THE CLONING OF THE HUMAN TUMOR SUPPRESSOR GENE ING1: DNA CLONING INTO PLASMID VECTOR AND DNA ANALYSIS BY RESTRICTION ENZYMES

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Abstract

DNA cloning is one of the most important techniques in the field of molecular biology, with a critical role in analyzing the structure and function of genes and their adjacent regulatory regions. DNA cloning is helpful in learning fundamental molecular biological techniques, since DNA cloning involves a series of them, such as polymerase chain reaction (PCR), DNA ligation, bacterial transformation, bacterial culture, plasmid DNA extraction, DNA digestion with restriction enzymes and agarose gel electrophoresis. In this paper the cloning of the human tumor suppressor gene ING1 has been used to illustrate the methodology. The gene was amplified by PCR, cloned into a TA-cloning vector, and restriction enzyme mapping was used to distinguish the sense ING1 construct from the antisense ING1 construct.

Introduction

One of the most important techniques in the field of molecular biology is DNA cloning, which is used to obtain large quantities of specific DNA regions.

DNA cloning involves many fundamental molecular biological techniques, such as polymerase chain reaction (PCR), DNA ligation, bacterial transformation, bacterial culture, plasmid DNA extraction, DNA digestion with restriction enzymes and agarose gel electrophoresis. In this paper the cloning of the human tumor suppressor gene ING1 is used to illustrate the methodology.

There are numerous cloning vectors for different purposes. TA-cloning vector has recently been designed for cloning PCR products directly from the PCR reaction using an overnight ligation step. Taq DNA polymerase is a thermostable DNA-dependent DNA polymerase and has an activity to polymerize one adenosine monophosphate additionally at the 3' end of PCR product (Fig 1).
survive on an agar plate containing antibiotic like ampicillin (Fig 1). These cloning vectors also carry an amino-terminal portion of the β-galactosidase gene on the multiple cloning sites (Fig 1). The IPTG induces the β-galactosidase in active form, and can catalyze X-gal to develop blue color. However, the plasmid bearing an insert DNA in multiple cloning sites produces an inactive enzyme, and colony retains white color. This blue-white selection system is easy to use for selecting the plasmid vector containing an insert DNA.

After transformation of E. coli, plasmid vectors containing an insert DNA are selected and cultured in LB-broth with ampicillin. Then the plasmid DNA is prepared by the alkaline lysis method. Cell wall of E. coli bearing the plasmid is lysed by SDS (sodium dodecyl sulfate detergent; Fig 2). By adding NaOH, E. coli DNA and plasmid DNA are denatured to single-stranded DNA. Addition of acidic solution brings renature of plasmid DNA, leading to conformation of double stranded DNA, while the E. coli DNA with high molecular weight is not renatured and forms an aggregate with the denatured proteins.²⁵

After centrifugation, plasmid DNA is collected in the supernatant. Restriction enzymes can bind specifically to and cleave double-stranded DNA at sites within or adjacent to a particular sequence known as the recognition sequence (Fig 3). In this way the restriction enzymes digestion is used to confirm the successful insertion of the specific DNA.

Materials and Methods

1. Materials

The most important materials for DNA cloning and analysis are listed in Table 1.
Table 1. Materials used for DNA cloning and analysis.5

<table>
<thead>
<tr>
<th>Material</th>
<th>Contents</th>
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</thead>
<tbody>
<tr>
<td>LB-broth</td>
<td>1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl</td>
</tr>
<tr>
<td>Agar plates (+Ampicillin)</td>
<td>1.3% agar in LB-broth containing 50 μg/ml ampicillin</td>
</tr>
<tr>
<td>Agarose gel</td>
<td>0.7 % agarose in 1X TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) containing 0.5 μg/ml of ethidium bromide</td>
</tr>
<tr>
<td>ALM solution A 1)</td>
<td>50 mM glucose, 25 mM Tris-HCl, 10mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>ALM solution B 1)</td>
<td>0.2N NaOH, 1% SDS</td>
</tr>
<tr>
<td>ALM solution C 1)</td>
<td>3 M potassium acetate (pH 4.8)</td>
</tr>
<tr>
<td>40 mg/ml X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethyl-formamide</td>
</tr>
<tr>
<td>40 mg/ml IPTG</td>
<td>Isopropylthio-β-D-galactoside in dH2O</td>
</tr>
</tbody>
</table>

1) ALM = alkaline lysis method

![Fig. 2 Plasmid DNA Preparation by Alkaline Lysis Method](image)

![Fig. 3 Restriction Enzymes and Multicloning sites of pT7Blue vector](image)
2. DNA Amplification by PCR

The chosen gene (here human ING1 gene) is amplified by PCR. The PCR mixture contains 100 ng of normal cDNA, 1X PCR buffer, 200 μM of each dNTP, 20 pmole of each primer for ING1 gene and 1.25 units of Taq DNA polymerase (Takara, Tokyo, Japan) in 50 μl volume. Initial denaturation at 94°C for 3 minutes is followed by 35 cycles of a denaturation step at 94°C for 30 seconds, an annealing step at 58°C for 1 min and an extension step at 68°C for 90 second. A final extension step at 72°C is added.

3. Confirmation of The DNA Size of PCR Products on Agarose Gel

After the PCR reaction, 3 μl of the PCR mixture is mixed with 1 μl of 6X loading buffer (40% sucrose, 0.25% bromophenol blue), applied on 0.7% agarose gel, and electrophoresed in 1X TAE buffer. A size marker DNA is also applied on the gel. After the electrophoresis, the gel is photographed on ultraviolet-transilluminator, and the DNA size of the PCR product is confirmed.

4. Ligation and Transformation

An amount of 4 μl of the PCR mixture is mixed with 1μl of 10X ligation buffer, 2 μl of TA-cloning vector pT7blue (12.5 ng/μl), 2μl of dH2O and 1 μl of T4 DNA ligase, and incubated at 16°C for 1 h. Then 2 μl of the ligated DNA solution is added to 40 μl of competent cells (E coli DH 5α), and chilled on ice for 30 min. After heat-shock at 42°C for 30 seconds, 0.5 ml of LB-broth is added in the sample tube, and the tube is shaken gently in 37°C incubator for 30 min. After centrifuging the tube at 6,000 rpm for 2 min at room temperature, the supernatant is discarded. The E. coli cells are suspended in 100 μl of LB-broth and spread on an agar plate containing 50 μg/ml X-gal and 80 μg/ml IPTG. The plate is incubated upside down at 37°C in an incubator.

5. Culture of Colonies

A white colony is picked up by a sterilized toothpick, transferred into 2 ml of LB-broth containing 50μg/ml ampicillin, and shaken at 37°C overnight. (A saturated culture contains about 10⁸ live E. coli cells per ml. A high copy plasmid, like pT7blue, can replicate to 500-700 copies in one E. coli cell).

6. Preparation of Plasmid DNA by The AlkalineLysis Method

The saturated culture is transferred into a sample tube, and centrifuged at 6,000 rpm for 3 minutes at room temperature. After discarding the supernatant, the cells are suspended in 100 μl of alkaline lysis method solution A and vortexed gently. 200 μl of alkaline lysis method solution B is added and mixed gently by inverting the tube. After keeping at room temperature for 5 minutes, 150 μl of alkaline lysis method solution C is added and mixed by shaking the tube several times. Samples are chilled on ice for 5 minutes, and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant is transferred to a new tube with 400 μl of phenol / chloroform / isoamylalcohol (25:24:1), mixed well for 5 minutes, and centrifuged at 12,000 rpm for 5 min at 4°C. The aqueous phase is transferred to a new tube, and 900 μl of ethanol is added, mixed and kept on ice for 5 minutes. After centrifuging at 12,000 rpm for 5 min at 4°C, the supernatant is discarded. The DNA is rinsed with 300 μl of 70% ethanol, centrifuged at 12,000 rpm for 2 min, and dried up at room temperature after discarding the supernatant. The DNA is dissolved in 50 μl of 1mM Tris-HCl/0.1mM EDTA/50 μg/ml Rnase A.
7. DNA Analysis With Restriction Enzymes and Agarose Gel Electrophoresis

The plasmid DNA is digested by suitable restriction enzymes. The reaction mixture contains 2 μl of 10X reaction buffer, 2 μl of 10X BSA (bovine serum albumin), 13.5 μl of dH₂O, 2 μl of plasmid DNA and 0.5 μl of the restriction enzyme. The reaction mixture is made independently for each restriction enzyme, such as EcoRI, Bam HI, or HindIII. After incubation at 37°C for 1 h, 5 μl of the reaction mixture is applied on 0.7% agarose gel containing 0.5 μl/ml of ethidium bromide, and electrophoresed with size marker DNA. Restriction enzyme map of insert DNA (human INGI gene) is constructed depending on the position of multiple cloning sites in the plasmid vector.

Results and Discussion

Human INGI cDNA was amplified by PCR with INGI specific primers, and the PCR product was confirmed for the size (938 bp) on agarose gel. After cloning in pT7Blue vector, the PCR product was digested with selected restriction enzymes, and reconfirmed for the size and the orientation in the plasmid vector (Figs 4 and 5).

The popular TA-cloning plasmid vector was used in this cloning step. The vector must be selected according to the purpose of DNA cloning. A plasmid, bacteriophage, cosmid, P1 phage, BAC (bacterial artificial chromosome) or YAC (yeast artificial chromosome) vectors can be selected and used depending on the size of DNA to be cloned. The plasmid vectors for prokaryotes or eukaryotes, and viral vectors such as retroviruses and adenoviruses are available.
for gene expression. The primary tools used by the molecular biologists in manipulating DNA are restriction enzymes and other DNA/RNA modifying enzymes. Understanding the properties and use of these enzymes is important in application of DNA cloning in a variety of fields like gene therapy.

Acknowledgement

The authors wish to gratefully acknowledge the advice and access to resources provided by Prof. Noriyuki Nagai and Prof. Kenji Shimizu of the Okayama University. This work was supported by Japan Society for Promotion of Science.

References


