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Development of a Nuclease Protection Assay With Sandwich Hybridization (NPA-SH) to Monitor *Heterosigma akashiwo*

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Heterosigma akashiwo is a globally distributed raphidophyte that forms blooms and causes significant losses to the aquaculture industry in many coastal countries. The development of a fast and sensitive detection method is therefore required to facilitate the appropriate warning of harmful algal blooms. In this study, a nuclease protection integrated with sandwich hybridization (NPA-SH) assay was developed to both qualitatively and quantitatively detect *H. akashiwo*. The NPA, capture and signal probes were designed by nucleotide sequencing of *H. akashiwo*. The applicability of NPA-SH was evaluated using cultured *H. akashiwo* cells and field samples collected at Goseong Bay, Korea. The results show that this method has good applicability and effectiveness in analyzing cultured cells and field samples. A linear regression equation for the quantitative analysis of *H. akashiwo* was obtained, and the lower detection limit of the assay was 1×10^4 cells/ml. There was no statistically significant difference in the results of *H. akashiwo* quantitation using NPA-SH compared to those obtained using a microscope. These results indicate that NPA-SH can be a good alternative to the traditional microscopic method used to monitor *H. akashiwo*.

Key words : Algal blooms, field monitoring, HABs, *Heterosigma akashiwo,* NPA-SH

Introduction

Harmful algal blooms (HABs) caused by toxic species have a negative effect on fisheries, human health, tourism and aquatic ecosystems. *Heterosigma akashiwo* (Hada) (Chromophyta: Raphidophyceae) is a eurythermal and euryhaline flagellated golden-brown marine microalga with a global distribution [1]. This harmful microalga has been associated with blooms that kill fish and shellfish on the coasts of many countries. Therefore, *H. akashiwo* monitoring is needed to optimize the early warning of HABs and fish loss [7, 16].

Like many microalgal species, *H. akashiwo* is not easy to identify by optical microscopy because of its high morphological diversity [2, 16]. *H. akashiwo* cells are small (8-25 μm in length and 6-15 μm in width), fragile, and vary in shape depending on various sea conditions [2, 14]. Also, the morphological features, size and color of *H. akashiwo* could be changed when exposed to fixative [4]. Therefore, substantial experience and expertise are needed to microscopically identify natural *Heterosigma* species. Consequently, a fast and accurate detection method is required for effective monitoring of *H. akashiwo*. Molecular probes have replaced classical microscopy for identification at the species level [12]. Accordingly, various detection methods, such as sandwich hybridization assay (SHA), fluorescence in situ hybridization (FISH), DNA array, quantitative real-time PCR (qRT-PCR), and loop-mediated isothermal amplification (LAMP), have been applied for *H. akashiwo* [4, 5, 11, 12, 16].

H. akashiwo-specific SHA methods and comparative studies with qPCR for environmental samples have been reported [5, 8, 12]. However, SHA has been estimated to have low species specificity, sensitivity and reproducibility due to unstable reactivity [6]. To overcome these disadvantages, development of a nuclease protection (NPA-SH) assay based on sandwich hybridization was required. Previous researchers have developed NPA-SH methods to detect environmental samples of various HAB species. The NPA-SH methods upgrade specificity and sensitivity. Also, It can be checked the result visually, and reduce the time for preparation and hybridization [9]. Until now, NPA-SH methods for *Prorocentrum minimum*, *P. micans*, *P. donghaiense*, *Skeletonema*

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costatum, *Phaeocystis globosa*, *Cochlodinium polykrikoides*, and *Heterocapsa triquetra* [3, 9, 10, 15, 17] were developed. However, NPA-SH probes for *H. akashiwo* detection have not yet been developed.

In this study, we developed a species-specific NPA-SH probe set for the rapid and accurate detection of *H. akashiwo* and performed qualitative and quantitative analysis. The species specificity and sensitivity of the developed probes were analyzed, and *H. akashiwo* abundance was monitored by applying the probes to environmental samples.

Materials and Methods

Algal cultures

Six species of HAB-causing marine microalgae: *Chattonella marina*, *Cochlodinium polykrikoides*, *Heterocapsa triquetra*, *Heterosigma akashiwo*, *Prorocentrum minimum*, and *Scrippsiella trochoidea*, were obtained from the Library of Marine Samples, KIOST, Korea (Table 1). Microalgae were cultured axenically in f/2 medium at 20℃ under a 12 hr light–dark cycle with a photon flux density of 4,000 lx.

Primer design and RT-PCR

RNA was extracted using the modified method of Venugopalan and Kapoor [13]. Primer design, RT- PCR and LSU rRNA sequencing were performed for *H. akashiwo* [9, 10]. Primers for RT-PCR were designed using Oligo 6.0 in the most conserved regions of LSU rRNA (forward 5′-CGGAGGAAAAGAAACTAAC-3′, reverse 5′-AGCTACT AGATGGTTCGAT-3′) [18]. The primers were evaluated using Oligo software and chemically synthesized by Bioneer Corporation (Daejeon, Korea). RT-PCR amplification, cloning and sequencing were performed using primers and commercial kit (One-Step RNA PCR Kit; TaKaRa, Biotechnology, Seoul, Korea). All PCR products were separated by 1% agarose gel electrophoresis and the DNA bands were purified by using MEGA-spin™ Agarose Gel DNA Extraction Kit (Intron, Korea). The PCR products were cloned into pGEM-T-Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5α competent cells. Cloned LSU rRNA genes were sequenced by Bioneer Corporation (Daejeon, Korea) and LSU rRNA sequences for *H. akashiwo* were obtained.

Table 1. Algal species

Species	Collection site
Chattonella marina	Tongyeong, Korea
Cochlodinium polykrikoides	Masan, Korea
Heterocapsa triquetra	brackish Lake Shihwa, Korea
Heterosigma akashiwo	Tongyeong, Korea
Prorocentrum minimum	Tongyeong, Korea
Scrippsiella trochoidea	Tongyeong, Korea

Probe design and synthesis

The LSU rDNA sequence of *H. akashiwo* was identified by searching with BLASTn (http://www.ncbi.nlm.nih.gov/Blast.cgi). To design *H. akashiwo*-specific NPA-SH probes, the nucleotide sequences of the six microalgae used in this study, as well as other microalgae belonging to the same genus, were analyzed. The specificity of NPA probes was verified using Clustal W in MEGA6, and capture probe and signal probes were designed (Table 2). The capture probe was complementary to the 3′-end of the NPA probe and the 5′-end was labeled with biotin. The signal probe was complementary to the 5′-end of the NPA probe and the 3′-end was labeled with fluorescein. All designed probes were chemically synthesized by Bioneer Corporation (Daejeon, Korea).

NPA-SH assay

NPA-SH was performed to detect *H. akashiwo* qualitatively and quantitatively [3, 9]. Capture probe immobilization, microalgae collection and lysis, protection with S1 nuclease and sandwich hybridization with the capture probe were carried out. After that, the absorbance was measured at 450 nm and 620 nm using a plate reader (FLUOstar, BMG Thermo Fisher Scientific Inc., USA) and the A450 nm/A620 nm ratio was calculated.

Specificity

Five species of microalgae (Table 1): *Chattonella marina*, *Cochlodinium polykrikoides*, *Heterocapsa triquetra*, *Prorocentrum minimum* and *Scrippsiella trochoidea* were selected to test the specificity of the assay for *H. akashiwo*. All six species are common in Korean coastal waters. The cultured microalgae were collected from the stationary growth phase, lysed, and the NPA-SH assay was performed. The experiment was carried out in triplicate. The probe specificity for *H. akashiwo* was evaluated by comparing the absorbance values of *H. akashiwo* with other microalgae.

Calibration curve

For the quantitative NPA-SH assay, calibration curves

were constructed. *H. akashiwo* cells in the stationary growth phase were collected, serially diluted, and analyzed using both NPA-SH and microscopy. The experiment was carried out in four replicates. The absorbance value of NPA-SH and the number of cells obtained from the microscope were plotted to establish a calibration curve. The correlation between absorbance value and cell number was calculated.

Cultured and field sample tests

To evaluate the applicability of NPA-SH to the field samples, natural sea water was collected at Goseong Bay, Korea contained with *H. akashiwo* cells. The samples were evenly divided into two parts. One was analyzed with the NPA-SH probe for *H. akashiwo*, while the other was counted using a microscope. All experiments were repeated three times.

For NPA-SH analysis of mixed cells, samples of pure cultured *C. marina*, *C. polykrikoides*, *H. triquetra*, *P. minimum*, *S. trochoidea* and *H. akashiwo* were collected in similar cell counts and RNA was extracted. The extracts were mixed with *H. akashiwo* NPA probes and analyzed using NPA-SH. Each assay was performed in triplicate. NPA-SH was applied to monitor the abundance of *H. akashiwo* in field samples. Natural seawater was sampled from January to December 2014 near Tongyeong, Korea. Samples were collected by microfiltration with a 0.45 μm Millipore membrane and stored at -70℃ until NPA-SH analysis.

Statistical analysis

The NPA-SH assay was performed with at least 3 replicates per experiment. All data are expressed as means ± SE. Differences between the control and each experimental group were analyzed using Student's t-test. One-way ANOVA (Tukey's multiple comparison test) was applied to evaluate the difference between the control and each experimental group. Statistical significance was assigned to $p<0.05$ and $p<0.01$.

Result and Discussion

Probe specificity

For the results of NPA-SH analysis of mixed microalgal species, the A450 nm/A620 nm value of each sample was expressed as a relative value to the negative control group (Fig. 1A). The absorbance value (1.402) for *H. akashiwo* was clearly higher than that for other microalgae (<0.98) ($p<0.01$). In addition, when analyzing samples with (+mix) or without (-mix) *H. akashiwo*, the NPA-SH absorbance value was found only in the sample including *H. akashiwo* (+mix) (Fig. 1B, $p<0.01$). These results indicate that the NPA-SH oligonucleotide probe can distinguish *H. akashiwo* from five other microalgae.

Probe sensitivity

H. akashiwo samples were collected, serially diluted, and compared using the NPA-SH method and microscopic cell counting method. The absorbance value of NPA-SH was plotted against the number of cells determined microscopically (Fig. 2A). NPA-SH was performed at concentrations

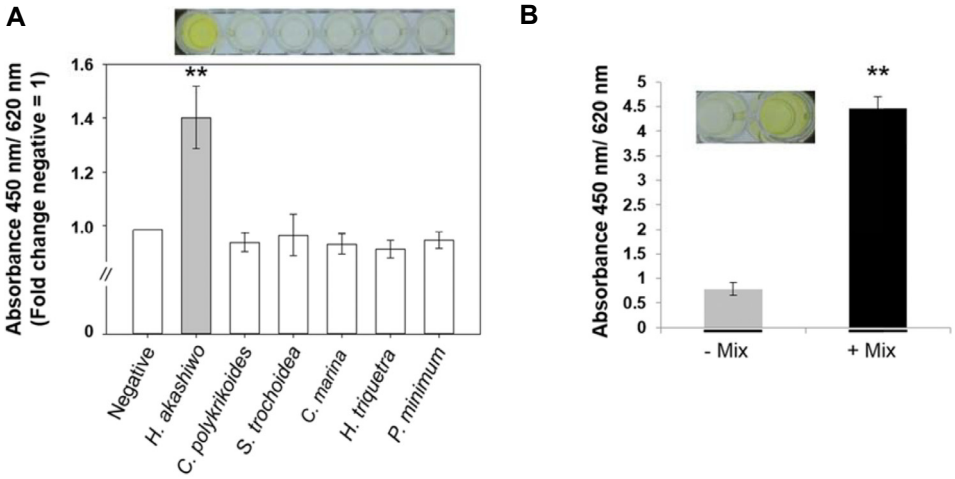

Fig. 1. Specificity of the sandwich hybridization integrated with nuclease protection assay (NPA-SH) probes for *H. akashiwo*. A. Comparison of NPA-SH assay on the six species of microalgae. Negative control is the microalgae mix with all species except *H. akashiwo*. B. Comparison of NPA-SH assay on the samples with and without *H. akashiwo*. Data are expressed as means ± SE and obtained from triplicate experiments. ** denotes a significant difference compared with the controls ($p<0.01$).

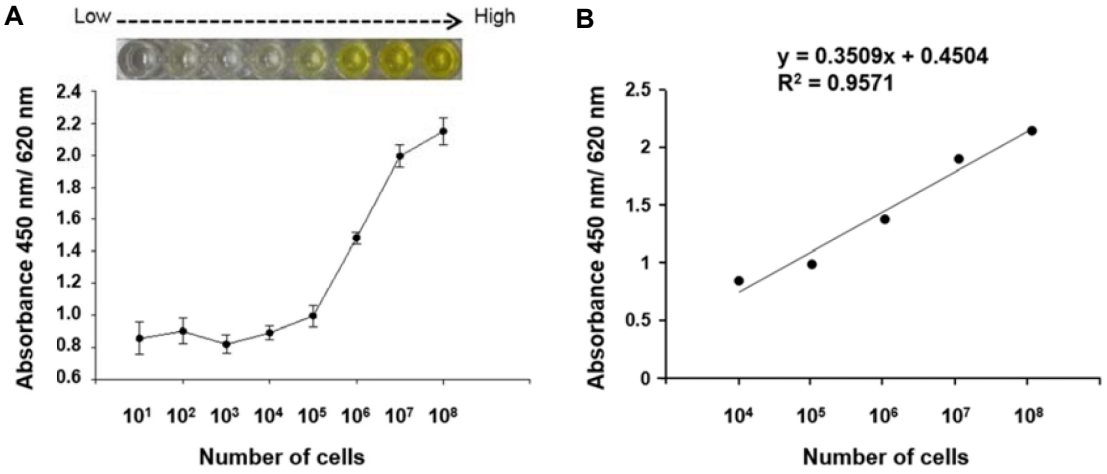

Fig. 2. Absorbance value (A) and regression curve (B) for the detection of *H. akashiwo* by NPA-SH assay. Data are expressed as means ± SE and obtained from triplicate experiments.

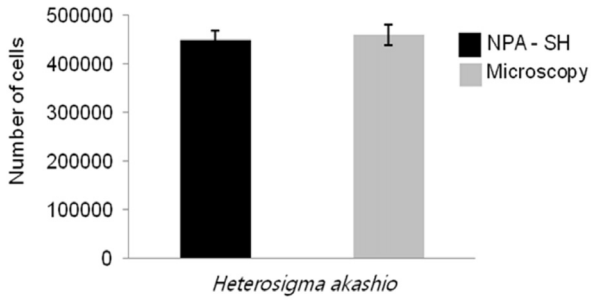

Fig. 3. Comparison of NPA-SH vs. microscopy assay. Data are expressed as means ± SE obtained from triplicate experiments.

between 1×10^1 and 1×10^8 cells/ml and a standard curve was established between 1×10^4 and 1×10^8 cells/ml. The fitted linear regression equation was y = 0.3509x + 0.4504 and R^2 = 0.9571 (Fig. 2B), where x is the cell density (number of cells per ml of seawater) and y is the absorbance value (A450 nm/A620 nm). The linearity of the regression equation indicates that it can be used to transform NPA-SH absorbance values to cell numbers. The results show that the detection limit of NPA-SH is 1×10^4 cells/ml, which is lower than that required for a red tide alert (3.0×10^4 cells/ml) by the Korea Ministry of Oceans and Fisheries in 2015.

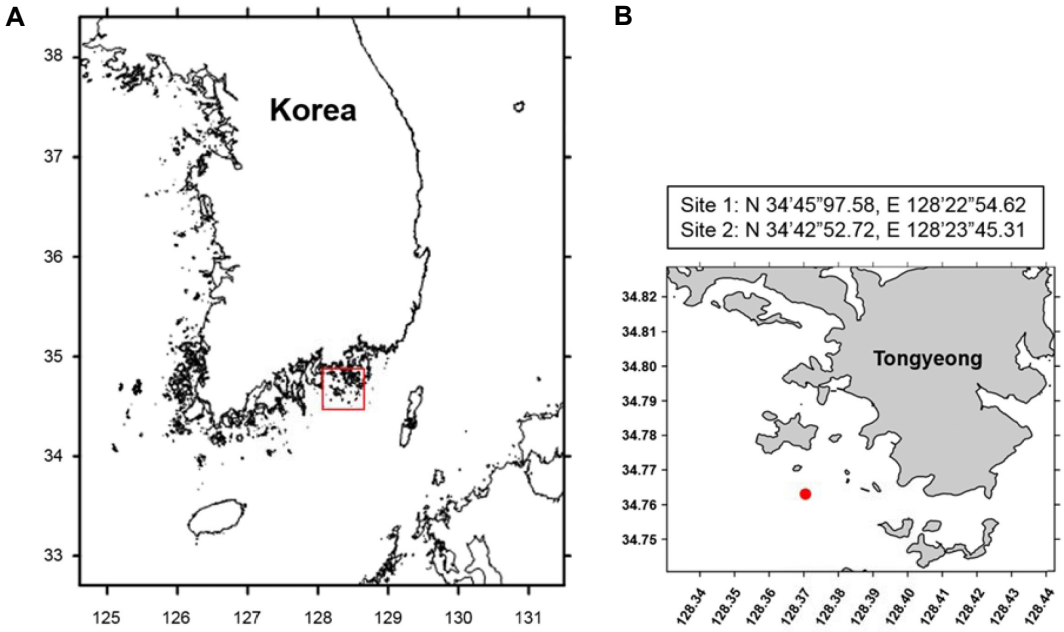

Fig. 4. Sampling locations near Tongyeong, in the South Sea of Korea. A. The red square shows the location of Tongyeong. B. Expansion of Tongyeong region. The red circle shows the sampling location.

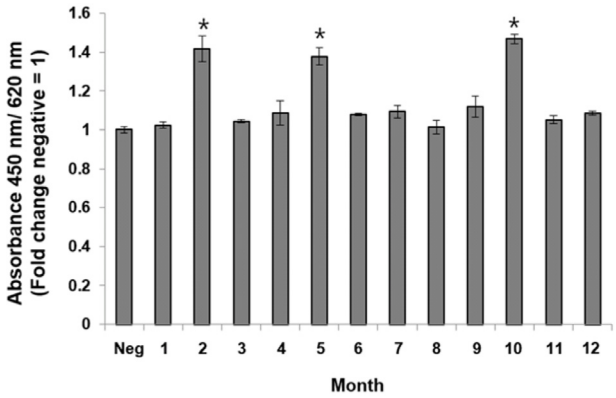

Fig. 5. Application of NPA-SH probes for detection of annual field samples from Tongyeong, Korea. Data are expressed as means ± SE and obtained from triplicate experiments. Neg is a negative control using natural seawater without phytoplankton. * denotes a significant difference compared with the controls (p<0.05).

Comparison of NPA-SH and microscopic cell counts

The results obtained from NPA-SH and microscopic analysis of *H. akashiwo* cells in natural sea water were compared (Fig. 3). The number of *H. akashiwo* cells estimated with NPA-SH and the number of cells counted using a microscope were similar (Fig. 3). There was no statistically significant difference (p<0.05) and the mean deviation between the two methods was 5.4%. These results indicate that the substances present in natural seawater do not affect the qualitative and quantitative NPA-SH detection of *H. akashiwo*.

NPA-SH Probe testing in field samples

Finally, the NPA-SH detection method was applied to monitor the annual occurrence of *H. akashiwo*. Each 100 ml of the site samples were collected three times a month in Goseong Bay, Korea (Fig. 4) in 2014 and analyzed using NPA-SH probes capable of detecting *H. akashiwo*. When the occurrence of microalgae was analyzed for one year, *H. akashiwo* appeared in February, May, and October, and no statistically significant (p<0.05) microalgae were observed in the other months (Fig. 5). Based on the standard regression equation, the numbers of *H. akashiwo* cells in February, May, and October were 2,752, 2,641, and 2,900 cells/ml, respectively. Taken together, these results suggest that the NPA-SH probe for *H. akashiwo* detection in this study is very useful for in situ monitoring in marine environments.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : *Heterosigma akashiwo*를 모니터하기 위한 뉴클레아제 보호 분석이 통합된 샌드위치 혼성(NPA-SH)의 개발

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*Heterosigma akashiwo*는 전세계적으로 분포된 침편모조류이며, 대발생을 형성하여 많은 나라의 양식산업에 커다란 손실을 유발시킨다. 따라서 빠르고 민감한 검출방법을 개발하는 것은 유해조류 대발생에 대한 적절한 경보를 위해서 필요하다. 이 연구에서는 *H. akashiwo*를 정성 및 정량적으로 검출하기 위하여 뉴클레아제 보호 분석이 통합된 샌드위치 혼성(NPA-SH)을 개발하였다. NPA-SH 프로브는 여섯 종의 미세조류 핵산 서열을 이용하여 디자인 후, 특이성을 확인하여 capture 프로브와 signal 프로브를 선정하였다. 배양시료와 현장시료를 이용하여 NPA-SH의 적용성을 평가한 결과, NPA-SH의 좋은 적용성과 효과를 확인하였다. *H.akashiwo*의 정량분석을 위한 선형회귀식을 확인하였으며, 최소 검출한계는 1×10^4 cells/ml이었다. NPA-SH를 사용하여 얻은 *H. akashiwo*의 정량결과와 현미경을 사용하여 얻은 결과 사이에는 통계학적으로 유의한 차이는 없었다. NPA-SH 분석은 환경시료에 잘 적용되었다. 이러한 결과는 NPA-SH가 *H. akashiwo*의 모니터링에 사용되어 왔던 전통적인 현미경적 방법에 대한 좋은 대안이 될 수 있음을 보여준다.