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# Reporting the limits of detection and quantification for environmental DNA assays

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## Abstract

**Background:** Environmental DNA (eDNA) analysis is increasingly being used to detect the presence and relative abundance of rare species, especially invasive or imperiled aquatic species. The rapid progress in the eDNA field has resulted in numerous studies impacting conservation and management actions. However, standardization of eDNA methods and reporting across the field is yet to be fully established, with one area being the calculation and interpretation of assay limit of detection (LOD) and limit of quantification (LOQ).

**Aims:** Here, we propose establishing consistent methods for determining and reporting of LOD and LOQ for single-species quantitative PCR (qPCR) eDNA studies.

**Materials & Methods/ Results:** We utilize datasets from multiple cooperating laboratories to demonstrate both a discrete threshold approach and a curve-fitting modeling approach for determining LODs and LOQs for eDNA qPCR assays. We also provide details of an R script developed and applied for the modeling method.

**Discussion/Conclusions:** Ultimately, standardization of how LOD and LOQ are determined, interpreted, and reported for eDNA assays will allow for more informed

interpretation of assay results, more meaningful interlaboratory comparisons of experiments, and enhanced capacity for assessing the relative technical quality and performance of different eDNA qPCR assays.

**KEYWORDS**

assay optimization, eDNA, qPCR, standardization

## 1 | INTRODUCTION

Environmental DNA (eDNA) studies commonly use quantitative real-time polymerase chain reaction (qPCR) for the detection of low levels of target species' eDNA found in complex environmental samples (e.g., water, soil, or air). Detection of low-concentration DNA by qPCR and the specificity of the technique provide a high level of confidence that DNA from the target has been identified. Currently, eDNA techniques are in the process of becoming a part of the standard fishery and wildlife management toolkit for population detection, assessment, and monitoring (Bohmann et al., 2014; Goldberg, Sepulveda, Ray, Baumgardt, & Waits, 2013; Hunter et al., 2015; Piaggio et al., 2014; Rees et al., 2014). Because erroneous detection or nondetection of a target organism can lead to costly management actions or ecological and economic impacts, accurate eDNA detection and appropriate interpretation of results are critical (Goldberg et al., 2016; Hunter et al., 2017). Likewise, understanding the quantitative precision of eDNA assays is paramount because qPCR measurements are being used to assess target species abundances (Yates et al., 2019). Robust quality control metrics and clear reporting of those metrics and the methodologies used to determine them are required to ensure that results are comparable across studies and can be defensibly interpreted (Bustin et al., 2009).

Since its development in the 1990s (Higuchi et al., 1992; Kubista et al., 2006; Wittwer et al., 1997), qPCR has become widely used for detection of nucleic acids in many fields, including clinical studies, forensics, water quality monitoring, gene expression, and genetically modified organism product identification (Borchardt et al., 2017; Di Domenico, Di Giuseppe, Wicochea Rodriguez, & Camma, 2017; Rasmussen & Morrissey, 2008; Russell et al., 2013; Scholtens et al., 2017). Motivated by the absence of standardization in fields employing the qPCR method, Bustin et al. (2009) developed the minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines to improve assay reliability, data interpretation, and laboratory transparency. The MIQE recommendations are widely cited, and application-specific adaptations and guides have been published (Bustin et al., 2010; Dooms, Chango, Barbour, Pouillart, & Abdel Nour, 2013; Edmunds, McIntyre, Luckenbach, Baldwin, & Incardona, 2014; Huggett et al., 2013; Johnson, Bibby, Wong, Agrawal, & Bustin, 2012; Taylor & Mrkusich, 2014). The MIQE guidelines are a good reference for the eDNA community to use in developing and using qPCR assays. In particular, the guidelines address the testing and reporting of an assay's efficiency, linear dynamic range, and precision, which are parameters critical to well-performing eDNA qPCR assays. However, the MIQE

guidelines also include recommendations that are irrelevant for eDNA applications, such as gene expression, while lacking guidance on other topics pertinent to the analysis of environmental samples.

One topic essential to eDNA studies is assay performance at low target DNA concentrations. However, robust assessments of parameters associated with low-concentration detection by a qPCR assay are often omitted or poorly described in studies. This may be due to confusion in how to define parameters such as the limits of detection (LOD) and quantification (LOQ) for qPCR studies. Some of the earliest efforts to standardize measurement and reporting of low-quantity analytes were developed for analytical chemistry, and the definitions given for LOD and LOQ have become the convention (Currie, 1999). However, these conventional definitions do not fit qPCR data as they require a linear response between the analyte and the signal of response and they assume a level of background noise in blank samples (i.e., the limit of the blank [LOB]) from which the analyte must be distinguished (Armbruster & Pry, 2008). Data from qPCR analyses do not meet these assumptions because the response is not linear and negative samples do not produce a signal distinguishable from background signal of the thermocycler (Forootan et al., 2017; Hunter et al., 2017). Therefore, LOD and LOQ require different definitions and interpretation for qPCR. From a qPCR perspective, LOD can be defined as the lowest concentration of target analyte that can be detected with a defined level of confidence, with a 95% detection rate as the standard confidence level (Burd, 2010; Burns & Valdivia, 2007; Bustin et al., 2009; CLSI, 2012; Forootan et al., 2017; Furlan, Gleeson, Hardy, & Duncan, 2016; Wolk & Marlowe, 2011). This is accomplished by running a large number of replicate standard curves including low-concentration standards and determining the lowest standard concentration at which 95% of the replicates produce positive amplification of the target DNA.

The LOD is based on detection/nondetection criteria and describes an assay's ability to detect the target sequence at low levels. In contrast, measurement of concentration is addressed by the LOQ, which reflects the assay's capacity to precisely quantify copy number. The LOQ plays a critical role when studies attempt to determine predictive relationships between eDNA concentration and target species biomass or relative abundance. The Clinical and Laboratory Standards Institute (CLSI) defines LOQ as the lowest amount of analyte in a sample that can be quantitatively determined with a stated precision, under stated experimental conditions (2012). For qPCR assays, precision can be assessed using the coefficient of variation (CV) of the measured concentrations of standards (Kubista, 2014; Taylor, 1987). The concentration of target DNA at an assay's LOQ

may be greater than or equal to the concentration identified as the LOD but cannot be less than the LOD (Armbruster & Pry, 2008; Kralik & Ricchi, 2017).

The ability to detect and quantify low target DNA concentrations is essential to eDNA work, but the eDNA field lacks clear definitions for assay LOD and LOQ. Currently, these metrics are applied inconsistently in assay development, and eDNA studies rarely report these parameters and the methods used to measure them. This lack of clarity in defining LOD and LOQ can be problematic as described in a study on microbial source tracking (Stewart et al., 2013). The Stewart et al. (2013) interlaboratory study comparing qPCR assays showed that differing concepts and definitions of LOD/LOQ produced inconsistent data analysis and reporting. For example, laboratories obtained the same result for a given sample (e.g., detection) but reported it differently based on their LOD (e.g., detection or nondetection), producing a false discrepancy. Because presence/absence is crucial for studies of invasive and imperiled species, the reporting conventions stemming from LOD and LOQ must be clear and consistent.

As the field moves forward, it is becoming increasingly important that we be able to analyze samples consistently across laboratories. This requires not only standardization of reporting on assay conditions (as per the standard MIQE; Bustin et al., 2009) but also confirming that the results are comparable or improved across laboratories as the application of DNA analytical methods can vary (e.g., types of thermocyclers, enzymes, and reagents) and new technologies become available. Indeed, there is an acknowledged need for introducing eDNA standard practice (Helbing & Hobbs, 2019). Our goal is to help establish standard LOD and LOQ definitions and guidelines to improve communication, inform data interpretation, and facilitate cross-study comparisons, all of which support conservation and management decisions. In addition, by determining their assay's LOD and LOQ as defined here, practitioners will be able to optimize the quantitative precision of their assay and better understand its capabilities and limits. We present a simple method based on discrete thresholds for determining the LOD and LOQ for an eDNA qPCR assay, which should be performed on each assay being validated for use or adopted by a new laboratory. Alternatively, calculations to determine LOD and LOQ based on curve fitting can be performed by an R script also provided here. We present the results from an interlaboratory and cross assay assessment utilizing these methods and address ways to improve the accuracy and precision of eDNA qPCR assays. Finally, we provide guidance on reporting LOD and LOQ for eDNA studies and some of the unique considerations required within this context.

## 2 | MATERIALS AND METHODS

Seven independent laboratories participated in an interlaboratory comparison of LOD and LOQ for eDNA assays for a variety of species. Each laboratory performed a series of replicate standard curves (totaling 20–96 replicates per standard concentration) for each of

their tested assays; a total of 36 assays were tested (Appendix S1). All standard curves were created using templates derived from synthetic double-stranded DNA of the target region, except one assay which used a nonlinearized plasmid standard. Each laboratory also included both positive and negative controls.

Data from the replicate standard curves were evaluated as the binary, qualitative outcome for LOD (detection/nondetection) and as the CV for LOQ. The CV was calculated for each standard by the equation derived by Forootan et al. (2017):

$$CV_{ln} = \sqrt{\left(1+E\right)^{\left(SD(C_q)\right)^2 * ln(1+E)} - 1}$$

where $E$ is the qPCR efficiency and $SD(C_q)$ is the standard deviation of replicate $C_q$ values. We determined the LOD and LOQ using both a discrete threshold method and a model fitting approach.

To determine LOD and LOQ for an assay, standard concentrations must span both parameters. For LOD, they must also include a mix of positive and negative replicates. The accuracy and precision of LOD and LOQ calculations increase with replication; however, there is not a definitive level of replication, and recommendations vary (e.g., CLSI, 2012; Kubista, 2014; Wolk & Marlowe, 2011). We relied on data available from routine standard curve analysis by the seven laboratories, and therefore, standard curve replicates varied among laboratories and assays.

### 2.1 | Discrete threshold methods

The discrete threshold approach identifies the lowest concentration meeting the LOD or LOQ criterion, and values for LOD and LOQ are restricted to the standard concentrations included in the curve. The LOD was the lowest standard concentration of template DNA that produced at least 95% positive replicates. The LOQ was the lowest standard concentration that could be quantified with a CV value below 35%. There is currently no standardized maximum CV for qPCR LOQs, but studies have utilized LOQ threshold CV values ranging from 25% to 35% (Forootan et al., 2017; Kralik & Ricchi, 2017).

### 2.2 | Curve-fitting methods

We also determined the LOD and LOQ for each assay using curve-fitting methods. Curve-fitting methods can provide more accurate determination of LOD and LOQ because less bias results from the particular standard concentrations being tested. To determine the LOD, qualitative, binary detection results for the standards were fit to a sigmoidal curve using the drc package in R (Ritz, Baty, Streibig, & Gerhard, 2015). We fit sigmoidal models with all 15 available logarithmic functions and selected the best fitting model based on log likelihood values, Akaike's information criterion, lack of fit, and residual variance using the mselect function from the drc package in R (Ritz et al., 2015). We also fit a linear model in R with the formula LOD ~ Assay + FunctionType + NumberParamet

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**TABLE 1** Limits of detection (LOD) and limits of quantification (LOQ), in copies per reaction Number indicates the sequential assay number that is used in all figures

| Number | Assay | Modeled LOD | Discrete LOD | Modeled LOQ | Discrete LOQ | Lab |
|---|---|---|---|---|---|---|
| 1 | CID | 51.0 | 192 | 184 | 1,920 | CERC |
| 2 | CID[a] | NA[b] | 15.6 | 15.6 | 15.6 | CERC |
| 3 | MYPI6 | 260 | 250 | 260 | NA | CERC |
| 4 | MYPI6[a] | 7.18 | 15.6 | 55 | 62.5 | CERC |
| 5 | ELNU2 | 4.05 | 10 | 110 | 1,250 | ERDC |
| 6 | MYPI2 | 4.92 | 10 | 50 | 50 | ERDC |
| 7 | Hno | 2.19 | 5 | 11 | 10 | MNRF |
| 8 | D-loop | 2.81 | 10 | 6[c] | 10[c] | NWRC |
| 9 | AD-BHC | 6.33 | 10 | 9 | 10 | UMESC |
| 10 | AD-SVC | 6.33 | 10 | 10 | 100 | UMESC |
| 11 | Dre16s | 22.5 | 100 | 839 | 1,000 | UMESC |
| 12 | SS | 2.80 | 10 | 27 | 100 | UMESC |
| 13 | YPC | 7.73 | 10 | 40 | 100 | UMESC |
| 14 | eASMO9 | 5.74 | 20 | 50 | 100 | UVIC |
| 15 | eASTR4 | 9.59 | 20 | 130 | 100 | UVIC |
| 16 | eFISH1 | 22.1 | 20 | 128 | 500 | UVIC |
| 17 | eLIPI1 | 4.49 | 4 | 62 | 20 | UVIC |
| 18 | eMIDO1 | 2.69 | 4 | 49 | 100 | UVIC |
| 19 | eMISA2 | 5.92 | 20 | 159 | 100 | UVIC |
| 20 | eONKI4 | 6.82 | 20 | 370 | 500 | UVIC |
| 21 | eRAAU1 | 6.86 | 20 | 44 | 100 | UVIC |
| 22 | eRACA2 | 8.57 | 20 | 69 | 100 | UVIC |
| 23 | eRALU2 | 6.29 | 20 | 32 | 100 | UVIC |
| 24 | eRAPR2 | 5.92 | 20 | 39 | 20 | UVIC |
| 25 | ACTM1 | 2.21 | 10 | 10 | 10 | WGL |
| 26 | ACTM3 | 2.20 | 10 | 9 | 10 | WGL |
| 27 | BHTM1 | 5.13 | 10 | 32 | 50 | WGL |
| 28 | BHTM2 | 9.04 | 10 | 56 | 50 | WGL |
| 29 | GCTM10 | 2.93 | 10 | 25 | 50 | WGL |
| 30 | GCTM22 | 2.48 | 10 | 135 | 250 | WGL |
| 31 | GCTM32 | 2.44 | 10 | 239[c] | 250[c] | WGL |
| 32 | Goby | 3.60 | 10 | 12 | 100 | WGL |
| 33 | SCTM4 | 2.96 | 10 | 20 | 50 | WGL |
| 34 | SCTM5 | 2.77 | 10 | 13 | 50 | WGL |
| 35 | BRK2 | 7.16 | 10 | 24 | 50 | WSU |
| 36 | NZMS | 7.60 | 10 | 24 | 50 | WSU |

*Note:* Assay indicates the name of the assay tested. Modeled LOD indicates the 95% limit of detection for a single replicate as determined by sigmoidal modeling using our generic LOD/LOQ calculator script. Discrete LOD indicates the lowest standard tested with 95% or greater positive detections among all replicates tested. LOQ indicates the limit of quantification as determined by using our generic LOD/LOQ calculator script. Laboratory indicates where the testing was done: CERC = U.S. Geological Survey—Columbia Environmental Research Center, ERDC = U.S. Army Corps of Engineers—Engineer Research and Development Center, MNRF = Ontario—Ministry of Natural Resources and Forestry, NWRC = U.S. Department of Agriculture Animal and Plant Health Inspection Service—National Wildlife Research Center, UMESC = U.S. Geological Survey—Upper Midwest Environmental Sciences Center, UVIC = University of Victoria, British Columbia, WGL = U.S. Fish and Wildlife Service—Whitney Genetics Laboratory, WSU = Washington State University.
[a]Same assay as above, but with TE and tRNA added to template DNA standards.
[b]Not solvable, because only standards with 100% detection were tested.
[c]Precision threshold adjusted to 0.783 CV for assay 8 and 0.512 CV for assay 31.

ers to evaluate the effect of choosing different logarithmic functions as the LOD models.

Furthermore, we used the selected model to determine the effective LOD to assess how the LOD changes with increasing numbers of PCR replicates per sample. If a given concentration of target DNA sequence can be detected in a single qPCR with some probability ($p$), then analyzing that sample in $n$ replicates would result in a probability of detection in at least 1 of $n$ replicates given by $1 - (1 - p)^n$. Seeking to achieve 95% detection probability with $n$ replicates, we can determine the required single reaction probability by taking the $n$th-root of 0.05 using: $p = 1 - 0.05^{(1/n)}$. Solving the sigmoidal detection model for this adjusted $p$ results in the effective LOD, or the concentration that can be detected with 95% probability when analyzing the sample with $n$ replicates.

For LOQ, we modeled the CVs using base R functions for exponential decay, linear, and polynomial models (R Core Team, 2019). We then selected the model with the lowest residual standard error and visually confirmed that the models had good fit using the ggplot2 package in R (Wickham, 2016). We used a threshold of 35% CV to determine LOQs, except in one case where variability in even the highest standards yielded CVs above 35%. In that case we set the threshold at 1.5 times the lowest CV obtained for any standard. As accurate quantification requires reliable detection in all replicates, we also stipulated that the LOQ could not be lower than the LOD.

As part of this study, we developed an R script that will read user data provided in a comma-separated values (*.csv) format and analyze the LOD and LOQ using the curve-fitting modeling approach. The script provides some data suitability checks that are helpful for troubleshooting potential problems such as improperly formatted data, inadequate range of standards tested, or potential outliers. The script also automatically generates three figures that are useful for understanding and evaluating the results: a calibration curve, a plot of the LOD model, and a plot of the LOQ model. This generic script code can be found at https://github.com/cmerkes/qPCR_LOD_Calc, and our additional analysis code can be found at https://github.com/cmerkes/LOD_Analysis. The data used in this study are available at https://doi.org/10.5066/P9AKHU1R

## 3 | RESULTS

### 3.1 | Interlaboratory results

The 36 assays we tested had LODs ranging from 4 to 250 copies per reaction for the discrete threshold methods, while using the curve-fitting method, LODs ranged from 2.19 to 260 copies per reaction (Table 1). For the LOQ, results from the discrete threshold method ranged from 10 to 1,920 copies per reaction, whereas LOQ results derived from the curve-fitting method ranged between 6 and 839 copies per reaction (Table 1). As expected, precision (indicated by narrower confidence intervals) generally increased with replication. Likewise, most assays followed a similar trend of decreasing effective LOD values as the number of replicates increased (Figure 1). Moving from one analytical replicate to eight, our assays showed a mean 11.1-fold drop
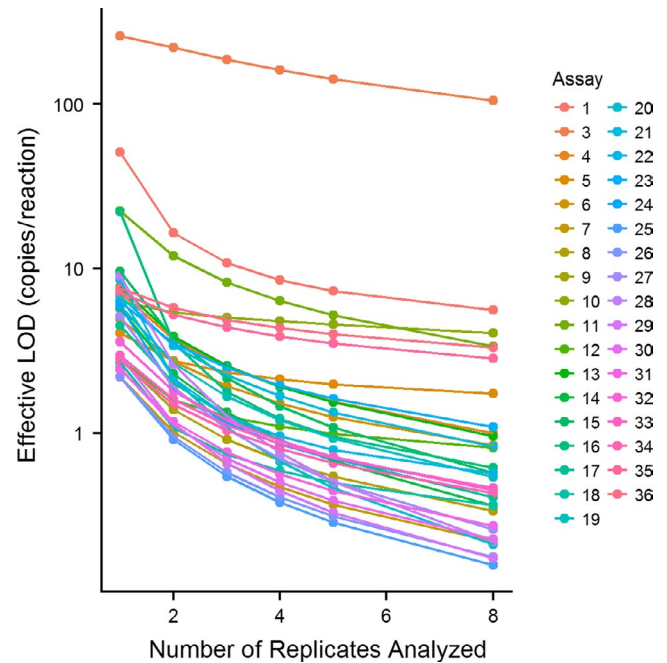


**FIGURE 1** Effective limits of detection with increasing analytical replicates. The effective LOD is shown on the y-axis for the number of analytical replicates on the x-axis. Each assay is represented by a different color, and lines are drawn between for readability. Assay numbers are listed in Table 1

in effective LOD ($SD$ = 9.24). Assays 3, 5, 9, 10, 35, and 36 exhibited less than a 3-fold reduction, while assays 16, 20, and 28 stood out as they showed 36-, 32-, and 41-fold reductions, respectively (Figure 1).

### 3.2 | LOD and LOQ script output

Our LOD/LOQ calculator script generates three plots and a number of outputs. An example of the three plots generated for the BHTM1 assay (assay 27) is in Figure 2, and the LOD and LOQ plots for all assays can be found in Figures S1–S10. The calibration curve plots all points (Figure 2a) for identifying potential outliers. This plot is for diagnostic purposes only, and the linear regression displayed is not used in any LOD or LOQ calculations. The LOD plot (Figure 2b) shows the relative detection rates for each standard as well as the LOD model curve. The 95% LOD is identified, and effective LODs with confidence intervals for analyzing samples with multiple replicates are displayed. The logarithmic function that was used to determine the LODs is shown in the plot subtitle along with the $p$-value for a lack of fit test on the model. The LOQ plot (Figure 2c) shows the CV of $C_q$ values for each standard as well as the curve for the LOQ model. The LOQ is represented by a gray polygon with the upper limit as the defined precision threshold and the right limit, where the polygon intersects the curve, as the calculated LOQ. The LOD is also plotted as a vertical line to provide a visual comparison to the LOQ.

In addition to the three plots shown in Figure 2, the LOD/LOQ calculator script also generates four output files. The first output is a text document titled "Analysis Log" that documents notes from the script including a time stamp of when the analysis was started, notes
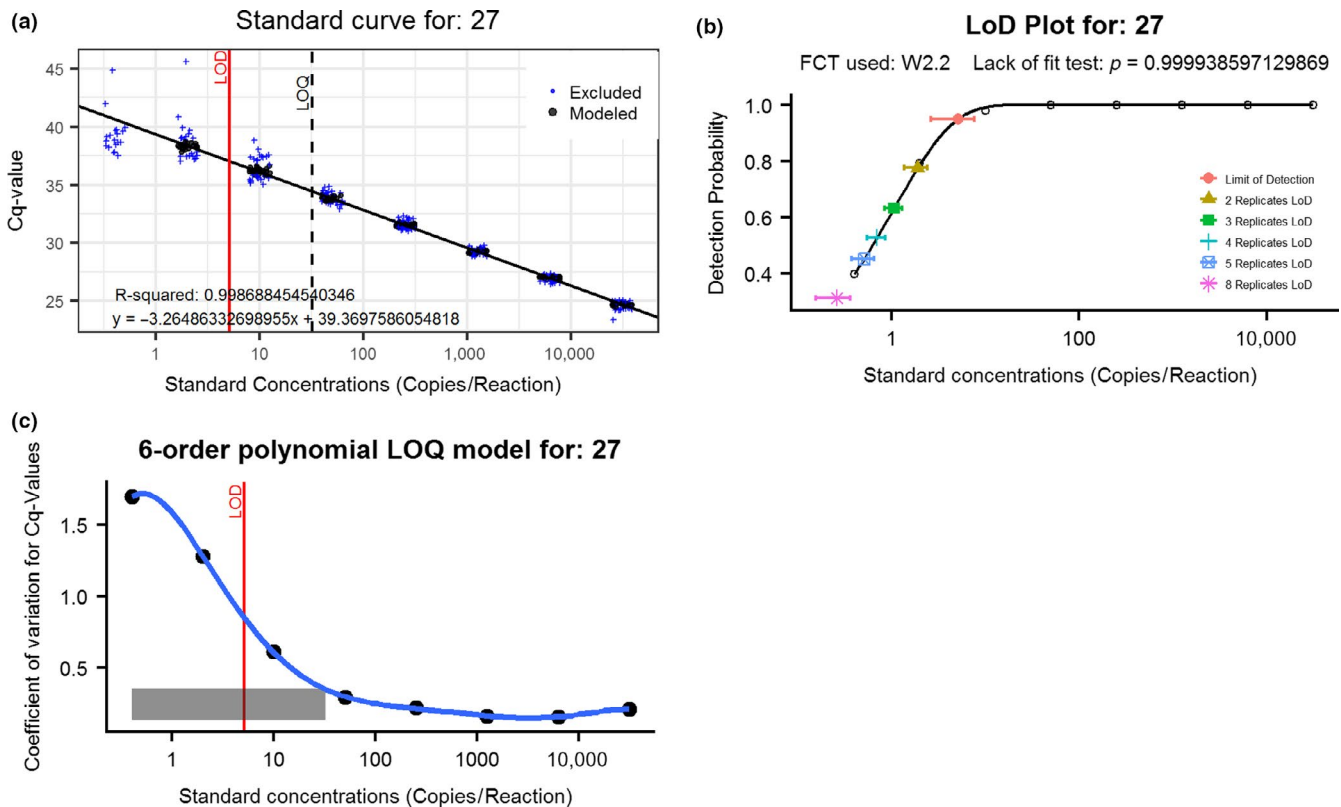
**FIGURE 2** Demonstration of figures automatically generated by LOD/LOQ calculator script. (A) Calibration curve plot with $C_q$ value on the *y*-axis and standard concentration on the *x*-axis. Points drawn with black circles are the middle 2 quartiles of standards with ≥50% detection and are included in the linear regression calculations. Points drawn with blue pluses (+) are outside the middle 2 quartiles or for standards with <50% detection and are not included in the linear regression calculations. (B) LOD plot with detection probability on the *y*-axis and standard concentrations on the *x*-axis. Points are drawn with open circles for the detection rates of each standard tested, and the line represents the LOD model. Colored points with 95% confidence intervals are drawn to represent the LOD and effective LODs for multiple replicate analyses. Logarithmic function used and lack of fit test results are shown in the subtitle. (C) LOQ plot with CV on the *y*-axis and standard concentrations on the *x*-axis. Points are drawn for the CVs of each standard tested. The vertical red line is at the LOD for reference. The blue line represents the LOQ model. The LOQ is represented by a gray rectangle with the upper limit as the defined precision threshold (0.35 CV for this study except as noted) and the right limit as the calculated LOQ (where it hits the curve)

about any data abnormalities detected for the user to review, a note if any potential outliers were detected, standards that may not be included in the calibration curve regression, a summary of the raw data, the lowest standards with 95% or greater actual detection, model exceptions that may have occurred, and a description of the headings for the second output. The second output is a comma-separated values (*.csv) file titled "Assay Summary" that contains the calibration curve regression information, the lowest standard analyzed with 95% or greater detections, the LOD, the LOQ, and effective LODs for analysis with multiple replicates. The third output titled "LOD Confint" is the 95% confidence interval information for the LOD and effective LODs. The fourth output is the raw data for any suspected outliers and is named "Potential Outliers." Potential outliers are flagged if the $C_q$ value is less than 90% or greater than 110% of the median $C_q$ value for that standard. These are flagged for closer scrutiny by the user, but the script does not remove any data points before completing its analysis. The user must review the potential outliers and reanalyze the data if outliers are removed.

## 3.3 | Logarithmic functions/model selection

The 15 logarithmic functions that were evaluated to calculate the LOD produced slightly different results ranging from 1.07-fold to 8.72-fold changes between highest and lowest modeled LODs (Figure 3, Tables S1–S4). The Asymptotic Regression function with three parameters and the Weibull type II function with two or four parameters fit our data the best and were selected most frequently by the LOD/LOQ calculator script (Figures S1–S5). The lowest LODs overall were generated by these two functions, but they were not significantly different from each other ($p = .868$). Log-logistic and Michaelis–Menten functions resulted in slightly higher LODs overall, but the difference was not significant ($p > .211$ and $p > .088$, respectively). Weibull type I functions resulted in significantly higher LODs overall compared to other function types ($p < .01$), except Michaelis–Menten where the difference was not significant ($p = .108$). We found that increasing the number of parameters included in the model lowered the LODs overall ($p = .0186$).
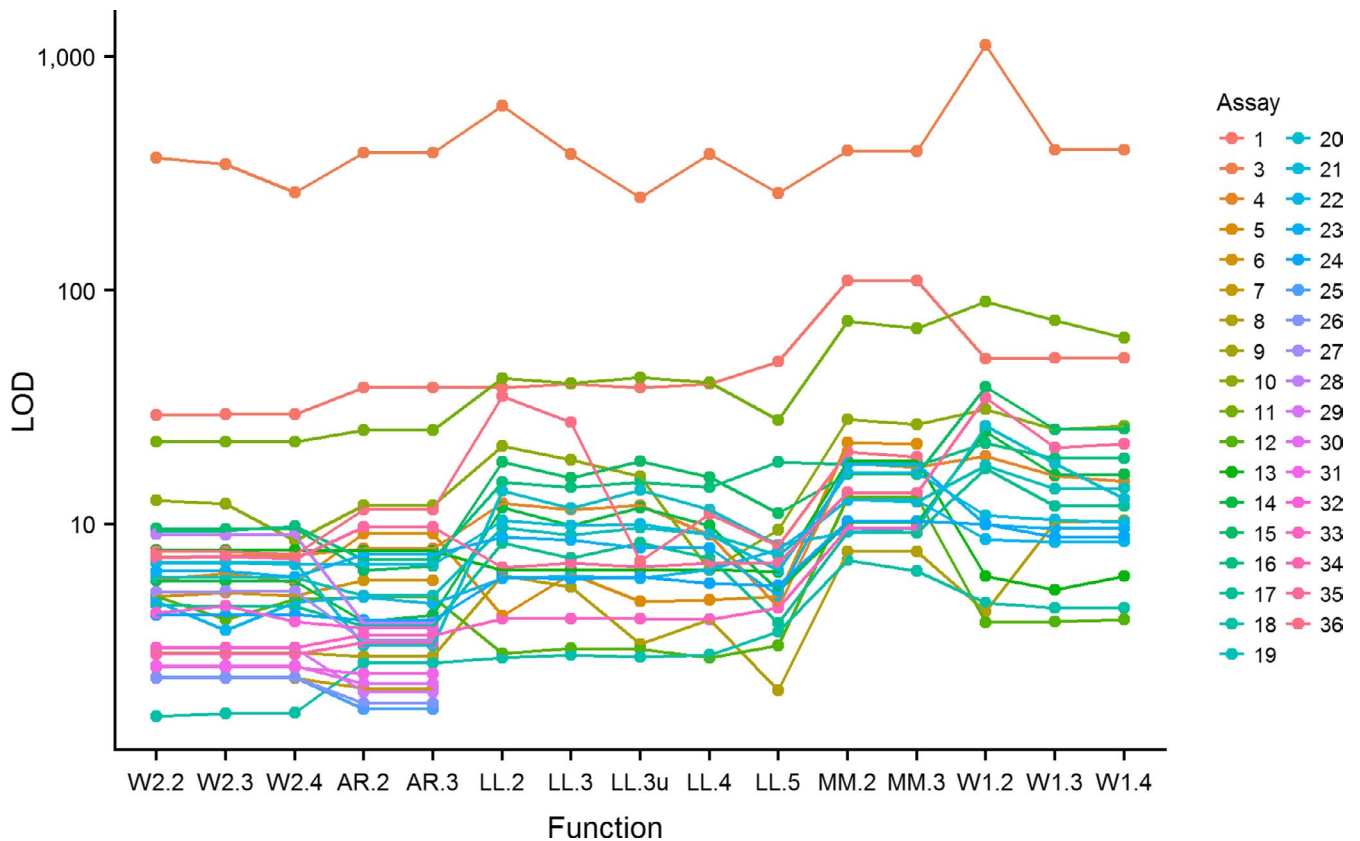
**FIGURE 3** Limits of detection by logarithmic function used. The different logarithmic functions tested are on the *x*-axis denoted by type and number of parameters (XX.#). AR = Asymptotic Regression, LL = Log-Logistic, MM = Michaelis–Menten, W1 = Weibull type I, W2 = Weibull type 2. The 3u number of parameters for LL.3u indicates a 3 parameter function with the upper limit set at 1, whereas the rest of the 3 parameter functions set the lower limit at 0 instead. Limit of detection as determined is on the y-axis in copies per reaction. Each assay tested is represented by a different color, and lines are drawn between to help identify shifts from 1 function to the next. Missing points for a given assay-function combination indicate that the model could not be solved for that data and function combination. Assay numbers are listed in Table 1

Five of the logarithmic functions were not able to fit all of our data sets. Of 36 assays tested, Weibull type II and Asymptotic Regression functions solved models for 35, Log-Logistic and Michaelis–Menten functions solved models for 24, and Weibull type I functions solved models for 21. In addition to conforming to the common practice of selecting the best model for fitting a data set (Akaike, 1973), this was also a consideration for testing multiple models for each data set instead of choosing one model to fit them all.

## 4 | DISCUSSION

### 4.1 | Defining and measuring LOD and LOQ

Our goal was to clarify definitions of LOD and LOQ within the context of qPCR assays for eDNA and provide simple, consistent methods for measuring these parameters for eDNA single-species assays. With LOD, we address the question "How many target DNA copies per reaction can one reliably detect with 95% confidence?" and with LOQ "How few target DNA copies per reaction can one reliably quantify with a defined precision?" These questions form the basis

for asking larger ecological questions as well as provide the precision required to infer occupancy and density from an eDNA data set.

Here, we defined the LOD as the lowest concentration at which we get 95% detection and LOQ as the lowest standard concentration with a CV value below 35%. These parameters were measured by running multiple replicate standard curves of an assay and then applying a 95% detection level or CV calculation. We recommend that future studies describing the development of qPCR-based eDNA assays adopt these definitions and report these parameters. It should be noted that the LOD and LOQ of an assay should be determined after the assay has been optimized for annealing temperature, primers/probe concentrations, etc. as these changes will affect the PCR efficiency as well as detection and quantification limits. When transferring an assay to a new laboratory, the LOD should be quantified to confirm similarity to the assay's published LOD before use to confirm that any changes in laboratory equipment, reagents, probe chemistry, or pipetting precision are not negatively affecting results.

The measurement of LOD and LOQ, using either the discrete threshold method or the curve-fitting modeling method, is a simple and straightforward procedure. The discrete threshold method

produces useful results; however, modeling the data to determine LOD and LOQ is a considerable improvement because rigorous testing of a large number of DNA concentrations is impractical and the selected standards analyzed can have a substantial subjective influence on the outcome. Furthermore, using models allows for the calculation of confidence intervals around effective LOD estimates. Various model forms can be applied to the sigmoidal data typically produced for LOD experiments, and our approach was to evaluate multiple models and select the best for each individual data set. Previously published LOD methods use probit-based approaches (CLSI, 2012; Stokdyk, Firnstahl, Spencer, Burch, & Borchardt, 2016; Wolk & Marlowe, 2011), but broadening the available models ensures that the selected model fits the data best and uses the full range of data.

## 4.2 | Application of the LOD/LOQ calculator

Our R script facilitates the calculation of LOD and LOQ and includes the flexibility of researcher inputs and decisions in several ways. The precision threshold is a user-defined setting in the LOD/LOQ calculator script. We set our precision threshold at 35% CV for determining LOQ (except as noted in Table 1), but we do not recommend a standardized maximum CV for qPCR LOQs. Instead, we suggest that researchers evaluate the decline in precision (i.e., increase in the CV) across standards, as this will vary depending on assay and laboratory conditions (e.g., standard curve material) and report their precision threshold and LOQ. For example, one of the high copy standards in assay 31 (Figure S10) was above our precision threshold for determining LOQ. However, there was reasonably stable precision for at least four standards tested, so we adjusted our precision threshold to more accurately estimate the LOQ where consistent precision begins to decline. In this way, visualization of the data informs selection of the CV threshold, and it also supports selection of the best model.

Additionally, we encourage eDNA researchers to visualize their results to verify model selection because poorly fit models can produce inaccurate results. For example, in assay 23 some fluctuation was being modeled that does not exist in the data (Figure S8, bottom panels). This resulted in the LOQ being mistakenly estimated at over 4,000 copies per reaction with 3 lower standards consistently showing greater precision than our 35% CV threshold (bottom-left, Figure S8). Adjusting the LOQ model to a 7th-order polynomial reduced the noise to fit within our precision threshold, and the model more accurately estimated the LOQ where precision begins to decline. Visualization of data can reveal other issues as well. For example, in our 36 data sets there was typically a drastic decline in precision as concentration decreased (Figures S6–S10), but in some cases the lowest copy standard had greater precision than higher-copy standards (Assays 1, 3, and 24 Figures S6 and S8). This feature is an artifact of successful amplification in fewer replicates, which means the CV is calculated from fewer values (Forootan et al., 2017), and this important detail could only be discerned through careful visual evaluation of the plotted results.

Because visualization of the data is important, we wrote our LOD/ LOQ calculator script to plot the data even if LOD or LOQ models cannot be determined (Assay 2, Figures S1 and S6). It is commonly accepted that qPCR does not have highly accurate quantifications at extremely low template concentrations, so it may not be intuitive for an eDNA researcher to test concentrations as low as necessary for accurate calculations. For example, in Assay 2, we did not test standards below 15.625 copies per reaction. At the lowest concentration tested, we observed 100% detection and quantified copy number with greater precision than the 35% CV threshold we set for determining LOQ. These plots can demonstrate to the researcher that their assay is performing better than expected and suggests additional lower-copy standards should be analyzed.

Determining an assay's LOD and LOQ provides an opportunity for assay optimization, laboratory improvements, and field survey design improvements. For instance, we identified two assays (1 and 3) that had high LOD and LOQs relative to assays run in other laboratories. This laboratory reran those assays using TE buffer and tRNA in their standards (now Assays 2 and 4), and the LOD and LOQs dropped to values similar to those observed in other laboratories. We hypothesize that the original standard working stock, which had been diluted in nuclease-free water, degraded or adsorbed onto the plastic tubes, making the true standard concentrations in the qPCRs less than the nominal concentrations, which were based on the measured absorbance of the concentrated stock solution. The addition of TE and tRNA may have stabilized the standard stocks and dilution series and allowed the concentrations in the reactions to remain closer to nominal (Green & Sambrook, 2012; Stürzenbaum, 1999; Wang, Xioa, Mindrinos, & Davis, 2002). Thus, we recommend the use of stabilizers in all DNA standard solutions, except to the initial stock whose concentration will be measured (Bustin et al., 2009). Further, low-copy standards have the potential to lose a larger proportion of their DNA copies to nonspecific adsorption on plastic of the vials or pipette tips. The use of tRNA in standard dilutions alleviates this problem by competing with the template DNA for adsorption to surfaces, leaving more of the template in solution and available for amplification. Adsorption can be further reduced by the use of low retention tubes and pipet tips, and consistent performance is aided by creation of fresh standard dilutions at least weekly.

Finally, our script provides information on the effective LOD which can be useful for designing an eDNA study or survey. The number of field and technical replicates can have a direct effect on the ability to detect DNA in the field, and on the ability to accurately calculate detection probabilities (Hunter et al., 2015; Erickson et al., 2019). As demonstrated in our study, the number of replicate amplifications affects the lowest target concentration one can reliably detect (Figure 3). In this way, determining LOD is a useful tool for helping researchers choose an appropriate balance between field replicates, volume of water per sample, and technical replicates.
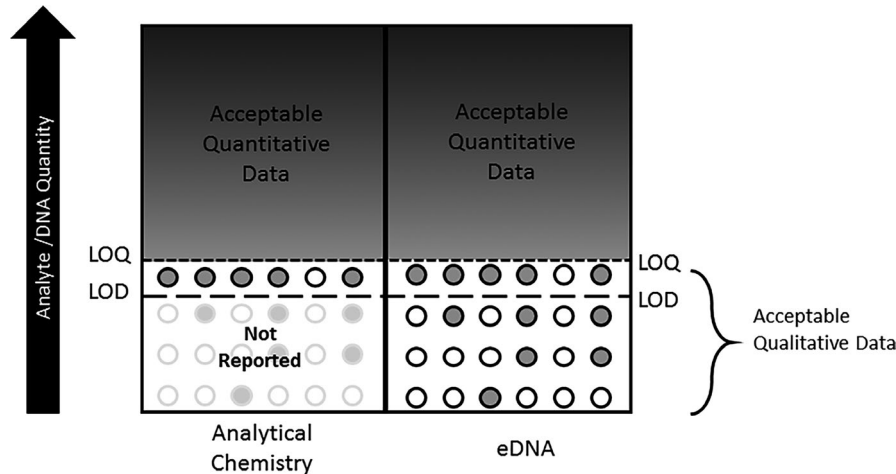
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**FIGURE 4** Conceptual diagram of interpretation of limit of detection (LOD) and limit of quantification (LOQ) in most analytical chemistry applications compared to quantitative eDNA assays. For most analytical chemistry applications, data above the LOQ are reported as quantitative data, data between the LOQ and LOD are reported as qualitative or semi-quantitative data, and data below the LOD are reported as nondetects. For eDNA analysis, data above the LOQ are reported as quantitative data, and data below the LOQ or below the LOD are reported as qualitative data. For eDNA assays, the interpretation of the LOD is that below this concentration, there is less than a 95% chance of detecting the target DNA sequence even if it is present at this low concentration

## 4.3 | Interpreting the LOD and LOQ

An important distinction between PCR-based measurements of eDNA and most other analytical chemistry techniques is that detections below the LOD should not simply be considered "noise." There is not a signal response (i.e., $C_q$ value) for truly negative samples ("noise"), so even low-concentration detections are meaningful. With eDNA studies, for example, detections of target eDNA at copy numbers below those of the LOD may be expected due to the rarity of target species. Such detections may still be true positives and are too important to disregard (Ellison, English, Burns, & Keer, 2006; Hunter et al., 2017; Kralik & Ricchi, 2017). The observation of eDNA detections below the LOD concentration indicates that there is less than the desired confidence level of detecting the target sequence when it is present at that low concentration (Figure 4). This is generally different from a chemical analyte where presence at concentrations below the LOD can often be ignored. For eDNA analyses, LOD is used as a measuring stick for comparing results across different assays or results from the same assay but different sampling locations or sample processing laboratories. Given the LOD is based on the probability of detection, detection rates for samples below the LOD are informative and detections below the LOD could be considered true positives given the criteria below (Ellison, English, Burns, & Keer, 2006; Hunter et al., 2017). For accurate qPCR detections to occur, we suggest that detections be made at no more than 40 cycles, the curve morphology needs to be uniform, and negative template controls show no amplification (also see Bustin et al., 2009). Quantitative PCR runs with evidence of contamination in the no-template PCR controls should be repeated.

Limit of quantification plays a critical role when attempting to determine predictive relationships between estimated target eDNA concentration or flux and target species biomass or numbers. Estimated eDNA concentrations below an LOQ should be evaluated qualitatively, as detections and nondetections. Robust and realistic interpretation of eDNA data can have important implications for eDNA monitoring programs, and it is critical that results be accurately communicated to wildlife managers, who can then decide whether such positive eDNA detections warrant further investigation in the field.

## 4.4 | Reporting LOD and LOQ

To facilitate the evaluation of eDNA assays and their results, the LOD and LOQ must be interpreted properly and easily, which requires a clear explanation and complete reporting. At a minimum, along with other parameters identified by Bustin et al. (2009) and Goldberg et al. (2016), we suggest that the reported LOD and LOQ values are accompanied by

1. The concentration range and number of replicate standards per concentration used for calculating LOD and LOQ,
2. The determination approach used (i.e., either the discrete threshold or curve-fitting modeling method) and,
3. The specific criteria for LOD probability of detection (e.g., 95%) and LOQ precision (e.g., 35% CV) that were applied.

In addition, given the diversity of LOD and LOQ concepts, we also suggest specifying the definitions that underlay LOD and LOQ to avoid misinterpretation or misuse (e.g., censoring positive results below the LOD). For qPCR-based eDNA studies, clear and complete descriptions of LOD and LOQ constitute only one part of methodological documentation that should also include primer design, sample collection, laboratory processing, and experimental

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design (Bustin et al., 2009; Mize et al., 2019; Strickland & Roberts, 2018; Wilcox et al., 2013, 2015). Additional details required for thorough descriptions of assays include the sequence and form of the standard template, efficiency of the amplification, and the modeling function and software used to compute the concentration values and report assay parameters (readers are directed to Bustin et al. (2009) and Goldberg et al. (2016)). It should be noted our methods are specific for assessing the LOD and LOQ of the assay in ideal conditions and are intended to provide baseline data of an assay's capabilities. The sensitivity of markers within actual environmental samples can be tested using in situ validation trials with known amounts of eDNA spiked into "samples" of the same or surrogate environmental matrices (Goldberg et al., 2016; Guan et al., 2019). Internal positive controls should be used with environmental samples to assess inhibitor compounds that will affect marker sensitivity (Goldberg et al., 2016; Wilson, Wozney, & Smith, 2016). As the field of eDNA grows, standardization of laboratory methods, assay development, and data analysis will continue to evolve (Dorazio & Erickson, 2018; Goldberg et al., 2016; Helbing & Hobbs, 2019). Following such standard reporting should facilitate easier implementation of assays across laboratories, allow better interpretation of results by managers making decisions about resource allocation when positive eDNA results are reported, and overall provide clear evaluation of assay performance.

# 5 | CONCLUSION

Management decisions are likely to be increasingly made using results from eDNA surveys; thus, data generated by eDNA studies must be reliable, defendable, and executed with high-quality assurance standards. Environmental DNA assay development, testing, and validation are critical steps in the process, and clear definitions of assay performance are needed in the eDNA community. Reporting assay quality metrics of performance in ideal conditions with known concentrations is a first step in any eDNA study and must be followed by additional demonstration of the assay using field samples. Understanding assay limits provides a solid base on which to build the rest of the eDNA survey protocols. We describe a cohesive set of definitions and determination methods that can be applied to eDNA studies, and the definitions and approaches in turn guide the interpretation and use of these metrics. Clear reporting of these and other qPCR performance metrics facilitates the evaluation and comparison of qPCR data across studies and provides resource managers with a sound basis for decision-making.

## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTION

Katy E. Klymus involved in conception and design of study, data acquisition, data analysis, data interpretation, writing of the manuscript, and all those phone calls. Christopher M. Merkes involved in conception, design of study, data acquisition, data analysis, data interpretation, and writing of the manuscript. Michael J. Allison performed data acquisition and data analysis. Caren Goldberg performed data acquisition, data analysis, data interpretation, and writing of the manuscript. Caren C. Helbing performed data acquisition, data analysis, data interpretation, and writing of the manuscript. Margaret E. Hunter involved in data analysis, data interpretation, and writing of the manuscript. Craig A. Jackson performed data acquisition and data analysis. Richard F. Lance performed data acquisition, data analysis, data interpretation, and writing of the manuscript. Anna M. Mangan performed data acquisition, data analysis, data interpretation, and writing of the manuscript. Emy M. Monroe involved in conception, data acquisition, data analysis, data interpretation, and writing of the manuscript. Antoinette J. Piaggio performed data acquisition, data analysis, data interpretation, and writing of the manuscript. Joel P. Stokdyk involved in design of study, data analysis, data interpretation, and writing of the manuscript. Chris C. Wilson performed data acquisition, data analysis, data interpretation, and writing of the manuscript. Catherine Richter involved in conception, data acquisition, data analysis, data interpretation, and writing of the manuscript.

## DATA AVAILABILITY STATEMENT

The R script code can be found at https://github.com/cmerkes/qPCR_LOD_Calc (https://doi.org/10.5066/P9GT00GB), and our additional analysis code can be found at https://github.com/cmerkes/LOD_Analysis (https://doi.org/10.5066/P9G4MPVQ). The data used in this study are available at https://doi.org/10.5066/P9AKHU1R.

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## SUPPORTING INFORMATION

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