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Environmental DNA (eDNA) Quantitative Polymerase Chain Reaction-Based Assays for Surveying 125 Taxa of Importance to North America

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ABSTRACT

Timely and accurate assessment of the presence of at-risk or invasive species is critical for effective responses to climate change and human impacts. For example, at-risk species are often difficult to find, while invasive species are often well established before their infiltration is detected using conventional surveying methods. However, all organisms release genetic material such as DNA into their surroundings, leaving traces of themselves that can be detected using environmental DNA (eDNA) methods. These approaches are powerful tools in the conservation toolbox, as they are transforming how risk assessments and the evaluation of mitigation and remediation effectiveness are done. Despite this, poorly performing tools hinder broad adoption of eDNA-based detection methods, due in part to their associated high false negatives and false positives that can impair effective management decision-making. iTrackDNA is a multi-year, large-scale applied research project that is addressing these concerns with researchers and end users from various sectors across North America. It is building end-user capacity through innovative, accessible, socially responsible genomics-based analytical eDNA tools for effective decision-making by publishing 125 quantitative real-time polymerase chain reaction (qPCR) primer/probe sets designed to detect key invertebrates, fish, amphibians, birds, reptiles, and mammals in coastal and inland ecosystems important to North America, with an emphasis on Canada. These 125 assays were designed to meet or exceed the new Canadian Standards Association (CSA) consensus-based and multi-stakeholder national standards for eDNA (CSA W214:21 and CSA W219:23). Herein, we describe how we applied eDNA assay design and validation approaches across a wide range of animal taxa to achieve compliance.

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1 | Introduction

Natural resource decisions require reliable information on animal taxa to assess, monitor, and mitigate the deleterious impacts on biodiversity and water quality (Ulicsni et al. 2019). The use of environmental DNA (eDNA), that is, the genetic material released by organisms into their surroundings, for environmental monitoring and risk assessments can provide non-destructive, cost-effective, and accurate information on species biodiversity. Moreover, certain conventional methods are being replaced by eDNA standardized protocols. For example, this has already been established in some mandatory protocols to inventory key species requested by governments (e.g., great crested newt (Buxton et al. 2022), turtles (Gouvernement du Québec 2022), endangered fish (Gouvernement du Québec 2023), at-risk snakes (Gouvernement Du Québec 2024a), and some salamander species (Gouvernement Du Québec 2024b, 2024c)). These eDNA-based protocols will continue to gain popularity as they are robust and cost-effective for conservation biology.

Advances in molecular technologies have greatly improved our ability to survey global biodiversity in natural ecosystems and impacted areas. Rising societal pressure to balance environmental protection with natural resource extraction and increasingly stringent regulatory requirements necessitate that countries support various stakeholders (i.e., regulators, industry, Indigenous Peoples, and non-governmental organizations (NGOs)) with new tools to monitor the biosphere, build overlapping datasets to monitor entire ecosystems, and decrease time to action (Fitzgerald et al. 2021). In addition, ecological effects assessment and trend monitoring systems need overhauling to increase effectiveness, resolution, and efficiency. Reliable, socially responsible, and timely monitoring techniques of targeted taxa population status in coastal and inland ecosystems are not readily accessible (Van Der Heyde et al. 2020).

Several large international-based research projects, such as GEN-FISH (<https://gen-fish.ca/fr/>), BIOSCAN (<https://ibol.org/bioscan/>), and iTrackDNA (<https://itrackdna.ca/>) are contributing to eDNA methods development to improve biodiversity survey results with reduced invasiveness. GEN-FISH is working on toolkits for the identification of freshwater fish species and the expression of stress-related genes in parts of Canada and the USA. For over a decade, BIOSCAN has built DNA barcode reference libraries to assess global biodiversity. iTrackDNA provides international leadership in genomics and the social sciences to ensure rigor and reliability with transformative, socially responsible eDNA methods. iTrackDNA also builds capacity by bringing innovative tools to support the standardization of environmental data that can be fed into industry operational and risk assessment models to de-risk decisions while decreasing time to action. Indeed, all these large-scale genomic projects aim to overcome the current barriers to eDNA uptake by end-users, such as inconsistent practices, poor-quality eDNA detection tools, and unacceptably high false negatives and false positives that can compromise effective management decision-making (Ruppert et al. 2019; Langlois et al. 2021).

The most widely used method for detecting eDNA of targeted species or taxonomic groups is quantitative real-time polymerase

chain reaction (qPCR). qPCR equipment is widely available and relatively accessible in comparison with other eDNA methods. While targeted species assay results are the most directly translatable for regulatory purposes, genus- or family-level assays can act as general screens for rapid, cost-effective analyses in scenarios such as determining if fish inhabit an impacted area, if mouse-eared bats inhabit abandoned mines, or if a parasite host (deer for brainworm) or invasive mussels are present (all these examples have been covered by eFish1, eMyotis1, eOdocoileus2, and eDrsp7 assays, respectively, described below).

However, there is a wide range in the quality of qPCR-based assays within the published literature and in practice (Taylor et al. 2019). That is why most research projects are attempting to address important quality assurance/quality control (QA/QC) aspects that are required for confident uptake of targeted eDNA detection assays, namely standardization and assay validation within the context of qPCR kits. The iTrackDNA project received feedback from 50+ project participants representing provincial and federal regulators, industry, First Nations, and NGOs on priority species for which eDNA assays were desired. As a result of this consultation, a list of over 100 species was generated. From this priority list, 125 qPCR-based eDNA assays were designed and validated using a workflow described previously (Langlois et al. 2021). These assays cover the detection of 59 distinct fish, 25 mammals, 21 herptiles (amphibians and reptiles), 18 invertebrates, and two bird taxa, including at-risk, indicator, invasive, and socio-economically important organisms.

The eDNA assays reported herein were designed to meet two Canadian national eDNA standards. These two documents, published by the Canadian Standards Association (CSA), present consensus-based, multi-stakeholder national standards to define minimum criteria that need to be met to have confidence in eDNA data (Gagné et al. 2021; Abbott et al. 2023). The first standard applies to the reporting of information regarding the conducting of eDNA biological surveys or assessments, while the second defines the performance criteria of targeted qPCR assays used in eDNA surveys. According to the CSA standard, those qPCR assays must be comprised of primers and a probe rather than primers combined with an intercalating agent, as the latter assay configuration does not have the desired level of performance (Abbott et al. 2023).

The degree of inclusion of eDNA in regulatory protocols depends on the availability of appropriate and quality tools, standards, and regulatory approvals (Langlois et al. 2021). This critical need to develop standards-compliant eDNA assays will help fill knowledge gaps in eDNA research so that the use of eDNA can be included in regulatory decisions to improve biodiversity monitoring and conservation management efforts worldwide. The aim of the present paper is to provide an example of the approach taken by the iTrackDNA project to generate 125 robust eDNA assays that are compliant with the CSA standards and generally meet the highest validation level as previously described (Langlois et al. 2021; Thalinger et al. 2021). While we continually strive to ensure robust assays and validation activities are ongoing, it is important to note that some assays herein are presented with limited validation due to constraints in mitochondrial sequence information, access to voucher specimens, and/or field samples.

For example, we were not yet able to establish that the yellow perch (*Perca flavescens*) ePEFL1 assay does not detect other Percidae species that could be confounders in vitro due to lack of access to appropriate voucher specimens (File S2). As with any eDNA assay, it is imperative that users ensure that appropriate checks are incorporated within the specific contexts in which the assay is to be used and that appropriate detection of target species within the survey area is established.

2 | eDNA Assay Design and Validation

Robust assays for detecting eDNA are a keystone for environmental surveys and risk assessments. There is an inherent expectation that the results of targeted assays provide a high level of performance and reliability. To attain this, validation of a targeted eDNA assay requires clarity on what constitutes acceptance criteria of key factors defining assay specificity, sensitivity, and quantitative assay performance (Klymus et al. 2020; Lesperance et al. 2021). At a fundamental level, these factors need to be defined in a way that facilitates comparisons between studies and laboratories (Sivaganesan et al. 2022).

To accomplish qPCR-based assay design and validation, a general workflow includes identifying phylogenies of target and confounding taxa, sequencing and alignment of mitochondrial DNA sequences, primer and probe design, systematic specificity validation using voucher specimens, sensitivity determination with synthetic DNA, and field validation of sites with known target species' occupation and negative controls (Langlois et al. 2021). Herein, we feature the use of whole mitochondrial genome (mitogenome) sequences within this workflow and put out a call for the augmentation of these valuable resources. To facilitate ease of use, our design approach features having the same stringent amplification conditions (where possible) to allow assay runs targeting different species on the same plate (thus, using the same PCR conditions) to accommodate broad stakeholder needs.

2.1 | Specificity

The development and validation of a targeted eDNA assay requires steps for determining and reporting assay specificity (Thalinger et al. 2021). These steps serve to establish the assay's ability to distinguish target DNA sequences from confounding non-target (e.g., sympatric/allopatric) organism sequences to ensure that there is a reasonable expectation of the assay to detect target taxon DNA to the exclusion of non-target taxa within an eDNA survey. Gathering natural history information of target and possible confounder populations was accomplished through early communication between field and lab practitioners involved in specific surveys. To effectively accomplish this, access to appropriate DNA sequences from target and non-target taxa is critical. Ideally, this is in the form of complete mitogenome sequences from multiple individuals per taxon from diverse geographic locations to provide the maximal amount of sequence from which to select the best DNA sequences that satisfy the requirements for specificity and sensitivity of eDNA assays (Langlois et al. 2021). Public databases such as the National

Center for Biotechnology Information (NCBI) GenBank are invaluable resources for obtaining the required sequence information. In preparation for multi-mitogenome alignments as described below, we downloaded and utilized thousands of sequences from the NCBI database. For example, for the eFish1 assay, we used 4442 mitogenomes—4211 fish and 231 non-fish.

Despite the apparent high number of mitogenomes that were used, there are still substantial gaps in mitogenome sequence resources. Throughout the iTrackDNA project, we successfully sequenced and assembled the complete mitochondrial genomes (mitogenomes) of several species of interest using *mtGrasp* (Lopez et al. 2025). This significantly enhanced flexibility in identifying target regions for assay design, moving beyond the constraints of limited metabarcoding regions. Despite an increase in full mitogenome sequences, there are still many species that are not directly represented, and intraspecies or regional variation is still not yet adequately captured, as access to appropriate voucher specimen DNA is a limiting factor.

To address this, cost-effective whole genome shotgun sequencing of genomic DNA (gDNA) from voucher specimens and mitogenome assembly are facilitated through genome skimming using 150bp paired-end Illumina NovaSeq 6000 reads, e.g., (Hoban et al. 2022; Lopez et al. 2025). Curated collections from museums and institutions are invaluable sources of material, and the application of whole genome amplification techniques, such as through *GoEnrich* (Lopez et al. 2024), can faithfully augment sparse gDNA resources for sequencing and subsequent eDNA validation. Using *GoEnrich* and *mtGrasp*, we assembled ~400 mitogenome sequences from voucher specimens representing ~120 species. These sequences have been deposited in GenBank (BioProject PRJNA1139757) to fill gaps in sequence database knowledge. An example of the impact of this new knowledge is in iterative field use and further gDNA validation of the eFish1 assay that was designed to broadly detect bony fish. Initial designs with available mitogenomes revealed a broad fish assay, but novel mitogenome alignments and voucher specimen gDNA validation indicated that DNA from some amphibian species (*Ascaphus*, some *Ambystoma* and *Spea*) could also be detected (Files S1 and S2). This should be considered when performing field surveys.

2.1.1 | In Silico Screening Against Target and Confounding Non-Target Taxa

The first step in our workflow involves creating alignments of mitogenome DNA sequences of the target species to available confounding non-target taxa using MAFFT (Katoh et al. 2019) with default settings and visualized using Geneious Prime (Biomatters Ltd., Auckland, New Zealand) as described previously (Allison et al. 2023). Consistent with the CSA standard (Abbott et al. 2023), we paid particular attention to taxa that are closely related to the target taxa that may confound the results. Additionally, as human, domestic dog, and domestic cat DNA ubiquitously contaminate environmental samples (Clare et al. 2022), we routinely included these species in DNA sequence alignments. Moreover, we considered additional non-target organisms' DNA that the practitioner has significant contact with (e.g., mice, hamsters, goldfish, cattle, etc.), when relevant, as a

possible cofounder to ensure that the chosen sequences are unlikely to generate a false positive signal.

We found that querying complete mitogenome sequences with *unikseq* (Allison et al. 2023) was beneficial in identifying DNA regions possessing unique characteristics in an unbiased way from which primers and probes may be selected for testing. In contrast to the propensity for eDNA assay design to historically be based on the *cytochrome oxidase 1 (co1)* gene, we found that *mt-co1* was not a commonly chosen gene from which to derive fully validated eDNA assays (Figure 1). Rather, the mitogenome regions that supported successful eDNA assay designs spanned 18 genes overall ($n=125$; Figure 1A). The most represented genic regions were *mt-cyb*, *mt-nd1*, *mt-nd2*, and *mt-nd4* ($n=125$; Figure 1A). The most represented genic regions by animal group were: *mt-nd1* and *mt-nd2* ($n=59$; fish), *mt-cyb* and *mt-nd5* ($n=25$; mammal), *mt-co1* ($n=18$; invertebrates), and *mt-cyb* ($n=21$; herptile) (Figure 1B–E). The number of bird assays designed was too low to make a general statement ($n=2$; Figure 1F).

Once unique DNA regions were identified by *unikseq*, we used the Beacon Designer (PREMIER Biosoft, California, USA) primer/probe design software on those regions to identify the best forward/reverse primer-probe combination for in vitro screening based upon the guiding principles for primer/probe selection defined by the software (Table 1). The primers/probe candidates that have desirable characteristics were then examined in Geneious Prime with respect to the alignments to ensure that they were sufficiently different from DNA sequences of non-target taxa (Figure 2 and File S1). Any overlapping sequence identity to non-target taxa was identified, and assay designs were iteratively evaluated to obtain the most favorable assay performance characteristics.

To aid in determining assay component specificity *in silico*, we also used NCBI's Basic Local Alignment Search Tool (BLAST) for short sequences against the non-redundant (nr) nucleotide database. While this was a powerful tool, we realized the output only shows the first 100 results according to the level of different proxies (e.g., coverage, percent identity, E value). If the values of these proxies are equal, then the results are presented in random order. If the percent sequence identity remains high in those results, there are likely other sequences with high sequence identity that did not make the list and, hence, may be missed in the specificity evaluation. For species-specific and animal group (e.g., *Cervus* spp., *Odocoileus* spp., Salmonids, or bony fish) assays, this is particularly important when evaluating cross-hybridization from common contaminating DNA such as human, cat, and dog, or other relevant confounders. When this occurred, we queried each primer and probe candidate sequence against narrower categories (e.g., human, bony fish, etc.) rather than the whole NR database.

2.1.2 | In Vitro Screening Against Target and Confounding Non-Target Taxa

While the final eDNA assay will always be comprised of a primer pair and hydrolysis probe, both SYBR and Taqman

chemistry are used at this empirical validation stage; the former for initially testing the primer sets, and the latter for testing promising primer sets in combination with a hydrolysis probe containing a fluorescence dye such as FAM at the 5' end and a quencher (e.g., ZEN/Iowa Black FQ) at the 3' end. This two-phase approach is cost-effective to evaluate different designed assays as primers are much cheaper than hydrolysis probes and allows for more powerful troubleshooting during assay design.

We generally first test at least four technical replicates for each gDNA sample from target and non-target taxa voucher specimens and, once we establish that the desired amplification was achieved (see below), then we expanded the evaluation to between 12 and 25 technical replicates. This approach is more stringent than the minimum of four required by the CSA standard (Abbott et al. 2023). We adopted this approach to more effectively determine off-target detections.

We routinely normalize the gDNA samples to a low concentration (e.g., 10pg/reaction). A typical 15µL reaction contains 2µL (10pg) gDNA that is expected to reliably produce a detection in all technical replicates (e.g., a cycle threshold (Cq) value < 30) using the run conditions indicated in Table 2 on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). While not presented herein, other thermocyclers have been used and assays tested in multiple labs with similar results. While a number of enzymes and reaction mixes could be used, we used the QIacuity EG PCR [Cat. 250111, Qiagen, Hilden, Germany] or SensiFAST SYBR [Cat. BIO-98020, FroggaBio Scientific Solutions, Buffalo, New York, USA] kits for the SYBR reactions and the QIacuity Probe PCR [Cat. 250101, Qiagen, Hilden, Germany] or Immolase [Cat. BIO-21048, FroggaBio] for the Taqman probe-containing reactions. Further information on the enzymes and reaction conditions has been published in detail previously (Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a).

As intraspecies sequence variation can exist (Bernatchez et al. 2024), we attempted to evaluate at least five different individuals from the target taxon, preferably from different relevant geographic locations. However, this often was not possible due to limited availability of voucher specimen material. For non-target taxa, at least one individual of available confounding taxa was included when possible. There should not be any amplification of non-target taxa DNA observed within 50 cycles. In some cases, non-target taxon gDNA did produce hits, and these were recorded, and the assays were assessed to determine if those assay performance caveats were appropriate for the intended use. We routinely used eight technical replicates for the no template controls (NTCs), and all assays returned no observed detection within 50 cycles. The taxa tested and the results of the specificity testing were recorded in a technical bulletin for each assay (Figure 3 and File S2) to ensure that relevant performance criteria are consistently recorded and communicated between eDNA practitioners and end-users. The technical bulletin provides an accessible way for practitioners to provide information regarding the extent of validation of a given assay, irrespective of its origin, that can be provided in reports while supporting the ongoing process of assay validation. When some relevant species

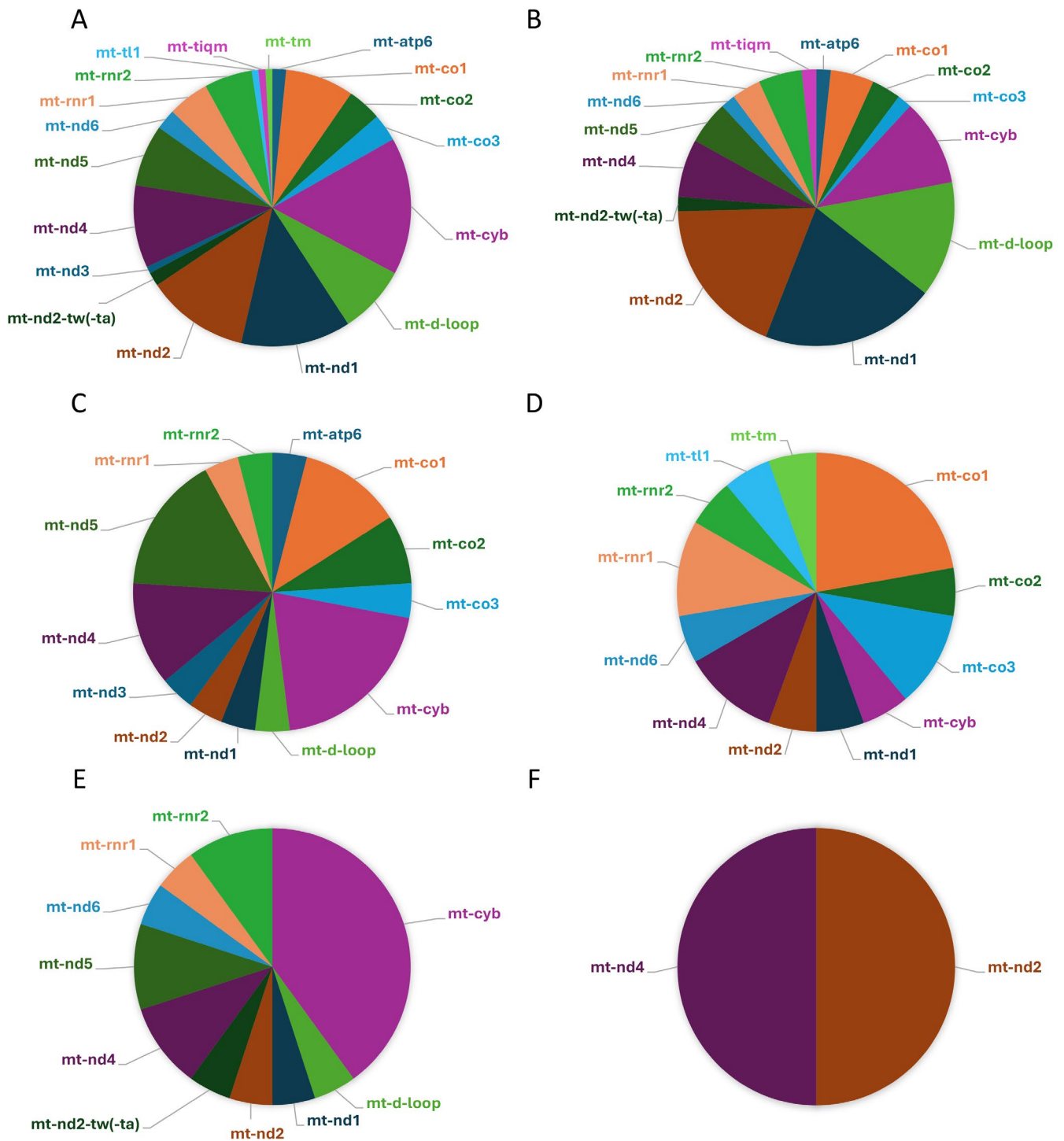


FIGURE 1 | Pie charts illustrating the identity and proportion of mitochondrial genes chosen for eDNA assay design for (A) all assays in the present work ($n = 125$), and by animal grouping (B) fish ($n = 59$), (C) mammal ($n = 25$), (D) invertebrate ($n = 18$), (E) herptile ($n = 21$), and (F) bird ($n = 2$). A major contributing factor to genic region choice was unbiased selection of unique sequences from whole mitogenome assemblies using *unikseq*. Gene name abbreviations: *Cl-23S*, chloroplast 23S ribosomal subunit; *mt-atp6*, mitochondrial ATP synthase membrane subunit 6; *mt-co1*, mitochondrial cytochrome c oxygenase 1; *mt-co2*, mitochondrial cytochrome c oxygenase 2; *mt-co3*, mitochondrial cytochrome c oxygenase 3; *mt-cyb*, mitochondrial cytochrome b; *mt-d-loop*, mitochondrial displacement loop; *mt-nd1*, NADH-ubiquinone oxidoreductase chain 1; *mt-nd2*, NADH-ubiquinone oxidoreductase chain 2; *mt-nd2-tw(-ta)*, NADH-ubiquinone oxidoreductase chain 2, tRNA-tryptophan, and tRNA-alanine; *mt-nd3*, NADH-ubiquinone oxidoreductase chain 3; *mt-nd4*, NADH-ubiquinone oxidoreductase chain 4; *mt-nd5*, NADH-ubiquinone oxidoreductase chain 5; *mt-nd6*, NADH-ubiquinone oxidoreductase chain 6; *mt-rnr1*, mitochondrial 12S ribosomal RNA; *mt-rnr2*, mitochondrial 16S ribosomal RNA; *mt-tl1*, mitochondrial tRNA-leucine 1; *mt-tiqm*, mitochondrial tRNA-isoleucine, tRNA-glutamine, and tRNA-methionine; *mt-tm*, mitochondrial tRNA-methionine.

TABLE 1 | Criteria and Beacon designer settings used for primer and probe design in the present study.

<p>Primer design</p> <ul style="list-style-type: none"> • Each primer should be between 18 and 23 nt long • Amplicon length between 80 and 400 bp with preference to <200 bp • 3' ends of primers should be selective for the species of interest • 3' end of primer should contain a C or G preferably in the two end positions to make a GC clamp • T_m target should be $59^{\circ}\text{C} \pm 5^{\circ}\text{C}$ • Maximum primer pair T_m mismatch 3°C • GC content target is 50% • No/minimal primer dimer or hairpin (max default cross dimer $\Delta G = 9$ kcal/mol) • Beacon Designer pair rating should be "Good" or higher <p>Probe design</p> <ul style="list-style-type: none"> • Probe areas are within the confines of the selected primer sequences • Probes should be between 25 and 30 nt long • 5' ends of probes should be selective for the species of interest • Probes CANNOT start with a "G" at the 5' end due to fluorophore dye chemistry constraints • The 5' end should start with a C then preferably followed by a C or G to create a GC clamp • T_a target should be $\sim 55^{\circ}\text{C}$ • GC content target should be $\sim 50\%$ • No/minimal hairpin [max default cross dimer $\Delta G = -6$ (internal) kcal/mol] • Beacon Designer pair rating should be "Good" or higher <p>Advanced design settings (primer set and probe combination)</p> <ul style="list-style-type: none"> • Hairpin maximum ΔG: -6.0 kcal/mol • Self-dimer maximum ΔG: -10.0 kcal/mol • Run/Repeat maximum length: 4 bp • Multiplexing maximum ΔG: -8.0 kcal/mol • Maximum bases between primer and probe: 20 • GC%: 30–80 • Probe should not have more G than C bases • Avoid G base at the 5' end of probe • 3' end maximum ΔG: -12.0 kcal/mol • G/C clamp – Consecutive G/Cs at the 3' end: 1 • Maximum ambiguous bases in amplicon: 0 • Maximum primer pair T_m mismatch: 3°C • Cross dimer maximum ΔG: -9.0 kcal/mol
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are absent from the bulletin, this should prompt the user to ensure that the assay is tested on voucher specimens of the missing species.

While the use of a hydrolysis probe provides an essential assay component to ensure specificity of a robust eDNA assay (Abbott et al. 2023), further verification of the detection of the correct DNA template can be accomplished through multiple means. In

some cases, if the amplicon was large enough (e.g., ~ 200 bp), we confirmed the amplicon sequence through Sanger sequencing. Additionally, we routinely performed melting curve analysis to establish that primer dimerization was not occurring, and agarose gel electrophoresis provided an indication regarding the number and sizes of bands generated during amplification. The checklists that we routinely used for phase 1 (SYBR) and phase 2 (Taqman) validation performance are provided in Table 3.

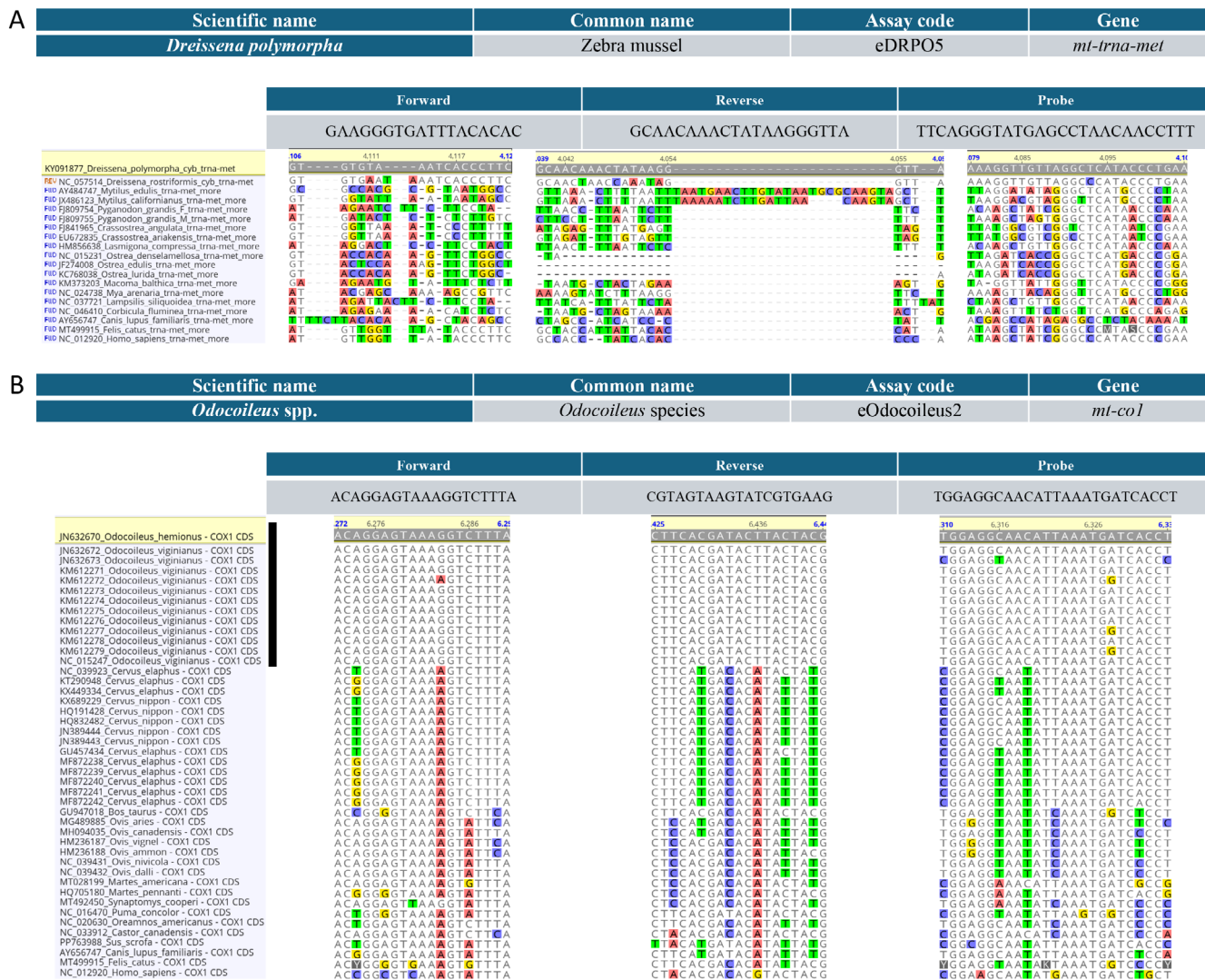


FIGURE 2 | Examples of forward primer, reverse primer, and probe alignments for the (A) eDRPO5 eDNA assay designed to detect *Dreissena polymorpha* DNA and (B) eOdocoileus2 eDNA assay designed to detect *Odocoileus* deer (e.g., mule and white-tailed deer) using Geneious. For these assays, the primers and probes are indicated below one target species' sequences (light yellow rectangle) and nucleotides that differ from this sequence are highlighted in color. For alignments of additional assays, refer to File S1.

TABLE 2 | Run parameters for each thermocycle for the described eDNA assays in the present work.

Thermocycle temperature sequence (°C)	Time sequence (s)	Cycles	MgCl ₂ (mM)	Reaction volume
95, 64 ^a , 72	15, 15, 30	50	3	2 µL input in 15 µL reaction

^aFive eDNA assays require a modified annealing temperature of 62°C. See Table 4 for details.

Given the complex and varied nature of environmental samples, we have elected to apply a high level of stringency to mitigate false positives. Stringency refers to the strictness with which Watson-Crick-Franklin base pairing is required for annealing to their complementary target DNA sequence. Stringency can be most easily influenced by annealing temperature and magnesium concentration, with lower annealing temperatures or higher magnesium concentrations each reducing stringency. We chose to routinely utilize a higher stringency annealing temperature of 64°C rather than the less stringent 60°C that is often the default recommendation and

keep the magnesium concentration constant (3 mM MgCl₂ (Table 2)), as temperature manipulation is easier to accomplish and the assays demonstrated strong performance at the higher annealing temperature (Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a). We also chose to avoid using minor groove binder (MGB) probes in preference to maximizing sequence selectivity within the assay, as MGB probes require shorter probe designs to maintain a T_m similar to regular probes (Kutyavin et al. 2000). This approach was successful for this research project (Table 4).

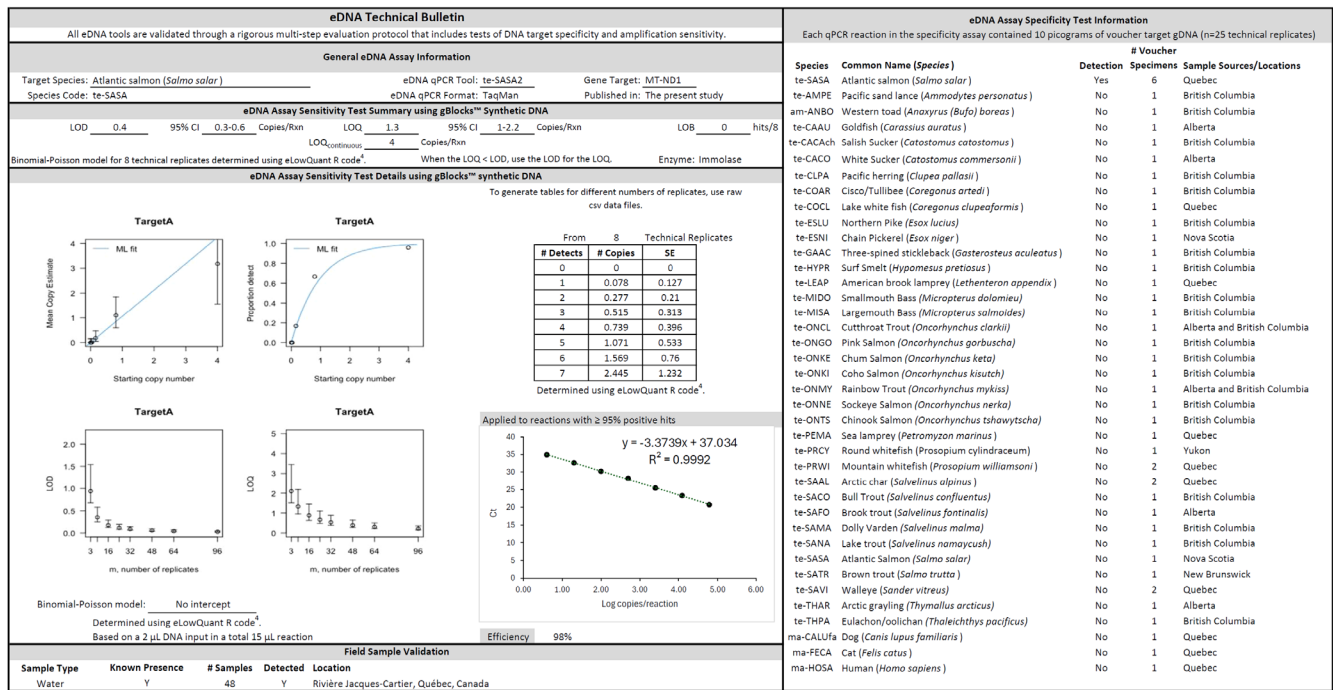


FIGURE 3 | Example of the eDNA assay sensitivity test details using synthetic double stranded DNA shown on a technical bulletin for the eSASA2 eDNA assay designed to detect *Salmo salar* DNA. Technical bulletins for additional assays in Table 4 are available in the File S2. CI, confidence interval; Cq, cycle threshold; LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantification; ML, maximum likelihood; MT-ND1, mitochondrial NADH dehydrogenase subunit 1; rxn, reaction.

TABLE 3 | Checklist for the phase 1 validation performance for primer pair candidates using SYBR chemistry and phase 2 validation performance for primer pair and probe candidates using Taqman chemistry.

Phase 1	Phase 2
<ul style="list-style-type: none">• Produces signal before 30 cycles for the target voucher specimen gDNA• Single tight melt peak• Single band on agarose gel of expected amplicon size• NTCs are negative• Human, dog, and cat gDNA are negative• Sympatric/confounder taxa gDNA are negative	<ul style="list-style-type: none">• 100% hits for target taxon gDNA across multiple individuals

Note: Each reaction contained 10 pg voucher specimen gDNA.

Despite this, we noticed that a few assays had consistency issues in amplifying 20 copies/reaction of the appropriate synthetic double-stranded synthetic DNA (e.g., gBlocks: IDT, Iowa, USA; Strings DNA fragments: ThermoFisher Scientific, Mississauga, Ontario, Canada). Twenty copies/reaction of double-stranded synthetic DNA were routinely used as a positive plate control (see Section 4 below). We performed an eight-point thermal gradient for annealing temperatures between 60°C and 64°C (specifically 60°C, 60.3°C, 60.8°C, 61.6°C, 62.6°C, 63.4°C, 63.8°C, and 64°C) on a Bio-Rad CFX96 Real-Time PCR Detection System to determine the role of annealing temperature on the inconsistent performance. To do this, we compared the performance of assays with consistency issues with a typically robust assay (e.g., eONMY5; Figure 4). For the robust assay, amplification success remained consistent across the eight tested annealing temperatures between 60°C and 64°C. However, it is

evident that the assays with inconsistent performance have difficulty producing amplicons at the 64°C annealing temperature despite the same template input amount (three examples are in Figure 4). Gradually reducing the annealing temperature reveals a common point of inflection at 62°C, under which lower annealing temperatures maintain the same strong amplification signal. Therefore, for those assays with inconsistent amplification at 64°C annealing temperature, reduction of the annealing temperature to 62°C enhanced assay performance (Table 4).

However, a reduction in stringency by decreasing the annealing temperature bears the increased risk of non-target DNA amplification. Thus, it is best that specificity is tested at the desired annealing temperature to identify any instances where a non-target taxon begins to show some hits. Generally, specificity was not compromised for the affected assays when the annealing

TABLE 4 | One hundred and twenty-five species- or animal group-specific validated qPCR assays from the iTrackDNA project.

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Accipiter gentilis</i>	Northern goshawk	eACGE3	<i>mt-nd4</i>	TGGTGACTTTTAGCAAC	GGTAGATCAGTTGAATAGTG	CTCTCCACCAACTA CAAAACCTGA	(Lopez et al. 2024)
<i>Acipenser fulvescens</i>	Lake sturgeon	eACFU1	<i>mt-nd1</i>	TGGCAATTTCACACTCTACAC	TTGGCGTATTACAGCTAAG	AACCTGGTCTCCGGCTTCAA CGTAGAATATG	The present study
<i>Acipenser medirostris</i>	Green sturgeon	eACME3	<i>mt-d-loop</i>	GGAACATTTATAACCTGCC	GAGTCATGTGAGGGTAAG	ATGGTACAATGACATATCC TTGACGCTA	The present study
<i>Acipenser transmontanus</i> *	White sturgeon	eACTR2 ^b	<i>mt-d-loop</i>	CTACCATTAAATGTTATACACC	CCCTCATCTCAITTAACITTA	CATATCTCCATGTTT GGCTCTTACTAC	The present study
<i>Acipenser transmontanus</i> *†	White sturgeon	eACTR4	<i>mt-d-loop</i>	GGTACAAATGACATATCCC	CCATGATTGTATGTGAA	AAACCAGGCTCTGT GAAACATCC	The present study
<i>Alces alces</i>	Moose	ma-eALAL4	<i>mt-nd3</i>	GGCACCATACTCACTATG	GGCATAAGAGAGAGGATATG	TATTAGCTGCAA GCCTAGCCTATGAAT	The present study
<i>Ambystoma macrodactylum</i>	Long toed salamander	eAMMA3	<i>mt-nd2</i>	CACCTCTCTTCACTATTAAAGTTTA	GGTTGTTTTGTGCTCAAA	CATACCTCAATTTCTCT TACAACCTTCACCCA	The present study
<i>Ambystoma mavortium</i> †	Western tiger salamander	eAMMV4	<i>mt-d-loop</i>	TATCTGCCACACGACTATTT	AATTGATGAATGAATCTTCG	CGCCACTGGTTAAA ATCTATGGAC	The present study
<i>Ammodytes personatus</i>	Pacific sand lance	eAMPE5	<i>mt-rnr2</i>	AGACGCCAAGACAGACC	CTGTTTCAATTAGAGCTGTAGC	CCATAAGGAGCCCA AACCATAATGGT	(Robinson et al. 2022)
<i>Anaxyrus (Bufo) boreas</i>	Western toad	eANBO5	<i>mt-nd1</i>	TACCCATCTCACTCTCTGAC	CTCAGATTCTCCTTCAGTAAGG	CAGACAAATTTCTTATGAA GTCACCCCTG	(Acharya-Patel, Groenwold, Allison, and Helbing 2024b ; Acharya-Patel, Groenwold, Lemay, et al. 2024a)
<i>Anguilla rostrata</i> [#]	American eel	eANRO1	<i>mt-cyb</i>	CATCTCAACTGCCTTCTCC	ATGGAGGAATAGGAGGTGA	CAACCTACTATCTG CCGTTCCATAC	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Anoplopoma fimbriatum</i>	Sablefish, black cod	eANF16	<i>mt-nd2</i>	AGCAGCAATACTTCTCTTT	CAAGGTGATTAAGGTGATG	ACTACTAATGCGTGACTAAC AGGACAATG	(Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a)
<i>Ascapshus montanus</i>	Rocky Mountain tailed frog	eASMO9	<i>mt-cyb</i>	ACTTTATTACGGCTCTTACTTGG	GTACGTTTCCGATGTAAGGGA	ATACGTATTACCATG AGGACAAATATC	(Veldhoen et al. 2016; Hobbs et al. 2019; Klymus et al. 2020; Lesperance et al. 2021)
<i>Ascapshus truei</i>	Pacific (coastal) tailed frog	eASTR4	<i>mt-cyb</i>	GAACATTGGCATTATCCTACTT	AGCGAAAAAATCGTGTAAAC	CGCTTTTGTAGGTAT GTGTTACCG	(Veldhoen et al. 2016; Hobbs et al. 2019; Klymus et al. 2020; Lesperance et al. 2021)
<i>Bison bison</i>	Bison	eBIB14	<i>mt-nd4</i>	AGCTATTTCTAATAATGTCAAC	GTATATACAGAGATATAGGG	CACTCCCATTAGAATAATT GTAATGTTAGA	(Lopez et al. 2024)
<i>Cancer productus</i> [†]	Red rock crab	eCAPR4	<i>mt-co1</i>	CGCGTATAAACAAACATAAGG	GGAGGGTAGACAGTTTCAG	TTCCAACACCTCTTTCTACT AATCCTCTT	The present study
<i>Canis lupus</i> ^{††}	Wolf	eCALU1	<i>mt-cyb</i>	CCTAGTATTCTCCATCCTAATC	GGCCGGAATATTATGCTG	TGGCAATTTCAT TCCACTCCTCCA	The present study
<i>Carassius auratus</i>	Goldfish	eCAAU5	<i>mt-cyb</i>	CTATACATTACACCTCAGACA	GAATTAGTCAGCCCGTAGT	TTCAACCGCATTC TCATCCGTTACC	The present study
<i>Carcharodon carcharias</i>	Great white shark	echCACA2	<i>mt-nd5</i>	CCGAGTAGGAGATATTGG	GGTAGGGTTAAGTTTATGTC	CTAATTTCTTAGCATAGC TTGACTGGCT	The present study
<i>Carcinus meanus</i>	European green crab	earCAMA1	<i>mt-co2</i>	ATGGCAACTTGATCTTATC	GCACATCATATAACCTACAA	CGGATTCCAGGATA GTGCTTCTC	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Castor canadensis</i>	Beaver	emaCACA3	<i>mt-col</i>	GCAGTCCTTTTACTACTC	CGAAGAAAAGTGGTGTTTA	CCTTACCACTCC TAGCAGCCG	The present study
<i>Catostomus catostomus</i>	Longnose sucker	eCACA2	<i>mt-cyb</i>	GTAATAATGACTGCCTTCGTAG	GAGGTGGATGATAGTTGCT	CTTATCCGCAGTTCCCTA TGTAGGA	The present study
<i>Catostomus commersonii</i>	White sucker	eCACO4	<i>mt-nd2</i>	CTAACTCTCCTTGCCCTA	TGGTAGTAACATATAATTGGACTT	TTCATAACATCAGCAGCATT CCTTACACT	The present study
<i>Catostomus macrocheilus</i>	Largescale sucker	eCAMA2	<i>mt-nd2</i>	AGAGCCCCATTATAGTCA	GCAGAATAAGTCACCTTAGGTA	ACTGCCCTTGGTCTTACTA TCTCTTGG	The present study
<i>Cervus</i> spp.	Cervus species	eCervus3	<i>mt-col</i>	ATATAA ACCTCTGTCTTTAGATT	ACGAATCAGTAGGGCTTAA	TACTCTGTATCTATTATTGG TGCCTGAGC	The present study
<i>Chelydra serpentina</i>	Snapping Turtle	eCHSE1	<i>mt-nd6</i>	CCACTAAAAATCAAAACGAATA	GAGCATGAAAGTGAGAAAG	CCATCACCATCAACT GTAGCAACAAC	The present study
<i>Clemmys guttata</i>	Spotted turtle	eCLGU3	<i>mt-cyb</i>	ACTTCTTACTACCGTTCAC	TCACTCAGGCTTGATATG	CCTAACAAATAACACCTGC TCTTCTTACA	The present study
<i>Clupea pallasii</i>	Pacific herring	eCLPA1	<i>mt-rnr2</i>	TTACTACTGAGCCATCCTA	CCGATCCAACCTTACACTT	AGTTCATTCTTCTTGTTACTC GTTCTAGCA	The present study
<i>Contia tenuis</i>	Sharp-tailed snake	eCOTE3	<i>mt-nd4</i>	CACATAGGCTTAGTCAATTGC	TTATTAGGCTGGTTAGGAGTC	CTCCTCAGCACT CTTCTGCTTAGCCAAACAC	(Mathias et al. 2021)
<i>Corbicula fluminea</i>	Asian clam	eCOFL13	<i>mt-rnr2</i>	GATCTGAAATGGTTTAAACGG	CCCCAATTAAAGGGCAAA	TGCTACCTTAGTAC GGTTTCAATCGC	The present study
<i>Coregonus artedii</i>	Cisco/Tullibee	eCOAR7	<i>mt-nd6</i>	CACCACAAATAGCGTTAG	GTAGCCCTAATATACTCTTTCA	CACACACCACCAACAGTCCC	(Lopez et al. 2023)
<i>Coregonus clupeaformis</i>	Lake whitefish	eCOCL1	<i>mt-nd2</i>	CATCAATTCTCTCATAGCA	ATTGGGTGGTTAATTGT	CCATTCTCCAACCAAGTCAA GCATTAGT	(Lopez et al. 2023)
<i>Coregonus zenithicus</i>	Shortjaw cisco	eCOZE1	<i>mt-col</i>	ACGGGCATTGTCTCTAGCAA	TGCTGTGAAGAGTGATCCGG	TCGATAGGGGGCTGTTTCGC	The present study
<i>Cottus cognatus</i>	Slimy sculpin	eCOCO3	<i>mt-nd1</i>	CTCCTGGCTCCAATTCTA	GGGCGAGAAATAACAAAATAC	ACCCTTGGCCCTCACTCTATGG	The present study
<i>Crassostrea gigas</i>	Pacific oyster	eCRGI2	<i>mt-tli</i>	CCAAGAAATTGGGCCTTTA	GCTGAATTAACTTTAAT AATTTAAG	CTAAGCCCAACACC AGTATTCTCATG	The present study
<i>Crotalus oreganus</i> [†]	Northern Pacific (western) rattlesnake	eCROR1	<i>mt-cyb</i>	TCCTCTATTACACACATCTCC	GGATGTGAAGTGAGGAGATAG	CATCTATTACGGCTC CTACCTAAAC	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Ctenopharyngodon idella</i>	Asian grass carp	eCTID1	<i>mt-nd4</i>	CCTACTCCAACAATCTACA	CTAGGAATGCGATTAAAGC	AATTCAATACTC ACAACCACACTACTCCTAGA	The present study
<i>Culaea inconstans</i>	Brook stickleback	eCUIN2	<i>mt-nd2</i>	CCTAGCTTACTCTTCAGTC	GTGGCTAGTGAATTAATAGTG	CCACCTCGGCTGAA TAACACTAAT	The present study
<i>Decamastus gracilis</i>	Decamastus	eDEGR2	<i>mt-nd4</i>	CGCCTATCATCCTATTCTG	CTCAACAACGACACCATC	CGAACACAGACACTA CACAGATACAC	(Acharya-Patel et al. 2025)
<i>Dreissena bugensis</i>	Quagga mussel	eDRBU5	<i>mt-rnr1</i>	CGGTTAAACCGATGTTCC	CTCCTATGCGGAGACTAC	TTTCTTTTCTTAACATACCG CCGTTGAC	The present study
<i>Dreissena polymorpha</i>	Zebra mussel	eDRPO5	<i>mt-tm</i>	GAAGGGTGATTACACAC	GCAACAACACTATAAGGGTTA	TTCAGGGTATGAGC CTAACAACCTTT	The present study
<i>Dreissena</i> spp.	Zebra/quagga mussel	eDRspp7	<i>mt-rnr1</i>	GCGGGGACTATGAACAAAAACG	GGGTAAATTGTCAACGGCGG	AAAAACCAAGAGG CTTGGCG	The present study
<i>Emydoidea blandingii</i>	Blanding's turtle	eEMBL4	<i>mt-nd5</i>	AGGCTTCATCAAAAATCTATA	CACAGGTAAGTTCTTTG	ATAACCTCATTCCT CCTAACACTACTACT	The present study
<i>Engraulis mordax</i>	Northern anchovy	eENMO2	<i>mt-co2</i>	AGGCATGATACTGTCTATTA	GAGAGTTGGAATAAAGCAC	TTATCGCCTTGTCAC TCTGGTCTCT	The present study
<i>Enhydra lutris</i>	Sea otter	eENLU6	<i>mt-cyb</i>	TTCATCATTTTCAGCACTG	GGATAGGATTAAGGCTAGTA	ATAATCCACCTCCTA TTCCTCCACGA	The present study
<i>Esox americanus</i>	American pickerel	eESAM2	<i>mt-d-loop</i>	CTAGGCGATTCTTAAATATCC	GTAGGGCTGAATTTAAACAA	ACCCCAAAACCA GGACTATCACTT	The present study
<i>Esox americanus vermiculatus</i>	Grass pickerel	eESAMve1	<i>mt-d-loop</i>	TGCTCTACTTGTATCTATACTA	GATGGATAATTGATGGGAAA	AGCTCGCATCAC TAACCTCAA	The present study
<i>Esox lucius</i>	Northern pike	eESLU1	<i>mt-cyb</i>	TCTCCACAGCCTTCTCATC	CCGCCTCAGATTCAATTGG	CTCCTCCTAAACAATAAATAC CGCCTTCGT	(Lopez et al. 2023)
<i>Esox niger</i> [†]	Chain pickerel	eESNI3	<i>mt-nd2</i>	ACAGGTGAATGAGATATTAAC	AGGTGGATAAAATAAGTCCA	CGTGACTTCCTCAATGA TTCTACTAGC	The present study
<i>Eubrachichius oregonus</i>	Oregon fairy shrimp	eEUOR1	<i>mt-col</i>	GGACTGGATGAACAGTAT	AATAGAAAGATACACCTGCTAA	CTCCACTATCTTC AGGTATTGCTCACG	The present study
<i>Exoglossum maxillingua</i>	Cutlip minnow	eEXMA1	<i>mt-nd5</i>	CACCTGTCATAACAATACC	GCTGATTGTCTAGAAATTAG	CTCTTATCGTGACTATTCTTG GACTTCTGA	The present study
Fish ^{#†}	Bony fish	eFish1	<i>mt-rnr1</i>	CACCTAGAGGAGCCTGTTCTA	CTACACCTCGACCTGACGTT	TATATACCRCCGTC GTCAGCTTACCC	(Klymus et al. 2020)

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Glycyera nana</i>	Glycyera	eGLNA3	<i>mt-nd6</i>	ACTGCTATTCAACCAAAC	GAAGCGTAATTGTTGTTATAG	TCCTGACGCGCTACAATCGCC	(Acharya-Patel et al. 2025)
<i>Glyptemys insculpta</i> *	Wood turtle	eGLIN1	<i>mt-cyb</i>	GCTCTTCTACACGAAACTG	GATGTATGTAGGGTGGGTATC	CTCATCTTAATATTAACCT TCCTACT	(Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a)
<i>Haliaeetus leucocephalus</i>	Bald eagle	eHALE3	<i>mt-nd2</i>	GGAGGATGAATAGGCTTA	GACATATAAGTAGAAGGTTAGAA	TAGACGATTAC TGCGGTTATCCATCC	(Lopez et al. 2024)
<i>Heteromastus filibranchus</i>	Heteromastus	eHEFI7	<i>mt-cyb</i>	CGGACTTCATGTAATTATCG	TATTCTTACTTCAGGCACATA	AACCTATTCCGA ACTTATTGCTGCCAT	(Acharya-Patel et al. 2025)
<i>Hiodon alosoides</i>	Goldeye	eHIAL6	<i>mt-nd2</i>	CCTGTACACTTCTGGATA	GTGTGAGATTTTGGCATAA	TACTACAAGGC TTAGACCTAACCCACAG	(Lopez et al. 2024)
<i>Hippoglossus stenolepis</i>	Pacific halibut	eHIST2	<i>mt-rnr2</i>	TTTCAAGCAAAAGAGAACCTA	GTGCATGAGTGGAGAAATC	CTTCTATTCAAATTCACAGG CAACCAGC	The present study
<i>Holbrookia species</i> [†]	Spot-tailed lizard	eHolbrookial	<i>mt-nd1</i>	CGCACACTACTACTTACA	GGTCTTACAGGAGCTAG	AACACTACGACATCACT ACTAATACTCC	The present study
<i>Hypomesus pretiosus</i>	Surf smelt	eHYPR4	<i>mt-col</i>	AGCTTCCGTAGATTTTGACC	CCGAAGAATCAGAAATAGATGC	CCATCTTGCAGGAATCTCC TCTATT	(Robinson et al. 2022)
IntegritE-DNA	Endogenous DNA	ePlant5	<i>cl-23S</i>	TCTAGGGATAACAGGCTGAT	TGAACCCAGCTCACGTAC	TTTGGCACCTCGATGTCGG	(Veldhoen et al. 2016; Hobbs et al. 2019)
<i>Lepus americanus</i>	Snowshoe hare	eLEAM1	<i>mt-nd5</i>	CCTCATTTCCTAATCTTATCTCA	GTTGGTTTAGTTCTATGGC	ATCCCTCAAAATAACC ATACCCCTTATACAT	(Lopez et al. 2024, 2025)
<i>Lithobates (Rana) catesbeiana</i>	North American bullfrog	eLICA5	<i>mt-nd5</i>	ATACACCGCACTATTACT	AAGAGGACTGATAGGTAAG	CCTTAACCTCAGCCTGACA ACTTATT	(Allison et al. 2023)
<i>Lithobates (Rana) piptiens</i>	Northern leopard frog	eLIP11	<i>mt-rnr1</i>	AGCTTACCATGTGAACGCTTT	TACTACTAAATCCACCTTCGGCT	CAATTGGCTACAATTTT TAATATAGAACA	(Klymus et al. 2020)
<i>Lota lota</i>	Burbot	eLOLO4	<i>mt-nd4</i>	CTTGCTGCTGTATTACTAA	ATAAACTATTTCCTTGGAGAG	ATGAGTCGTATTATACC ATAAACCGCCTAGT	(Allison et al. 2023)

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—I ² DT 5' FAM with ZEN quencher (5'-3')	References
<i>Metacarcinus gracilis</i> [#]	Graceful rock crab	eMEGR3	<i>mt-col</i>	ACTCCTGCTAAGTGAAG	CCTCCTTAAACATTACTCCTTA	ATCAACTGAGGCTCCAGCGT	The present study
<i>Metacarcinus magister</i>	Dungeness crab	eMEMA3	<i>mt-col</i>	CGGGTCATCTTTTATTAACTC	GAGAGAAAAATTAATAA TGAAGTAA	AGGAAACACCGGAGCCCTCTC	The present study
<i>Micropterus dolomieu</i>	Smallmouth bass	eMIDO1	<i>mt-cyb</i>	CAACTACGGCTGGTTAATC	AGCGTCAGAGTTCAATCCT	AACCTCCTCTCAGCC GTTCTCTTACATT	(Klymus et al. 2020)
<i>Micropterus salmoides</i>	Largemouth bass	eMISA2	<i>mt-col</i>	CCTACTCCTTCTGTCACTTC	TCATGGCTCAAACTATACCC	TCCTTACAGAC CGAAACCTCAACAC	(Klymus et al. 2020)
<i>Morone saxatilis</i>	Striped bass	eMOSA1	<i>mt-atp6</i>	CGCTTACTTGTCTCTTCAA	CAAGCATATTTAGG GTAATAAGAA	TTTACACAACAACT GCTTCTTCCTT	The present study
<i>Myotis</i> spp.	Myotis bats	eMyotis1	<i>mt-rnr1</i>	TTCGCCAGAGTACTACTA	GCTGATTAGCAAAGATTG	TCGATTATAGAA CAGGCTCCTCT	The present study
<i>Myotis lucifugus</i>	Little brown myotis	eMYLU4	<i>mt-nd5</i>	CCTACTCATCCGATTCTTAC	GGTGGTAAATTGCTCCTAG	AGAGCAATAAAATAAT CCAATCCTTAGCCC	The present study
<i>Myotis septentrionalis</i>	Northern myotis	eMYSE3	<i>mt-co2</i>	CGAACTAACAAATCCGTATG	CAGTGTGTCTGTGATTAGG	TATTGCACTCCTGA GCTGTTCCTTC	The present study
<i>Myotis yumanensis</i>	Yuma myotis	eMYU7	<i>mt-nd4</i>	ATCGCATACTCATCTGTTAG	CCATGAAACTTCAGGGAC	TGAATTAAGACAGCC ATAATCACAAAGCG	The present study
<i>Odocoileus</i> spp.*†	<i>Odocoileus</i> species	eOdocoileus2	<i>mt-col</i>	ACAGGAGTAAAGGTCTTTA	CGTAGTAAAGTATCGTGAAG	TGGAGGCAACATTA AATGATCACCT	The present study
<i>Oncorhynchus clarkii</i> [‡]	Cutthroat trout	eONCL4	<i>mt-nd1</i>	TTGCACATATCCAGCCTAGCT	GGCTGGTACGAGCAGTCAA	TACAGGAGGATTTACTC TTCAAACCTTCAA	The present study
<i>Oncorhynchus gorbuscha</i>	Pink salmon	eONGO5	<i>mt-nd1</i>	CAAGCCTGGCAGTATATTC	ACCAGTAGTCAGATGCTTTCC	CATTATCACAGGAGGA TTCACCTCTCAAAAC	(Su et al. 2023)
<i>Oncorhynchus keta</i>	Chum salmon	eONKE9	<i>mt-nd4</i>	GGGAAATGGCTATGAACC	CGATCACTTAAGTCAGGTTA	TCAAAGTCTAATTATC GCCCTAGCAAGT	The present study
<i>Oncorhynchus kisutch</i>	Coho salmon	eONKI4	<i>mt-nd1</i>	CCCTTTTGACCTCACAGAG	GGTTCAGGGCAGTTAGTTCT	TGCCCTCTTTTTCCTA GCCGAGTACG	(Klymus et al. 2020; Lesperance et al. 2021; Hocking et al. 2022)
<i>Oncorhynchus mykiss</i>	Rainbow (steelhead) trout	eONMY5	<i>mt-nd2</i>	GTCTCTCCCTGTATATCG	GAGTATTGATGGTTAAAGAGT	TGACATCTTCAGCC TTCTCTCACAT	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—I ² DT 5' FAM with ZEN quencher (5'-3')	References
<i>Oncorhynchus nerka</i>	Sockeye salmon	eONNE2	<i>mt-cyb</i>	TGAGGACAAATATACCTTCTGG	GCAATGACGAAAGGGAAC	TCGTACGTCGGGGGGCGCC	(Su et al. 2023)
<i>Oncorhynchus tshawytscha</i>	Chinook salmon	eONTS5	<i>mt-nd1</i>	ACTATTTATTAAAGAACCCGTC	GCTCAGCCTGATCCCAA	TCCTGTTACAGATCTTAACCTAGGGGTAC	(Su et al. 2023)
<i>Ophelina acuminata</i>	Ophelina	eOPAC1	<i>mt-nd2</i>	CAACCATATAGGATGAATACTA	GAGGATAAGGATTGAGGA	TCGTATCCGCCCATTCGCTA	(Acharya-Patel et al. 2025)
<i>Oreannos americanus</i> [#]	Mountain goat	eORAM2	<i>mt-co3</i>	CCTTCACAATCTCAGATG	CAGACGATTAAAGAAAGGTG	TTCTTCGTAGCCA CAGGATTCCA	The present study
<i>Ostrea lurida</i>	Olympia oyster	eOSLU2	<i>mt-co3</i>	CAGTCTTCTAATTTTACGCATA	TTCATCCATACACAACCATTA	CACCTTCTCATATAC TTCTCTCGTTCTCCT	The present study
<i>Ovis canadensis</i>	Bighorn sheep	eOVCA1	<i>mt-d-loop</i>	TCTGCTAAAACTTCCAAAC	ACGTGTTTCATACATTCAAG	TTCCACTCCACA AGCTCACAC	The present study
<i>Pantherophis vulpinus</i> (<i>P. gloydi</i>)	Eastern foxsnake	ePAVU5	<i>mt-nd4</i>	AAACCCGAATTATAATCCTTAC	TTGGACACCAATTAATAATAGTG	CTGCGGATACTA ACAACCTGATGG	The present study
<i>Parelaphostrongylus tenuis</i>	Brainworm	ePATE1	<i>mt-col</i>	GGGACTAGATGAACTGTG	CATAAAATTAATACCGCCGAG	TCTTCTACCAACATGA CCTAAAGTACTCAA	The present study
<i>Pekania pennanti</i>	Fisher	ePEPE2	<i>mt-cyb</i>	CCTACTTCACACCTCAAA	GCCAAATGATAATGAATGG	TTCCACTGGTT GTCCTCCGATT	The present study
<i>Perca flavescens</i>	Yellow perch	ePEFL1	<i>mt-nd5</i>	CTCCCATGTTAAAACTGC	TCAGGTTTCAGGAAGTTAC	TTCTTAGTCAGCC TTCTTCCGTTATT	The present study
<i>Percopsis omiscomaycus</i>	Trout Perch	ePEOM6	<i>mt-nd2</i>	CCTACTTACATACTTTCATCATAA	GGATATTGTTGTAGAGATTGT	TTCAAGACCAACAACA CAACAAGCATTA	The present study
<i>Pimephales promelas</i>	Fathead minnow	ePIPR2	<i>mt-nd2</i>	GAGGCGGATTAAACCAAA	GCAGATCTTATTAGGATGTATG	ATTCTGGCTTACT CCTCAATCGC	The present study
<i>Platygobio gracilis</i>	Flathead chub	ePLGR2	<i>mt-nd1</i>	GCCTATATTGTCCCAGTC	CCAGGTTAAGGTTAATGAC	TCTTAGCAGTAGC CTTCTTGACCTTG	The present study
<i>Prionospio (Minuspio) lighti</i>	Prionospio 2	ePRLI4	<i>mt-nd4</i>	TATACTTCAACGGCAACA	ATCTGACCCTGTAAATTCG	TTGCTAAGGCTAA TGCTGCTTCACA	(Acharya-Patel et al. 2025)
<i>Prosopium williamsoni</i>	Mountain whitefish	ePRWI4	<i>mt-nd1</i>	CAGAGTACTCCAACATCC	GAGGTTTATTGCCGTGAG	TACACTCTCGACCA TCCTCTTCCCTAG	The present study
<i>Pseudacris</i> spp. [#]	Boreal and spotted chorus frogs	ePSpp2	<i>mt-rnr2</i>	GAAAGCGGGGATAAACTTA	TGTGGAGGTTTATTTTACTC	ACGAGAAAGACCCTAT GGAGCTTTAAACTA	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Ptychocheilus oregonensis</i>	Northern pikeminnow	ePTOR1	<i>mt-nd1</i>	CCCTTTGCTTTATTCTTC	CGTTGTTAATTCTGGTATAC	CCCTATAAATAACA CGGGCGGACAG	The present study
<i>Puma concolor</i>	Cougar	ma-ePUCO13	<i>mt-co2</i>	GCCATCATCCTAATTCTC	GGGATTATGTAGGAGTCAAAG	AAGCTACGAGTACACT GACTACGAAGA	The present study
<i>Rana aurora</i>	Northern red-legged frog	eRAAU1	<i>mt-rnr2</i>	TGAAGAAGCGGGAATCAAA	GCATACAGATTCTTGTGTGTG	TAAACTCATCATAC ACCTCTGTGCTC	(Klymus et al. 2020; Lesperance et al. 2021)
<i>Rana cascadae</i>	Cascades frog	eRACA2	<i>mt-cyb</i>	CCTAATTGCCCAATCGCT	CTCAAAATGACATCTGGCCC	TGGCTGGTCCCTTC GTAATTTACATG	(Klymus et al. 2020; Lesperance et al. 2021)
<i>Rana luteiventris</i> [†]	Columbia spotted frog	eRALU2	<i>mt-nd1</i>	TTCTCTACCAATCCCCTAT	CCAGGAAAAACAGTGCATAAG	CAGCCTAACCGTTT ACACAAATTTTGGG	(Klymus et al. 2020; Lesperance et al. 2021)
<i>Rana pretiosa</i> [†]	Oregon spotted frog	eRAPR2	<i>mt-cyb</i>	GTAACCTCCATGCTAACGGT	CTATCACTAGGAATAGGAGGATC	TTTCCACATCGGC CGAGGCCTC	(Klymus et al. 2020; Lesperance et al. 2021)
<i>Rangifer tarandus</i>	Reindeer	ma-eRATA3	<i>mt-nd5</i>	CTGAGCCCTCTTAATAACA	GCGAATAGACTTCCGATTA	ATTGCCACCTCCTTCACAGC	The present study
<i>Rhinichthys cataractae</i>	Longnose (nooksack) dace	eRHCA4	<i>mt-nd4</i>	TAGCCAAAACCACATCAA	AGCCATGATTAGGAAAAGC	CGGCAACGTCTTT ACCTCACAC	The present study
<i>Rhinichthys umatilla</i> [#]	Umatilla dace	eRHUM4	<i>mt-d-loop</i>	GTGTAGCATTACATGCGTATAG	TTATCCGAGGGTGGAGTC	CACACTGTGAACATAT TCCTTGCATC	The present study
<i>Salmonidae</i> [†]	All Salmonids	te-Salmo2	<i>mt-itqm</i>	CCCTCAATTCTAGAGAGA	CTGACCCTTACTAGAAAAGTG	TCGAACCCCATCCT CAAGAGATCAAA	The present study
<i>Salmo salar</i>	Atlantic salmon	te-eSASA2	<i>mt-nd1</i>	CCCATACTTGCCCTTACG	CGCTGAGTAAAGATTAGTCCAA	CCCCATGCCCATCC CTTACCCCATTA	The present study
<i>Salvelinus alpinus</i> [†]	Arctic char	eSAAL1	<i>mt-co2</i>	TCCGAATCCTATATCTCATG	TCGTAATCGGTGACTCG	CGCATCTTACTATTAA AGCAATGGGCCACC	The present study
<i>Salvelinus fontinalis</i>	Brook trout	eSAFO6	<i>mt-co3</i>	GCATCATAGAAGGAGAGC	GGCTTCGTATACTCCAT	CGTCCAAGCTCTTA CTCTCACCATC	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Salvelinus namaycush</i>	Lake trout	eSANA1	<i>mt-nd2</i>	GGGCTTATCCTGTGTCTACATG	CCCAGGGATAGAAAGCACTA	TGACTCTTCCTTA ATTATCGCATTA	(Allison et al. 2023)
<i>Sander canadensis</i>	Sauger	eSACA1	<i>mt-nd2-tw</i>	CTCTCTCTCAGCCGATAG	AGCCTAAGTCTCTTTAAAG	ATTCTTCTACTACCA ATAACTCCAGCCATT	The present study
<i>Sander vitreus</i>	Walleye	eSAV12	<i>mt-nd1</i>	CTCGGGATCTTGTTTCTA	CTGATACTAATTCCGGATTCTG	CCTATCAAGCCTAG CAGTCTACTCTATTCT	(Lopez et al. 2023)
<i>Sebastes maliger</i>	Quillback rockfish	eSEMA3	<i>mt-d-loop</i>	CGAAGGTATTACATAAAGCA	GAGTGTTTGTGTTAGGTCCTTA	CCAACAATCATTTA TAAGGACTGAGCGAAT	(Allison et al. 2023; Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a)
<i>Sebastes paucispinus</i>	Boccacio rockfish	eSEPA9	<i>mt-d-loop</i>	CCAGTAAACAATACAAAACTAC	CCTAGAAATTAATAATTCAGCTTG	TACCCACCACTT TCTCCAACATGCTA	(Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a)
<i>Sebastes ruberrimus</i>	Yelloweye rockfish	eSERU5	<i>mt-d-loop</i>	CTACCAGTCCACTCATAA	TGGGATTTCATATTCTACTTG	AACATAAGATATA CGCCCATCAACCC	(Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a)
<i>Sorex bendirii</i>	Pacific water/marsh shrew	eSOBE7	<i>mt-nd4</i>	CCCCTTATACTCATAGCT	CGAGTGATAATAATTAAGGTTG	ATCACCTAATAC ACGAGACAGCGAC	The present study
<i>Sorex cinereus</i>	Masked shrew	eSOC11	<i>mt-rnr2</i>	TACATTCTCTAATCCTTAATCC	GTTATTGATCTGACATAAGC	CACATTAATCCTA ACCCACAACCTGGACTA	The present study

(Continues)

TABLE 4 | (Continued)

Scientific name	Common name	Assay code	Gene ^a	Forward sequence (5'-3')	Reverse sequence (5'-3')	Probe sequence—IDT 5' FAM with ZEN quencher (5'-3')	References
<i>Sorex fumeus</i>	Smoky shrew	eSOFU1	<i>mt-nd1</i>	CCAAGAATACTCATGATTACTG	GAAATGCACCTAGAAATAGAAC	ACGTGAATAT GCCGCAGGACCAATT	The present study
<i>Sus scrofa</i> ^{#†}	Wild boar	eSUSC1	<i>mt-cyb</i>	ACCAGAAATGATATTCTTATTTCG	AGGCTATTAGAGCTAGCA	CTACGGCTATCCTAC GTTCAATTCCCTAATAA	The present study
<i>Synaptomys cooperi</i>	Southern bog lemming	eSYCO1	<i>mt-nd2</i>	TAAACAGGACCTTTAATCACC	TGTTTCATAGTCCTAGTTGC	AACCTACCCTAACCC CACGCTCTACAGAAG	The present study
<i>Tadarida brasiliensis</i>	Brazilian free-tailed bat	eTABR1	<i>mt-cyb</i>	GCTGATTACTACGCTACCTAC	GTGATGACTGTGGCTCCT	CTATTACGGAT CTTACACCTACACA	The present study
<i>Thalichthys pacificus</i>	Eulachon/Olrichan	eTHPA6	<i>mt-nd1</i>	CTTCCTATTCTCGCTAC	AGAACAATAAGATCCCTAGA	CATACTAGCCCT GACACTTGCCCTC	The present study
<i>Thamnophis sauritus</i>	Eastern ribbonsnake	eTHSA2	<i>mt-cyb</i>	AACTCGGAGGAACAATCG	CAATCAAGGTGAATGGTGC	CAACTCACATT CTGACTCCCTAATCG	The present study
<i>Thymallus arcticus</i>	Arctic grayling	eTHAR1	<i>mt-rnr1</i>	GAGAGGCCCAAGTTGATAGT	ACCCATTTTGCTTACTATATGA	CCCTGAAAC CCACGACAGCTATG	The present study
<i>Thysanocardia nigra</i> [*]	Segmented marine worm	eTHNI3	<i>mt-nd1</i>	ACCCTCAACATATCCTTTA	CGGAGATTAGTCTGAGAA	CACATTCTCTTCT TATTCTCTCTTCTTCT	(Acharya-Patel et al. 2025)
<i>Ursus americanus</i>	American black bear	eURAM1	<i>mt-atp6</i>	GCCTATTCAATCCAAACCTA	GCTGTAATGGTGCTAATAC	CGACTAACCG CTAACATTACTGC	(Lopez et al. 2025)
<i>Ursus arctos</i>	Grizzly bear	eURAR2	<i>mt-nd5</i>	CCACAATATTACACCCCACTA	ATGCCAGGATAAAACCTAA	CCCACAAACAACCAT ACCCTACTATCTAA	The present study

Note: Amplicon size and sequence information are in Table S1. ^aRequires a modified annealing temperature of 62°C. Some specificity limitations have been identified: [#]Refer to alignments in File S1 and [†]Refer to the technical bulletin in File S2 for details.

^{*}See Figure 1 legend for gene abbreviations.

^bThe eACTR4 assay is the better overall performing assay. eACTR2 is shown for comparative purposes only and is not recommended for use.

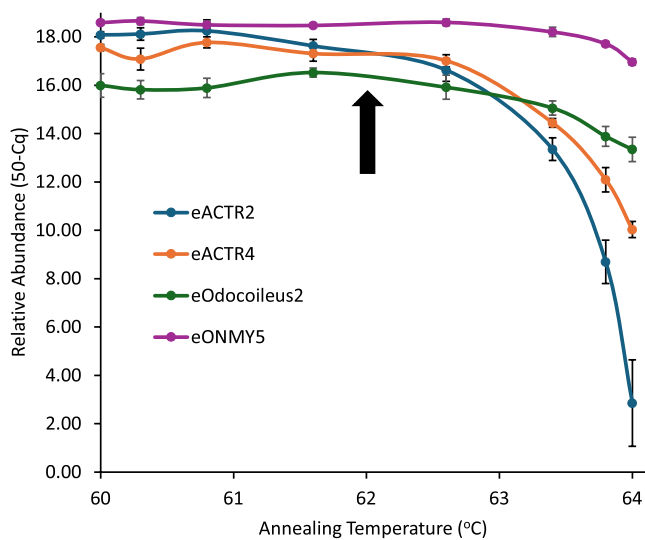


FIGURE 4 | Example of the impact of annealing temperature on eDNA assay amplification performance. Twenty copies/reaction of double stranded synthetic DNA appropriate for the indicated assays were used as a template. Eight annealing temperatures were simultaneously tested between 60°C and 64°C on an example of a robust assay (eONMY5, *Oncorhynchus mykiss*, $n=4$ technical replicates) and three assays that performed inconsistently at 64°C: eACTR2 (*Acipenser transmontanus*; old eDNA assay design), eACTR4 (*Acipenser transmontanus*), and eOdocoileus2 (*Odocoileus* spp. deer) assays ($n=4$ technical replicates). The robust assay performs equally well at all annealing temperatures (purple horizontal arrow). Gradually reducing the annealing temperature reveals a point of inflection at 62°C (black vertical arrow) where lower annealing temperatures maintain a consistent strong signal. The circles depict the mean relative abundance expressed as 50 minus the measured Cq value (“50 – Cq”) where a higher value indicates higher abundance. The whiskers depict the standard error of the mean.

temperature was reduced to 62°C. However, there was a notable exception—the eOdocoileus2 assay, which was designed to detect *Odocoileus* deer (e.g., mule and white-tailed deer) but exclude *Cervus* deer (e.g., elk and red deer). This was a challenging assay to design due to the high sequence identity of the mitogenomes between groups. The best DNA sequence regions were selected through *unikseq* and primer/probe designs were created to best satisfy the criteria of a high-performing qPCR assay. However, there was a compromise, and examination of primers and probe alignments indicates that the 3' ends of the forward and reverse primers have sequence identity of seven and five nucleotides with *Cervus* sequences, respectively (Figure 2). While its amplification efficiency using a synthetic double-stranded DNA template increases from 81% to 85% to 97% for annealing temperatures of 64°C, 63°C, and 62°C, respectively, the propensity to hit closely related non-target sequences also increases at the lower annealing temperatures compared to 64°C (Figure 5). Thus, in this case, a trade-off between these performance parameters needs to be made to enhance the confidence in the use of the eDNA assay, taking into consideration the intended study location. Therefore, balancing off-target amplification with amplification efficiency, the recommended annealing temperature is 62°C for the eOdocoileus2 assay if elk are not abundant in the study region.

Despite the extensive efforts to generate/use full mitogenome sequences for assay design and empirically validate assay

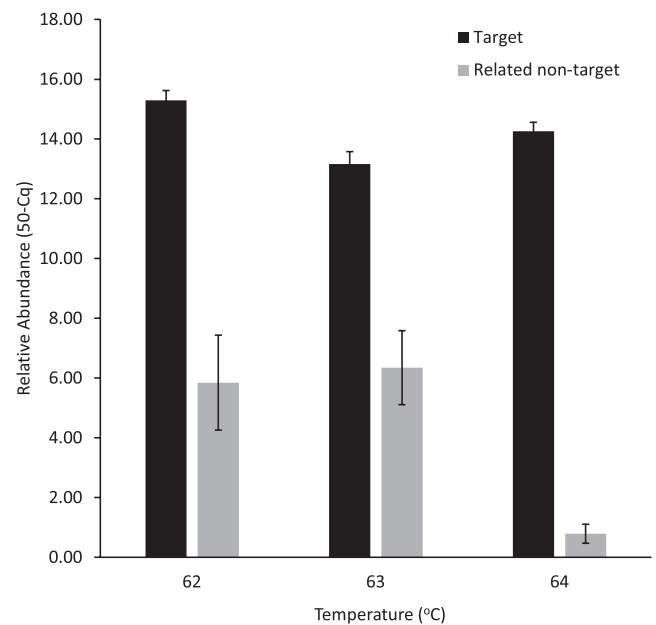


FIGURE 5 | Impact of annealing temperature on assay specificity. The eOdocoileus2 eDNA assay was run with the indicated annealing temperatures (62°C, 63°C, and 64°C) using 10 pg/reaction gDNA from white-tailed deer (*Odocoileus virginianus*; “Target”; $n=6$ technical replicates) or elk (*Cervus canadensis*; “Related non-target”; $n=16$ technical replicates). The mean relative abundance is depicted as 50-Cq where a higher value indicates higher abundance. The whiskers depict the standard error of the mean.

specificity performance in vitro with voucher specimens, there are still gaps in these important resources. In a few cases, we were unable to distinguish between closely related co-occurring species through sequence alignment and/or validation with voucher specimen DNA. These assays are marked in Table 4. This was the case for the white sturgeon (eACTR4), Western tiger salamander (eAMMV4), American eel (eANRO1), wolf (eCALU1), red rock crab (eCAPR4), chain pickerel (eESNI3), spot-tailed lizards (eHolbrookia2), graceful rock crab (eMEGR3), cutthroat trout (eONCL4), mountain goat (eORAM2), chorus frogs (ePSpp2), umatilla dace (eRHUM4), Arctic char (eSAAL1), wild boar (eSUSC1), and Oregon spotted frog (eRAPR2). In those cases, the user should determine if the assay is appropriate within their desired survey context.

In some cases, the use of a combination of assays can provide valuable insight. For example, assays to detect DNA from the at-risk Oregon spotted frog (eRAPR2) had to be designed with very limited sequence resources. While the assay had many strong characteristics, we were unable to differentiate between DNA from the Oregon spotted frog and the least concern Columbia spotted frog. Fortunately, the Columbia spotted frog assay (eRALU2) only detects its desired target and not Oregon spotted frog DNA, so a combination of the two assays' results still enables inference on whether Oregon spotted frog occupancy has occurred.

One surprise was discovering that the eFish1 assay can detect some but not all amphibians (e.g., tailed frogs, some salamanders, and toads; see Files S1 and S2). This may reveal some interesting evolutionary relationships that were not apparent

before. Extensive attempts at assay redesigns have not yielded a better-performing assay. As full mitogenome resources increase, especially for amphibians, it may be possible to identify another suitable region that can distinguish bony fish DNA from other animal groups. Despite the shortcomings of the eFish1 assay, it remains a powerful tool for eDNA surveys. In many cases, gradually filling in the gaps for missing mitogenome sequences and better access to voucher specimens will enable effective assay redesign efforts to address these issues. However, in some cases, even full mitogenome sequences may not be sufficient to create an assay that is sufficiently robust, specific, and sensitive. In these situations, assay designs using nuclear genomes may provide a possible solution.

2.2 | Sensitivity and Quantitative Assay Performance

Sensitivity refers to the minimum number of copies in a sample that can be measured accurately in the assay (Bustin et al. 2009). It is expressed as a limit of detection (LOD; Klymus et al. 2020; Lesperance et al. 2021), the concentration that can be detected with reasonable certainty (a 95% confidence interval is usually used; (Burns and Valdivia 2008)). Standard curves generated with synthetic double-stranded DNA can reveal the assay's sensitivity, allow the calculation of the LOD and limit of quantification (LOQ), and allow for the estimation of DNA copy numbers within a sample. These measures and associated 95% confidence intervals for an eDNA assay based on targeted qPCR are expressed in the number of copies per reaction. To generate standard curves, the CSA performance criteria require a serial dilution "of at least eight concentrations of a defined template, of which five concentrations have a detection rate of $\geq 95\%$ and three have a detection rate of $< 95\%$ " (Abbott et al. 2023). We routinely perform a 10-point serial dilution of synthetic double stranded DNA template ranging from 62,000 to 0.032 copies/reaction, exceeding the CSA standard, as we found that this approach aptly provided the desired data coverage needed depending upon assay performance. Technical replication differed depending upon the number of copies, with higher technical replication at the lower concentrations ($n=8$ for 62,000–500 copies/reaction; $n=24$ for 100–0.032 copies/reaction) to ensure greater confidence in the estimation of assay performance characteristics. Consistent with the CSA standard, we also had 24

NTC technical replicates that all showed no detection (limit of blank (LOB)=0).

While the LODs can be calculated by multiple approaches with similar results (Klymus et al. 2020; Lesperance et al. 2021), one of the robust methods for calculating the LOQ, eLowQuant, utilizes an adapted binomial-Poisson distribution (Lesperance et al. 2021). We therefore used eLowQuant to calculate both LOD and LOQ as assay performance indicators using synthetic double-stranded DNA that contains the amplicon sequence (Table S1) (Klymus et al. 2017). We chose synthetic DNA over plasmid or PCR amplicons that are also permissible according to the CSA standard, as reproducibility between batches and labs tends to be better. It should be noted that gDNA is not desirable for generating standard curves, as mitogenome copy number can vary widely between tissue types, physiological states, and individuals (Garrido-Sanz et al. 2022).

With the generated data set, two standard curves are produced: a log linear curve with continuous data for the higher DNA concentrations and a maximum likelihood curve derived from the binomial data for low DNA concentrations (Lesperance et al. 2021) (Figure 3). A $LOQ_{\text{continuous}}$ defines the transition point between the two standard curve types. These, along with the LOD and LOQ, were determined and recorded along with 95% confidence intervals in the technical bulletins (File S2), satisfying the reporting criteria of the CSA standard (Gagné et al. 2021). We generated separate technical bulletins for the same assay when different enzymes (Qiacity or Immolase) were tested. We generally found highly comparable results for these sensitivity parameters (File S2) (Acharya-Patel, Groenwold, Allison, and Helbing 2024b; Acharya-Patel, Groenwold, Lemay, et al. 2024a). Overall, for the 125 assays presented herein, the median LOD was 0.4 copies/reaction, $LOQ=1.7$ copies/reaction, and the $LOQ_{\text{continuous}}$ was 4 copies/reaction ($n=125$) (Table 5). All CSA performance criteria were met or exceeded apart from five assays that were slightly below the 80% efficiency cut-off for continuous data (Table 5).

3 | Reporting Data

Specific considerations are required when determining the performance of assays used to detect or quantify eDNA contained within complex matrices. eDNA assay data may be reported as "detected"

TABLE 5 | Performance summary of the 125 eDNA assays.

Category	LOD ^a	LOQ ^b	$LOQ_{\text{continuous}}$ ^c	Efficiency ^d	R^2 value ^e
Median	0.4 (0.3–0.8)	1.7 (1.2–2.9)	4	93%	0.9987
Maximum	5.1	19.4	100	110%	1.0000
Minimum	0	0.09	0.8	71%	0.9827

Note: The 95% confidence intervals for the LOD and LOQ are shown in brackets. Individual details are recorded in the technical bulletin for each assay (File S2).

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; $LOQ_{\text{continuous}}$, limit of quantification for continuous data.

^aAll 125 assays exceed the CSA requirement of having a $LOD \leq 10$ copies/reaction.

^bAll 125 assays exceed the CSA requirement of having a $LOQ \leq 20$ copies/reaction.

^cAll 125 assays met or exceeded the CSA requirement of having a $LOQ_{\text{continuous}} \leq 100$ copies/reaction.

^dEfficiency is calculated for continuous data only and is required to be between 80% and 110% to meet the CSA standard. All but five assays satisfied this requirement. eCAAUS, eEMBL4, eSalmo2, eSANA1, and eSERU5 assays, while meeting or exceeding all other performance criteria, had amplification efficiencies $< 80\%$. See technical bulletins in File S2 for details.

^eAll 125 assays exceeded the required R^2 of the continuous data standard of ≥ 0.95 .

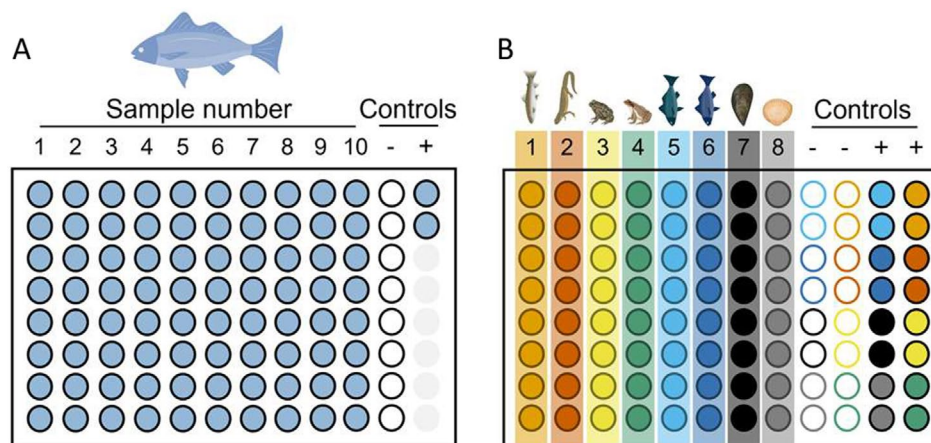


FIGURE 6 | Examples of sample plate set-ups for eDNA detection of (A) single species from multiple environmental samples or (B) multiple species from a single sample. The different colors in the plate wells represent different eDNA assays used on a plate. Eight technical replicates are run in each column per sample in A or per target species in B. A minimum of two NTCs per assay per plate (“–”) and a minimum of two replicates of 20 copies/reaction of positive controls (synthetic DNA, gDNA or PCR amplicon; “+”) are run on each plate. Animal images created with Biorender with permission.

or “not detected” to generate qualitative results. For quantitative results, we calculated copy number from Cq values when the frequency of detection was $\geq 95\%$ of the technical replicates using the linear regression equation of the continuous standard curve (Figure 3 and File S2). For samples with $< 95\%$ detections in a given number of technical replicates (e.g., $n=8$, see rationale in Section 4), we calculated a copy number estimate with eLowQuant (Lesperance et al. 2021) along with an estimated standard error (Figure 3 and File S2). Functional examples of applying these estimation approaches on field samples can be found in the references presented in Table 4. The higher the number of technical replicates, the smaller the 95% confidence intervals and standard error estimates and the higher the chances of detection (Lesperance et al. 2021). This becomes very important to end-users if they wish to have a high confidence that an at-risk or invasive species may not be present at a given site. There are different ways to express the sample analysis results depending upon the matrix used [e.g., copies/L (water), copies/g (sediment or soil), or copies per sample (surface swabs)]. Individual assay results from field sample replicates are reported to facilitate data interpretation.

4 | Routine Testing of Environmental Samples

As indicated by Thalinger et al. 2021 and in the CSA standard (Abbott et al. 2023), the highest validation level includes testing on field samples. We note that the procedure can be done in situ against well-characterized environmental samples. Moreover, eDNA samples from actual field sites known to host the target species with great abundance were tested to assess the false negative rates for many assays presented herein. Field validation is an iterative process that may require expansion depending upon survey needs, and, in some cases, finding an appropriate site may be difficult. In the same vein, field sites where the targeted taxon is known to be absent should also be harvested to establish associated false positive rates. This field validation completes the robustness of the targeted qPCR-based assay by demonstrating that it is possible to detect the target taxon DNA from field samples (Abbott et al. 2023). Ideally, field validation

is incorporated within an eDNA study, wherever possible, to account for variable environmental sample and population effects. We have reported those instances where the assays have been field validated in the technical bulletins (Figure 3 and File S2), and field validation is ongoing.

Finally, the CSA standards address approaches to take to identify false negatives and positives (Gagné et al. 2021; Abbott et al. 2023). False negatives can come from failure to detect targeted species that are present on the site or because of the poor quality of the samples. False positives can come from contamination or incorrect analytical detection of non-targeted species (Rees et al. 2015; Evans et al. 2017). To avoid false positive (type 1) errors, we include field blanks in the sampling sets (e.g., one per sampling day) that are randomized along with other samples prior to sample processing (Figure 6). The number of technical replicates used for field surveys can vary from lab to lab as there may be different needs and capacities, but the minimum number of technical replicates required by the CSA standard is $n=3$ (Abbott et al. 2023). However, it is important to note that the number of technical replicates chosen impacts the LOD and LOQ and their confidence intervals (Lesperance et al. 2021). We routinely use eight technical replicates (Figure 6) as there is a marked improvement on LOD and LOQ from $n=3$ to $n=8$, and it is a good compromise between sensitivity and cost (Lesperance et al. 2021). This decision is further supported by the eLowQuant-generated estimates of the impact of technical replicate number on LOD and LOQ shown on all technical bulletins (Figure 3 and File S2) and in previous publications appearing in Table 4.

As plate negative controls, NTCs must show no fluorescence. We routinely run $n=8$ on every plate and situate the NTCs at the end of the plate immediately beside the positive plate controls (Figure 6). This is logistically easiest to set up while expecting the highest level of pipetting prowess to identify plate contamination issues. To avoid false negative (type 2) errors (Thomsen and Willerslev 2015; Evans et al. 2017), we include the required two replicates of positive controls on every 96-well qPCR plate

(Figure 6). These positive controls are at a concentration expected to produce 100% detection. As suggested by the standard, we use a low copy number per reaction of the positive controls that produces reliable detection (e.g., 20 copies/reaction) to reduce the risk of contamination.

Samples can contain material that affects the DNA integrity, or the DNA may be damaged by bleach or ultraviolet light. Thus, to evaluate the probability and reduce the likelihood of a type 2 error, extracted DNA is routinely evaluated for quality and integrity (Abbott et al. 2023). The CSA standard recommends using an endogenous sample positive control (ESPC) or an internal positive control (IPC) (Abbott et al. 2023). The former is “a nucleic acid fragment that is not derived from the target taxon and expected to be ubiquitously present within an eDNA sample,” whereas the latter is “an exogenous synthetic nucleic acid fragment added to an eDNA sample either during extraction or to the extracted DNA prior to qPCR setup” (Abbott et al. 2023). Examples of published ESPC use are detection of chloroplast 16S or 23S ribosomal RNA gene sequences or using a general fish assay (Veldhoen et al. 2016; Furlan and Gleeson 2017; Hobbs et al. 2019; Zhu et al. 2024). We have elected to use a chloroplast gene-based assay as an ESPC for routine use in the context of animal taxon eDNA assays. The IntegratE-DNA assay, also known as ePlant5 (refer to Table 4; for open access details see Veldhoen et al. 2016; Hobbs et al. 2019), has been extremely useful in determining if the DNA sample is too degraded, inhibited, or otherwise compromised to affect the interpretation of the results. If a sample fails the integrity test, then we routinely subject the affected sample to an inhibitor clean-up step using a OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA). Often this resolves the issue if the reason for detection failure was inhibition, and the cleaned-up sample is then tested for the target DNA of interest. If the clean-up step does not result in an improved chloroplast DNA signal, then the sample is deemed poor quality, and caution is exercised in interpreting the target DNA detection result. The reader is encouraged to refer to the references in Table 4 for examples of the application of the IntegratE-DNA assay. While unable to detect sample degradation, an IPC is particularly useful for specifically determining enzyme inhibition and we refer the reader to excellent resources on this topic in the literature (Hartman et al. 2005; Goldberg et al. 2016; Hunter et al. 2019).

Another important consideration in interpreting eDNA surveys is the deposition of DNA by the target species. It is well known that organisms can vary greatly in their rate of DNA release into the environment (Allan et al. 2021). Previous studies and work herein have found that some high-quality assays developed to detect reptilian eDNA have trouble detecting their targets in environmental samples apparently for this reason (Matthias et al. 2021; Samuels et al. 2025) (e.g., spot-tailed lizard (eHolbrookia2), Northern Pacific (western) rattlesnake (eCROR1); see File S2).

5 | Conclusions

Herein, we present 125 eDNA assays particularly relevant to North American species that were generated through the iTrackDNA research project that meet or exceed the recent CSA

W214:21 and CSA W219:23 standards to detect target taxa by qPCR (Gagné et al. 2021; Abbott et al. 2023). These standards are increasing confidence in the use of eDNA methods, and federal and provincial regulators are incorporating them into operations and assessments. In developing the assays, we incorporated useful tools and best practices, including full mitogenome sequence evaluation where possible to maximize the likelihood of designing high-performance eDNA assays that meet reliability standards. While these assays perform well in certain contexts, it is important that users validate each individual assay on voucher specimens and field samples that are relevant to their survey needs. Recognizing that full validations of assays and high levels of technical replication may be unfeasible for resource-limited laboratories, we emphasize that our workflow seeks to fulfill best practices, and modifications can be made to prioritize the most crucial elements of assay validation prior to use. Regardless, these new eDNA tools will aid stakeholders, which include regulators, Indigenous peoples, non-governmental organizations, and those in academia and industry, to monitor biodiversity, protect ecosystems, improve environmental assessments, and ameliorate biosphere conservation.

Author Contributions

V.S.L., I.B., and C.C.H. conceived and designed the research; M.L.D.L., M.J.A., J.J.I., J.C., N.A.-P., L.C.B., M.T.B., M.-P.B., M.-L.C., L.C., A.H.D., E.T.G., H.L., I.G.M., Y.R., G.K.K., F.S., T.A.T., R.L.W., C.L.Y. acquired the data; V.S.L., M.L.D.L., M.J.A., J.J.I., J.C., N.A.-P., and C.C.H. analyzed and interpreted the data; V.S.L., M.L.D.L., J.C., J.J.I., and C.C.H. drafted the manuscript. All authors read and approved the final manuscript.

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The authors wish to dedicate this work to the late Dr. Louis Bernatchez, who was a scientific co-lead along with V.S.L. and C.C.H. on the iTrackDNA project.

Conflicts of Interest

C.C.H. is coinventor on U.S. patent #11,479,813 issued to the University of Victoria that includes the ePlant5 aka IntegratE-DNA assay for commercial use. Otherwise, the authors have nothing to report.

Data Availability Statement

Raw data are available upon request to the corresponding author. Mitogenome assemblies are publicly available through the National Center for Biotechnology Information (NCBI)'s GenBank Bioproject PRJNA1139757.

References

- Abbott, C., D. Bright, H. Bryant, et al. 2023. "W219:23 Performance Criteria for the Analyses of Environmental DNA by Targeted Quantitative Polymerase Chain Reaction." *National Standard of Canada, Canadian Standards Association*, 23 pp.
- Acharya-Patel, N., L. C. Bergman, K. Cram, et al. 2025. "Monitoring Marine Pollution Effects Through Targeted Environmental DNA (eDNA) Testing in the Pacific Northwest." *Marine Pollution Bulletin* 216: 118036. <https://doi.org/10.1016/j.marpolbul.2025.118036>.
- Acharya-Patel, N., E. Groenwold, M. A. Lemay, et al. 2024a. "Comparison of Environmental DNA and SCUBA Diving Methods to Survey Keystone Rockfish Species on the Central Coast of British Columbia, Canada." *Ecological Indicators* 160: 111830. <https://doi.org/10.1016/j.ecolind.2024.111830>.
- Acharya-Patel, N., E. Groenwold, M. J. Allison, and C. C. Helbing. 2024b. "Systematic Evaluation of the Influence of Taq Enzyme Choice and Amplification Conditions on Targeted Environmental DNA Assay Performance and Detection in Field Samples." *Environmental DNA* 6: e578. <https://doi.org/10.1002/edn3.578>.
- Allan, E. A., W. G. Zhang, A. C. Lavery, and A. F. Govindarajan. 2021. "Environmental DNA Shedding and Decay Rates From Diverse Animal Forms and Thermal Regimes." *Environmental DNA* 3: 492–514. <https://doi.org/10.1002/edn3.141>.
- Allison, M. J., R. L. Warren, M. L. D. Lopez, et al. 2023. "Enabling Robust Environmental DNA Assay Design With "Unikseq" for the Identification of Taxon-Specific Regions Within Whole Mitochondrial Genomes." *Environmental DNA* 5: 1032–1047. <https://doi.org/10.1002/edn3.438>.
- Bernatchez, L., A. L. Ferchaud, C. S. Berger, C. J. Venney, and A. Xuereb. 2024. "Genomics for Monitoring and Understanding Species Responses to Global Climate Change." *Nature Reviews. Genetics* 25: 165–183. <https://doi.org/10.1038/s41576-023-00657-y>.
- Burns, M., and H. Valdivia. 2008. "Modelling the Limit of Detection in Real-Time Quantitative PCR." *European Food Research and Technology* 226: 1513–1524. <https://doi.org/10.1007/s00217-007-0683-z>.
- Bustin, S. A., V. Benes, J. A. Garson, et al. 2009. "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments." *Clinical Chemistry* 55: 611–622. <https://doi.org/10.1373/clinchem.2008.112797>.
- Buxton, A., A. Diana, E. Matechou, J. Griffin, and R. A. Griffiths. 2022. "Reliability of Environmental DNA Surveys to Detect Pond Occupancy by Newts at a National Scale." *Scientific Reports* 12: 1295. <https://doi.org/10.1038/s41598-022-05442-1>.
- Clare, E. L., C. K. Economou, F. J. Bennett, et al. 2022. "Measuring Biodiversity From DNA in the Air." *Current Biology* 32: 693–700.e695. <https://doi.org/10.1016/j.cub.2021.11.064>.
- Evans, N. T., P. D. Shirey, J. G. Wieringa, A. R. Mahon, and G. A. Lamberti. 2017. "Comparative Cost and Effort of Fish Distribution Detection via Environmental DNA Analysis and Electrofishing." *Fisheries* 42: 90–99. <https://doi.org/10.1080/03632415.2017.1276329>.
- Fitzgerald, A., J. Halliday, and D. Heath. 2021. "Environmental DNA as Novel Technology: Lessons in Agenda Setting and Framing in News Media." *Animals (Basel)* 11: 2874. <https://doi.org/10.3390/ani11102874>.
- Furlan, E. M., and D. Gleeson. 2017. "Improving Reliability in Environmental DNA Detection Surveys Through Enhanced Quality Control." *Marine and Freshwater Research* 68: 388–395. <https://doi.org/10.1071/MF15349>.
- Gagné, N., L. Bernatchez, D. Bright, et al. 2021. "W214:21 Environmental DNA (eDNA) Reporting Requirements and Terminology." *National Standard of Canada, Canadian Standards Association*, 31 pp.
- Garrido-Sanz, L., M. A. Senar, and J. Pinol. 2022. "Relative Species Abundance Estimation in Artificial Mixtures of Insects Using Mito-Metagenomics and a Correction Factor for the Mitochondrial DNA Copy Number." *Molecular Ecology Resources* 22: 153–167. <https://doi.org/10.1111/1755-0998.13464>.
- Goldberg, C. S., C. R. Turner, K. Deiner, et al. 2016. "Critical Considerations for the Application of Environmental DNA Methods to Detect Aquatic Species." *Methods in Ecology and Evolution* 7: 1299–1307. <https://doi.org/10.1111/2041-210X.12595>.
- Gouvernement Du Québec. 2022. "Protocole Standardisé Pour l'inventaire des Tortues d'eau Douce à l'aide de l'ADNe au Québec." (ed. Ministère des Forêts de la Faune et des Parcs), p. 39. Gouvernement du Québec, Québec.
- Gouvernement Du Québec. 2023. "Protocole Standardisé d'inventaire Pour les Espèces de Poissons de Petite Taille En Situation Précaire au Québec." (ed. Ministère des forêts de la faune et des parcs), p. 56. Gouvernement du Québec, Québec.
- Gouvernement Du Québec. 2024a. "Protocole Standardisé Pour les Inventaires de Couleuvres et la Recherche d'hibernacles au Québec." (ed. Ministère des forêts de la faune et des parcs), p. 26. Gouvernement du Québec, Québec.
- Gouvernement Du Québec. 2024b. "Recueil des Protocoles Standardisés d'inventaires de Salamandres à Quatre Orteils au Québec." (ed. Ministère des forêts de la faune et des parcs), p. 33. Gouvernement du Québec, Québec.
- Gouvernement Du Québec. 2024c. "Recueil des Protocoles Standardisés d'inventaires de Salamandres de Ruisseaux au Québec." (ed. Ministère des forêts de la faune et des parcs), p. 47. Gouvernement du Québec, Québec.
- Hartman, L. J., S. R. Coyne, and D. A. Norwood. 2005. "Development of a Novel Internal Positive Control for Taqman Based Assays." *Molecular and Cellular Probes* 19: 51–59. <https://doi.org/10.1016/j.mcp.2004.07.006>.
- Hoban, M. L., J. Whitney, A. G. Collins, et al. 2022. "Skimming for Barcodes: Rapid Production of Mitochondrial Genome and Nuclear Ribosomal Repeat Reference Markers Through Shallow Shotgun Sequencing." *PeerJ* 10: e13790. <https://doi.org/10.7717/peerj.13790>.
- Hobbs, J., J. M. Round, M. J. Allison, and C. C. Helbing. 2019. "Expansion of the Known Distribution of the Coastal Tailed Frog, *Ascaphus truei*, in British Columbia, Canada Using Robust eDNA Detection Methods." *PLoS One* 14, no. 3: e0213849. <https://doi.org/10.1371/journal.pone.0213849>.
- Hocking, M. D., J. C. Macadams, M. J. Allison, et al. 2022. "Establishing the Signal Above the Noise: Accounting for an Environmental Background in the Detection and Quantification of Salmonid Environmental DNA." *Fishes* 7: 266. <https://doi.org/10.3390/fishes7050266>.
- Hunter, M. E., J. A. Ferrante, G. Meigs-Friend, and A. Ulmer. 2019. "Improving eDNA Yield and Inhibitor Reduction Through Increased Water Volumes and Multi-Filter Isolation Techniques." *Scientific Reports* 9: 5259. <https://doi.org/10.1038/s41598-019-40977-w>.
- Katoh, K., J. Rozewicki, and K. D. Yamada. 2019. "MAFFT Online Service: Multiple Sequence Alignment, Interactive Sequence Choice and Visualization." *Briefings in Bioinformatics* 20: 1160–1166. <https://doi.org/10.1093/bib/bbx108>.
- Klymus, K. E., N. T. Marshall, and C. A. Stepien. 2017. "Environmental DNA (eDNA) Metabarcoding Assays to Detect Invasive Invertebrate Species in the Great Lakes." *PLoS One* 12: e0177643. <https://doi.org/10.1371/journal.pone.0177643>.
- Klymus, K. E., C. M. Merkes, M. J. Allison, et al. 2020. "Reporting the Limits of Detection and Quantification for Environmental DNA Assays." *Environmental DNA* 2: 271–282. <https://doi.org/10.1002/edn3.29>.
- Kutyavin, I. V., I. A. Afonina, A. Mills, et al. 2000. "3'-Minor Groove Binder-DNA Probes Increase Sequence Specificity at PCR Extension

- Temperatures." *Nucleic Acids Research* 28: 655–661. <https://doi.org/10.1093/nar/28.2.655>.
- Langlois, V. S., M. J. Allison, L. C. Bergman, T. A. To, and C. C. Helbing. 2021. "The Need for Robust qPCR-Based eDNA Detection Assays in Environmental Monitoring and Species Inventories." *Environmental DNA* 3: 519–527. <https://doi.org/10.1002/edn3.164>.
- Lesperance, M., M. J. Allison, L. C. Bergman, M. D. Hocking, and C. C. Helbing. 2021. "A Statistical Model for Calibration and Computation of Detection and Quantification Limits for Low Copy Number Environmental DNA Samples." *Environmental DNA* 3: 970–981. <https://doi.org/10.1002/edn3.220>.
- Lopez, M. L. D., M. Bonderud, M. J. Allison, et al. 2023. "qPCR-Based eDNA Workflow for Humic-Rich Lake Sediments: Combined Use of Sedimentary DNA (sedDNA) and Indigenous Knowledge in Reconstructing Historical Fish Records." *Ecological Indicators* 155: 111014. <https://doi.org/10.1016/j.ecolind.2023.111014>.
- Lopez, M. L. D., M. T. Bonderud, I. G. Ma, V. C. Thompson, and C. C. Helbing. 2024. "GoEnrich: Creating High Quality Genomic DNA Resources From Limited Voucher Specimen Tissues or Museum Specimens of At-Risk Species for Conservation-Friendly Use in the Validation of Environmental DNA Assays." *BMC Research Notes* 17: 255. <https://doi.org/10.1186/s13104-024-06936-z>.
- Lopez, M. L. D., C. L. Yang, L. Coombe, et al. 2025. "mtGrasp: Streamlined Mitochondrial Genome Reference-Grade Assembly and Standardization to Enhance Mitogenome Resources and Improve the Development of Environmental DNA Assays." *Methods in Ecology and Evolution* 16: 668–677. <https://doi.org/10.1111/2041-210X.14506>.
- Matthias, L., M. J. Allison, C. Y. Maslovat, J. Hobbs, and C. C. Helbing. 2021. "Improving Ecological Surveys for the Detection of Cryptic, Fossorial Snakes Using eDNA on and Under Artificial Cover Objects." *Ecological Indicators* 131: 108187. <https://doi.org/10.1016/j.ecolind.2021.108187>.
- Rees, H. C., K. C. Gough, D. J. Middleditch, J. R. M. Patmore, and B. C. Maddison. 2015. "Applications and Limitations of Measuring Environmental DNA as Indicators of the Presence of Aquatic Animals." *Journal of Applied Ecology* 52: 827–831. <https://doi.org/10.1111/1365-2664.12467>.
- Robinson, C. L. K., L. C. Bergman, M. J. Allison, J. Huard, J. Sutherst, and C. C. Helbing. 2022. "Application of Environmental DNA as a Tool for Detecting Intertidal Habitat Use by Forage Fish." *Ecological Indicators* 142: 109306. <https://doi.org/10.1016/j.ecolind.2022.109306>.
- Ruppert, K. M., R. J. Kline, and M. S. Rahman. 2019. "Past, Present, and Future Perspectives of Environmental DNA (eDNA) Metabarcoding: A Systematic Review in Methods, Monitoring, and Applications of Global eDNA." *Global Ecology and Conservation* 17: e00547. <https://doi.org/10.1016/j.gecco.2019.e00547>.
- Samuels, L. R. N., H. C. Chandler, M. Hoffman, et al. 2025. "Persistence of Reptile DNA in a Terrestrial Substrate: A Case Study Using the Eastern Indigo Snake." *Environmental DNA* 7: e70053. <https://doi.org/10.1002/edn3.70053>.
- Sivaganesan, M., J. R. Willis, M. Karim, et al. 2022. "Interlaboratory Performance and Quantitative PCR Data Acceptance Metrics for NIST SRM(R) 2917." *Water Research* 225: 119162. <https://doi.org/10.1016/j.watres.2022.119162>.
- Su, G. Y., M. J. Allison, J. Beblow, et al. 2023. "Enumeration Potential of Environmental DNA for Pacific Salmon Stock Assessments." *Environmental DNA* 6: e508. <https://doi.org/10.1002/edn3.508>.
- Taylor, S. C., K. Nadeau, M. Abbasi, C. Lachance, M. Nguyen, and J. Fenrich. 2019. "The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time." *Trends in Biotechnology* 37: 761–774. <https://doi.org/10.1016/j.tibtech.2018.12.002>.
- Thalinger, B., K. Deiner, L. R. Harper, et al. 2021. "A Validation Scale to Determine the Readiness of Environmental DNA Assays for Routine Species Monitoring." *Environmental DNA* 3: 823–836. <https://doi.org/10.1002/edn3.189>.
- Thomsen, P. F., and E. Willerslev. 2015. "Environmental DNA – An Emerging Tool in Conservation for Monitoring Past and Present Biodiversity." *Biological Conservation* 183: 4–18. <https://doi.org/10.1016/j.biocon.2014.11.019>.
- Ulicsni, V., D. Babai, C. Vadasz, V. Vadasz-Besnyoi, A. Baldi, and Z. Molnar. 2019. "Bridging Conservation Science and Traditional Knowledge of Wild Animals: The Need for Expert Guidance and Inclusion of Local Knowledge Holders." *Ambio* 48: 769–778. <https://doi.org/10.1007/s13280-018-1106-z>.
- Van Der Heyde, M., M. Bunce, G. Wardell-Johnson, K. Fernandes, N. E. White, and P. Nevill. 2020. "Testing Multiple Substrates for Terrestrial Biodiversity Monitoring Using Environmental DNA Metabarcoding." *Molecular Ecology Resources* 20: 732–745. <https://doi.org/10.1111/1755-0998.13148>.
- Veldhoen, N., J. Hobbs, G. Ikonomou, M. Hii, M. Lesperance, and C. C. Helbing. 2016. "Implementation of Novel Design Features for qPCR-Based eDNA Assessment." *PLoS One* 11: e0164907. <https://doi.org/10.1371/journal.pone.0164907>.
- Zhu, X. C., K. L. Bell, M. L. Rourke, H. W. Wu, and D. Gopurenko. 2024. "Generic qPCR Assays for Quality Control in Environmental DNA Research." *Environmental DNA* 6: e536. <https://doi.org/10.1002/edn3.536>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.