH 5.2. The PT provider adds 15 mL of simulated pond water to each of the six blinded sample tubes. The simulated pond water samples are composed of GCN DNA-free buffer (weak buffer solution), which is spiked with a known amount of GCN DNA and/or PCR inhibitor to mimic potential conditions in the field. The participant laboratory receives the pond water

and follows a protocol to perform a DNA extraction (Biggs et al. 2014) and qPCR analysis (Biggs et al. 2014). The protocol is highly prescriptive, specifying the DNA extraction kit (Qiagen DNeasy Blood and Tissue Kit; QIAGEN), PCR master mix (Applied Biosystems Taqman Environmental Mastermix 2.0; ThermoFisher Scientific), hydrolysis assay (Biggs et al. 2014), and PCR cycling conditions. The participant laboratory reports their test results (“negative,” “positive,” and “inhibited”) to the PT provider. An inconclusive result can also be reported when there is inhibition detected in a sample and no GCN eDNA detected, since the sample may contain GCN eDNA which inhibition rendered undetectable.

The PT provider gives participants a score of “satisfactory” or “not satisfactory” based on their proficiency in detecting GCN eDNA and PCR inhibitors in the simulated pond water samples (Table 1). While Natural England requires that GCN eDNA analysis providers participate in PT, it does not mandate a minimum percentage of “satisfactory” results for tested samples. Although not required, laboratories with poor PT performance often take steps to improve, motivated by the incentive to demonstrate satisfactory performance to clients (e.g., GCN mitigation license applicants). As this PT scheme has evolved, substantial improvements have been observed, and it is now uncommon for participating laboratories to receive non-satisfactory results. However, it is important to note that in some instances, laboratories may fail to detect very low concentrations of GCN eDNA due to the stochastic nature of PCR.

3.1.2 | Australian National eDNA Reference Centre Proficiency Testing

In 2022, the Australian Government Department of Agriculture Fisheries and Forestry (DAFF) established the Australian National eDNA Reference Centre (NRC) to support the development of eDNA testing capability, capacity, and competency under the National eDNA Testing Program for both regulatory and non-regulatory applications in Australia. To achieve this, the DAFF signed a Memorandum of Understanding with the Australian National Association of Testing Authorities (NATA), enabling eDNA testing facilities to undergo ISO/IEC 17025: testing and calibration accreditation. This accreditation ensures that facilities meet the standards required to provide testing services to the Australian Government. Within this framework, the NRC is responsible for maintaining a National eDNA Collaborative Testing Network, which consists of eDNA testing facilities in Australia and New Zealand, designated as eDNA Collaborative Centres. These facilities provide services for both regulatory and non-regulatory DAFF biosecurity applications, the latter being the most common service in Australia. To become an eDNA Collaborative Centre, testing facilities must participate in the PT scheme annually and demonstrate competency. The Proficiency Testing Charter of the NRC developed in accordance with ISO/IEC 17043 evaluates proficiency based on the accuracy and precision of tests conducted on 10–20 blind samples while measuring uncertainty across participating labs (Table 2, data in Table S1).

The NRC prepares test samples by spiking eDNA extracts with known concentrations of DNA from a pre-selected target species.

TABLE 1 | Great crested newt (GCN) annual proficiency test (PT) results from 2017 and 2022 showing the performance of the laboratories taking part.

Year	Lab	Inhibited		Not inhibited										% Correct
		Negative		Positive low			Positive medium	Positive high	Negative					
2017	1	0/12 i	0/12 n	0/12 n	—	—	3/12 n	7/12 n	0/12 n	0/12 n	—	—	86	
	2	0/12 i	0/12 i	2/12 n	—	—	11/12 n	12/12 n	0/12 n	0/12 n	—	—	100	
	3	0/12 i	0/12 i	1/12 n	—	—	5/12 n	3/12 n	0/12 n	0/12 n	—	—	86	
	4	0/12 i	0/12 i	1/12 n	—	—	11/12 n	10/12 n	0/12 n	0/12 n	—	—	100	
	5	0/12 i	0/12 i	9/12 n	—	—	11/12 n	0/12 n	7/12 n	0/12 n	—	—	71	
	6	0/12 i	0/12 i	1/12 n	—	—	11/12 n	1/12 n	0/12 n	0/12 n	—	—	100	
	7	0/12 i	0/12 i	0/12 n	—	—	4/12 n	12/12 n	0/12 n	0/12 n	—	—	86	
2022	1	0/12 i	0/12 i	10/12 n	2/12 n	3/12 n	8/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	100	
	2	0/12 i	0/12 i	2/12 n	3/12 n	2/12 n	10/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	100	
	3	0/12 i	0/12 i	1/12 n	1/12 n	1/12 n	6/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	100	
	4	0/12 i	0/12 i	5/12 n	0/12 n	4/12 n	9/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	90	
	5	0/12 i	0/12 i	6/12 n	1/12 n	2/12 n	9/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	100	
	6	0/12 i	0/12 i	5/12 n	4/12 n	5/12 n	11/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	100	
	7	0/12 i	0/12 i	2/12 n	1/12 n	3/12 n	3/12 n	—	0/12 n	0/12 n	0/12 n	0/12 n	100	

Note: Laboratories are randomly assigned a number, which may change across PTs. Dashes indicate no testing occurred for a given sample type. The shaded cells illustrate results that were classified as not satisfactory. For the low positives “n” indicates that no inhibition was found, “i” indicates that inhibition was found. It should be noted that in some instances, laboratories are unable to detect very low concentrations of GCN DNA due to the stochastic nature of PCR (e.g., Lab 7 in 2017 and Lab 4 in 2022). In 2017, seven samples were provided; however, this number increased to 10 from 2018 onwards.

These eDNA extracts are typically collected from aquaria or natural environments where the target species is absent. The blinded samples are distributed to each participating facility, along with a contextual scenario, recommended qPCR cycling conditions, and clear proficiency expectations. Participants are not required to follow a standardized protocol; instead, they use their own laboratory procedures and qPCR protocols for testing. Each participant must report the overall outcome of each sample (i.e., positive, not detected, or undetermined) and the qPCR cycle threshold values for all technical replicates. Participants have 2 months to submit their test outcomes along with a brief explanation of their methodologies. To be considered proficient, the following must be achieved: (1) 100% accuracy across all samples expected to be negative, (2) over 90% accuracy in all samples considered to be positive with low-high DNA concentrations, and (3) attain < 2% mean relative standard deviation of Cq values across all samples tested. Testing facilities that do not meet these proficiency standards receive feedback on workflow improvements and are given an opportunity to re-submit results using a different set of blind eDNA samples. In the case of the 2024 PT, one participant (Laboratory 2, Table 2) requested a second round of analysis, significantly improving their results and ultimately achieving proficiency in eDNA testing (Table 3).

3.2 | Research That Informs Environmental DNA Proficiency Tests

Recently, the EU-funded COST Action DNAqua-Net community of researchers (17 labs, 14 countries) compared DNA

extraction and PCR amplification PT frameworks for a metabarcoding workflow. Using a provider laboratory that oversaw all aspects of the testing, the study compared diatom community indices inferred from eDNA metabarcoding output between laboratories using a standardized eDNA protocol across multiple labs to those results from laboratories using their own choice of protocol (Vasselon et al. 2025). They found a high consistency among laboratories in output results, regardless of workflow protocol. They concluded that protocol variation is not necessarily a barrier to the operationalization of eDNA metabarcoding methods. This is especially important since laboratory-specific protocols are often optimized to a site or to the equipment a laboratory has on hand. However, as long as test results (i.e., performance) are benchmarked against a standardized approach and have critical quality assurance and control components, outputs can be used effectively for decision-making. While this study assessed a PT scheme for eDNA metabarcoding, this research is still useful in the context of targeted PT schemes since it demonstrates how prescriptive and performance-based approaches can be jointly evaluated in a single PT scheme.

The iTrackDNA project led by a pan-Canadian team of university scientists (<https://itrackdna.ca/>) aims to establish a PT framework for targeted taxon eDNA assays. This is being achieved through a multi-phase, inter-laboratory comparison exercise designed to identify workflow elements contributing to variability in results. With the involvement of 17 experienced academic, government, and commercial laboratories in Canada and the United States, the iTrackDNA project systematically

TABLE 2 | The 2024 Australian National Environmental DNA (eDNA) Reference Centre Proficiency Test involved nine participating laboratories which measured the relative standard deviation of quantitative PCR Cq values.

Sample	Expected test result	Target amount	Inhibition	Laboratories (1–9) and Cq relative standard deviation values									% Correct
				1	2	3	4	5	6	7	8	9	
1	Positive	Low	Low	0.73	ND	1.3	ND	4.03	0	ND	0.19	ND	67
2	Positive	Very low	None	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
3	Negative	Non-target	None	ND	ND	ND	ND	ND	ND	ND	ND	ND	100
4	Positive	Very low	None	ND	ND	0	ND	ND	0	ND	ND	ND	0
5	Positive	Moderate	Low	2.67	0.59	2.42	1.33	0.93	2.86	0	1.1	1.12	100
6	Negative	Blank	None	ND	ND	ND	ND	ND	ND	ND	ND	ND	100
7	Positive	Moderate	High	2.93	ND	0.36	0	1.8	ND	3.77	1.93	1.5	78
8	Positive	Low	High	0	ND	1.07	0	2.48	0.16	0.86	1.94	ND	78
9	Negative	Blank	None	ND	ND	ND	ND	ND	ND	ND	ND	ND	100
10	Negative	Non-target	None	ND	ND	ND	ND	ND	ND	ND	ND	ND	100
% Correct				80	60	80	70	80	70	70	80	60	

Note: Participants were required to test 10 blind samples for the presence of *Varroa destructor* eDNA. A “Pass” (no shading) is given if relative standard deviation is lower than 5% for a sample expected to be “Positive,” or to a non-detection (ND) for any known negative sample. A “Fail” (shading) is given if these criteria are not met. Data associated with these PT can be found in the Table S1.

TABLE 3 | Comparison of measurements of uncertainty for facility 2, which chose to repeat proficiency assessment following quality control and assurances advice from the Australian National eDNA Reference Centre.

Test order	Accuracy of expected negatives	Accuracy of expected positives	Mean RSD (%)
Test 1	3/3 (100%)	1/6 (16.67%)	0.59
Test 2	3/3 (100%)	6/7 (85.71%)	2

Note: This test required the participant to test a separate set of 10 blind samples for the presence of *Varroa destructor* eDNA.

addressed issues of the influence of equipment, reagents and reagent preparation, targeted taxon assay type, protocol clarity/adherence, and filter handling, shipping, and processing. With the goal of informing a PT framework, particular attention was focused on taking a phased approach to identifying pinch points that contribute to variation in results between labs. The details and results of this inter-laboratory validation exercise are currently being prepared for publication. In the first phase, participating laboratories followed a shared protocol and a standardized data analysis template. A single provider laboratory premade and aliquoted all reagents necessary (including pre-made synthetic double stranded DNA [dsDNA] template dilutions) for the same targeted eDNA assay and distributed them to all participating laboratories. In the second phase, participating laboratories purchased and prepared their own reagents for three different eDNA assays and performed their own dsDNA template dilutions, but used the same enzyme in both phases. Each laboratory analyzed the assay using their own equipment,

which varied. Preliminary findings indicate that laboratories produce results highly comparable to those of the reference laboratory, even when using different equipment. The few observed deviations were addressed through targeted technician training.

The third phase involved testing of filters prepared with RM developed by a designated laboratory (the Pacific Yukon Laboratory for Environmental Testing [PYLET] at Environment and Climate Change Canada). The RM consisted of a calibrated slurry derived from a fish fin clip that was diluted to two concentrations (low and high). Each participating laboratory received two filters per concentration and was instructed to process each quarter filter using the same standardized eDNA extraction protocol.

Preliminary results showed that calculated copy numbers per quarter filter extraction were consistent within replicates for most laboratories. However, while all laboratories were able to detect target eDNA on the filters, the copy numbers between labs deviated over 2+ orders of magnitude. These results offer insight into establishing realistic expectations for PT testing and RM development. To date, PT testing efforts in Canada have been limited to activities such as Bureau Veritas (renamed Mérieux NutriSciences), which has been supporting accreditation under ISO/IEC 17025 through blind evaluation of eDNA samples verified by an academic laboratory.

In the literature for the field of eDNA detection, there are numerous case studies that report on inter-laboratory comparisons. Most of these studies evaluated the reliability of an eDNA method to generate repeatable and reproducible results. However,

because these inter-laboratory studies lacked a provider, they do not meet the definition of PT. Regardless, these studies provide examples of how to perform inter-laboratory comparison studies and highlight considerations for future eDNA PT schema. For example, Amberg et al. (2015) and Rodriguez et al. (2025) evaluated the performance of different extraction methods for eDNA detections of invasive carp (*Hypophthalmichthys* spp.) and several marine megafauna, respectively, across multiple laboratories. Additionally, Guan et al. (2019) and Sepulveda et al. (2020) evaluated the performance of different assays for eDNA detections of invasive carp and invasive mussels (*Dreissena* spp.) across multiple laboratories. All studies found that results were generally robust to a wide range of laboratory conditions, such as the use of different equipment (e.g., different platforms of PCR thermocyclers, multichannel vs. robotic pipette type), and that discrepancies in inter-laboratory results were traceable and relatively easy to resolve. For example, Rodriguez et al. (2025) found that errors were associated with a laboratory's extraction method and that slight modifications to this method improved performance. In the studies discussed above, errors or discrepancies across laboratories could largely be resolved through more extensive technician training and consistent production of RMs, underscoring how the development of eDNA PT RMs can help to more clearly understand variance and enable formal PT assessments.

4 | Considerations for Environmental DNA Best Practice Proficiency Testing

Here we have discussed PT applied to different molecular diagnostic needs as well as provided examples of eDNA PT in practice. These include both prescriptive (GCN) and performance-based (Australian) scenarios, as well as additional research projects that can inform eDNA PT. To promote the use of PT in the eDNA community, we provide a *best practice, performance-based* PT schema for targeted species detection in the section *How to administer a proficiency test*. Other schemas may be developed for specific laboratory networks and those involved in regulations; however, here we present a best practices PT that can be applied to any laboratory network for the improvement of data reproducibility and quality assurance.

For broad-scale PT implementation, such as that undertaken at the Australian National eDNA Reference Centre and in SARS-CoV-2 monitoring, flexible schemes adhering to justified and reported performance-based criteria (Rees et al. 2023) are best suited to accommodate variations in methods and the definition of “detections” across laboratories (e.g., Dávila et al. 2023; Singh et al. 2025). This flexibility can allow for the inclusion of diverse molecular platforms (e.g., digital PCR, high throughput qPCR) and varied detection criteria to minimize method lock-in and promote broader application. However, a more prescriptive or formal approach may be required for regulatory and non-regulatory applications to inform management decisions. As eDNA tools advance to make inferences on abundance and other concentration-relevant methods, quantitative metrics (e.g., the target DNA concentration reported in copy numbers within the range of acceptable values) may be a useful PT criterion; however, eDNA CRM produced by an accredited laboratory would be needed.

4.1 | Identifying Proficiency Testing Workflow Steps

Like many molecular procedures, eDNA workflows can be complicated to assess because they are composed of many steps, including field collection, DNA extraction, laboratory analysis, and data interpretation, that may not be standardized. However, PT schemes need not necessarily encompass every step. For example, in clinical laboratories under CLIA, PT evaluates the laboratory's ability to extract nucleic acids and conduct molecular analysis but does not evaluate sample handling (e.g., preparation, shipping) or patient sampling. Therefore, building on lessons learned from the iTrackDNA project, a practicable and informative path forward for broader adoption of eDNA PT is to focus on a select number of key steps rather than attempting to standardize the entire workflow. As needs are identified and understanding of the protocols and processes continue to evolve, PT could be progressively extended to additional steps within the workflow. For example, a PT including DNA extraction could be used to assess variation due to template degradation or extraction efficiency but will require protocol development and the production of RM or CRM (Trujillo-González et al. 2021). As a starting point for supporting PT in eDNA practice, we suggest that initial key steps and criteria to assess are: (1) PCR performance (i.e., correct classification of DNA detection in a sample) and (2) detection of PCR inhibition. These are important criteria because end-user concern over false positive and false negative error types can prevent eDNA method adoption (e.g., Stein et al. 2024).

4.2 | Defining “Detection” for Proficiency Testing

A common goal for PT across disciplines is to ensure the accurate and reproducible inference of analyte detection or non-detection. There are currently no standardized criteria for scoring detection/non-detection of aquatic organism eDNA, and practices vary across individual laboratories (Goldberg et al. 2016; Serrao et al. 2018). For example, the U.S. Fish and Wildlife Service invasive carp program considers a detection valid when one or more PCR replicates amplify (USFWS 2024), whereas the eDNA results hosted in the U.S. Geological Survey's Non-Indigenous Aquatic Species database require the amplification of two or more PCR replicates across samples for detections to be reported and displayed (Ferrante et al. 2022, 2024). Others define detections based on assay properties including the limit of detection (LOD), a cycle threshold cutoff (e.g., $C_q < 50$, CSA W219:23), or statistical methods like receiver operator characteristics (Serrao et al. 2018) to set detection thresholds. As our current framework focuses on PCR performance, we define detection with respect to the assay and not to the entire process, which includes sample collection and processing. With this in mind, only fully optimized assays should be considered in PT to ensure meaningful and reliable results. Per the CSA W219:23, a fully optimized assay has the following characteristics: amplification efficiency between 0.8 and 1.1, R^2 of the standard curve ≥ 0.95 , limit of detection < 10 copies/reaction, and limit of quantification ≤ 20 copies/reaction. Given these parameters, 40 qPCR cycles are a maximum threshold for detections in PT, especially given that RM concentrations may be near LOD (discussed below).

4.3 | Testing Materials

Ideally, PT schemes would generate positive samples using CRMs. However, a CRM does not yet exist for eDNA testing and it may be challenging for CRMs to be produced across the range of intended uses for eDNA sampling. When a CRM is not an option, a RM that has been demonstrated to be sufficiently homogeneous and stable, but lacks a formal certificate, may be used, depending on the level of accreditation required. Various RMs for the purpose of eDNA PTs are discussed in Trujillo-González et al. (2021) and include the use of synthetic oligonucleotides or the use of RM collected from live or recently deceased specimens. The iTrackDNA project selected a tissue slurry created from a fish fin clip for its PT assessment because it more closely represented a field sample. Here, we propose that a RM composed of either synthetic DNA or tissue-derived DNA follow best practices that are compliant with key standards in ISO 17034, which are (1) an analyte with a known reportable concentration range that retains stability and homogeneity through production and distribution, and if applicable; (2) is traceable to a specimen(s) identified by an expert (e.g., voucher specimen) with full details on the production and quantification of the material and supporting metadata indicating pertinent details like geographic origin, life history, etc. Importantly, RM composition will be agreed upon by the provider and participating laboratories in line with the goals of the PT.

The test sample concentrations need to be within the dynamic range of a well-performing assay and able to withstand the stochastic variation associated with low concentration eDNA analyses. Concentrations in an “optimal zone” are slightly above a typical optimized qPCR assay LOD [~3–10 copies/reaction] but less than the LOQ [~10–100 copies/reaction] (Klymus et al. 2020; Lesperance et al. 2021). For example, testing at lower concentrations in the GCN PT has resulted in some false negative results (Table 1), likely due to PCR stochasticity. It is also important that the samples are provided at a “low” DNA concentration to mimic eDNA from the environment. Best practice methods include testing target DNA concentrations near the limit of detection to challenge the analytical performance, as demonstrated in SARS-CoV-2 PTs (Chik et al. 2021; Buchta et al. 2023). However, the concentration needs to be high enough for accurate eDNA detection with and without inhibition.

One aim of PT should be to evaluate lab performance using sample types that reflect typical field conditions rather than concentrations being artificially elevated or supraphysiological. The iTrackDNA project found that target concentrations ranging between 1000 and 2000 copies per filter (equivalent to 3–6 copies/reaction) were optimal for that assay. Given the challenges of accurately measuring target concentrations at the lower eDNA levels and the Poisson distribution associated with pipetting and PCR, we underscore that “low” target concentrations should be measured in an optimal zone for that assay rather than a specific value. Additional “medium” and “high” target and/or inhibitor concentrations could be incorporated to further assess accuracy and precision, as demonstrated by the Australian National eDNA Reference Centre PT.

Due to the potential of PCR inhibition to produce false negative results in environmental samples, it is important to test the

presence and relative concentration (i.e., high vs. low) of inhibitors, ensuring that these values are representative of those found in the environment (McKee et al. 2015). High concentrations of inhibitors are especially challenging to overcome and may require specialized protocols (Hunter, Ferrante, et al. 2019). The addition of purified inhibitors or mixes of inhibitors, such as humic acid or tannins, can allow for controlled and standardized concentrations to be tested (Uchii et al. 2019).

4.4 | How to Administer a Proficiency Test

We envision a voluntary PT scheme composed of multiple participant laboratories and a single provider (Figure 1). The provider may not necessarily hold ISO/IEC 17043 accreditation but could still demonstrate *compliance* with ISO/IEC 17043 standards or even ISO 17034 through documented procedures and quality assurance practices. The provider would supply blinded individual samples in tubes that contain a target species' DNA generated from a RM (as described previously) to participating laboratories. A variety of sample types could be tested including DNA extract or samples in need of extraction such as filters containing cells and/or free DNA. To balance the risks of false positive and negative errors (Table 4), it is recommended to include at least two negative and two positive samples, with one positive sample containing a spiked PCR inhibitor. In line with the GCN and Australian PT schemes, the provider laboratory would spike the RM into multiple matrices, such as a matrix including an ideal use case (e.g., deionized water), a similar environment (e.g., lake water or stormwater, potentially with environmental inhibitors), and a more challenging use case that contains common inhibitors added in known amounts (e.g., purified humic acids; Table 4). The low number of tested samples is in line with other PTs used in molecular diagnostics (e.g., Stang and Anderson 2013) and encourages participation by lowering the time and cost associated with testing. Indeed, several SARS-CoV-2 PT schemes consisted of only a few samples to maximize participation given constrained resources (e.g., Edson et al. 2020; Ranadheera et al. 2023). Our PT scheme only evaluates detection accuracy; however, networks aiming to assess quantitative precision of an assay should include a larger number of samples to account for the substantial technical variability associated with pipetting errors, particularly of low concentrations (e.g., Furlan et al. 2016; Guri et al. 2024; Augustine et al. 2024).

In this PT scheme, participating laboratories would be required to test all samples, submit the protocol used and the target detection/non-detection and inhibition results (reported as none, low or high, or detection/non-detection) for each sample. The goal of the PT is to obtain 100% detection accuracy. Participation in voluntary PT should occur at least annually to ensure that protocols remain compliant; however, more frequent testing may be warranted when results inform consequential decision-making (Figure 2).

Similar to laboratories participating in the GCN PT, participant laboratories that do not achieve 100% accuracy (i.e., non-proficiency) can retain the autonomy to determine appropriate follow-up actions, such as protocol optimization and retesting. Demonstrating proficiency provides both external and internal benefits. Those that do not meet proficiency standards can work

TABLE 4 | A proposed four filter proficiency test (PT) consists of one blank, one negative, and two low concentration target eDNA filter samples.

Filter number	Target eDNA on filter	Matrix	Detection	Inhibition
1	Blank	Deionized water	No	No
2	Non-target DNA	Deionized water	No	No
3	Target DNA	Environmental water	Yes	No
4	Target DNA	Environmental water with inhibitor	Yes	Yes

Note: This represents the minimum recommended scale for ISO compliance and information content on lab performance. Participants are blinded to the presence or absence of DNA on each filter and whether the sample has been treated with inhibitors (e.g., humic acid) or not. While the blank and low concentration filters are standard for each PT, the presence or absence of inhibitors per sample is randomly assigned.

with other laboratories or the provider to identify areas for improvement and retesting.

5 | Conclusions

The establishment of eDNA PT schemes presents opportunities and challenges. A primary benefit of PT is building trust and reproducibility within the scientific and regulatory communities. By demonstrating that laboratories are competent and capable of producing reliable results, PT helps foster confidence in eDNA-based measurements and decision-making processes. Increased confidence in eDNA methods and a clear expectation of what is considered satisfactory performance is also likely to create more favorable conditions for investments in eDNA-based monitoring (Lodge 2022).

Proficiency Testing is not designed to exclude underperforming laboratories, but rather to provide them with opportunities to improve their competency. It serves as a constructive tool for testing the laboratory and its quality assurance practices. This approach ensures that all participating laboratories, regardless of their initial performance level, have a pathway for growth and improvement, as demonstrated by the Australian National eDNA Reference Centre PT scheme. Improving competencies could include revisiting general laboratory best practice guidelines (Goldberg et al. 2016) and reassessing various metrics in the protocols, such as the standard curve parameters and LOD and inhibition assessment and mitigation (Hunter, Ferrante, et al. 2019; Hunter, Meigs-Friend, et al. 2019). Demonstrably proficient laboratories can support the anticipated increase in environmental monitoring strategies using eDNA surveillance over the next decade (Goodwin et al. 2024). Meeting this growing demand may require exploration of support mechanisms or incentives to encourage broader participation by both public agencies and commercial laboratories.

Our PT schema is proposed as a starting point for networks of laboratories that are beginning to scale up sample numbers for high throughput biomonitoring (Jones et al. 2024). Initially, these networks would be responsible for selecting the provider laboratory, overseeing PT administration, and securing funding. However, a key challenge for the future is determining who will manage and sustain the system, ensuring its fairness, transparency, and long-term effectiveness.

Continuous oversight, adaptability, and support all contribute to maintaining the quality and reliability of formal PT

systems. One approach is to establish a long-term managing body to ensure that the PT system remains current, fit-for-purpose, flexible, and reliable, as has been proposed in the U.S. National Aquatic eDNA Strategy (Goodwin et al. 2024). Such a body would be responsible for revising PT protocols as new technologies and methodologies emerge, thereby keeping the system relevant and effective. An alternative approach for *best practice* eDNA PT is the establishment of collaborative laboratory networks, as demonstrated by the iTrackDNA project (<https://itrackdna.ca/>), the DNAqua-Net diatom research community (Vasselon et al. 2025), and the invasive species research communities (Amberg et al. 2015; Sepulveda et al. 2020). An additional approach may be vesting oversight to governmental agencies or professional societies, as has been done for other DNA-based PT by the Society for Wildlife Forensic Science since 2004 (Hawk 2012). We anticipate that broad adoption of PT will provide economies of scale as increased participation by laboratories will lower the cost per laboratory to support provider expenses (Miller et al. 2011). This creates an incentive to make PT participation open and accessible. Proficiency testing alone is not a panacea for errors in eDNA analysis and is one component of a larger quality management system along with general good laboratory practices (Maloney 2005).

Although eDNA-based standards are still maturing, the establishment of PT systems is valuable as a clear pathway to improve rigor and reliability. In the rapidly evolving field of eDNA assessments, we propose this voluntary, non-regulatory PT schema as a starting point for others to customize and enhance. By maintaining flexibility and allowing for continuous updates, both formal and best practice PT systems can deliver substantial benefits without becoming overly rigid. The development of PTs for LODs and extraction methods offers a key opportunity for future growth and standardization within the field, along with quantitative eDNA studies and metabarcoding methods. There are no significant drawbacks to setting up a PT, as it serves as a framework for improvement and quality assurance. With thoughtful implementation and a commitment to adaptability, PT systems can greatly enhance the credibility and utility of eDNA-based methodologies, paving the way for broader acceptance and increased applications.

Author Contributions

Conception or design of the study (M.E.H., A.J.S., K.E.K.); acquisition, analysis, or interpretation of the data (M.E.H., A.J.S., A.T.-G., D.M.G.,

C.C.H., H.C.R., R.M., C.V.R., K.E.K.); and writing of the manuscript (M.E.H., A.J.S., A.T.-G., C.C.H., H.C.R., D.M.G., D.N.J.-S., R.C.M., T.M., S.T., T.W., H.Y., C.V.R., K.E.K.).

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Conflicts of Interest

M.E.H., T.M., and T.W. are on the *Environmental DNA* Advisory Board.

Data Availability Statement

Data are available in article [Supporting Information](#).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370189-sup-0001-supinfo.docx.