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Rapid detection of *Chattonella marina* by PCR combined with dot lateral flow strip

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Abstract

In this study a novel technique referred to as PCR combined with dot lateral flow strip (PCDS) is proposed and its application to the detection of harmful microalgae was explored. For this purpose, using *Chattonella marina* as a test algal species, PCR targeting the D1–D2 region of large subunit ribosomal gene of this alga was performed with the tagged specific primers. The amplicons were then analyzed with the manually prepared dot lateral flow strip, and the strip could produce a test dot and a control dot that are naked-eye detectable, indicating the successful establishment of PCDS. The established PCDS assay does not require expensive instruments for the detection, and the results can be observed visually after adding 7.5 μL of PCR amplicons in combination with 92.5 μL of chromatography buffer to the sample pad of the strip for about 10 min. The PCR conditions were optimized to enhance the effectiveness of detection. The cross-reactivity test with 23 microalgae species, including *Chattonella marina*, showed good specificity of the PCDS. The detection limit of PCDS was 1.25×10^{-2} ng μL^{-1} for genomic DNA and 10^1 cells mL^{-1} for crude cell extracts, which can meet the detection needs. In summary, the PCDS proposed in this study has low cost, clear, and intuitive detection results and good specificity and sensitivity, providing a novel detection method for *C. marina*.

Keywords *Chattonella marina* · Raphidophyceae · PCR · Dot lateral flow strip · Detection

Introduction

Harmful algal blooms (HABs) refer to natural phenomena of mass propagation of algal cells in a short period due to the eutrophication of seawater, which usually can cause massive mortality of aquatic animals (Foss et al. 2019). In recent years HABs have become an increasingly serious environmental problem and pose a threat to human health (Huang et al. 2018) and sustainable development of the marine economy (Wang et al. 2017a). Therefore, it is essential to establish effective and convenient techniques for the early detection of harmful algae to take timely and effective measures to reduce the potential economic damage caused by HABs.

Chattonella marina, as a member of Raphidophyceae, is a highly adaptable marine harmful microalga. This algal species can live in a wide range of temperatures (15–30 °C) and salinities (10–35 psu) (Yamaguchi et al. 1991). *Chatonella marina* is highly resistant to harsh environmental conditions and even can survive under an irradiance of up to about 1000 μmol photons m^{-2} s^{-1} (Qiu et al. 2013). *Chatonella marina* can produce a variety of metabolites such as neurotoxins, hemolysins toxins, and reactive oxygen, and thus the HABs triggered by this alga can usually cause mass mortality of fish (Shimasaki et al. 2021). In recent years, great economic losses to aquaculture caused by *C. marina*–forming HABs have been reported in Korea (Kim et al. 2007), Japan (Onitsuka et al. 2011), Europe (Waite and Lindahl, 2006), and China (Wang et al. 2017b). Therefore, it is important to establish efficient and sensitive detection methods for *C. marina*.

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To date, the internationally recognized standards for the identification of microalgae are based on the morphological characteristics of the cells, including traditional microscopic observation and automatic image recognition by combining microscopic observation with computer technology. However, the traditional microscopic observation that is dependent on morphological structures has several limitations, such as low efficiency, poor timeliness, and high requirements for working personnel. Although these drawbacks have been overcome to some extent by automatic image recognition, this technique is not suitable for point-of-care testing (POCT) as it relies on flow cytometry and is therefore costly.

Since the 1980s, nucleic acid-based molecular techniques have been gradually applied to detect harmful algae. Several molecular detection techniques for HABs have been developed so far, including fluorescence in situ hybridization (FISH) (Miller and Scholin 2000; Liu et al. 2020), sandwich hybridization (SH) (Diercks et al. 2008; Zhu et al. 2012), polymerase chain reaction (PCR) (Lyu et al. 2017; Elleuch et al. 2020), isothermal amplification (IA) (Toldrà et al. 2019a; Zhang et al. 2019), microarrays (Taylor et al. 2013; Noyer et al. 2015), biosensors (Diercks-Horn et al. 2011; Toldrà et al. 2019b), and real-time qPCR (Nishimura et al. 2016; Zhang et al. 2016). These molecular detection techniques have, to a certain extent, overcome the disadvantages of traditional morphological identification methods, greatly improved the detection efficiency, and provided new ideas for the monitoring and early warning of HABs.

The lateral flow strip is a relatively new molecular detection technique that has been applied in many different areas due to its simplicity and sensitivity. In the field of food detection, Suryoprabowo et al. (2021) achieved the semi-quantitative detection of tadalafil, an illicit drug in coffee samples, using a lateral flow immunoassay based on a fluorescently labeled monoclonal antibody. Yao et al. (2021) used chromatographic strips for the simultaneous detection of fipronil and its metabolites in eggs and cucumbers. In the medical field, Takarada et al. (2020) combined the chromatographic strip technique with loop-mediated isothermal amplification (LAMP) for the rapid detection of rifampicin-resistant *Mycobacterium tuberculosis*. Zhang et al. (2020) developed a lateral flow strip for the rapid detection of SARS-CoV-2 based on a double antibody sandwich principle, which can be used as a diagnostic aid for the COVID-19 pandemic. Based on the above examples, the lateral flow strip can be combined with a variety of nucleic acid detection techniques to achieve qualitative or even semi-quantitative detection results. Therefore, combining the lateral flow strip with existing detection techniques for HABs is a promising idea.

Up to now, the molecular detection methods targeting *C. marina* mainly include multiplex polymerase chain reaction (mPCR) (Sun et al. 2019), quantitative polymerase chain reaction (qPCR) (Park et al. 2012), hyperbranched rolling circle amplification (HRCA) (Nie et al. 2017), loop-mediated isothermal amplification (LAMP) (Qin et al. 2019), double probes rolling circle amplification (dpRCA) (Qin et al. 2020), and membrane-based DNA array (Zhang et al. 2018). To initially establish the lateral flow strip technique for the detection of *C. marina*, PCR amplification combined with the lateral flow strip was chosen in this study.

Although the lateral flow strip technique is efficient and intuitive for nucleic acid analysis, the preparation of lateral flow strips requires special instruments and is relatively costly, which can significantly increase the detection cost especially when the number of samples to be tested is large. Therefore, in this study, we developed a hand-prepared dot lateral flow strip (DLFS) and investigated its application to detect harmful microalgae in combination with PCR. To achieve these objectives, the PCR combined with DLFS (PCDS) was firstly developed by using the sequence of large subunit D1–D2 (LSU D1–D2) as the target gene. Secondly, the PCR system and the detection conditions of the DLFS were optimized. Finally, the specificity, sensitivity, and practicality of the developed PCDS were also evaluated.

Materials and methods

Algal species and culture

Chatonella marina and control microalgal species used in this study are listed in Table 1. These algal species were obtained by micropipette isolation, purchased from commercial companies, or kindly donated as gifts. The algal culture conditions were as follows: (1) the culture medium was *f*/2 (for diatoms) or *f*/2-Si medium (Guillard 1975) with a pH of 8.2 prepared from filtered and sterilized seawater; (2) all of the algal cultures were grown at 20–24 °C, with a light intensity of 50–100 μmol photons m^{-2} s^{-1}, and a light-to-dark ratio of 12 h:12 h; and (3) all of the cultures were shaken regularly once or twice per day, with culture medium renewed every 2–3 weeks for good growth.

DNA extraction

Aliquots (45 mL) of *C. marina* cultures in the logarithmic growth phase were subjected to centrifugation (4000 × *g* for 10 min at 4 °C), and then the resulting cell pellets were washed once under the same centrifugation conditions as above. Genomic DNA was extracted from the obtained algal cells using the Ezup Column Plant Genomic DNA Extraction Kit (Sangon Biotech, China). The concentration of the extracted genomic DNA solution (1.25×10^2 ng μL^{-1}) was determined using a NAS-99 microspectrophotometer (Thermo Fisher Scientific, USA). The purity of genomic DNA was evaluated by the ratio of absorbance at 260 nm

Table 1 List of algal species used in this study and results of PCR-dot chromatography strip (PDCS) using the specially designed probes/primers for *Chattonella marina*

Species	Taxonomy	Geographic origin	Analysis by PDCS
Chattonella marina	Raphidophyceae	Daya Bay, South China Sea	+[a]
Heterosigma akashiwo	Raphidophyceae	South China Sea	–[b]
Phaeocystis globosa	Prymnesiophyceae	South China Sea	–
Dierateria zhanjiangensis	Prymnesiophyceae	Xiamen Bay, East China Sea	–
Isochrysis galbana	Eustigmatophyceae	East China Sea	–
Alexandrium catenella	Dinophyceae	East China Sea	–
Alexandrium tamarense	Dinophyceae	East China Sea	–
Prorocentrum lima	Dinophyceae	Daya Bay, South China Sea	–
Prorocentrum minimum	Dinophyceae	Daya Bay, South China Sea	–
Alexandrium minutum	Dinophyceae	Hongkong, South China Sea	–
Ellipsoidion sp.	Dinophyceae	East China Sea	–
Prorocentrum micans	Dinophyceae	Weihai Bay, Yellow Sea	–
Scrippsiella trochoidea	Dinophyceae	South China Sea	–
Karlodinium veneficum	Dinophyceae	East China Sea	–
Prorocentrum donghaiense	Dinophyceae	Zhejiang, East China Sea	–
Amphidinium carterae	Dinophyceae	East China Sea	–
Karenia mikimotoi	Dinophyceae	Wenzhou,East China Sea	–
Chlorella vulgaris	Chlorophyceae	Bohai Sea	–
Chaetoceros muelleri	Bacillariophyceae	Weihai Bay, Yellow Sea	–
Pseudo–nitzschia pungens	Bacillariophyceae	Zhujiang Estuary, East China Sea	–
Nitzschia closterium f.minutissima	Bacillariophyceae	Weihai Bay, Yellow Sea	–
Thalassiosira pseudonana	Bacillariophyceae	Taiwan Strait, West Pacific Ocean	–
Phaeodactylum tricornutum	Bacillariophyceae	East China Sea	–

[a] "+" represents a positive PDCS result

[b] "–" represents a negative PDCS result

(A_{260}) to that at 280 nm (A_{280}). The obtained DNA samples were subjected to 1% agarose gel electrophoresis (AGE) and stored at – 20 °C.

Primers and probes

The primers used for the PCDS assay consist of three parts, i.e., tag sequence, spacer, and specific sequence. The specific sequences for the forward and reverse primers were obtained from Sun et al. (2019), and the tag sequences Tgn and Tsp, as well as the probes cTsp, Tgn, and cTgn, were obtained from Nagai et al. (2016). All the probes and primers used in this study are summarized in Table 2 and were synthesized by Sangon Biotech (Shanghai) Co., Ltd. In particular, the gold nanoparticle probe cTgn was labeled with a sulfhydryl (SH) group at the 5′ end to allow it to bind to the gold nanoparticles. In addition, a poly (A_{16})-tail labeled with biotin was added to the 5′ end of the detection probe cTsp and the control probe Tgn. The purpose of the biotin labeling is to enable the probes to bind to the anti-biotin antibody to form large molecules for attachment to the detection membrane of the test strip, and the addition of the poly (A_{16}) tail is to prevent encapsulation of the probe sequence when the biotin binds to the anti-biotin antibody.

Principle and detection process of the PCDS

The schematic diagram principle of the PCDS is shown in Fig. 1. The detection process consists of two stages, namely, PCR amplification and nucleic acid chromatography. In the PCR amplification phase, since both the forward primer Cm-Fp with Tgn and the reverse primer Cm-Rp with Tsp contain Spacer 9 that can effectively prevent the extension of DNA polymerase when the specific sequence triggers PCR amplification, they will produce the PCR products with double tags (Tgn and Tsp). In the nucleic acid chromatography phase, the PCR products are first mixed with chromatography buffer and added to the sample pad. Due to capillary action, the mixture flows laterally to the conjugate pad, where the tag sequence Tgn can be complementarily bound to the gold nanoparticle-labeled probe (cTgn), thus allowing the PCR products to form a complex with the gold nanoparticle. Next, the unbound gold nanoparticle-labeled probe and the complex mentioned above continue to laterally flow to the test

Table 2 Summary of primers/probes used in this study

Primer/probe	Sequence (5–3′)	Reaction	Sources
D1	ACCCGCTGAATTTAAGCATA	PCR	Scholin et al. (1994)
D2	CCTTGGTCCGTCTTTCAAGA	PCR	
Cm–F[a]	CTTGGTTGTTGTAGCGTCTT	PCR	Sun et al. (2019)
Cm–R[b]	GAGAGAGTGAGGTCAGCAGA	PCR	
Cm–Fp[c]	atttttcactgggtttatagt–iSp9– CTTGGTTGTTGTAGCGTCTT	PDCS	This study
Cm–Rp[c]	tcgagtgacagctaatgtgtgatt–iSp9– GAGAGAGTGAGGTCAGCAGA	PDCS	This study
cTgn[d]	SH–ACTATAAACCCAGTGAAAAAT	PDCS	Nagai et al. (2016)
Tgn[e]	Bio–Poly(dA)–ATTTTTCACTGGGTTTATAGT	PDCS	
cTsp[f]	Bio–Poly(dA)–AATCACACATTAGCTGTCACTCGA	PDCS	

[a]Cm–F is forward primer

[b]Cm–R is reverse primer

[c]The uppercase letters of primer sequence represent the necessary part for PCR amplification of the LSU rDNA of *Chattonella marina*, and the lowercase letters represent tag sequence

[d]The probe cTgn is labeled with SH at the 5′ end

[e]The probe Tgn is labeled with biotin at the 5′ end

[f]The probe cTsp is labeled with biotin at the 5′ end

dot site on the detection membrane, where the test probe cTsp can complementarily bind to the tag sequence Tsp, producing a red test dot due to the aggregation chromogenic effect of gold nanoparticles. Finally, the free gold nanoparticle-labeled probe continues to flow laterally to the control dot site, where cTgn binds to the preset control probe Tgn, again producing a red control dot due to the aggregation of gold nanoparticles.

For the practical application of the PCDS, 7.5 µL of PCR amplification products were first mixed with 92.5 µL of chromatography buffer. Then, the sample pad of the prepared DLFS was dipped into the mixture. After 5–10 min, the detection result was determined by observing whether red dots can be produced at the test and control dot sites or not. A positive result is determined when two red dots are produced at both the test and control dot sites, and a negative result is determined when only one red dot is produced at the control dot site. If no red spot appears in both the test and control spot sites, the test strip detection system is proven to be not working effectively.

Preparation of DLFS

Preparation of the sample pad

The glass fiber SB06 (Shanghai Liangxin Technology Co., Ltd.) used as the sample pad was immersed in sample pad buffer (0.05 M Tris–HCl, 0.15 M NaCl, 0.25% Triton X-100; pH 8.0) for 20 min, dried at 37 °C for 2 h and then stored at room temperature (RT).

Preparation of the conjugate pad

The solution containing gold nanoparticles with an average diameter of approximately 30 nm was prepared by the citrate reduction method (Turkevich and Enüstun 1963; Yeh et al. 2012). Aliquots (1 mL) of gold nanoparticle solution were centrifuged at 12,000 × *g* for 20 min at RT. After removing the supernatant, 6 µL of 100 µM gold nanoparticle probe (cTgn) and 94 µL of 1 × TE buffer were added to the precipitate, mixed well, and incubated at 4 °C for 12 h. An equal volume (100 µL) of PBS solution (0.2 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4; pH 7.0) was added, mixed quickly, and incubated at 4 °C for 24 h. The solution was centrifuged at RT for 10 min at 2000 × *g*. After removing the supernatant, the precipitate was resuspended with 200 µL of ultrapure water and centrifuged again under the same conditions to remove the unlabeled probes. Finally, the resultant gold nanoparticle-labeled probe was resuspended with 1 mL of embedding buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Triton X-100, 8% sucrose) and stored at 4 °C away from light. When in use, the above-stored gold nanoparticle-labeled probe solution was first thoroughly dispersed by ultrasound under lightproof conditions, then uniformly sprayed onto glass fiber RB65 (Shanghai Liangxin Technology Co., Ltd.) (ca. 0.18 mL cm^{-2}), dried at 37 °C for 2 h, and stored at RT.

Preparation of detection membrane

The nitrocellulose membrane (NCM) (Sartorius UniSart CN140 Membrane) used as the detection membrane was successively immersed in ddH_2O for 10 min and in 20 × SSC

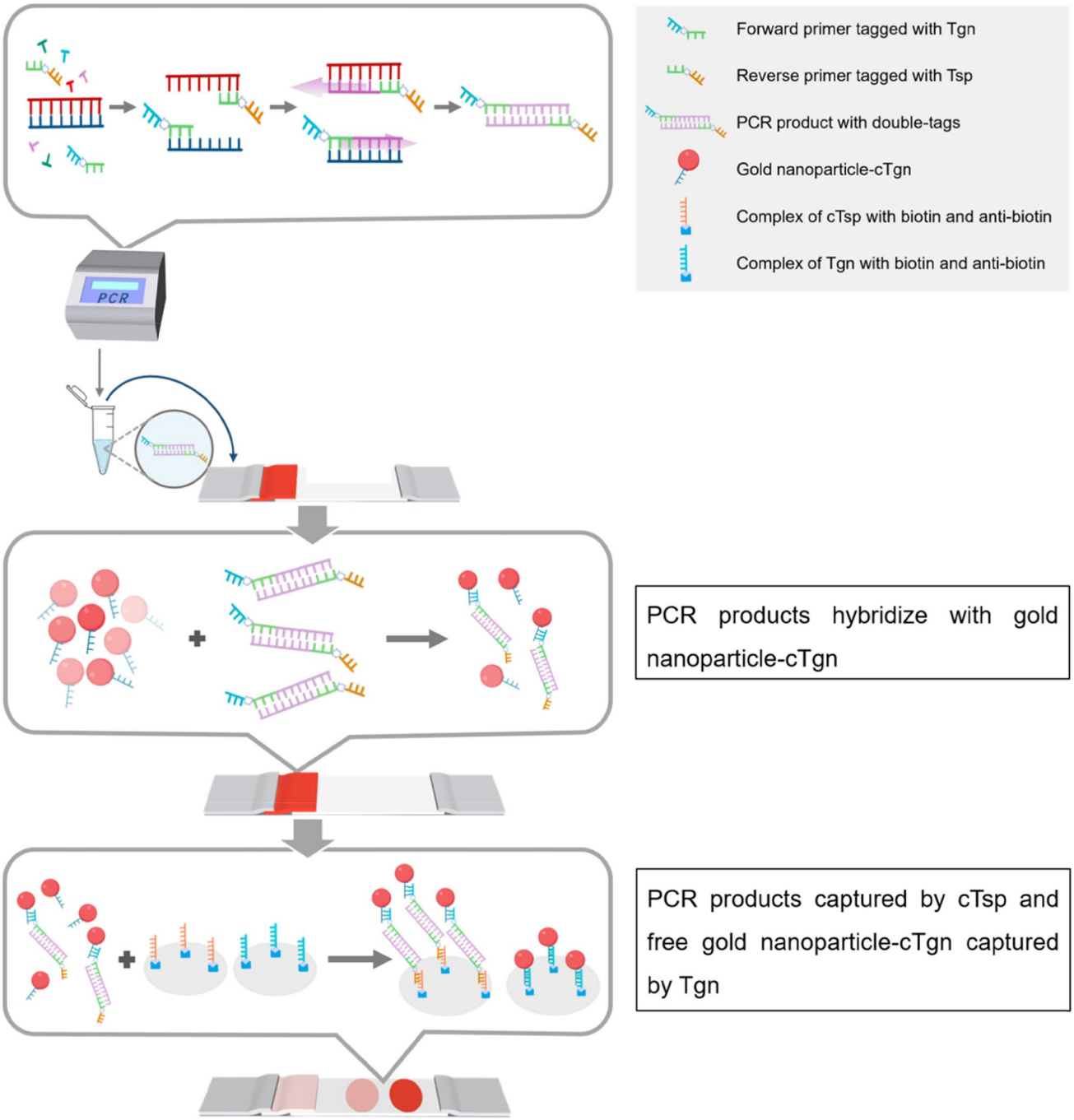

Fig. 1 Schematic diagram of detection principle of PDCS

solution (NaCl, 17.5322 g; $Na_3C_6H_5O_7.2H_2O$, 8.823 g; ddH_2O, 100 mL; pH 7.0) for 5 min, and then dried in the air. Next, anti-biotin antibody (Cell Signaling Technology) was mixed, respectively, with the test probe (cTsp) and the control probe (Tgn) (10 μM) with a volume ratio of 1 to 10, and aliquots (1 μL) of the mixture was spotted onto the test and control sites (approximately 7 mm apart). The spotted NCM was then UV cross-linked (UV cross-linker setting: wavelength, 254 nm; intensity, 90 J cm^{-2}) for 2 min to bind the probe to the NCM firmly.

Test strip assembly

The schematic diagram of the assembly of DLFS is as shown in Fig. 2. In brief, the detection membrane was first pasted on the PVC base (SM31–40) (Shanghai Liangxin

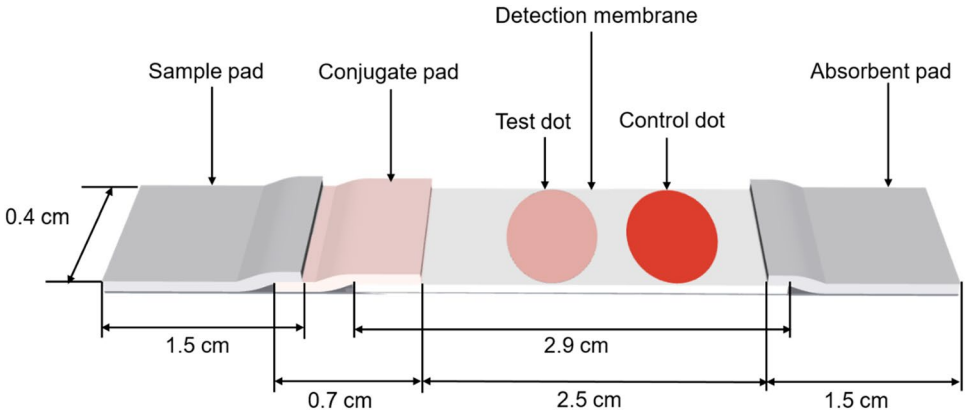

Fig. 2 Schematic diagram of the structure of DLFS

Technology Co., Ltd.). Then, the conjugate pad near the test spot and the absorbent pad (CH27) (Shanghai Liangxin Technology Co., Ltd.) near the control spot were attached to each end of the detection membrane with a 2 mm overlap. In addition, the sample pad was paste on the other side of the conjugate pad with a 2 mm overlap.

Optimization of specific PCR conditions and detection system

Specific PCR was performed in a thermal cycler (Bioer Technology, China) with a total volume of 12.5 μL consisting of 1 × PCR buffer (1.25 μL), 2.5 mM $MgCl_2$ (0.75 μL), 0.4 mM dNTP mix (1 μL), 0.4 mM primers (Cm–Fp and Cm–Rp, 0.25 μL each), 0.2 U *Taq* polymerase (Sangon Biotech, China) (0.1 μL), and 12.5 ng genomic DNA (1 μL). The amplification procedure was as follows: 94 °C for 5 min; 29 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 50 s; and a final extension at 72 °C for 7 min. The PCR products were analyzed by 1.5% AGE.

After the initial establishment of the PCR system, various PCR conditions, including annealing temperatures (55 °C, 57 °C, 59 °C, 61 °C, 63 °C, and 65 °C), primer concentrations (0.05 μM, 0.10 μM, 0.15 μM, 0.20 μM, 0.25 μM, and 0.30 μM), Mg^{2+} concentrations (0 mM, 0.5 mM, 1.0 mM, 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM) were optimized by comparative testing. Each parameter was tested with other conditions unchanged. The optimized conditions were used in the subsequent tests.

Specificity test of PCDS

A total of 23 microalgal species (Table 1), including the target species, were selected for a cross-reactivity test to verify the specificity of the established PCDS. In brief, universal PCR amplification was first performed using the genomic DNA from all the test species as templates with the previously reported primers D1 and D2 (Scholin et al. 1994) (Table 2) to determine whether the template quality was suitable for subsequent specific PCR with the primers Cm–Fp and Cm–Rp. Second, the same genomic DNA mentioned above was used as test samples for specific PCR and PCDS, respectively. The PCR products were analyzed by 2% AGE, and the results of the PCDS were judged by observing the presence or absence of colored dots on the test and control spot sites of DLFS.

Sensitivity test of PCDS

The sensitivity of PDCS was tested using the genomic DNA of *C. marina* as a template. The genomic DNA prepared in the subsection "DNA extraction" was diluted tenfold with nuclease-free water (Sangon Biotech (Shanghai) Co., Ltd.) to prepare DNA test samples in a gradient of seven concentration dilutions (1.25×10^{2}–1.25×10^{-4} ng μL^{-1}). The test samples were analyzed by PCDS, with the specific PCR as control. The specific PCR was performed with primers Cm–Fp and Cm–Rp. The PCR products were analyzed by 1.5% AGE, and the results of the PCDS were determined by observing the coloration results of DLFS.

Analysis of spiked samples by PCDS

The natural seawater that was used to prepare spiked samples was collected from Jinhai Bay (Weihai, Shandong, China) in the Yellow Sea, in which no microalgal target cell could be observed by microscopic examination. Aliquots of *C. marina* cultures in the exponential growth stage were fixed with 1.5% Luger's solution, and the cell concentration was determined by counting with a plankton counting chamber under light microscopy. The *C. marina* cells that were collected from 50 mL of algal culture by centrifugation (4 °C, 4000 × *g*, and 10 min) were resuspended in 50 mL of natural seawater, which was used as the initial spiked sample with a concentration of 10^5 cells mL^{-1}. Then, the initial sample was tenfold diluted with natural seawater to result in a series of spiked samples with the concentration range of 10^5–10^{-1} cells mL^{-1}. Algal cells were, respectively,

collected from these spiked samples by centrifugation using the same conditions as above, and DNA extracts were prepared using One-tube Plant Genome DNA Extraction Kit (Sangon Biotech, China) according to the manufacturer's instructions. The obtained crude DNA extracts were used as templates for specific PCR and the PCDS, respectively.

Results

Optimization of PCR conditions and probe amount

The PCR conditions, including annealing temperature, primer concentration, and Mg^{2+} concentration were optimized to gain high amplification efficiency with a minimum yield of primer dimer. The optimization results of annealing temperature are shown in Fig. S1. When the annealing temperature was higher than 63 °C, no target band could be detected by AGE, indicating that the PCR could not occur. In comparison, when the annealing temperature was between 55 and 59 °C, clear target bands could be detected by AGE, indicating that efficient PCR could occur. Therefore, the optimal annealing temperature was finally set at 55 °C to reduce primer dimers. Next, the primer concentration was optimized by setting the annealing temperature at 55 °C (Fig. S2). Successful PCR amplification could occur with all primer concentrations. In addition, a much higher amplification efficiency could be detected with the primer concentration of 0.15, 0.20, 0.25, and 0.30 μM, respectively. The optimal final primer concentration was set to 0.15 μM to save costs and reduce primer dimer. Finally, the Mg^{2+} concentration was optimized by setting the annealing temperature at 55 °C and the primer concentration at 0.15 μM (Fig. S3). Because a much higher amplification efficiency could be detected with the Mg^{2+} concentrations of 1.5, 2.0, and 2.5 mM, respectively, the optimal Mg^{2+} concentration was set to 1.5 mM to save costs.

Based on the above results, the optimal PCR conditions (Table 3) were applied to perform PCR amplification (Fig. 3A). The resultant PCR products were subjected to DLFS (Fig. 3B) in triplicate by setting the probe amount at both test and control spot sites to 10 pM. Three independent tests consistently showed that although DLFS could work well, i.e., the positive samples could produce red dots at both test and control spot sites of the strips, the blank control samples could also unexpectedly produce two light red dots at both test and control spot sites of the strips, which is actually a "false positive" result. Therefore, to further eliminate the "false positive" results, three parallel PCDS tests were performed by adjusting the amount of test probe to 7.5 pM (Fig. 3C). Fortunately, all the tests produced the same results

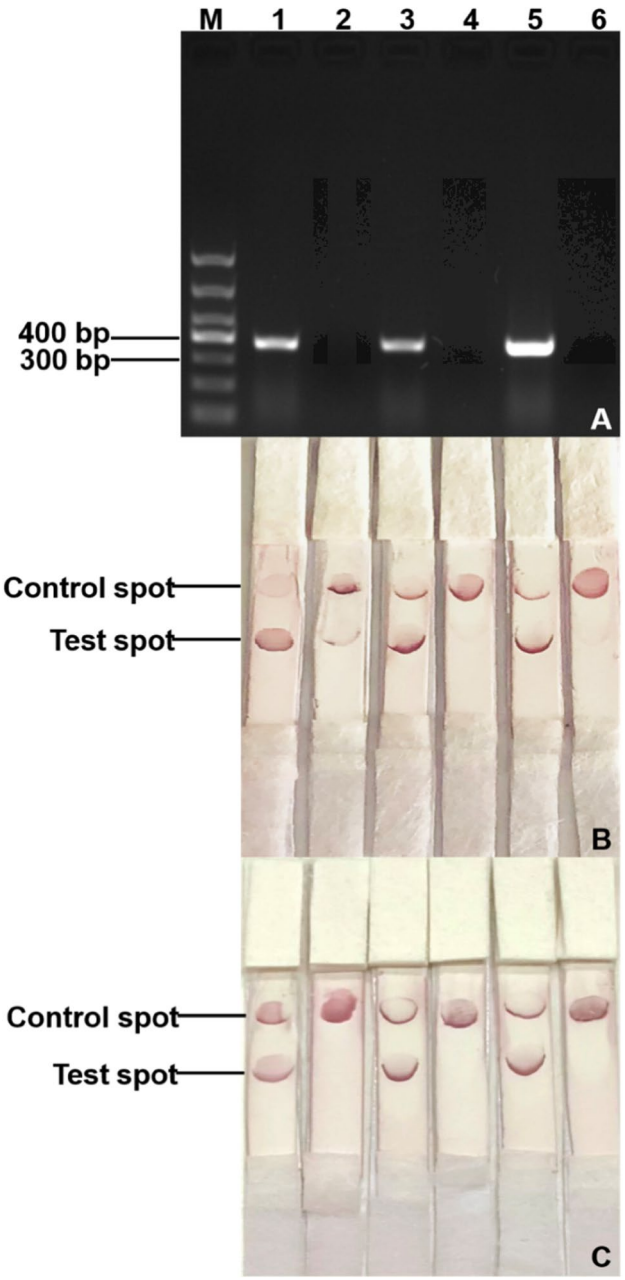

Fig. 3 Optimization of PCR conditions and probe amount for DLFS. **A** AGE analysis of PCR products; **B** PCDS analysis using the preliminarily prepared DLFS by setting the probe amount to 10 pM; **C** PCDS analysis using the optimized DLFS by setting the probe amount to 7.5 pM. M, DL 1000 DNA marker; lanes 1, 3, and 5 are PCR products; lanes 2, 4, and 6 are blank controls

Table 3 Summary of the optimized conditions for PCR

Conditions	Result
Annealing temperature	55 °C
Cm–Fp concentration	0.15 μM
Cm–Fp concentration	0.15 μM
Mg^{2+} concentration	1.5 mM

that indicate that "false positive" has been successfully eliminated, i.e., PCR amplification products could produce red dots at both test and control spot sites of DLFS, while blank control could produce red dots only at control spot sites.

Specificity analysis of PCDS

A total of 23 marine microalgal species, including *C. marina*, were selected for the cross-reactivity test to confirm the specificity of the established PCDS assay. First, the universal PCR amplification of the LSU rDNA D1–D2 region using the genomic DNA of all the algal species as the template consistently showed positive results, indicating that the quality of the DNA template for each species could meet the requirements of the amplification test (Fig. 4A). Second, specific PCR amplification using the *C. marina*–specific primers Cm–Fp/Rp was carried out, showing that successful PCR amplification could exclusively occur with only the genomic DNA from the target species (Fig. 4B). Finally, all the specific PCR amplification solutions (with or without PCR products) were subjected to a DLFS assay. The results showed that only the target species produced two red dots on the strips for test and control spot sites, whereas the non-target species and the blank control produced only one red spot on the strips for the control site (Fig. 4C). In conclusion, the PCDS assay developed in this study is specific for the detection of the target algal species.

Sensitivity analysis of PCDS

A series of tenfold dilutions of the target algal genomic DNA was used for comparative PCR-AGE and PCDS analysis using the specific primers Cm–Fp/Rp (Fig. 5), respectively. PCR-AGE and PCDS displayed a detection limit of 1.25×10^{-1} ng μL^{-1} and 1.25×10^{-2} ng μL^{-1} for genomic DNA. Therefore, PCDS is 10 times more sensitive than PCR-AGE.

Assessment of the application of PCDS

To assess the practicability of the established PCDS, spiked natural water samples containing target algal cells with the concentration range of 10^5–10^{-1} cells mL^{-1} were prepared and used as test samples for the comparative analysis by PCR-AGE and PCDS (Fig. 6). Positive PCR-AGE and PCDS results could be detected with the spiked samples with the

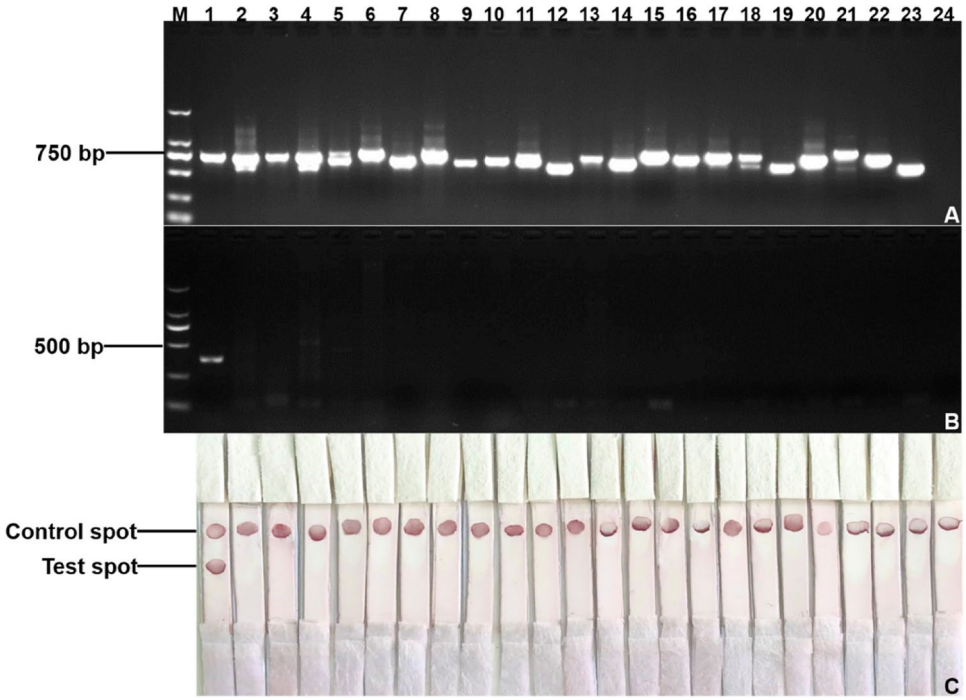

Fig. 4 Specificity validation of PCR primers (Cm–Fp/Rp) and specificity test of PCDS. **A** AGE analysis of PCR products with the universal primers (D1/D2); **B** AGE analysis of PCR products with the specific primers (Cm–Fp/Rp); **C** PCDS analysis. M, DL2000 DNA marker; lanes 1–23, the test algal species are *Chattonella marina*, *Alexandrium catenella*, *Heterosigma akashiwo*, *A. tamarense*, *Chaetoceros muelleri*, *Pseudo–nitzschia pungens*, *Prorocentrum lima*, *P. minimum*, *A. minutum*, *Chlorella vulgaris*, *Ellipsoidion* sp., *Nitzschia closterium f.minutissima*, *P. micans*, *Scrippsiella trochoidea*, *Karlodinium veneficum*, *P. donghaiense*, *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, *Amphidinium carterae*, *Phaeocystis globosa*, *Isochrysis galbana*, and *Dierateria zhanjiangensis*, respectively; lane 24, blank control

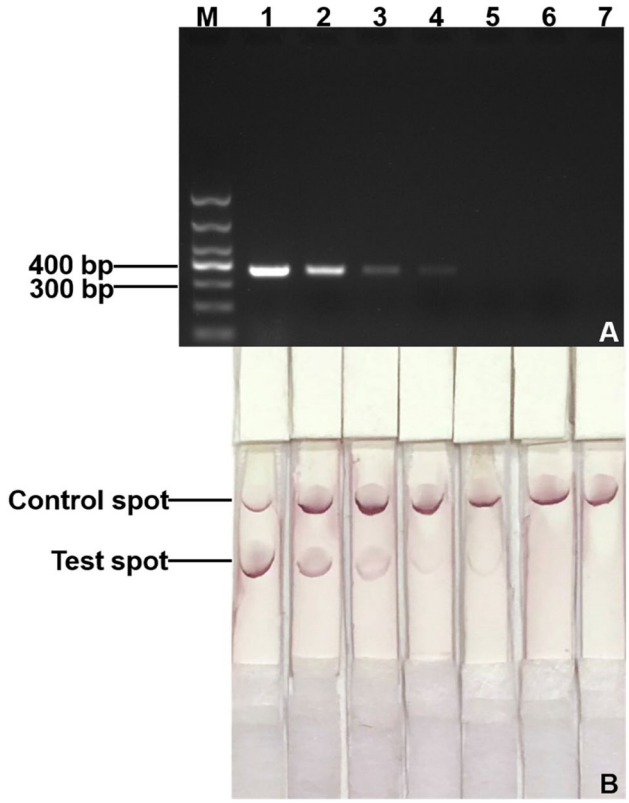

Fig. 5 Comparison of the sensitivity of PCR-AGE (A) and PCDS (B) with genomic DNA. M, DL1000 DNA marker; lanes 1–7, the concentrations of genomic DNA range from 1.25×10^{2} to 1.25×10^{-4} ng μL^{-1}

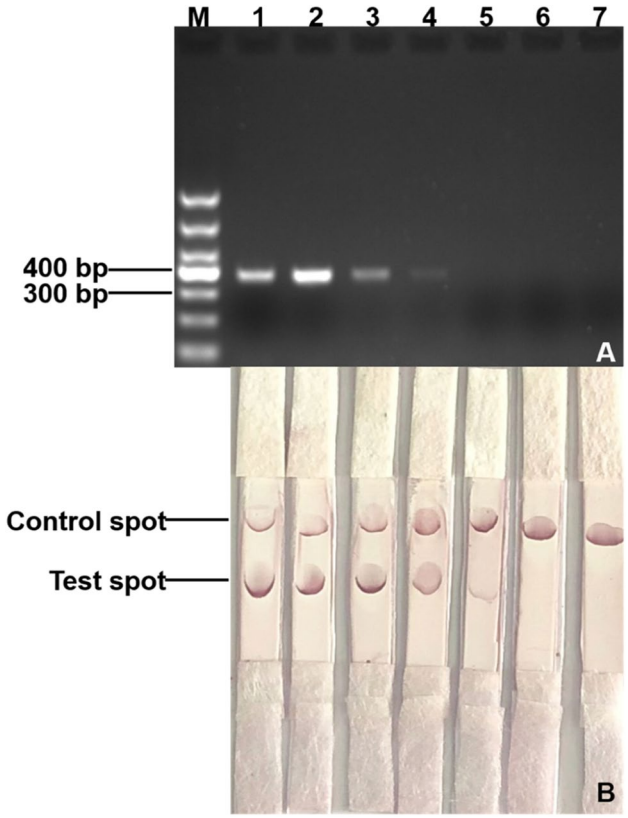

Fig. 6 Comparison of the sensitivity of PCR-AGE (A) and PCDS (B) with crude DNA extracts. M, DL1000 DNA marker; lanes 1–7, the crude DNA extracts prepared with the cell density ranging from 10^{5} to 10^{-1} cells mL^{-1}

concentration ranges of 10^{5}–10^{2} cells mL^{-1} (Fig. 6A) and 10^{5}–10^{1} cells mL^{-1} (Fig. 6B), respectively. In other words, PCR-AGE and PCDS displayed a detection limit of 10^{2} cells mL^{-1} and 10^{1} cells mL^{-1}, respectively. Again, the tests with spiked samples indicated that PCDS is 10 times more sensitive than PCR-AGE.

Discussion

Chatonella marina can produce several toxic metabolites such as reactive oxygen species (ROS), brevetoxin/brevetoxin-like compounds, free fatty acids (FFAs), hemagglutinins, and hemolysins (Shen et al. 2010), which may play a synergistic role in ichthyotoxicity and indirectly cause large-scale fish mortality (Qiu et al. 2020). In addition, *C. marina* may exert an allelopathic effect on other dinoflagellates, triggering larger-scale HABs and causing serious economic and ecological damage to the aquatic environment (Fernandez-Herrera et al. 2016). Therefore, it is important to establish a rapid detection method for *C. marina* to take effective measures to reduce costs before it causes serious damage.

In this study, we try to establish a convenient, sensitive, and practical detection method for *C. marina*. The existing molecular detection methods developed for *C. marina* including mPCR, qPCR, HRCA, LAMP, dpRCA, and membrane-based DNA array have been proven to be effective but still have some limitations. For example, mPCR and qPCR require relatively high requirements for primer design, and qPCR requires expensive fluorescent dyes, leading to a relatively high detection cost. Although the isothermal amplification techniques such as LAMP, HRCA, and dpRCA are independent of thermal cyclers, the need for the key components such as DNA polymerase with strand-displacement activity, *Bst* polymerase, and phosphorylated probes can also increase the cost of the assay. The membrane-based DNA array is not suitable for POCT because of the complex experimental process and long test time. By contrast, neither complex experimental procedures nor expensive components are required for PCDS. In addition, the cost for PCDS that is prepared by hand is much lower compared with the traditional chromatography strip technology, which makes PCDS more competent for POCT.

The selection of suitable target genes is vital for a molecular detection method. Noncoding internal transcribed spacer (ITS), LSU rDNA, and small subunit (SSU) rDNA sequences are often selected as molecular markers for the discrimination of algal species. The main reason for this is that these genes contain both conserved and highly variable regions, facilitating the design of primers or detection probes specific to different algal species (Xu et al. 2021). Also, both ITS and LSU rDNA have been used as the main target for most of the established molecular detection methods for *C. marina*. For example, Park et al. (2012) designed the primers targeting ITS for specific qPCR detection of *C. marina*. Kai et al. (2006) established a single-cell PCR assay using the LSU rDNA as a target to distinguish between two *Chattonella* species. In this study, the LSU rDNA sequence of *C. marina* was selected as the target, and based on the previously designed specific primers (Sun et al. 2019), the tag sequence was added and used for the establishment of the PCDS assay. The specificity experiments in this study in combination with previous studies again demonstrated the effectiveness of LSU rDNA for interspecies differentiation.

The overall optimization of the detection conditions is a crucial step in the development of an assay. According to the principle of PCDS, the production of primer dimers as a by-product of PCR can interfere with the determination of detection results, also known as "false positives." To eliminate the interference of "false positive," a series of control variables were optimized. First, the PCR conditions were optimized to avoid the production of primer dimer by successively determining the optimal annealing temperature (55 °C), primer concentration (0.15 μM), and Mg^{2+} concentration (1.5 mM). However, DLFS analysis of blank control with the optimized PCR conditions showed that "false positives" could still interfere with specific detection. Therefore, we tried to adjust the amount (sampling volume) of the test probe on the strip and tested again. Fortunately, "false positive" for the blank controls was successfully eliminated in this way.

Sensitivity is a critical factor that could reflect the practical application value of a detection method. Therefore, in this study, we evaluated the detection limit of the established PCDS assay. First, a comparative test was performed using the serially diluted genomic DNA as test samples, showing that the sensitivity of PCDS is one order of magnitude higher than that of PCRAGE. Spiked natural water samples were further employed to assess the detection limit of PCDS, which was determined to be 10 cells mL^{-1}. In general, the sensitivity test with both the genomic DNA and the crude cell extracts consistently displayed that PCDS is an order of magnitude more sensitive than PCR-AGE. According to the previously reported results (Lee et al. 1995), the early warning concentration for the outbreak of *C. marina*-forming HABs is 200 cells mL^{-1}, so the PCDS assay developed in this study can meet the practical needs. In addition, the sensitivity of PCDS is also comparable to that of the established molecular detection methods for *C. marina* including HRCA (Nie et al. 2017) and dpRCA-LFD (Qin et al. 2020).

In conclusion, a novel method referred to as PCDS was developed for the highly specific and sensitive detection of *C. marina* in this study. The developed PCDS could enable the efficient detection of *C. marina*, with the intuitive results visible to the naked eyes. In addition, the low cost of preparation of DLFS also gives PCDS an advantage in terms of in situ detection, making it an effective means of preventing HABs. In the future, DLFS could be combined with RPA to further shorten the detection time and make it suitable for use in resource-poor areas to really meet the requirements for POCT. In addition, DLFS can also be combined with mPCR or multiplex recombinase polymerase amplification to realize the multiple detections of harmful algae.

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Data availability All data generated or analyzed during this study are included in this published article.

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