Rapid detection of Cyanobacteria by recombinase polymerase amplification combined with lateral flow strips

Jingjing Li, Chunming Wang, Xin Yu, Huirong Lin, Chen Hui, Li Shuai and Shenghua Zhang

ABSTRACT

Cyanobacteria are one of the major groups of algae causing algal blooms. In this study, we developed a rapid method for detecting Cyanobacteria using a recombinase polymerase amplification (RPA) method coupled with lateral flow (LF) strips. After releasing cyanobacterial DNA from cells using a freeze-cracking method, DNA was amplified using the RPA method. Next, the RPA products were detected using the LF test. LF-RPA successfully amplified the DNA of eight cyanobacterial species and detected their presence in the sample with high specificity, distinguishing them from five non-cyanobacterial species. The method could detect cyanobacterial DNA in water samples containing as low as 0.01 cell/mL Cyanobacteria, making the method more sensitive than polymerase chain reaction (PCR), which required cell densities of at least 10^4 cell/mL. LF-RPA could amplify and detect cyanobacterial DNA at any temperature in the range 30–45 °C in just 30 min and without the need for a thermal cycler. The method developed in this study is simple, rapid, and effective for on-site testing of Cyanobacteria, which may become a routine measurement in efforts to detect and treat harmful algal blooms.

Key words | cyanobacteria, lateral flow strips, rapid detection, recombinase polymerase amplification

INTRODUCTION

Cyanobacteria, one of the major groups of algae causing algal blooms, are ubiquitous in aquatic habitats (Stone 2011; Paerl & Otten 2013). But current methods for detecting Cyanobacteria or other conditions that trigger blooms tend to be complex, time-consuming, or require precision instrumentation and are therefore unsuitable for rapid, on-site testing. These methods include microscopy for direct identification and cell counts (Scheifhacken et al. 2010); monitoring of environmental indicators, including dissolved oxygen, total nitrogen, total phosphorus, pH, chlorophyll a, and others (Brown et al. 2013; Chen et al. 2013); satellite remote sensing (Klemas 2012; Li et al. 2012; Wen et al. 2014); quantification of Cyanobacteria by detection of algal toxins using high performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS) and ELISA (Li et al. 2006; Foss & Aubel 2013); and polymerase chain reaction (PCR)-based detection methods, including PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) (Ouellette et al. 2006; Otten et al. 2012; Zhu et al. 2014).

Improved methods for the rapid and accurate detection of Cyanobacteria in aquatic habitats are needed to prevent algal blooms. Recombinase polymerase amplification (RPA) is an isothermal alternative to PCR for the amplification of nucleic acids from viruses (Mekuria et al. 2014; Babu et al. 2017; Natoli et al. 2018), bacteria (Chao et al. 2015; Ma et al. 2017; Raja et al. 2017), and parasites (Sun et al. 2016; Wu et al. 2017). RPA uses three enzymes in the reaction system: a recombinase capable of binding single-stranded nucleic
acids (oligonucleotide primers), single-stranded DNA binding protein (SSB), and strand displacement DNA polymerase (James & Macdonald 2015; Daher et al. 2016). These enzymes function optimally at 37–42°C (James & Macdonald 2015) and RPA primers are only around 30–35 bp (Daher et al. 2016). These characteristics of RPA make it a good candidate for replacing PCR when laboratory facilities are unavailable. Once the nucleic acids are amplified, species can be identified using lateral flow (LF) strips. The combined method (LF-RPA assay) is simple, rapid, and suitable for on-site testing.

In this study, the performance of an LF-RPA assay was investigated by detecting cyanobacterial DNA released by a freeze-cracking method. The results showed that the LF-RPA assay was reliable for detecting cyanobacterial DNA in aquatic field samples.

**MATERIALS AND METHODS**

**Cyanobacteria cultivation**

We used 12 species of algae, including Cyanobacteria (Cyanophyta), Diatoms (Bacillariophyta), Euglenophyta, and Chlorophyta, plus one model plant (Table 1). Eleven of the strains were purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB). All algae species were incubated in their adapted liquid medium at 25°C under a 12 h/12 h light/dark cycle with 1,000–2,000 lux of light intensity.

**Rapid cell disruption and DNA extraction**

A Cyanobacteria-laden water sample (1 ml) was centrifuged at 13,000 rpm for 10 min (Michinaka et al. 2016) in a desktop centrifuge (HERMLE Z216MK, Germany), the supernatant discarded, and the remaining pellets mixed with 50 μl sterile water. The resuspended pellet was frozen in liquid nitrogen or a −80°C freezer for 3 min and then thawed at 40°C for 1 min. This cycle was repeated three times to break open the cells. The sample was centrifuged at 13,000 rpm for 10 min and the DNA in the supernatant was used for the PCR, RPA, and LF-RPA experiments.

**Optimization of RPA assay and primer screening**

A number of RPA conserved primers were designed based on the 16S gene sequences of six of the cyanobacterial species (Genbank accession numbers: U40338.1, AY065975.1, MG282255.1, AB012335.1, FJ234897.1 and AF139329.1). A set of primers generating a 250 bp amplicon and named 16S-RPA-212F and 16S-RPA-461R (Table 2) were verified using a TwistAmp Basic kit (TwistDX, UK). A 50 μl reaction was prepared in a tube containing 2.4 μl of 10 μM 16S-RPA-212F, 2.4 μl of 10 μM 16S-RPA-461R, 29.5 μl supplied rehydration buffer, 12.6 μl H₂O, 1 μl DNA template, one supplied lyophilized enzyme, and 2.5 μl of 280 mM magnesium acetate (MgAc). The tubes were incubated at 37°C for 20 min. After gel electrophoresis (2% agarose), the purified fragments were checked by a gel imaging analysis system.

**LF-RPA assay development**

The LF-RPA assay was performed with the TwistAmp nfo kit (TwistDX, UK) using a normal forward primer, a 5’-biotin-labeled reverse primer, and a TwistAmp LF probe designed according to the TwistDX guidelines (Table 2). Each LF-RPA assay mixture contained 2.1 μl 16S-LF-212F (10 μM), 2.1 μl 16S-LF-461R (10 μM), 0.2 μl 16S-LF-P (10 μM), 29.5 μl supplied rehydration buffer, 12.6 μl H₂O, 1 μl DNA template, 2.5 μl of 280 mM MgAc, and one supplied Table 1 | Cyanobacteria and other strains used in the study

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Species/strains</th>
<th>FACHB no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanophyta</td>
<td>Nostoc sp.</td>
<td>FACHB-596</td>
</tr>
<tr>
<td></td>
<td>Anabaena cylindrica</td>
<td>FACHB-1038</td>
</tr>
<tr>
<td></td>
<td>Aphanizomenon flos-aquae</td>
<td>FACHB-1040</td>
</tr>
<tr>
<td></td>
<td>Microcystis flos-aquae</td>
<td>FACHB-1028</td>
</tr>
<tr>
<td></td>
<td>Cylindrospermopsis raciborskii</td>
<td>FACHB-1503</td>
</tr>
<tr>
<td></td>
<td>Microcystis aeruginosa</td>
<td>FACHB-905</td>
</tr>
<tr>
<td></td>
<td>Microcystis aeruginosa</td>
<td>FACHB-912</td>
</tr>
<tr>
<td></td>
<td>Microcystis wesenbergii</td>
<td>FACHB-1334</td>
</tr>
<tr>
<td>Bacillariophyta</td>
<td>Cyclotella meneghiniana</td>
<td>FACHB-1031</td>
</tr>
<tr>
<td></td>
<td>Navicula sp.</td>
<td>FACHB-1051</td>
</tr>
<tr>
<td>Euglenophyta</td>
<td>Euglena gracilis</td>
<td>FACHB-848</td>
</tr>
<tr>
<td>Chlorophyta</td>
<td>Scenedesmus obliquus.</td>
<td>/</td>
</tr>
<tr>
<td>Angiospermae</td>
<td>Arabidopsis thaliana</td>
<td>/</td>
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</tbody>
</table>
lyophilized enzyme. The reaction procedures were the same as those described in the preceding section. After incubation at 37°C for 15–25 min, 1 μl of the reaction mixture was added to 99 μl running buffer. A 10 μl sample of this solution was transferred to the LF strip of the Hybridetect-1 (Milenia Biotec, Germany) and the strip end was placed into 100 μl running buffer for 4-min incubation at room temperature. The color test band indicated that amplification of DNA was successful. A separate control band confirmed that the system was functioning properly.

**PCR assay**

PCR was carried out to evaluate the amplification performance of LF-RPA. The PCR reaction (25 μl total volume) contained 12.5 μl 2× Easy Taq mix buffer, 10.5 μl ddH2O, 0.5 μl of 10 μM primer 16S-118F, 0.5 μl of 10 μM primer 16S-414R, and 1 μl DNA template. Reactions were conducted using a thermal cycling protocol: 95°C for 4 min, 35 cycles of 94°C for 35 s, 56°C for 35 s, and 72°C for 45 s, and 72°C for 10 min. Products of 297 bp were subjected to 2% agarose gel electrophoresis and checked with a gel imaging analysis system.

**RESULTS AND DISCUSSION**

**Temperature of reaction**

To determine the optimal amplification temperature for LF-RPA, we carried out a set of LF-RPA reactions at six different temperatures using DNA extracted from a 10^3 cell/mL culture of *Microcystis aeruginosa* (FACHB-905). The LF-RPA assay worked optimally over a wide range of temperatures, from 30°C to 45°C (Figure 1). We chose 37°C as the reaction temperature for all of our analyses because this was the optimal temperature recommended by the kit.

The LF-RPA assay was conducted using DNA from a 10^3 cell/mL culture of *Microcystis aeruginosa* (FACHB-905). Amplification by RPA worked optimally at temperatures between 30°C and 45°C.

**Duration of incubation reaction**

To identify the optimal duration of the RPA reaction, we incubated six identical RPA reactions at 37°C for six different durations, from 0 min to 25 min (Figure 2). The test band colored in 5-min amplification time, and the color of the test band became more solid and darker with longer time. The color of the LF strips suggested that the optimal incubation was 15 min, which was long enough for the LF-RPA assay to

![Figure 1](https://iwaponline.com/ws/article-pdf/19/4/1181/593795/ws019041181.pdf)

**Table 2** Primers for amplification of cyanobacterial DNA

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer name</th>
<th>Sequence (5’ – 3’)</th>
</tr>
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<tbody>
<tr>
<td>PCR</td>
<td>16S-118F</td>
<td>ACTTGAGGAATCAGCCTCGG</td>
</tr>
<tr>
<td></td>
<td>16S-414R</td>
<td>TTTGCTCCCCCTAGTTTCG</td>
</tr>
<tr>
<td>RPA</td>
<td>16S-RPA212F</td>
<td>TCGGCCAAGGCTGCCGATCCAGGTTGC</td>
</tr>
<tr>
<td></td>
<td>16S-RPA461R</td>
<td>AAGCAGGCTGTATCCTGATCGTTACGGC</td>
</tr>
<tr>
<td>LF-RPA</td>
<td>16S-LF-212F</td>
<td>TACGCAAGTCTGCGTCAATCAGGTTGC</td>
</tr>
<tr>
<td></td>
<td>16S-LF-461R</td>
<td>Biotin-AAAGCAGCTGCTGATCCATCTGTTACGCC</td>
</tr>
<tr>
<td></td>
<td>16S-LF-P</td>
<td>FAM-CCGTTGAAACTGGCAGACTAGAGCAGTACGTTAGG-THF-GTAGCAGGAATCCAG- SpacerC3</td>
</tr>
</tbody>
</table>
detect Cyanobacteria (a colored test band indicated successful detection).

The LF-RPA assay was conducted at 37 °C for 0 min to 25 min using DNA from a 10³ cell/mL culture of *Microcystis aeruginosa* (FACHB-905). The test band began to appear after 5 min incubation, but the 15 min incubation produced the most product.

**Specificity of LF-RPA**

The LF-RPA primers and probe were designed for the 16S rRNA gene sequences of the cyanobacterial species. The specificity of LF-RPA was evaluated using the eight cyanobacterial species (lanes 1–8 in Figure 3) and five non-cyanobacterial controls (lanes 9–13 in Figure 3). As shown in Figure 3, the test band appeared only for the cyanobacterial species, suggesting that LF-RPA successfully detected all of the cyanobacterial species for which the primers and probe were designed. The test did not detect any of the negative controls, which included *Euglena intermedia*, *Euglena gracilis*, *Cyclotella meneghiniana*, *Navicula sp.*, *Scenedesmus obliquus*, and *Arabidopsis thaliana*. The results suggest that LF-RPA assays can accurately detect target cyanobacterial species with high specificity.

The test detected all eight cyanobacterial species (1–8), but none of the five non-cyanobacterial control species (9–13) used in this study. Lane 1: *Microcystis aeruginosa* (FACHB-905); Lane 2: *Nostoc* sp.; Lane 3: *Microcystis aeruginosa* (FACHB-912); Lane 4: *Microcystis flos-aquae*; Lane 5: *Anabaena cylindrica*; Lane 6: *Aphanizomenon flos-aquae*; Lane 7: *Cylindrospermopsis raciborskii*; Lane 8: *Microcystis wesenbergii*; Lane 9: *Euglena gracilis*; Lane 10: *Cylotella meneghiniana*; Lane 11: *Navicula* sp.; Lane 12: *Scenedesmus obliquus*; Lane 13: *Arabidopsis thaliana*.

**Detection sensitivity of LF-RPA compared with PCR**

The detection sensitivities of LF-RPA and PCR were assessed using DNA samples from different cell concentrations of *Microcystis aeruginosa* (FACHB-905): 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10, 1, 0.1, and 0.01 cell/mL. The detection limit of PCR was 10⁴ cell/mL (Figure 4(b)), while the detection limit of LF-RPA was 0.01 cell/mL (Figure 4(a)). The results suggest that LF-RPA can detect cyanobacterial DNA at much lower cell concentrations than PCR.

The DNA detection limit of LF-RPA is 0.01 cell/mL for *Microcystis aeruginosa* (FACHB-905), while PCR requires a much higher cell concentration (10⁴ cell/mL) to detect DNA. NC is the negative control.

**Analysis of lake water**

To verify the feasibility of using the LF-RPA assay on field samples, we tested the method on samples from a cyanobacterial bloom in a lake. The density of cyanobacterial cells in the lake samples was quantified using a microscope. The lake samples were then diluted as shown in Figure 5. The procedure and conditions used for DNA extraction and LF-RPA analysis were the same as described above. The
results showed that the LF-RPA method could successfully detect Cyanobacteria in natural water samples with the same sensitivity as was achieved in pure laboratory-grown cultures of Cyanobacteria (Figure 5).

The detection sensitivity of LF-RPA was the same for the lake samples as for laboratory-grown pure cyanobacterial cultures. NC is the negative control.

**CONCLUSIONS**

LF-RPA detects the DNA of Cyanobacteria more rapidly and simply than traditional PCR-based methods. We used a freeze-cracking method to release DNA from cyanobacterial cells for amplification using the RPA method, which shortens the time of detection of Cyanobacteria in a water sample. We found that LF-RPA works efficiently over a wide range of temperatures, from 30 °C to 45 °C, and the optimal amplification duration was 15 min. These results suggested that the LF-RPA reaction could proceed at a single temperature, without the need for a thermal cycler, and that it achieved adequate DNA amplification using a short incubation time.

The specificity of LF-RPA was validated by testing for eight cyanobacterial strains and five non-cyanobacterial strains. RPA successfully amplified the 16S rRNA gene of the cyanobacterial species with high specificity. The LF strip was then able to detect the amplification products, making it readily transferrable to field testing. The lower limit of detection by LF-RPA was 0.01 cell/mL *Microcystis aeruginosa*, making it more sensitive than the PCR method.

In summary, LF-RPA could detect Cyanobacteria quickly and accurately. The method has the advantages of simple operation, no need for advanced instrumentation, and real-time monitoring of Cyanobacteria in the field. This method can facilitate rapid, early detection of Cyanobacteria which can help improve efforts to prevent and treat cyanobacterial blooms.

**CONFLICTS OF INTEREST**

There are no conflicts of interest to declare.

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