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Optimization and Validation of Concentration-based Standard Curve for Quantification of *Alexandrium* spp. using eDNA

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In recent years, environmental DNA (eDNA) has emerged as a promising tool for detecting microorganisms in the natural environment. One specific application of eDNA technology is detecting and monitoring Harmful Algal Blooms (HABs), which can cause significant ecological and economic damage. In addition, quantitative analysis using eDNA is limited by the lack of optimized and validated Standard Curves. In this study, we aimed to optimize and validate a concentration-based Standard Curve for the quantitative analysis of HABs using eDNA. Instead of using DNA dilutions, which are commonly used in quantitative PCR (qPCR), the toxic red tide Alexandrium catenella was cultured and counted under a microscope, and Standard Curves were prepared by DNA extraction after dilution. By adjusting experimental conditions such as concentration, PCR amplification cycle, and specific primer design, we obtained an optimal Standard Curve with an R2 value close to 1. This Standard Curve showed similar results to those obtained using DNA dilution-based methods. Simultaneous quantification of eDNA from clonal Alexandrium catenella cultured in the laboratory and Alexandrium species from field samples showed a higher correlation with cell dilution than with DNA dilution. These results provide evidence that each Alexandrium spp. cells have a different amount of DNA. Consequently, when using a cell dilution-based Standard Curve to quantify Alexandrium spp. the Standard Curve is better determined by a dilution method based on cell counting. Overall, this study provides a useful method to optimize and validate concentration-based Standard Curves for eDNA-based quantitative analysis of HABs.

1. Introduction

Environmental DNA (eDNA) refers to the genetic material derived from organisms that are present in environmental samples such as water, soil, or air. This genetic material can come from various sources, such as shed skin cells, feces, mucus, or gametes, and can provide valuable information about the organisms present in a particular environment. In recent years, eDNA has emerged as a powerful tool for detecting and monitoring the presence of various species, including those that are difficult to detect using traditional survey methods. The analysis of eDNA has many applications, including biodiversity assessment, invasive species detection, and monitoring of endangered species, and eDNA has become an important tool in the study of aquatic ecosystems, where it has been used to detect and monitor Harmful Algal Blooms (HABs), aquatic invasive species, and fish populations (Thomsen and Willerslev, 2015).

Dinoflagellates belonging to the genus *Alexandrium* are a species of HABs found in oceans. These toxic microalgae are responsible for causing Paralytic Shellfish Poisoning (PSP), which is a global problem due to its impact on marine life and human health (Hallegraeff, 1993). *Alexandrium* spp. is distributed globally, and its wide distribution may be attributed to the potential transportation through ship ballast water. (Hallegraeff et al., 1990). Consequently, monitoring and early detection of PSP is important for governments, aquaculture practitioners, and scientists to reduce economic and health impacts. Traditional monitoring relies on counting cells under a microscope, which is time-consuming and subject to the researcher's ability to identify individual cells accurately (Coyne et al., 2005). Quantitative real-time PCR (qPCR) methods using eDNA have emerged as a faster and more accurate alternative to traditional cell counting methods for the quantitative detection of HABs species (Hatfield et al., 2019). Conventional qPCR methods typically extract a known density of target

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species, sequentially dilute the DNA, and generate a Standard Curve, which is then compared to a Ct (Threshold cycle) value to determine the abundance of the target species. Nevertheless, these conventional methods often show abundances that are lower than the actual cell count, which may be attributed to differences in the amount of intracellular DNA in cells of different sizes (Bahrenfeld et al., 2021). Consequently, there is a growing need for an improved qPCR method.

The primary objective of the study was to establish a Standard Curve for accurately measuring the appropriate cell density using qPCR analysis. To achieve this goal, the researchers compared DNA dilution methods with cell dilution methods in laboratory clone cultures and field samples. Due to the difficulty of diluting cells in the field, the researchers manually counted the number of target species in seawater under a microscope and used these counts to filter out eDNA from various volumes to use as a Standard Curve. By conducting this research and adopting a highly innovative approach that surpasses traditional DNA dilution methods, our study demonstrates the superiority of cell dilution methods in establishing an accurate Standard Curve. Through meticulous comparisons and analyses, we aim to identify the most accurate and reliable method for determining the appropriate cell density. This study aims to establish a foundation for future research to swiftly and precisely ascertain the suitable cell density.

2. Methods and Materials

2.1 Primer Set Design and qPCR Design for Quantitative Analysis

To specifically quantify the genus *Alexandrium*, we used the SYBR Green qPCR method and developed a new set of primers. The ITS2 region of *Alexandrium catenella* 16s rRNA (DQ785887.1) was selected from a gene bank (www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using the ClustalW program (www.genome.jp/tools-bin/clustalw), and species specificity was confirmed using NCBI BLAST (blast.ncbi.nlm.nih.gov). Primer sets for the target species were designed using Primer3 (Whitehead Institute and Howard Hughes Medical Institute, MD) and synthesized by Bioneer (Korea). The designed primers are listed in Table 1. To verify species specificity, we used *A. affine* (LIMS-PS-3447), *A. pacificum* (LIMS-PS-2611), and *Gymnodinium impudicum* (LIMS-PS-3373) were provided from Library of Marine Samples (LIMS, Korea), as well as *Skeltonema costatum*, *Nitzschia* spp., *Akashiwo sanguinea*, and *Thalassionema* spp. isolated in the field, as controls. All qPCR runs were amplified using a Thermal Cycler Dies Real-Time System III (TaKaRa, Japan) under the following conditions: Initial denaturation at 95°C for 5 min followed by 35 cycles of 95 °C for 30 s, 61 °C for 35 s, and 72 °C for 30 s. In addition, all experiments were performed in triplicate for reliability.

Table 1: The primer set was designed in this study.

Type	Name	Sequence (5′–3′)
Forward primer	Alx1F	TGGCATTGGAATGCAAAGTGG
Reverse primer	Alx1R	AATCCACACCCCGCAGGAAA

2.2 Standard Curve Method for Quantification

To determine a Standard Curve for field samples, *Alexandrium* spp. were sampled using a water sampler at 3 locations in Masan Bay, South Korea, in April 2023, when red tide occurred frequently (Figure 1). *A. catenella* (LIMS-PS-3427) provided from LIMS were cultured in f/2 medium for at least 6 weeks at 20±2 °C, 3,000-10,000 Lux illumination under fluorescent light. Cells fixed with Lugol's solution were enumerated using an SR chamber under a microscope. These cells were used for Figure 2a (DNA dilution method of the target species) and Figure 2b (cell dilution method of the target species). In Figure 2a, *A. catenella* 5,000 cells/mL were subjected to DNA extraction using the AccuPrep ® Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. The extracted DNA was diluted to concentrations of 100, 50, 25, 2.5, and 0.25 % using DNA elution buffer and used as a Standard Curve. Figure 2b shows a method with 5,000 cells/mL of *A. catenella* that were diluted to concentrations of 5,000, 2,500, 1,250, 125, and 12.5 cells/mL and were extracted using the same method as above for each concentration. The collected seawater was filtered in the laboratory using a 0.8 μm pore mixed cellulose ester filter (Advantec MFS, USA) in volumes of 500, 250, 125, 12.5, and 1.25 mL. The filter was then placed on a petri dish and aliquoted with 3 mL of PBS before being shaken at 200 rpm on a shaker for 2 h. The eDNA collected on the filter was then separated from the filter using a sterilized scalpel and tweezers, transferred to a 5 mL tube, and DNA was extracted as described above (Figure 2c). In Figure 2d, 500 mL of seawater was collected from Masan Bay, filtered through a 0.8 μm mixed cellulose ester filter, and the DNA was extracted in the same way as in Figure 2c. The extracted DNA was then diluted to concentrations of 100, 50, 25, 2.5, and 0.25 % using DNA elution buffer.

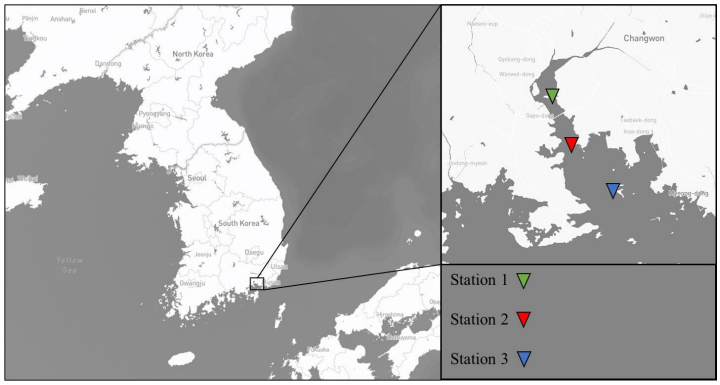

Figure 1: The field sample was taken from Masan Bay, Republic of Korea, in April 2023.

The DNA template obtained from all methods was used as a standard curve. To compare traditional and qPCR quantification methods, seawater (1,000 mL) was collected from each point (Figure 1) and stored in PVC bottles. This water was fixed with Lugol's solution and allowed to settle for 24 hours in the dark at 4 °C. Then 900 mL of the supernatant was removed and concentrated 10-fold. *Alexandrium* spp. were counted in triplicate using an SR chamber under a microscope.

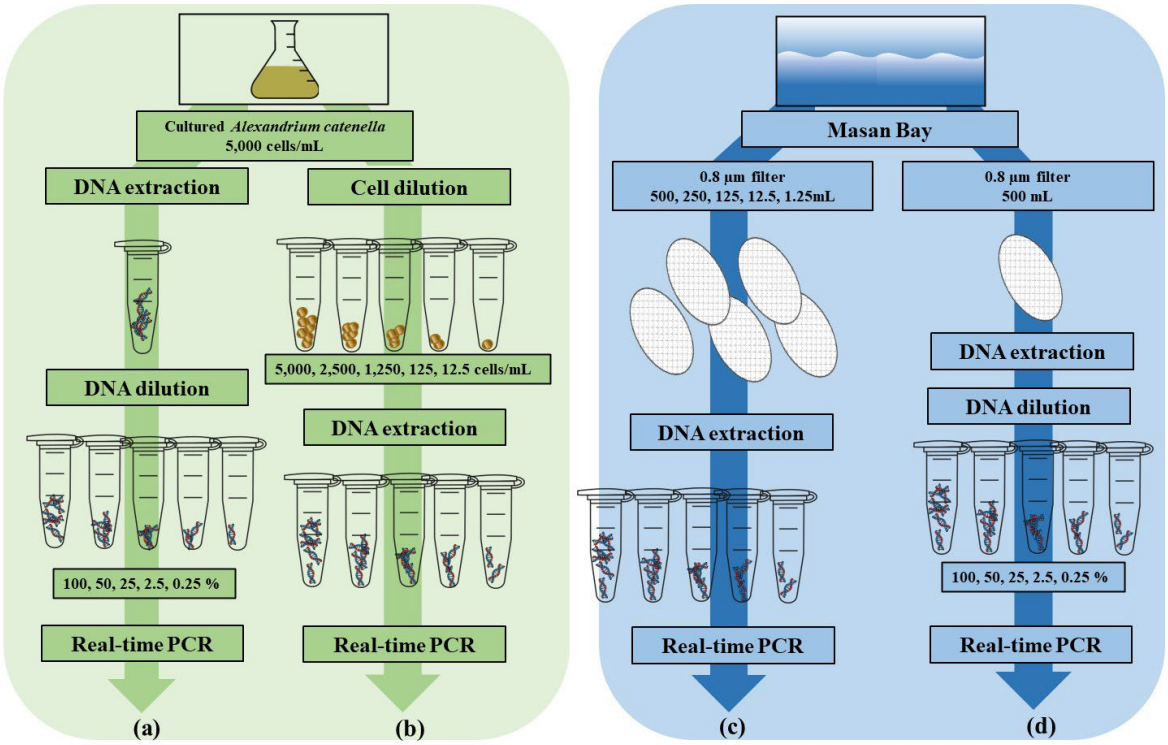

Figure 2: The process used to prepare the DNA and cell concentrations for the standard curve for the target species. (a) Dilution of DNA extracted from cultured Alexandrium catenella (b) Dilution of cultured cells (c) Dilution by volume in the field (d) Dilution of DNA extracted from samples obtained in the field.

3. Result

3.1 Confirmation of Species Specificity of Primers.

To confirm the species specificity of the primers used in the study, a qualitative analysis was performed using the same species that cause red tides during the same period and other Dinoflagellates algae (Table 2). The species specificity results showed that the Alx1 primer set specifically detected only *Alexandrium* spp.

Table 2: PCR assay results for species-specific primer validation

Species	Origin	Positive / Negative
Alexandrium catenella	LIMS	+
Alexandrium affine	LIMS	+
Alexandrium pacificum	LIMS	+
Gymnodinium impudicum	LIMS	-
Akashiwo sanguinea	Field	-
Thalassionema spp.	Field	-
Skeltonema costatum	Field	-
Nitzschia spp.	Field	-

3.2 Correlation Analysis of Cell Abundance and Ct Values

The correlation coefficient (R^2) value of the plot between the log of cell abundance and the critical cycle (Ct) values, after DNA extraction and dilution of laboratory cloned *Alexandrium catenella* (5,000 cells/mL), was 0.9967 (Figure 3a). The average abundance of *Alexandrium* spp. at stations 1, 2, and 3, represented by triplicate counting under a microscope, a traditional quantitative method, was 4.9±0.186, 5.533±0.247, and 4.667±0.067, each. Based on this, when a Standard Curve of Log (cell abundance) and Ct values were plotted, the R^2 values were determined to be 0.9729, 0.9977, and 0.9322, each (Figure 3).

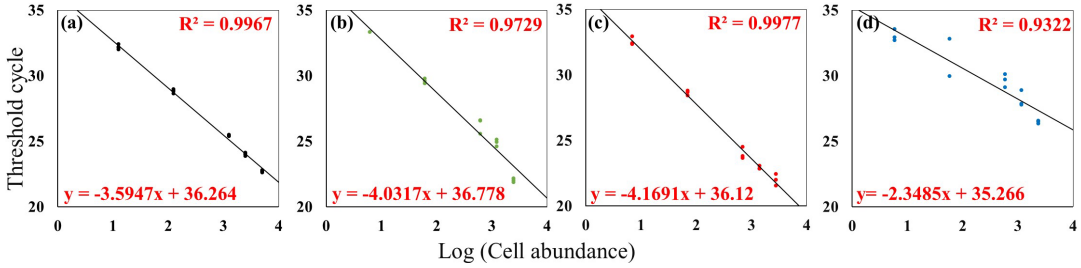

Figure 3: Standard curves ofDNA concentration and threshold cycle (Ct) as a function of DNA dilution for various samples. (a) Standard curve for laboratory culture of Alexandrium catenella; (b), (c), and (d) Standard curves for seawater samples taken from stations 1, 2, and 3, each case.

When the cell abundance of the laboratory clone culture was between 12.5-5,000 cells/mL, the R^2 value of the regression line of log (cell abundance) compared to (Ct) values was 0.9894, indicating correlation (Figure 4a).

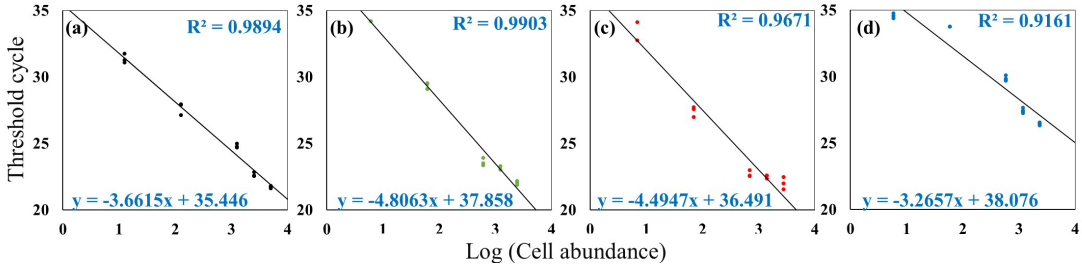

Figure 4: Standard curves for cell concentration and threshold cycle (Ct) as a function of cell dilution and seawater volume. (a) Cell dilutions ranging from 12.5-5,000 cells/mL for laboratory Alexandrium catenella clone cultures. (b), (c), and (d) are seawater volumes ranging from 1.25 to 500 mL for stations 1, 2, and 3, each case.

The cell concentration in the seawater at Station 1 ranged from 6.125-1225 cells/mL, with an R^2 value of 0.9903 for the Standard Curve (Figure 4b). The cell concentration at Stations 2 and 3 ranged from 6.917-1,383.333 cells/mL and 5.833-1,166.667 cells/mL, each, and the R^2 values were 0.9671 and 0.9161, each (Figure 4c, and d).

3.3 Correlation analysis between qPCR and microscopic quantification methods

Utilizing the slope of the DNA dilution method standard curve (Figure 3), the Ct values for the laboratory clone cells and Masan Bay vertices based on DNA concentration were calculated and correlated with the quantitative analysis performed under the microscope, resulting in an R^2 value of 0.8225 (Figure 5a). The qPCR Ct values obtained for the laboratory clone cells and each of the Masan Bay Stations, along with their corresponding cell

concentrations, were also correlated with the quantitative analysis under the microscope using the standard curve slope determined by the cell dilution method (Figure 4). This yielded an R^2 value of 0.8907 (Figure 5b).

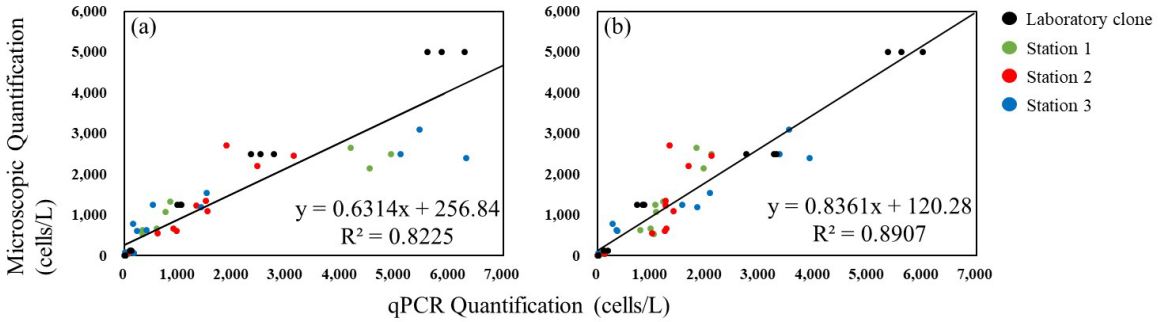

Figure 5: Correlation analysis between conventional microscopy quantification and qPCR quantification using Alexandrium spp. present at three stations in Masan Bay, in April 2023, and A. catenella cultured in the laboratory. (a) displays the correlations using the standard curve determined by the DNA dilution method, while (b) shows the correlations using the standard curve determined by the cell dilution method.

4. Discussion

Paralytic shellfish poisoning (PSP) can be caused by a neurotoxin called saxitoxin in most coastal farmed bivalves, and humans can die at a dose of about 14 mg (Genenah and Shimizu, 1981). Reported fatalities from PSP in Korea in 1984, 1986 (Chang et al., 1987), and 1996 (Lee et al., 1997). Early detection and prevention of these toxic red tides can help minimize their impact. Nevertheless, traditional monitoring methods can be time-consuming and the results can be erroneous depending on the knowledge of the expert. While traditional monitoring methods can be time-consuming and prone to errors due to expert knowledge, monitoring with eDNA has the potential to offer faster and more accurate detection if established protocols are adhered to. Based on these technologies, water quality can be analyzed in real-time to provide early warning of algal blooms, reduce water pollution, and ensure the cleanliness of water resources (Rong Lin, 2018).

Rapid detection and accuracy are critical to preventing toxic red tide organisms such as *Alexandrium* spp. in the marine environment. Many researchers have used eDNA present in seawater (Castro-Cubillos et al., 2022) or ship ballast water (Shaw et al., 2019) for monitoring. Quantitative monitoring using eDNA is still being developed in many studies. For such quantitative monitoring, an appropriate standard curve is important.

In this study, we aimed to validate a qPCR assay method for the quantitative analysis of *Alexandrium* spp. during a red tide event that occurred in April 2023 in Masan Bay, a region in the southeastern part of South Korea. In the experiment, qPCR analysis was performed using a DNA dilution method using a Standard Curve and a cell dilution method. The standard curves for the DNA dilution method and the cell dilution method used in conventional qPCR showed that both methods had R^2 values close to 1 for cloned cells in the lab and the field. Even so, the R^2 values for the DNA dilution method ranged from 0.9322-0.9977, which was closer to 1 than the R2 values for the cell dilution method of 0.9161-0.9903 (Figure 3 and 4). The DNA dilution method is likely to bring the amount of DNA in serial dilutions closer to the target DNA concentration than the cell dilution method. And it should be noted that the amount of DNA in flagellate algal cells can vary based on different factors such as the growth stage and physiological conditions of the cells (Gribble and Anderson, 2007). More, different cell sizes may contain different amounts of DNA in their cells (Bahrenfeld et al., 2021). The researchers compared results obtained using two standard curve methods based on DNA dilution and cell dilution and assessed the validity of each method, The correlation analysis between the qPCR and microscopy results using the DNA dilution method standard curve showed an R^2 value of 0.8225. When using the cell dilution method standard curve, the R^2 value increased to 0.8907, indicating a stronger correlation between the qPCR and microscopy results (Figure 5). Some studies reported that the amount and copy number of a certain portion of DNA in Dinoflagellate cells even in a culture may vary (Hou et al., 2010). So, the amount of DNA in each *Alexandrium* spp. cells may not be the same, so the standard curve determined by the DNA dilution method may not correspond to the actual cell count.

The researchers also tested the specificity of the primer set (Table 1) used in the study and found that they only detected *Alexandrium* spp., demonstrating their species specificity (Table 2). Overall, the study demonstrated the effectiveness of qPCR as a tool for the detection and quantification of *Alexandrium* spp. in seawater samples, with the cell dilution method being more effective than the DNA dilution method. This information can be useful for monitoring HABs in Masan Bay and other coastal areas. Besides, this study demonstrated that qPCR-based monitoring of HABs can be used as a complementary method to traditional microscopy-based monitoring, providing a rapid and sensitive means of detecting and quantifying HABs in marine environments. The results

of this study suggest that the use of qPCR can provide a reliable method for detecting and quantifying HABs, with the potential for widespread application in monitoring programs. Even so, further research is needed to optimize and standardize qPCR methods for specific HABs species and to determine the limits of detection and quantification for each species. Overall, this study compared the cell concentration-based standard curve for the quantification of *Alexandrium* spp. with the DNA dilution-based standard curve often used in previous studies and found that the cell concentration-based standard curve had R_2 values closer to 1 than the DNA dilution-based standard curve.

5. Conclusion

The results obtained in this study show that the standard curve plays a very important role in eDNA qPCR quantitative monitoring. In particular, the standard curve based on the correlation between Ct and cell abundance is one of the most important factors for accurate cell abundance measurement. For this reason, it is very important to select an appropriate standard curve and to check the accuracy of the standard curve.

Acknowledgments

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