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Rapid Detection of *Cochlodinium polykrikoides* by Sandwich Hybridization Integrated with Invertase Assay

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Invertase 분석이 통합된 sandwich hybridization에 의한 *Cochlodinium polykrikoides*의 신속 검출

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요 약 유해조류 대발생(harmful algal blooms, HABs)을 유발하는 해양 미세조류의 신속하고 정확한 종판별은 HABs을 예측하고 관리하기 위한 매우 중요한 도구이다. 우리는 이전 연구에서 적조 유발 미세조류인 *Cochlodinium polykrikoides*를 현장에서 검출하기 위한 nuclease protection assay sandwich hybridization (NPA-SH) 방법을 개발한 바 있다. 본 연구에서는 *C. polykrikoides* 검출을 위한 NPA-SH 방법의 반응 단계를 간소화시켜 현장 적용성을 향상시키는 것을 목적으로, signal probe에 형광 대신 invertase (INV)를 결합시켰으며, hybridization 과정에서 sucrose를 반응물로 사용하여 발색 반응을 유도하였다. INV와 signal probe의 결합 여부는 SDS-PAGE와 형광 현미경을 사용하여 확인하였으며, 반응 최적화를 위한 적정 프로브의 양, sucrose의 양 및 처리시간 등을 정립하였다. 본 연구의 결과 개발된 INV-SH는 NPA-SH와 비교하여 현장에서의 처리시간이 감소되었으며, 흡광도 측정기 뿐만 아니라, 상대적으로 부피가 작고 값 싼 개인 혈당계 사용이 가능하게 되었다. 본 연구에서 개발된 INV-SH는 INV가 적용된 최초의 *C. polykrikoides* 종판별 기술이며, 증진된 현장기술이 될 수 있다.

Abstract Rapid and accurate identification of marine microalgae causing harmful algal blooms (HABs) is a crucial tool for predicting and managing HABs. We previously developed a nuclease protection assay sandwich hybridization (NPA-SH) method for the in situ detection of blooming microalgae *Cochlodinium polykrikoides*. In this study, we improved the applicability of the NPA-SH method for the detection of *C. polykrikoides* by simplifying the reaction step. For this purpose, invertase (INV) was conjugated to the signal probe instead of using fluorescence, and sucrose was used as a reactant to induce a color reaction. The INV-signal probe conjugation was confirmed by SDS-PAGE and epifluoromicroscopy. The treatment time and appropriate amounts of the probe and sucrose that optimized the reaction were determined. As a result, the developed INV-SH reduced the treatment time in the field compared with NPA-SH, and also enabled the use of a relatively small volume and low-priced personal glucose meter, as well as an absorbance meter. INV-SH is the first *C. polykrikoides* species identification technology to which INV has been applied and could be an improved field technique.

Keywords : HABs, *C. polykrikoides*, NPA-SH, INV-SH, Invertase

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1. Introduction

The sandwich hybridization assay (SHA) is designed with capture and signal probes for rRNA, and the two probes react using the principle of sandwich hybridization. Since the SHA method generally has a unique nucleotide sequence in the 18S rRNA that is present as many copies in cells, multiple researchers have developed an SHA probe for detecting harmful algae such as *Heterosigma akashiwo, Alexandrium minutum*, and *Gymnodinium catenatum*[1-3].

Since the SHA is applied to relatively unstable RNA, the issues of low specificity and reproducibility has been raised. Therefore, nuclease has been used to degrade single-stranded nucleic acids present in the reaction mixture, while leaving only the double-stranded nucleic acid present[4,5]. The sandwich hybridization method using the nuclease protection assay (NPA-SH) has the advantage of being able to target a large number of rRNAs in the cell, and enhances the specificity and reproducibility[5]. The NPA-SH method has been applied by previous researchers to detect various microalgae such as *Prorocentrum minimum*, *Phaeocystis globosa*, *Cochlodinium polykrikoides*, and *Heterocapsa triquetra*[4,6-8].

Meanwhile, invertase (INV) is an enzyme that degrades sucrose into glucose and fructose, and the reaction product, glucose, can be measured using a commonly available personal glucose meter. Research on the use of invertase in the field of point-of-care diagnosis systems has recently become a focus of attention[9-12]. The developed invertase-conjugated DNA has adverse effects on the structure and activity of the enzyme, and the disadvantage in that the reproducibility is poor in the separation step has been reported[13]. Nonetheless, many studies have been carried out to apply to the detection of various molecules in many research groups[14-15].

The purpose of this study was to develop a sandwich hybridization method integrated with an invertase assay for easy and rapid utilization of the NPA-SH method developed for the detection of *C. polykrikoides*[7]. For this purpose, an INV conjugated signal probe was developed, sucrose was used as a final reactant, and a chromogenic system for detecting glucose was developed. The INV-SH technique contributes to the development of techniques for detecting harmful algae, such as red tide, quickly and accurately in the field.

2. Materials and Methods

2.1 Microalgal cultures

Cochlodinium polykrikoides to be used for the study was obtained from the Library of Marine Samples in KIOST. The microalgae were cultured in F/2 medium at 20 °C under 12 h light-dark cycle. *C. polykrikoides* were sub-cultured at intervals of about 2 weeks, and exponential growth cells were used for INV-SH experiments.

2.2 Probe design and synthesis

RNA was extracted from cultured *C. polykrikoides*. Then, rRNA primer design, RT-PCR, probe design, and synthesis were performed according to the method of Suh et al.[7]. Designed oligonucleotides were synthesized by Bioneer Inc. (Table 1). Capture probes were labeled with biotin and designed to be complementary to the 3'-terminal region of the NPA probe. On the other hand, the signal probe was synthesized without labeling for invertase (INV) conjugation.

Table 1. Designed probes for INV-SH (From Suh et al., 2016)

Probes	Nucleotide sequences	Size (mer)
NPA	5'-TTAATGCCAGGACGAACCACTCAAGG ATGGCGCGACAACATCGAGTACCCACCA AAGGTC-3'	59
Capture	3'-GTTGTAGCTCATGGGTGGTTTTCCAG -biotin	26
Signal	AATTACGGTCCTGCTTGGTGAGTTC-5'	25

2.3 INV-signal probe conjugation

The conjugation of INV (from baker's yeast) and signal probe was performed according to the method of Xiang and Lu[9] (Fig. 1). Briefly, 30 μL of thiol-signal probe (1 mM) and 2 μL of Tris (2-carboxy ethyl) phosphine hydrochloride (TCEP, 30 mM) were reacted at room temperature for 1 h to obtain a sulfhydryl-exposed signal probe. Then, 8 mg of INV and 250 μL of sulfosuccinimidyl-4- (N-maleimido methyl) cyclo hexane-1-carboxylate (sulfo-SMCC, 10 mM) were reacted at room temperature to bind the ester of sulfo-SMCC with the primary amine of INV. INV-S-DNA conjugation, in a thioether form, was obtained by reacting the signal probe mix and INV mix at room temperature for two days. The pure INV-signal probe was stored at 4 °C until it was used for the sandwich hybridization reaction.

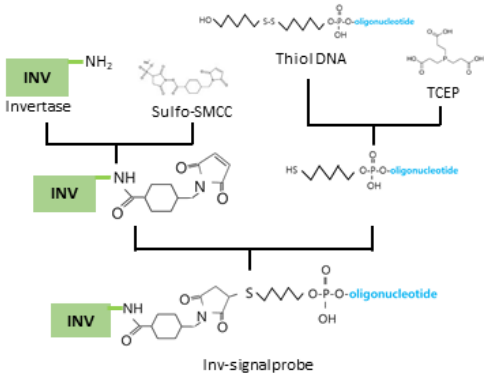

Fig. 1. Schematic diagram for INV-signal probe conjugation.

2.4 SDS-PAGE

SDS-PAGE was performed using 4-20% gradient polyacrylamide gels to determine whether the INV and the signal probe were well conjugated. The loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8) was added to the samples and boiled at 95 °C for 5 min. After loading 250 kDa size ladders, INV only, INV-thiol DNA and INV-sulfo-SMCC from the left of the 4-20% gradient, polyacrylamide gel electrophoresis was performed using a running buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS).

2.5 Magnetic beads preparation

First, 0.5 mg of streptavidin coated magnetic beads (BioLabs S1420S) was prepared in a 1.5 mL e-tube. The supernatant was removed using a magnetic bar rack, and then 1 mL of PBS (pH 7.2) was added and washed three times, leaving only magnetic beads. Then, 1 mL of capture probe (50 nM) was added and reacted at 37 °C for 2 h. The magnetic beads with capture probe were washed three times with PBST (0.5% tween-20) and stored at 4 °C until used for assays.

2.6 INV-SH assay and detection

The preparation of microalgal nucleic acid, the hybridization of the NPA probe, the treatment of S1 nuclease, the coating with biotin-labeled capture probes, and the hybridization of INV-signal probes for the INV-SH assay were performed as previously described[7,8]. Then, 200 μL of 1% sucrose dissolved in 100 mM NaAc, pH 5.5 were added to each well and reacted at 50 °C for 10 min in a shaking incubator (130 rpm). After this, 30 μL of the supernatant was transferred to a 96-well plate and the glucose assay kit (Sigma, Korea) was used according to manufacturer's instructions. Absorbance was measured at 405 nm using a plate reader (FLUOstar, BMG Thermo Fisher Scientific Inc, USA).

2.7 Statistical analysis

All results are shown as mean values of at least three experiments. Statistical analysis was performed using SPSS 18.0 software. Independent t-tests were performed for comparisons between the two groups. Values of *p $\langle$0.05 were considered statistically significant.

3. Results and Discussion

3.1 INV-signal probe conjugation

To confirm that the INV and signal probe were well conjugated, we performed gradient electrophoresis. Also, the INV conjugated with signal probe was observed under a fluorescence microscope (Fig. 2). In the gradient gel, INV was located between 98 and 148 kDa on the first lane and INV-signal probe was located between 148 and 250 kDa on the second lane. On the third lane, however, INV-sulfo-SMCC which is conjugated INV and sulfo-SMCC was also located between 148 and 250 kDa and was not distinguished from the INV-signal probe. Therefore, fluorescence microscopy was performed to distinguish the INV-sulfo-SMCC from the INV-signal probe. When the 148-250 kDa band of the electrophoresed gel was observed under a fluorescence microscope, the area where the signal probe was bound was green due to fluorescence (Fig. 2, b1). In contrast, the INV-sulfo-SMCC band did not show fluorescence expression (Fig. 2, b2), indicating that the INV-signal probe was well conjugated.

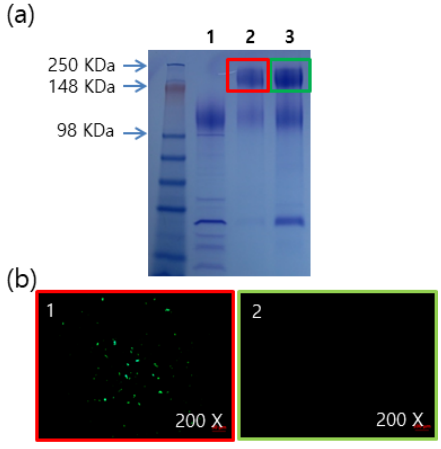

Fig. 2. Confirmation of INV-signal probe conjugation by gradient gel electrophoresis and epifluoromicroscope. (a) 4-20% gradient gel electrophoresis. (b) Epifluoromicroscopic photographs of INV-signal probe (1) and INV-sulfo-SMCC (2).

3.2 Optimal concentration of INV-signal probe

The absorbance of each INV unit was analyzed to determine the appropriate amount of synthesized INV-signal probe (Fig. 3). For INV-signal probe conjugation, 2,400 units (300 units/mg) were used. Then, 10 μL of INV-signal probe was used for enzyme assay after 1/10 dilution of the conjugated INV-signal probe. From the assay, the absorbance at 405 nm was 3.14, and when 3.14 was substituted for the absorbance equation, 13.79 units were calculated. Considering the dilution ratio of the INV-signal probe and the volume used in the INV enzyme assay, the amount of INV conjugated to the signal probe was between 1376-1876 units. When *C. polykrikoides* were detected, conjugated probes were diluted 200-fold and 500 μL were used, so that 38-52 units of INV per sample were used.

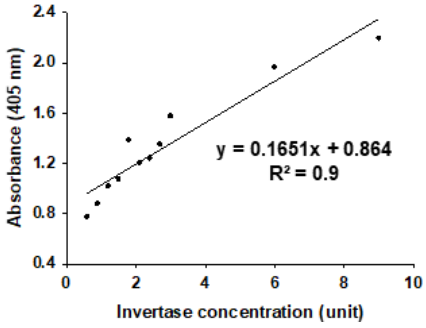

Fig. 3. Absorbance of glucose generated by treated INV units.

3.3 Streptavidin coating methods

Streptavidin-coated plates and streptavidin-coated magnetic beads used in NPA-SH were tested for efficiency. Since the probe's specificity had been verified in the previous NPA-SH [7], only *P. minimum* was used as a control (Fig. 4). When streptavidin was coated on a 96-well plate and assayed, an increase in absorbance with cell number of *C. polykrikoides* samples was not apparent. On the other hand, when streptavidin was coated onto magnetic beads, the absorbance

increased from 10^2 cells/mL, according to the number of cells (*p ⟨0.05). Therefore, streptavidin coating with magnetic beads was more effective than coating in 96-well plates.

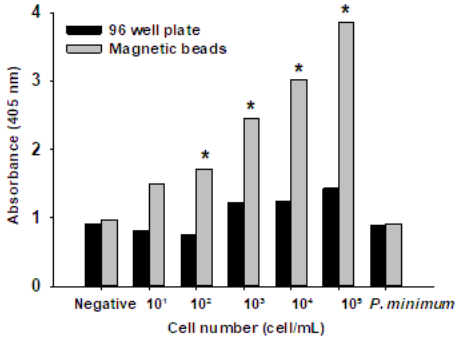

Fig 4. Absorbances analysis of straptavidin coating matrix. Black bar, streptavidin coated plate, gray bar, streptavidin coated magnetic beads. *p ⟨0.05 vs. control group.

3.4 Optimal sucrose treatment

In order to determine the optimal reaction time, the absorbance changes after sucrose treatment were analyzed at intervals of 10 minutes (Fig. 5). When reacted for 10 min and 30 min, there was almost no difference in absorbance of each sample. After 20 min of reaction, the absorbance of each sample was significantly different (*p ⟨0.05).

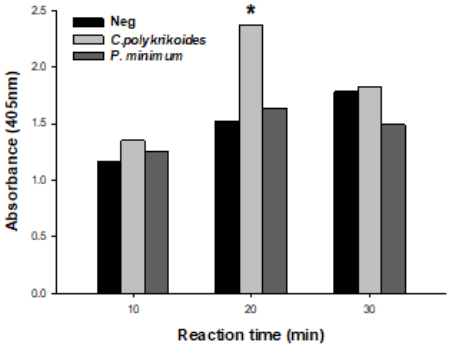

Fig 5. Changes of absorbance by sucrose reaction time. *p ⟨0.05 vs. control group.

4. Conclusion

In previous studies, we designed the NPA probe and the capture probe and signal probe to apply the NPA-SH method for the detection of *C. polykrikoides*, and performed specificity and sensitivity analyzes. The developed probes only responded to *C. polykrikoides,* and the NPA-SH probes were developed into an early warning kit for red tide surveillances.

In this study, we applied invertase to the NPA-SH technique for *C. polykrikoides*, and tried to utilize it in the field for faster and easier detection. We used species-specific capture probes, NPA probes, and signal probe sequences that were verified in previous NPA-SH experiments for *C. polykrikoides*. However, invertase was attached to the 3 'end of the signal probe instead of the existing fluorescence, so that the amount of glucose, the reaction product, could be measured. In the INV-SH method, the coloring process after the signal probe binding step was simplified (Fig. 6).

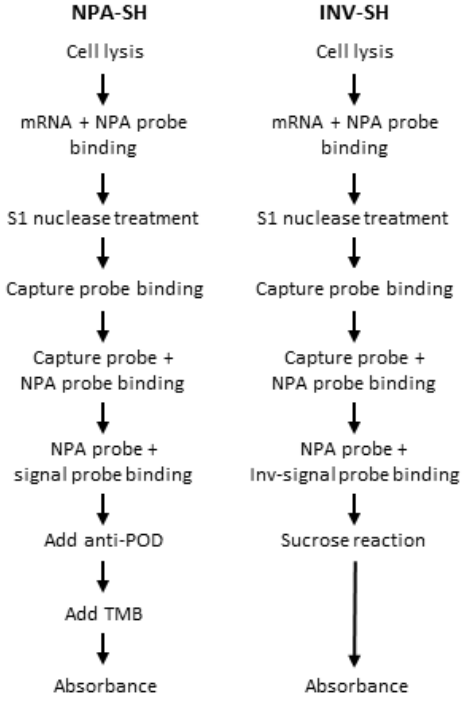

Fig 6. Schematic diagram for representing the reaction steps of NPA-SH and INV-SH.

Previous NPA-SH methods were able to detect the presence of *C. polykrikoides* as color development and absorbance, and a plate reader was required for absorbance measurement. The INV-SH method is expected to be capable of analyzing not only the absorbance analysis using a conventional plate reader but also a personal glucose meter. Therefore, the INV-SH method developed in this study could be more applicable to the field than the existing NPA-SH method.

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