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Library screening for D-amino-acid oxidase gene: Application of real-time PCR

Short Communication

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Summary. Quantitative real-time PCR shows the quantity in addition to the presence of the target sequence. This property seemed very useful for library screening. Then, real-time PCR was employed to screen for λ phages carrying D-amino-acid oxidase gene from mouse genomic library. Using stepwise dilution screening combined with real-time PCR, positive phages were isolated in a short time.

Keywords: Real-time PCR – D-Amino-acid oxidase – Library – Screening – Mouse

Introduction

D-Amino-acid oxidase (EC 1.4.3.3) catalyzes oxidative deamination of D-amino acids, stereoisomers of naturally occurring L-amino acids (Krebs, 1935). In higher animals, it is present in the kidney, liver and brain (Meister, 1965). It metabolizes D-amino acids of internal and external origin (Konno et al., 1993; D'Aniello et al., 1993). However, its physiological role is not clearly understood. Genetic manipulation of the D-amino-acid oxidase gene would help to elucidate this problem. For this purpose, the gene has to be cloned at first. Plaque hybridization has been the most frequently applied method for the library screening (see Sambrook and Russell, 2001). Later, a screening method that employs PCR was developed (Griffin et al., 1993; Israel, 1993; McAlinden and Krawetz, 1994; Yu and Bloem, 1996). The PCR-based screening is rapider, simpler and more sensitive than the plaque hybridization. Recently qualitative real-time PCR has been developed (see Sambrook and Russell, 2001). Real-time PCR seems more useful for the screening than the conventional PCR since it shows the relative abundance of positive phage carrying the target sequence in the library. This property seems useful especially for the stepwise dilution screening (Watanabe et al., 1997). Therefore, applicability of real-time PCR to library screening has been examined in this work.

Materials and methods

Materials

Mouse genomic library in the λ FIX II vector was purchased from Stratagene (La Jolla, USA). A forward primer (5′-GAG TGG AGC CAG CAA ACG TT-3′) and a reverse primer (5′-ATG GCT CAC CGG AAC TTC AT-3′) were synthesized from the sequence of D-amino-acid oxidase cDNA (Tada et al., 1990). AmpliTaq Gold DNA polymerase and SYBR Green PCR Core Reagents were purchased from PE Biosystems (Warrington, UK). GeneAmp PCR System 2400, ABI Prism 7700 Sequence Detection System and GeneAmp 5700 Sequence Detection System were from Applied Biosystems. SM buffer was 50 mM Tris-HCl (pH 7.5)/0.1 M NaCl/8 mM $MgSO_4$/0.01% gelatin. TE buffer was 10 mM Tris-HCl/1 mM EDTA (pH 8.0).

Conventional PCR

Two μl of the phage lysate was added to 23 μl of a PCR cocktail (1.5 mM $MgCl_2$, 0.2 mM dNTP mix with dUTP, 0.2 μM the forward primer, 0.2 μM the reverse primer, 1 × PCR buffer II solution, and 0.63 units of AmpliTaq Gold DNA polymerase). The solution was heated at 95°C for 10 min followed by 40 cycles of the denaturation (95°C, 15 sec) and the annealing/extension (63°C, 45 sec). The PCR product was electrophoresed on 2% agarose gel and visualized with ethidium bromide staining.

Real-time PCR

Two μl of phage lysate was added to 23 μl of a PCR cocktail (3 mM $MgCl_2$, 1 mM of dNTP mix with dUTP, 0.1 μM the forward primer, 0.1 μM the reverse primer, 1 × buffer solution, and 0.63 units of AmpliTaq Gold DNA polymerase) (SYBR Green PCR Core Reagents). The SYBR Green I double-stranded DNA chemistry was used since it had more versatility than the fluorogenic 5′ nuclease assay (TaqMan assay). The reaction tubes were applied to ABI Prism 7700 Sequence Detection System or GeneAmp 5700 Sequence Detection System. The PCR conditions were initial heating at 95°C for 10 min followed by 40 cycles of the denaturation (95°C, 15 sec) and the annealing/extension (63°C, 45 sec).

Results and discussion

Mouse genomic library was screened for phages carrying the D-amino-acid oxidase gene. First, a forward primer and a reverse primer were synthesized from the sequence of D-amino-acid oxidase cDNA. Conventional PCR using these primers and mouse genomic DNA as a template produced a 120-bp fragment (Fig. 1). Cloning and sequencing indicated that this fragment had a part of the D-amino-acid oxidase gene. This fragment was also amplified in the real-time PCR (Fig. 2).

An aliquot (100 μl) of a diluted λ library (1×10^6 pfu/ml) was mixed with host *Escherichia coli* (200 μl). The solution was mixed with melted top agarose and poured onto each of twenty 9-cm agar plates. The plates were incubated at 37°C until plaques started to fuse. Three ml of SM buffer were poured onto each of the plates. The plates were kept at 4°C for several hours and then the SM buffer was gathered. A 50-μl aliquot was mixed with 100 μl TE buffer and heated at 95°C for 2 min. The phage lysate was subjected to the conventional PCR and real-time PCR in order to examine the presence of the target sequence.

Some phage solutions from the twenty plates showed positive results indicating the presence of phages carrying the D-amino-acid oxidase gene. Figures 1 and 2 clearly illustrated the advantage of the real-time PCR over the conventional PCR in the screening. Conventional PCR indicated that positive phages were present in solution C and F (Fig. 1). However, it was not clear which solution contained more positive phages since the amplification

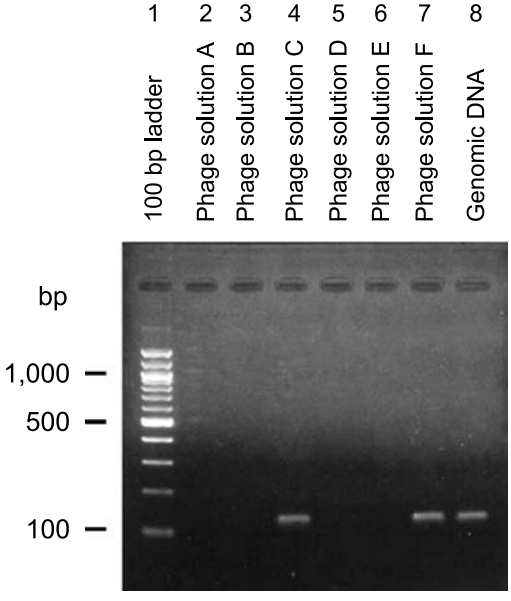

Fig. 1. Gel electrophoresis of the PCR products. Conventional PCR was carried out using the phage solution as templates and the products were electrophoresed on 2% agarose gel. Lane1: 100 base pair ladder. Lane 2: phage solution A. Lane 3: phage solution B. Lane 4: phage solution C. Lane 5: phage solution D. Lane 6: phage solution E. Lane 7: phage solution F. Lane 8: genomic DNA solution. This result indicates that positive phages carrying the target gene were present in solutions C and F

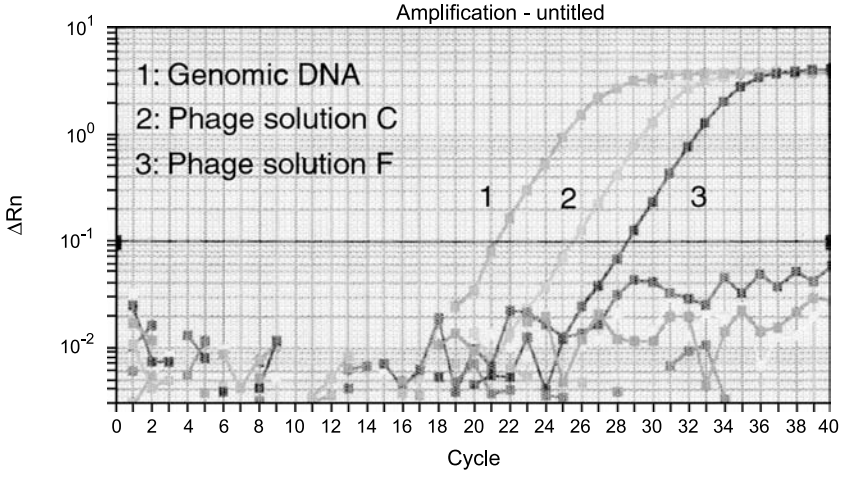

Fig. 2. Results of the real-time PCR. Amplification was monitored with SYBR Green I dye. Amplification occurred in phage solutions C (*2*) and F (*3*) and genomic DNA solution (*1*). From these results, the relative abundance of the target sequence in these solutions was calculated as 8 (solution C): 1 (solution F): 128 (genomic DNA solution)

reached the plateau. On the contrary, real-time PCR indicated that solution C contained approximately 8 times more positive phages than solution F (Fig. 2). It meant that solution C could be diluted 8 times more than solution F, which enabled to reduce the complexity of the library for the next screening step.

The phage solution was diluted with the SM buffer to 2×10^6 pfu/ml or less depending on the abundance of the positive phages. An aliquot (100 μl) of the diluted phage solution was mixed with *E. coli*. The solution was mixed with melted top agarose and poured onto a 9-cm agar plate. After a several hour-incubation at 37°C, the agarose-agar layer was cut with a scalpel into 16 equal pieces of a pie shape (Watanabe et al., 1997). Each top agarose layer was peeled with a flat spatula and transferred into a 15-ml tube containing 1.5 ml of the SM buffer. The solution was vigorously vortexed and centrifuged at 3,000 rpm for 10 min. The supernatant was collected into a new tube. A 50-μl aliquot was mixed with 100 μl TE buffer. This dilution step was included in order to avoid the possible inhibition of PCR with agar fragment (Sambrook and Russell, 2001) and to avoid the increase of Mg^{2+} ions in the PCR cocktail. The solution was heated at 95°C for 2 min. The phage lysate was examined for the presence and the relative abundance of the target sequence using the real-time PCR. From the results of the real-time PCR, the SM buffer containing the highest concentration of the positive phages was selected for the next screening. The phage titter of this buffer was determined.

Since at least one positive phage was calculated to be present among 1.25×10^4 phages ($2 \times 10^6 \times 0.1 \times 1/16$), the phage solution was diluted to 2.5×10^5 pfu/ml or less depending on the abundance of the positive phages. An aliquot (100 μl) was mixed with *E. coli* and poured with melted top agarose. The 16 pie-shaped pieces of the top agarose layer were collected as described above. Then the phage solutions were prepared and subjected to real-time PCR. From the results of the real-time PCR, the solution containing the highest concentration of the positive phages was selected for the next screening.

This stepwise dilution-amplification-selection method was repeated until one positive phage was found among 10–20 phages. Finally, single plaques were picked up with Pasteur pipettes and transferred into 500 μl SM buffer. Real-time PCR or conventional PCR was used to indicate which buffer solution contained the positive phages. Positive phage clone was plaque-purified.

The screening method employing the real-time PCR was so efficient that positive phages carrying the D-amino-acid oxidase gene were isolated in a short time.

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References

D'Aniello A, D'Onofrio G, Pischetola M, D'Aniello G, Vetere A, Petrucelli L, Fisher GH (1993) Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids. J Biol Chem 268: 26941–26949

Griffin HG, I'Anson KJ, Gasson MJ (1993) Rapid isolation of genes from bacterial lambda libraries by direct polymerase chain reaction screening. FEMS Microbiol Lett 112: 49–54

Israel DI (1993) A PCR-based method for high stringency screening of DNA library. Nucleic Acids Res 21: 2627–2631

Konno R, Oowada T, Ozaki A, Iida T, Niwa A, Yasumura Y, Mizutani T (1993) Origin of D-alanine present in urine of mutant mice lacking D-amino-acid oxidase. Am J Physiol 265: G699–G703

Krebs HA (1935) CXCVII. Metabolism of amino-acids. III. Deamination of amino-acids. Biochem J 29: 1620–1644

Meister A (1965) Biochemistry of the amino acids, vol 1, 2nd edn. Academic Press, New York, pp 297–304

McAlinden TP, Krawetz SA (1994) A practical method to screen libraries of cloned DNA. Anal Biochem 218: 237–238

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Tada M, Fukui K, Momoi K, Miyake Y (1990) Cloning and expression of a cDNA encoding mouse kidney D-amino acid oxidase. Gene 90: 293–297

Watanabe K, Sakai F, Orii H (1997) Stepwise dilution screening of a cDNA library by polymerase chain reaction. Anal Biochem 252: 213–214

Yu L, Bloem LJ (1996) Use of polymerase chain reaction to screen phage libraries. In: Harwood A (ed) Methods in molecular biology. Humana Press, Totowa, pp 335–339

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