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Development of strategy for competent cell preparation and high efficiency plasmid transformation using different methods

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Abstract

Current cloning technologies based on site-specific recombination are efficient, simple to use, and flexible. With the recent availability of complete genomic sequences of many organisms and plants, high-throughput and cost-efficient systems for gene cloning and functional analysis are in great demand. This study compares two different methods of preparation of competent cells using two strains of E.coli DH5α and HB101. From results the most efficient strain was found DH5α for cloning as it supports blue white screening utilizing galactosidase activity. The concentration of calcium chloride is another important factor; various concentrations of $CaCl_2$ were tried. Optimum concentration was found to be 75 mM. However PEG also has great influence on transformation efficiency, use of 40% PEG gave the best transformation efficiency. A convenient and rapid method for the genetic transformation of E.coli with appropriate plasmid is proposed which can be utilised for high efficiency transformation in normal laboratory conditions.

Key words: *Competent cells, Ligation, Molecular cloning, Transformation.*

1 Introduction

Molecular cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it (Sambrook *et al*., 1998) Generally, traditional $CaCl_2$-dependent transformation protocol is used for cloning of PCR product which consists of three steps (1) induce recipient cells using $CaCl_2$ to make them 'competent', (2) transform the competent cells with DNA in liquid by applying a heat-shock, (3) screening of transformants on selective plates (Sambrook *et al*., 1998). In early 1970's Cohen *et al*. (1973) successfully transformed R-factor and recombinant plasmids into *E.coli* cells using a calcium chloride method. Since that time this method has been widely used due to its convenience. More recently an alternative convenient and rapid method for the genetic transformation of *E.coli* with plasmids (Chen *et al.,* 2001) has proposed An alternative transformation method used is electroporation which results in a higher transformation efficiencies of up to 10^9 – 10^{10} transformants/µg DNA (Ryu *et al.,* 1990). Okamoto *et al.* (1997) also reported high efficiency transformation of *Bacillus brevis* by electroporation. However, special equipment is required for electroporation that many laboratories cannot provide. We have established an efficient system using *E.coli* competent cells for transformation plasmids and development of cloning based molecular markers in normal laboratory conditions. Cloning vectors are carrier DNA molecules. There are many possible choices of vectors depending on the purpose of cloning. The greatest variety of cloning vectors has been developed for use in the bacterial host *E.coli*. There are many different kinds of plasmids commercially available. All of them contain 1) a selectable marker (*i.e.* a gene that encodes for antibiotic resistance), 2) an origin of replication (which is used by the DNA making machinery in the bacteria as the starting point to make a copy of the plasmid) and 3) a multiple cloning site. The multiple cloning sites have many restriction enzyme sites and are used to insert the DNA of interest.

2 Materials and Methods

2.1 Preparation of Competent Cells

Competent cells using two stains of *E.coli* i.e. DH5α and HB101 were prepared using two methods one is given by Sambrook *et al.* (1998) using $CaCl_2$ (75 m*M*) and another was by Nishimura *et al* (1990) using LB broth (supplemented with 10 m*M* $MgSO_4.7H_2O$ and 0.2%-glucose). The competent cells were divided in aliquots of 0.1 ml each in eppendorf tubes and stored at -80ºC.

2.2 Restriction Digestion

Vector and PCR products (PCR amplified product of plant DNA containing single band) were restricted by *Eco*RI (Bangalore Genei) using 3U/µg of DNA/Vector. The reaction mixture was incubated at 37℃ for 1 hr.

2.3 Ligation

The vector and DNA sample was taken in ratio 1:3 for ligation with T_4 DNA ligase enzyme (1U). Ligation mixture was incubated overnight at 10ºC.

2.4 Transformation

One vial of ice thawed competent cells was taken and ligation mixture was added. PEG (40%) was also used. The samples were mixed properly and incubated on ice for 30 min. The heat shock treatment was given to competent cells at 42^0C for 90 sec. Then Luria broth was added in competent cell. The sample was incubated on shaker at 37^0C for 1 hr. at 200 rpm. Blue white screening of colonies was done using LB agar plates containing X-gal and IPTG. Transformation efficiency can be calculated using following formula:

$$\text{Transformant cfu} = \frac{\text{No. of bacteria colonies} \times \text{dilution ratio} \times \text{original transformation volume}}{\text{Plated volume}}$$

$$\text{Transformation efficiency (cfu/ µg)} = \frac{\text{Transformant cfu}}{\text{Plasmid DNA (µg)}}$$

2.5 Plasmid DNA Isolation

Isolation of plasmid from white transformed colonies was done using the procedure given by Sambrook *et al.* (1998).

3 Results

Competent cells of DH5α and HB101 strains were prepared with two different methods and then two types of plasmids i.e. pUC18 and pUC19 were introduced into different *E.coli* strains employing both methods. The transformants were selected on LB agar containing IPTG, X-gal and ampicillin. Blue and white colonies were observed after overnight incubation. Blue colonies indicate transformed colonies and white one indicate colonies without plasmid. Results show that that out of two strains, DH5α was giving best result (Plate 1 and 2). However, no transformants appeared with strain HB101 as it is rec A^- and is useful for cloning vectors that do not require α-complementation for blue/ white screening (Lacks and Greenberg, 1977).

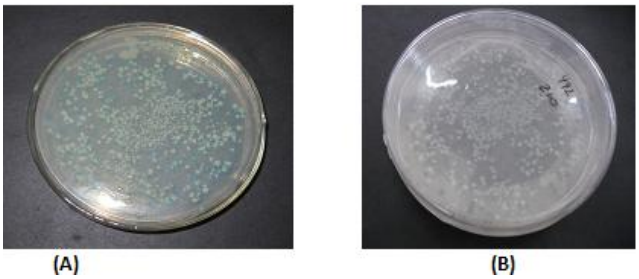

Plate 1. Transformed colonies of *E. coli* DH5α strain with pUC 18 (A) without use of PEG, (B) with PEG (Sambrook *et al.*, 1989 method).

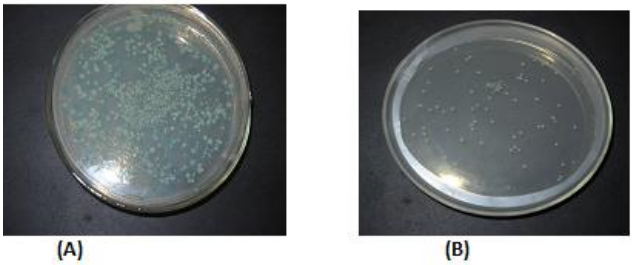

Plate 2. Transformed colonies of *E. coli* DH5α strain with pUC 18 (A) without use of PEG, (B) with PEG (Nishimura *et al.*, 1990 method).

Table 1. Transformation efficiency of different *E. coli* strains using different methods.

S. No.	No. of transformed colonies	Method 1 (Sambrook *et al.*, 1989)		Method 2 (Nishimura *et al.*, 1990)	
		DH5α	HB101	DH5α	HB101
1.	pUC 18	$1.1x10^6$	_	$2.0x10^6$	_
2.	pUC 19	_	_	_	_
3.	With PEG and pUC 18 vector	$2.1x10^6$	_	$4.2x10^6$	_

Furthermore out of the two methods used for competent cell preparation, the Nishimura *et al.* (1990), method of competent cell preparation shows higher rate transformation (Figure 2). Out of two vectors used for cloning, pUC 18 gives best result (Table 1 and Figure 1).

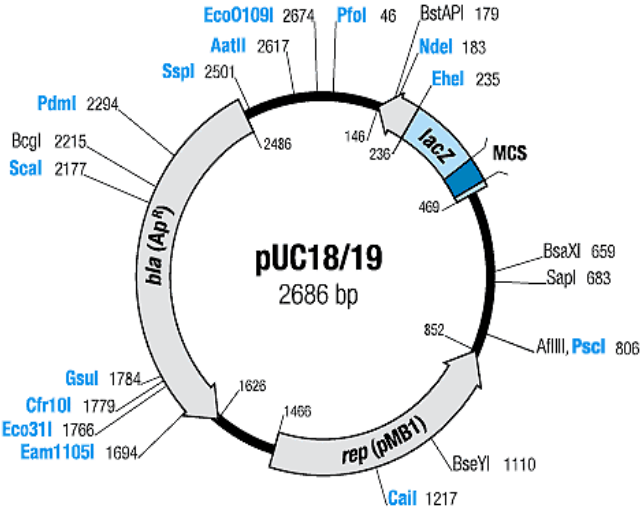

Figure 1. Genetic map of pUC18 & pUC19 vector.

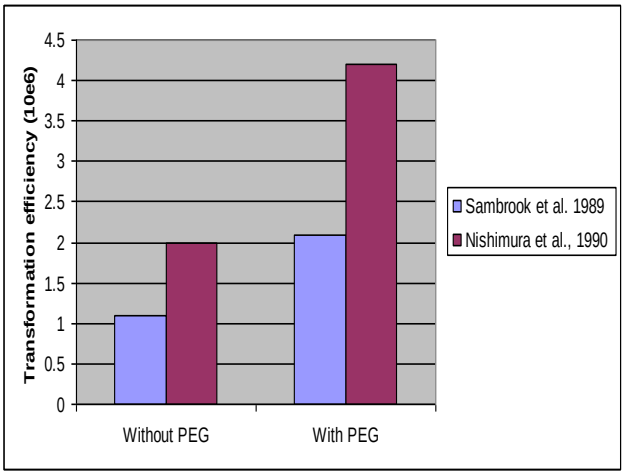

Figure 2. Effect of PEG on transformation efficiency of competent cells of *E.coli* DH5α prepared using different methods.

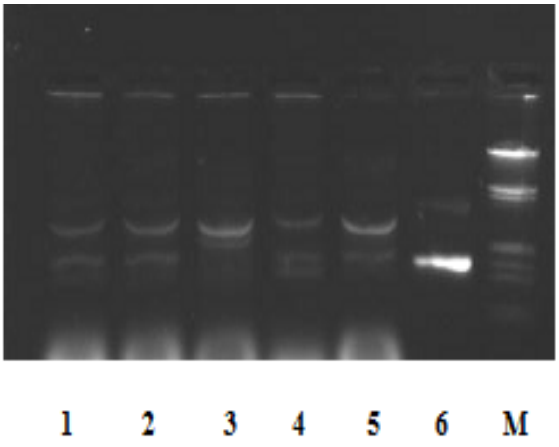

Figure 3- Lane 1-5 Plasmid DNA, Lane 6 -Vector, Lane M- λ DNA *EcoR* 1/*Hind* III double digested.

Since DNA is a very hydrophilic molecule, it doesn't normally pass through a bacterial cell's membrane. For insertion of plasmid in bacteria, they must first be made "competent" to take up DNA. This is done by creating

small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium chloride (Sambrook *et al*., 1998). Although 50-100 m*M* $CaCl_2$ can be used, but 75 m*M* $CaCl_2$ was found to be the optimum concentration which increases transformation efficiency. Randomly chosen transformants obtained from both methods were subjected to plasmid isolation for confirmation of DNA insertion in plasmid (Figure 3).

The addition of PEG during bacterial transformation can also affect transformation efficiency. Incubation of competent cells and DNA in a solution of PEG following a brief incubation and heat shock resulted in efficient uptake of DNA (Kurien *et al.*, 1995). Our experiments show that addition of 40% PEG during transformation process can give a transformation efficiency of two fold higher transformed colonies.

4 Discussion

In molecular cloning experiments transformation efficiency is very important and can be affected by many factors. A simple method of plasmid transformation of *E.coli* by rapid freezing has been reported by Takashashi *et al.* (1992). The majority of the efforts were directed toward optimizing the efficiency of transformation for different bacterial strains (Sambrook *et.al*. 1998; Dagert *et al*., 1979; Hanahan *et al*., 1991), the basic steps of this technique have undergone a few modifications. A number of transformation techniques have been described which are based on the observations originally made by Mandel and Higa (Mandel *et al*., 1998), who noticed that the uptake of lambda DNA by bacteria can be increased considerably in the presence of calcium chloride. Transformation methods are often strain dependent and may differ for members of the same species. Development of a transformation system also depends on having suitable DNA to transform (Dhingra *et al*., 2003).

pUC 18 and pUC19 vectors are small, high copy number, *E.coli* plasmids, 2686 bp in length (Figure 1). They are identical except that they contain multiple cloning sites (MCS) arranged in opposite orientations. The process of screening bacterial transformants for recombinant plasmids is made more rapid and simple by the use of vectors with visually detectable reporter genes. *Lac Z* system has been used extensively for this purpose in *E.coli* analogous systems for use in gram-positive bacteria remain uncommon. The *Lac Z* gene is one of three genes that make up the *E.coli* lac operon.

Bacteria that are able to take up DNA are called competent and competency can be induced by treatment with calcium chloride in early log phase of growth. The bacterial cell membrane is permeable to chloride ions, but is impermeable to calcium ions. The water molecules enter into the cell along with the chloride ions. The influx of water causes the cells to swell and is necessary for uptake of DNA; the exact mechanism of this uptake is unknown (Tu *et al.*, 2005). It is known that the effect of calcium chloride treatment can be enhanced if followed by a heating step, although there is some debate about whether the heat shock step is critical for the uptake of DNA (Cohen *et al*., 1973; Kimoto *et al.*, 2003). When *E. coli* is subjected to temperature of 42°C, a set of genes called heat shock genes are expressed, which enable the bacteria to survive at such temperature. However, at temperature above 42°C the bacterial ability to take DNA becomes reduced, and at more extreme temperature the bacteria will die. Although not essential, a heat shock can increase transformation efficiency.

In current scenario, the DNA markers become the marker of choice for the study of genetic diversity has become routine, to revolutionized plant biotechnology. Increasingly, techniques are being developed to more precisely, quickly and cheaply assess genetic variation. Cloning PCR products into plasmid vectors is a common downstream application of PCR for example for the development of cloning based markers such as CAPS, SCAR etc. it is often desirable to clone PCR products into plasmid vectors. This study could be useful to various weed flora, forestry in the southern and western Pacific and to various biological control parametric studies (Waterhouse *et al*., 2001; Waterhouse, 1993 and 1997; Whistler, 1998).

5 Conclusion

Individual reaction parameters for cloning purpose using competent cells were investigated to obtain such set of conditions which gave the highest efficiency in ligation and transformation of plasmids to bacterial cells.

Acknowledgements

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