OPTIMIZING DNA FOR TRANSFORMATION

Patricia V. King, Robert W. Blakesley
Bethesda Research Laboratories

T4 DNA ligase catalyzes the covalent joining of the ends of DNA molecules. The conditions for optimal enzymatic activity have been determined (1), but it is unclear whether these conditions are also optimal for the use of ligase in cloning experiments. For example, the efficient ligation of a DNA fragment to a vector DNA may require an incubation temperature or a cofactor concentration (i.e., ATP) significantly different from that needed to quantitate enzymatic activity. This is apparent from the many, sometimes conflicting sets of reaction conditions that are recommended in the literature. In many cases these parameters were chosen arbitrarily and do not represent the results of a systematic study.

The end result of using ligase is frequently the generation of a clone, that is, the transformation of a competent bacterial cell by the ligated (recombinant) DNA. Usually the progress of this ligation is monitored by gel electrophoresis of the reaction products. Identification of a higher molecular weight species and disappearance of monomer length substrate DNA in the gel is assumed to predict an efficient transformation. However, we have found this to be an unreliable correlation. Optimizing the ligase reaction for the appearance in a gel of very high molecular weight DNA often reduces the number of transformants obtained. Therefore, in order to improve the ligation reaction for efficient transformation, the ligase reaction products must be assayed directly for their ability to transform competent cells. The purpose of this study was to determine T4 DNA ligase reaction condition which increase the percentage of ligated DNA molecules that transform. Five reaction parameters were chosen as the most relevant for manipulation in order to optimize transformation: AT concentration, ligase concentration, time, temperature, and the molar ration of insert to vector. In addition, two compounds which stimulate DNA ligation, polyethylene glycol (2, 3) and hexamine cobalt chloride (4) tested.

Methods

The standard ligation reaction mixture (20 µl) contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCL_2$, 1 mM ATP, 170 ng (60 fmol) dephosphorylated EcoR V or Pvu II-cleaved pBR322 DNA, 13 ng (20 fmol) of a 1 Kb Tha I DNA fragment and 1 unit of BRL T4 DNA ligase in a sterile 0.5 ml polypropylene microcentrifuge tube. The components were mixed briefly, centrifuged for 5 seconds, then incubated at room temperature (23-26°C) for 4 hours. One µl of 0.5 M Na_2EDTA (pH 8.0) was added, the reaction was mixed, and then it was stored at +4°C.

The standard and small scale transformation assays were performed essentially as described for the BRL Frozen Component Cells (5). A 2 µl sample was removed from the completed ligase reaction and diluted to 10 µl with 10 mM Tris-HCl (pH 7.2), 1mM Na_2 EDTA. For the standard assay a 100 µl aliquot of thawed, competent E. coli HB101 cells from BRL was placed in a pre-chilled 17 x 100 mm polypropylene tube. One µl (1-2 ng) of the diluted ligase reaction mixture was added to the cells, mixed gently, then

incubated for 30 minutes on wet ice. The tube was placed at 42°C for 45 seconds and then immediately returned to the wet ice. After several minutes, 900 µl of S.O.C. media (5) were added, and the tube was incubated at 37°C for one hour in a shaking incubator. A 100 µl aliquot of the DNA/competent cell mixture was spread onto LB agar containing 100 µg/ml ampicillin (6). The agar plate was incubated overnight at 37°C. As a control, supercoiled pBR322 DNA subjected to this reaction and analysis routinely generated an efficiency of $\geq 1 \times 10^8$ transformants/µg. For the small scale transformation assay, 20 µl of competent cells, 80 µl of S.O.C. media and 50 µl plating volume were substituted. The smaller assay volume reduces the transformation efficiency 3 to 5-fold.

The products of the ligase reaction also were analyzed by agarose gel electrophoresis. A 20 ng aliquot from the completed ligase reaction was electrophoresed on a 2 x 50 x 75 mm (BRL Model H6, "Baby Gel") 1% agarose/Tris-acetate gel. The gel was soaked in aqueous ethidium bromide, and the DNA was detected by short wave UV irradiation of the gel.

RESULTS AND DISCUSSION

Usually fewer transformants/µg of input DNA are obtained from a ligation reaction involving blunt (paired) end insert and vector DNAs than from a reaction with sticky (unpaired) end DNAs. Improving the transformation of the blunt end DNA ligation reaction, therefore, was a primary goal of this study. For comparison, many of the experiments also were performed on sticky end insert with vector DNA, and blunt or sticky end phosphorylated vector DNAs without insert. Any new ligation conditions determined for blunt end DNAs must be compatible with these systems as well.

The general purpose cloning vector pBR322 was linearized with either EcoR V or PVu II to produce blunt ends and then dephosphorylated with bacterial alkaline phosphatase. The insert was a gel-purified 1 kb DNA fragment with Tha I (Blunt) ends or, in some instances a 349 bp DNA from a Rsa I (Blunt) cleavage. Generally, the same results were observed for either insert.

One of the parameters that dramatically influenced transformation was the temperature of ligation. The standard ligation reaction (see Methods) was incubated at either 4°C or room temperature (26°C). At the times indicated a 2µl aliquot was removed and then analyzed by the transformation assay. No transforming species were present after 2 hours of incubation at 4°C, and only a few were obtained after 4 hours. On the other hand, performing the ligation reaction at room temperature generated a large number of transformants rapidly, even in as little as one hour. In fact, the ligation performed at 26°C for 4 hours generated nearly 90% as many tranformants as were obtained after a 23 hour incubation, and approximately 25-fold more than that obtained after 4 hours at 4°C.

The amount of T4 DNA ligase in a blunt end reaction did influence the number of transformants. Approximately 1-2 units of enzyme were optimal. For comparison, EcoR I sticky end insert and vector DNAs examined in the same manner gave a maximum

number of transformants with only 0.1 unit of ligase. On the other hand, no effect on the transformation efficiency was observed when the ATP concentration of the blunt end ligation was varied from 10μM to 1mM. A similar result was obtained for the sticky end ligation reaction. Curiously, circularization of phosphorylated blunt end vector DNA without insert was influenced by the ATP concentrations, with a maximum near 0.1 mM. Increasing the molar ratio of dephosphorylated vector to blunt end insert DNA from 0.33 to 3.0 provided only a small (less than 3-fold) increase in the transformation efficiency. Finally, it is important to note that the ligation reaction mixture must be diluted at least 5-fold prior to adding the DNA to the competent cells in order to prevent inhibition of transformation (data not shown) and reference 5).

From this series of experiments, the largest number of transformants from a blunt end DNA ligation was obtained under the conditions given in the standard reaction (see Methods). Using this as a basis, polyethylene glycol 8000 and hexamine cobalt chloride were tested for their ability to increase the efficiency of ligation as measured by the transformation assay. Both compounds are reported to stimulate the linear polymerization of DNAs in a T4 DNA ligase catalyzed reaction as judged by gel electrophoresis (2, 4).

In these experiments, various volumes of a concentrated solution of polyethylene glycol 8000 (PEG) were added to the standard reaction mixture, and the resulting number of transformants was determined. The addition of PEG to 5% (w/v) consistently increased the transformation between 3 and 6-fold. At PEG concentrations greater than 5% at reduction in transformation efficiency occurred consistent with the observation by gel analysis that the DNA was converted to non-transforming highly-polymerized linear forms. In separate experiments the direct effect of PEG on competent cells was found to be less than a 50% enhancement of transformation. Thus, the addition of certain amounts of PEG to the ligation mixture improves the ligation and subsequently the transformation of blunt end DNA.

Hexamine cobalt chloride (HCC) also stimulates the ligation of blunt end DNA to produce linear multimers, and in the presence of 20 mM KCl, circular products were formed (4). When added at a concentration of 0.1 to 10mM to the standard reaction described here, HCC did not increase the number of transformants, even in the presence of 25 mM KCl. No transformants were obtained when the concentration of HCC in the ligation mixture was $\geq$ 3mM. Even though blunt end ligation appears to be stimulated by HCC, no improvement of transformation could be obtained by the addition of HCC to the ligation reaction.

Based upon the results of these experiments, the following conditions are recommended for ligation of blunt end insert into vector DNA with T4 DNA ligase to achieve high transformation efficiency.

50mM Tris-HCl (pH. 7.6)
10 mM $MgCl_2$
5% (w/v) polyethylene glycol 8000

1 mM ATP
1 mM dithiothreitol
1 unit T4 DNA LIgase
Vector/insert molar ratio=3
4 hours
Room temperature (23-26°C)
Dilute 3 to 5-fold before adding the DNA to competent cells.

For convenience the following 5x concentrated buffer can be prepared and stored at -20°C until use. (Starting February 17, 1986, 5X Ligase Reaction Buffer will be supplied with BRL T4 DNA Ligase).

5X Ligase Reaction Buffer
250 mM Tris-HCl (pH 7.6)
50 mM $MgCl_2$
25% (w/v) Polyethylene glycol 8000
5 mM ATP
5 mM dithiothreitol

This buffer is also compatible with ligation of inserts with sticky ends to vectors or addition of linkers to insert DNAs at 14°C (data not shown).

SUMMARY

Several ligation conditions were examined in order to improve the yield of transforming DNA in the T4 DNA ligase reaction. Surprisingly, more transformants were obtained by incubating the ligation reaction of blunt end DNAs at room temperature for 4 hours than in wet ice for up to a day. The addition of polyethylene glycol to the ligation reaction at 5% (w/v) stimulated the formation of transforming DNA. Higher concentrations of polyethylene glycol to the ligation reaction at 5% (w/v) stimulated the formation of transforming DNA. Higher concentrations of polyethylene glycol increase the amount of high molecular weight DNA observed by gel electrophoresis, but drastically reduced the number of transformants obtained. Hexamine cobalt chloride also stimulated the formation of multimers, but failed to increase the number of transforming DNA molecules. Generally, analysis of the ligase reaction products by gel electrophoresis proved unreliable in predicting the transformation efficiency of the ligated DNA. Finally, a concentrated ligase reaction buffer was described which optimizes these conditions to obtain more transformants from a DNA ligation reaction.

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