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Research Article

A Modified Colony PCR Method for Screening Recombinant *Chlamydomonas reinhardtii*

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ABSTRACT:
Chlamydomonas reinhardtii has been emerged as an interesting platform for production of recombinant and therapeutic proteins. However, there are some obstacles in wide usage of *Chlamydomonas reinhardtii*. Colony PCR is one of the first line diagnostic tools for determination recombinant strains but current protocols for colony PCR of *Chlamydomonas reinhardtii* are not as efficient as desired. This study is aimed to evaluate the present protocols and represent a simple modified method for colony PCR of *Chlamydomonas reinhardtii*.
wild type strain of *Chlamydomonas reinhardtii* was transformed by p_chlamy3 plasmid containing Hygromycin B resistance gene (aph7). Two primer pairs were used to amplify a sequence covering promoter and multiple cloning site and also amplify a fragment of aph7 gene in a multiplex PCR platform. Different extraction methods were used to screen recombinant colonies. PCR products were evaluated by agarose gel electrophoresis.
1060 and 500 bp fragments of the target sequence and aph7 were successfully amplified by PCR and our modified protocol led to the best results among different evaluated methods. Colony PCR is the first line screening method in recombination process. The present study introduced a simple modified and cost effective protocol for Colony PCR, which can be used to screen recombinant genes in *Chlamydomonas reinhardtii.*

Keywords: *Microalgae; Colony PCR; Chlamydomonas reinhardtii; recombination.*

[I] NTRODUCTION

Green microalgae, *Chlamydomonas reinhardtii*, has been used as a superb model organism to study a wide variety of cellular functions, photobiology and physiology of photosynthesis for many years [1]. *Chlamydomonas reinhardtii* is a freshwater biflagellate unicellular photosynthetic algae which can easily be cultivated in both autotrophic and heterotrophic conditions using acetate as organic carbon source [2, 3]. Nowadays, microalga, especially *Chlamydomonas reinhardtii*, are considered as the novel platforms for production of recombinant proteins and pharmaceuticals. *Chlamydomonas reinhardtii* is a "Generally Regarding as Safe" (GRAS) microorganism; can be grown in low price media; has a rapid growth rate, its nuclear, chloroplast

and mitochondrial genome are well studied and several well-known nuclear and chloroplast promoters are identified for it. It has also the ability to produce complex proteins with correct folding [4]. Polymerase chain reaction (PCR) is a fast method to amplify specific DNA fragments. Colony PCR is the first line technique for screening the presence of a desired sequence in a single colony without the need of DNA purification and provides a rapid and cost effective method [5]. Colony PCR is widely used in identification of recombinant microorganisms as well as diagnosis of unknown isolates [6]. The major problems of colony PCR in microalgae are low copy number of integrated sequence and rigid cell wall that limits the DNA extraction and secretion to reaction mixture. To date, several colony PCR methods have been emerged for microalgae; however, the majority of methods are time consuming, require special material and have low efficiency [7].

In the present study, we have compared the efficiency of several colony PCR methods and introduced a modified efficient technique for detection of recombinant gene in *Chlamydomonas reinhardtii*.

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[II] MATERIALS AND METHODS

2.1. Cells and culture condition

Wild type *Chlamydomonas reinhardtii* strain (137c) was obtained from the Chlamydomonas resource center, University of Minnesota, MN, USA. Cells were grown in TAP (Tris, acetate, Phosphate) broth or agar medium at 25°C under 16-8 hours light-dark cycle with light intensity equal to 50 $\mu E.m^{-2}.sec^{-1}$; Broth cultures were shaken at 120 rpm . Recombinant strains were cultivated in TAP medium containing 10 $\mu g.ml^{-1}$ hygromycin B (Invitrogen, USA).

2.2. Recombination of *C. reinhardtii*

Recombinant pChlamy_3 plasmid (Invitrogen, USA) containing a 180 bp sequence cloned in the multiple cloning site was used to integrate the target gene and aph7 (hygromycin resistance) gene to *Chlamydomonas reinhardtii* genome. The plasmid was linearized by Sca I restriction enzyme (Thermo Scientific, USA) and Transformed to *C. reinhardtii* cells via electroporation by Gene Pulser Xcell™ Electroporation System (Bio-Rad, USA) with the following settings: Voltage: 600V, capacity 50µF, resistance: infinity. Recombinant colonies were grown on TAP agar plates containing 10 $\mu g.ml^{-1}$ hygromycin B.

2.3. Colony PCR protocols

Recombinant colonies were grown on TAP-hygromycine agar plates and proceeded with different protocols as below: 1) the first method was done as described in the manual prepared by pChlamy_3 plasmid provider (Invitrogen, USA), a little portion of colony was directly added to PCR mixture and the reaction was performed on its conventional way; 2) a little portion of colony was added to 20 µl deionized water, boiled for 10 minutes and 2 µl of cell lysate was added to PCR mix; 3) a little portion of colony was added to 30 µl deionized water or TE (Tris-EDTA) buffer containing 0.1 gr glass beads with approximate 200 µm diameter (Sigma-Aldrich, USA), boiled for 10 minutes while it was vigorously shaken for seconds during boiling, centrifuged and then 2 µl of supernatant was added to PCR mix; 4) The recombinant colony was cultivated in TAP broth medium containing 10 $\mu g.ml^{-1}$ hygromycin B, as the culture reached 4-5 × 10^6 $cell.ml^{-1}$, 5 ml of culture was centrifuged at 4000 rpm for 5 minutes in room temperature. The cells were resuspended in 50 µl deionized water, TE buffer, deionized water containing 0.2% triton X-100 and TE buffer containing 0.2% triton X-100 and boiled for 10 minutes. The lysate was cooled on ice, centrifuged and 1-2 µl of supernatant was added to the PCR mix [5, 7-9].

2.4. Primers and PCR condition

In a multiplex PCR method, Hyg-F and Hyg-R primers were used to amplify a 500 bp fragment of aph7 gene. Sequences are: Hyg-F (Forward): 5'-

GATGACCGGCACCACCTGG-3' and Hyg-R (reverse): 5'-GAACACCTCGAAGTCGTGCAG-3'. The following primers were used to amplify a 1060 bp fragment covering promoter, cloned sequence and 3'-UTR sequence: Forward: 5'-TTGAGTGAGCTGATACCGC-3' and reverse: 5'- GACTGATCAGCACGAAACG-3'. Reaction mixture in the final volume of 25 µl, contained 2 units *Taq* DNA polymerase, PCR buffer and 0.2 mM of each dNTPs, all provided by Sinaclon, Iran; DMSO (Dimethyl sulfoxide) was also added to reaction mix with final concentration of 4%. Thermal profile of the reaction was initial denaturation at 94°C for 5 minutes, following by 35 cycles of 30 seconds on 94°C and 58°C, and 50 seconds on 72°C, and final extension at 72°C for 10 minutes. Purified plasmid was used as positive control. The PCR product was electrophoresed on 1% agarose gel (Merck, Germany) and visualized by DNA safe stain (Sinaclon, Iran).

[III] RESULTS

Both 1060 and 500 bp fragments were efficiently amplified by multiplex PCR. In the present study, we evaluated eight different colony PCR protocols (table 1) and the result is shown in figure 1. Protocol efficiency can be evaluated by sharpness of the bands on agarose gel. As it is shown in fig. 1, our home made protocol led to the best efficiency.

No.	Starting material	Procedure
1	Broth medium	Lysing the cells by boiling in TE buffer containing 0.2% Triton X-100
2	Colony on agar plate	Direct colony PCR
3	Colony on agar plate	Lysing the cells by boiling in TE buffer containing 0.2% Triton X-100
4	Colony on agar plate	Lysing the cells by boiling in TE buffer with glass beads
5	Broth medium	Lysing the cells by boiling in deionized water
6	Colony on agar plate	Lysing the cells by boiling in deionized water with glass beads
7	Broth medium	Lysing the cells by boiling in deionized water containing 0.2% Triton X-100
8	Broth medium	Lysing the cells by boiling in TE buffer

Table: 1. brief description of assayed methods.

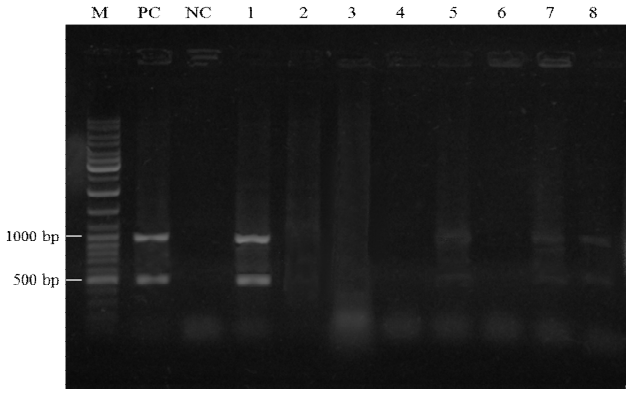

Fig 1. Agarose gel of PCR products:
M: Generuler DNA ladder mix, PC: positive control, NC: Negative control, lanes 1-8 are described in table 1.

[IV] DISCUSSION

Chlamydomonas reinhardtii has been considered as an emerging platform for production of recombinant proteins. Screening of recombinant colonies is a time consuming and somewhat troublesome step in recombination procedure. As mentioned, colony PCR is an efficient and cost effective method for screening special sequences in microorganisms.

By emerging of *Chlamydomonas reinhardtii* as a new platform for production of recombinant proteins, the demand for fast and cheap methods for first line screening has been raised up. However, obstacles such as high G+C content of *C. reinhaerdtii* genome, rigid cell wall, low copy number of gene of interest and potential PCR inhibitors in cell lysate could lead to low efficiency of colony PCR [5, 8]. In this regard, the protocol should slightly be optimized to prepare acceptable amounts of DNA for PCR as well as low concentration of potential PCR inhibitors to lead to appropriate results.

Here, we evaluated several amounts of cells, volume of buffer, boiling duration and composition of lysis buffers to find an optimized protocol for colony PCR which is schematically shown in figure 2.

Our results showed that boiling the cells that were harvested from 5 ml of 4-5 × 10^6 cell.ml^{-1} medium in TE buffer containing 0.2% Triton X-100 would result in the best efficiency for colony PCR.

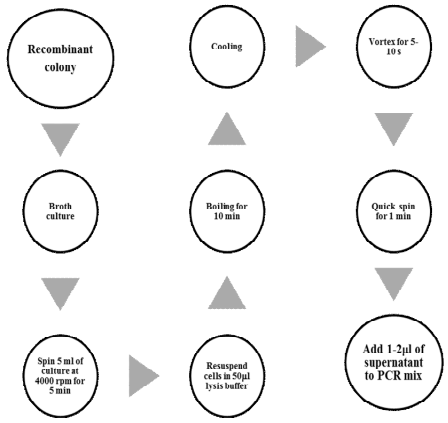

Fig. 2. Schematic diagram of optimized colony PCR. The best result achieved by TE buffer containing 0.2% triton X-100 as lysis buffer.

Amplifying a fragment of aph7 gene along with target sequence in a multiplex method, acts as an internal control for PCR, and enhances the reliability of method. The idea of using internal control in multiplex PCR may be modified by using genomic sequences as internal control for further researches. The presented method may also be useful to screen particular genomic sequences.

[V] CONCLUSION

In conclusion, the present study has assayed colony PCR technique for *Chlamydomonas reinhardtii* and introduced a new modified method for routine use in laboratory. Our method represents a reliable, cost effective and simple way to screen a large number of recombinant colonies of *Chlamydomonas reinhardtii*.

FINANCIAL DISCLOSURE

The authors have no other interests to declare.

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