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Full Length Research Paper

An improved calcium chloride method preparation and transformation of competent cells

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Transformation is one of the fundamental and essential molecular cloning techniques. In this paper, we have reported a modified method for preparation and transformation of competent cells. This modified method, improved from a classical protocol, has made some modifications on the concentration of calcium chloride and competent bacteria solution, rotation speed in centrifugation and centrifugation time. It was found that the optimal transformation efficiency were obtained when the concentration of $CaCl_2$ was 75 mmol/l, OD_{600} of the culture meets 0.35 to 0.45, the temperature of rotation was 4°C , rotation speed was 1000 g and rotation time was 5 min. Even more, we also found out that the transformation efficiency would increase about 10 to 30 times when adding 15% glycerine into $CaCl_2$ solution. The transformation efficiency, using our new method, reaches 10^8cf u/μg and higher than ultra-competent *Escherichia coli* method. This method will improve the efficiency in the molecular cloning and the construction of gene library.

Key words: Competent cells, $CaCl_2$, improved method, transformation, glycerine, transformation efficiency.

INTRODUCTION

Transformation, the heritable modification of the properties of competent bacteria by extraneous DNA, is one of the fundamental and essential molecular cloning techniques. The quality of competent cells is the most important factor of the transformation efficiency, which will have a direct impact on the posterior experiments. There are mainly two methods used to prepare the competent cells in great majority laboratory. The widely known procedure used for preparations of ultra-competent *Escherichia coli* uses techniques based on the work of Douglas Hanahan (Hanahan et al., 1991), in which many different parameters were optimized. By washing the cells in many different divalent cations and adding reducing agents, chemical transformation can be quite efficient. For these reasons, the transformation efficiency of competent cells is not low, usually yielding from 1×10^6 to 1×10^8 transformed colonies/μg of super-coiled plasmid DNA. However, this preparation procedure is complicated and unsuited for a common laboratory. The other conventional method is to prepare them using calcium chloride (Mandel and Higa, 1970; Cohen et al., 1972). This method has been widely used due to convenience. However, low efficient in transformation and difficult in preservation, could lead to a substantial loss of recombinants when efficiency is of the utmost importance, for example when constructing a random library.

Our research, based on the traditional $CaCl_2$ solution method, using DH5α as recipient bacteria, studied the influence of several factors on the transformation efficiency, such as different rotation speed, temperature and time, and the concentration of $CaCl_2$ solution and glycerine. Therefore, we found a new and highly efficient method for preparation of the competent cells by optimizing the various factors to increase the transformation efficiency. This new method protocol has made greatly improved the efficiency in transformation, additionally with less time, low cost and easy handling. It has been used to prepare batches

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of competent bacteria that yielded 10^8 transformed colonies/μg of super-coiled plasmid DNA. The presservation of competent cells is usually approximate one year at -70°C.

MATERIALS AND METHODS

Growth media, plasmids, strains and buffers and solutions

Luria–Bertani (LB; 10 g/l tryptone, 5 g/l yeast extract per liter, 10 g/l NaCl) medium was used in all experiments. For selection *E. coli* of transformants, Ampicillin (*Amp*^{r}) was added to LB agar plates at final concentrations of 100 Ag/ml. The pUC19 (0.1 μg /μl) plasmids (obtained from Tiangen) were used in this study. *E.coli* DH5α was used as recipient bacteria. These cells are compatible with *lacZ* blue/white selection procedures, are easily transformed, and good quality plasmid DNA can be recovered from transformants. $CaCl_2$ solution with different concentrations: 25, 50, 75, 100, 125, 150, 175 and 200 mmol/l were prepared as common buffers and solutions. The improved buffer was prepared as follows: add 150 ml of glycerine into 75 mmol/l $CaCl_2$ solution, and then add ddH_2O to bring the final volume to 1000 ml. Sterilize the solution by autoclaving at 121°C for 20 min. Divide the solution into aliquots and store them at -20°C.

Optimization of rotation speed and centrifugation time

Optimization of rotation speed and centrifugation time was carried out in this assay. The different rotation speed (500, 1000, 1500, 2000 and 3000 g) and centrifugation time (5, 10, 15 and 20 min), were tested to prepare competent cells by calculating the transformation efficiency. Other manipulation was performed according to tradition $CaCl_2$ protocol (Sambrook and Russell, 2001).

Optimization concentration of $CaCl_2$ solution

Different concentration of $CaCl_2$ solution (0, 25, 50, 75, 100, 125, 150 and 200 mmol/l) were tested to prepare and transform competent cells by calculating the transformation efficiency. Other manipulation was performed according to tradition $CaCl_2$ protocol (Sambrook and Russell, 2001).

Optimization of OD_{600} value

Optimized sector of OD_{600} value of *E. coli* from 0.2 to 1.0 were tested to prepare and transform competent cells by calculating the transformation efficiency. Other manipulation was conducted according to established $CaCl_2$ protocol (Sambrook and Russell, 2001).

Preparation of competent cells methods

Competent cells were prepared from *E. coli* DH5α by using three different methods. In this study, the M was a new method to prepare competent cells by improved buffer. M1 was traditional $CaCl_2$ solution method. M2 was ultra-competent method according to Douglas Hanahan (Hanahan et al., 1991). All the preparation was carried out in triplicates and all steps should be carried out on ice. The new detailed M manipulation protocol was as follows: The improved buffer and centrifuge tube was stored at -20°C overnight. Keep the solution at ice-cold temperature before use. First, pick a single bacterial colony (2 to 3 mm in diameter) from a plate that has been incubated at 37°C for 12 to 16 h and transfer the colony into 5 ml of LB medium for 12 to 14 h at 37°C with vigorous shaking, Second, imbibe 2 ml of the culture (1% of the medium) and add it into 200 ml LB medium for 1.5 to 2 h at 37°C with vigorous shaking (about 220 rpm). Then, measure the OD_{600} of the culture every 10 min after shaking 1 h and stop shaking when the OD_{600} of the culture meets 0.35 to 0.45; Third, transfer the bacterial cells to sterile, disposable, ice-cold 50 ml centrifuge tube and cool the cultures to 0°C by storing the tubes on ice for 5 min; Fourth, recover the cells by centrifugation at 1000 g for 5 min at 4°C, then, decant the medium from the cell pellets and stand the tubes in an inverted position on a pad of paper towels for 1 minute to allow the last traces of media to drain away; Fifth, resuspend the bacterial sediment gently in 20 ml of ice-cold solution containing 15% glycerine (improved buffer) and then store it in ice for 5 min and repeat the fourth; Sixth, resuspend the bacterial sediment gently in 20 ml of ice-cold improved buffer and recover the cells and harvest the cells by centrifugation at 1000 g for 5 min at 4°C ,then, stand the tubes in an inverted position on a pad of paper towels for 1 min to allow the last traces of media to drain away; At last, add 4 ml of chilled improved buffer and mix the bacterial suspension by swirling and then sub package it in 100 μl of each tube.

M1 was prepared according to the classical $CaCl_2$ method of Sambrook and Russell, (2001). M2 was prepared based on the work of Douglas Hanahan (Hanahan et al., 1991). All preparations were performed in triplicates and eluted in 100 μl of sterile Calcium Chloride Solutions. The quality of competent cells was assessed by calculating transformation efficiency.

Transformation of competent cells

To test the transformation efficiency of competent cells from each method, each method of transformation experiment was repeated 3 times to calculated the average value as the transformation efficiency and each times was selected three tubes of competent cells from each method and a negative control (competent cells without transforming plasmid) was carried out. The detailed manipulation protocol of transformation experiment was conducted according to transformation protocol (Sambrook and Russell, 2001).

Statistical analysis

Statistical analyses were performed by using GraphPad PRISM® software and SPSS13.0 software.

RESULTS

The effects on the transformation efficiency of rotation speed and centrifugation time

The activity of competent cells would be reduced by exceeded rotation speed, and result in reducing the transformation efficiency. The higher transformation efficiency was reached when collecting the precipitate with 1 000 g (Figure 1). In this study, we found that centrifugation time had little effect on transformation efficiency. However, in centrifugation time exceeding 10 min, some dead bacteria would form sediment together with activity bacteria, which could degrade transformation efficiency to a certain extent. Otherwise, it was also found that a great deal of active bacteria could form sediment when centrifugation time was five minute. These result implied that

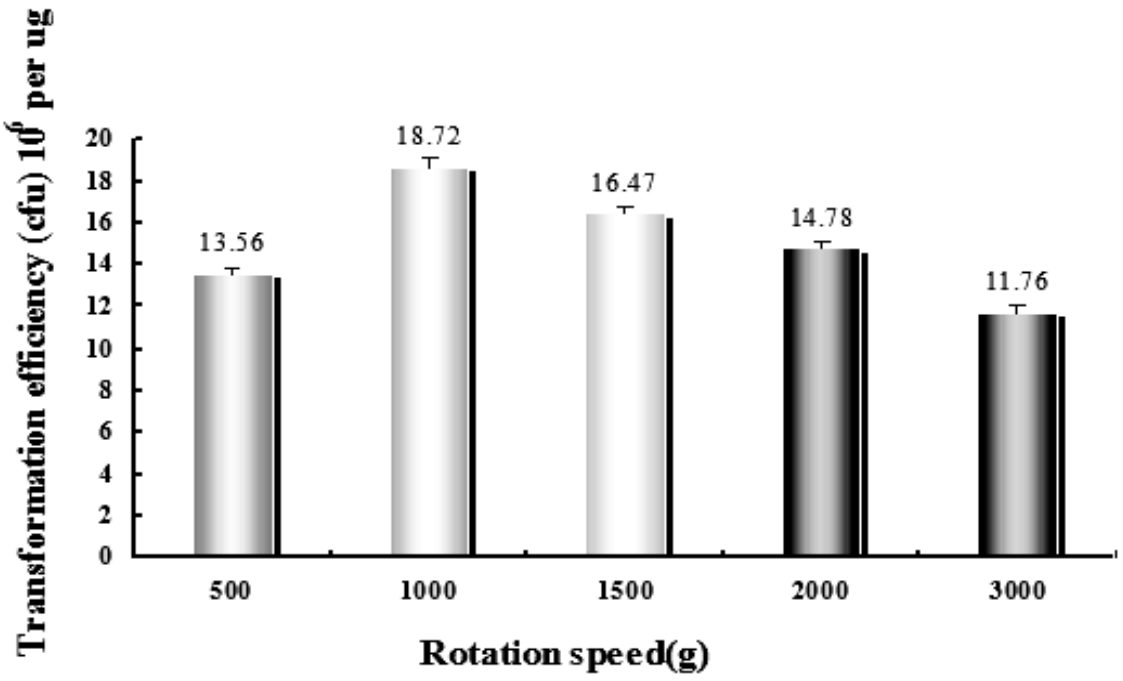

Figure 1. Average transformation efficiency of competent cells from different rotation speed of centrifugation (500, 1000, 1500, 2000 and 3000 g). Results shown are the average units for triplicate assays. Error bars represent standard deviation.

centrifugation time was reasonable from five to ten minutes for preparation of competent cells.

The optimized concentration solution for plasmid transformation

We used 0, 25, 50, 75, 100, 125, 150 and 200 mmol/l ice-cold $CaCl_2$ solution to prepare the competent cells and then transformed with pUC19 to test the transformation efficiency. In a certain concentration, the $CaCl_2$ solution will promote the growth of competent cells. Without $CaCl_2$ solution, competent cells couldn't be formed. As shown in Figure 2, the transformation efficiency of competent cells increased together with the concentration of $CaCl_2$ solution and reached the maximum when the concentration 75 mmol/l, then decreased rapidly when the concentration exceeded 100 mmol/l.

Evaluation of different OD_{600} value

The transformation efficiency of competent cells is significantly correlated with OD value. We calculated the efficiency from OD value 0.2 to 1.0 and found out that the highest transformation efficiency reached when the OD value between 0.35 to 0.45 (Figure 3),which suggest that competent cells was prepared in the OD value between 0.35 to 0.45 of bacteria liquid could gain the higher transformation efficiency.

Evaluation of preparation of competent cells

To compare three methods of preparation and transformation of Competent Cells, three tubes of competent cells were selected to calculate the transformation efficiency. This experiment was repeated 3 times to calculate the average value as the transformation efficiency (Table 1). The discrepancy of transformation efficiency among these three methods was significant ($F > F_{0.01}$, $P < 0.01$) by SPSS. Multiple comparisons, using least significant difference (LSD), shows that M was the best method of the three for preparation and transformation of competent cells, where the transformation efficiency could average reach as high as 1.5×10^8 and the transformation efficiency of M2 is higher than the M1. It was also shown that the transformation efficiency of competent cells using our new method (M) is about 10 to 30 times compared to the traditional method (M1).

DISCUSSION

Plasmid transformation into bacterial competent cells is a key technique in molecular cloning. In early 1970's Cohen successfully transformed R-factor and recombinant plasmids into *E. coli* cells using a calcium chloride method (Cohen et al., 1972). Since that time this method has been widely used due to its convenience. To date, there are many methods to prepare the Competent Cells in molecular cloning (Mandel and Higa, 1970; Cohen et

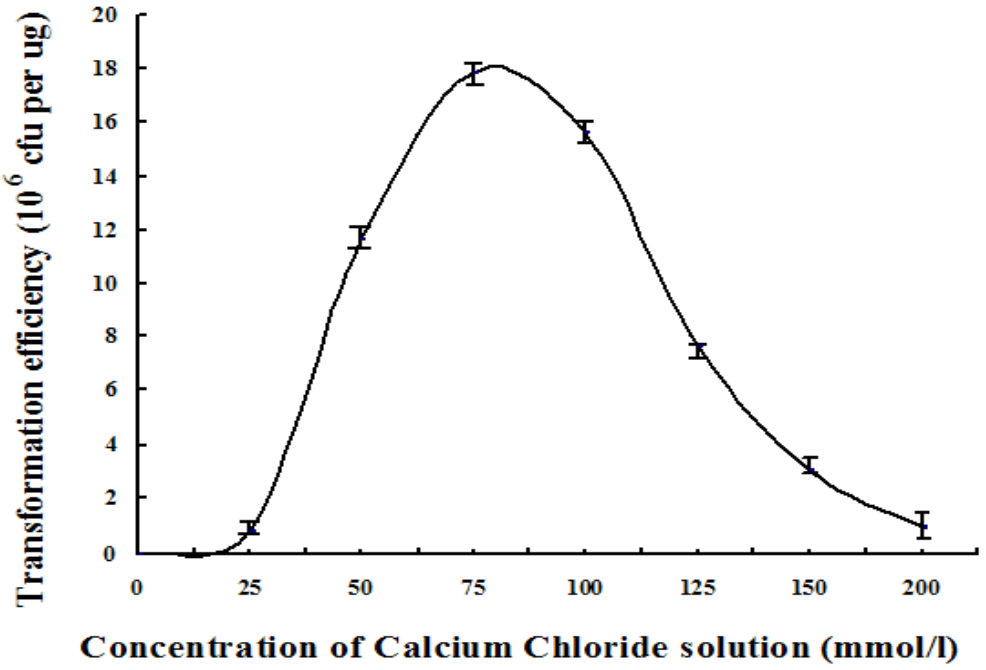

Figure 2. Average transformation efficiency of competent cells from different concentration of Calcium Chloride solution (0, 25, 50, 75, 100, 125, 150 and 200 mmol/l). Results shown are the average units for triplicate assays. Error bars represent standard deviation.

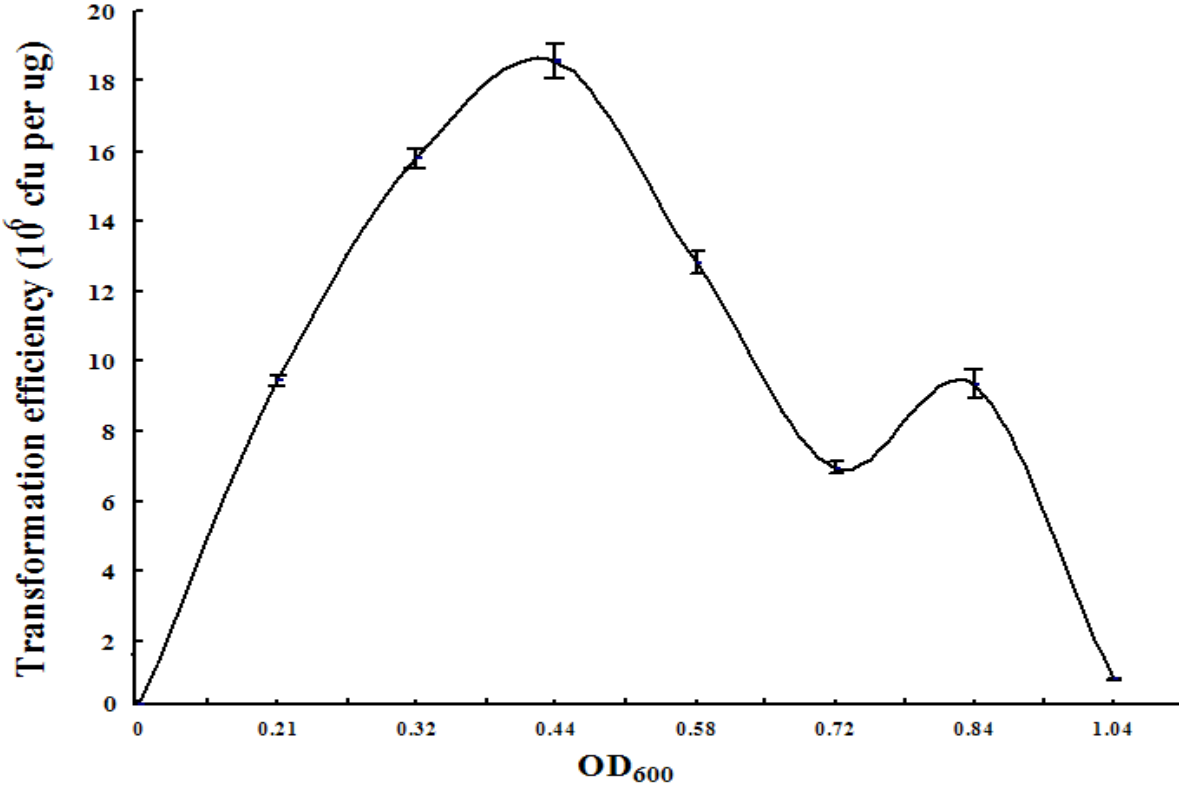

Figure 3. Average transformation efficiency of competent cells from different OD_{600} value (from 0.2 to1.0) Results shown are the average units for triplicate assays. Error bars represent standard deviation.

Table 1. The transformation efficiency of three methods.

Method	Transformation efficiency (10^6)									Max	Mean
M	109.83	123.56	117.87	264.15	98.76	174.89	152.71	167.55	147.86	264.15	150.8 ± 16.64^a
M1	11.39	14.82	15.07	12. 91	10. 21	14.36	14. 08	13.75	15. 27	15.27	13.54 ± 1.74^b
M2	42.16	47.13	82.37	32. 8 3	61 .41	56 .44	41 .45	68. 48	43.74	82.37	52.89 ± 5.22^c

M, The new protocol (this work); M1, classical $CaCl_2$ method of Sambrook et al. (2001); M2, based on the work of Hanahan et al. (1991). Different letters represent the significant difference at $P < 0.01$. Significant differences among these methods of M, M1 and M2 are shown.

al., 1972; Dower et al., 1988; Inoue et al., 1990; Nakata et al., 1997; liu et al., 2004). However, some of them are high cost, and some require special equipment, such as electroporation (Dower et al., 1988) which is not found in typical laboratories. In this study, based on the traditional calcium chloride solution method, we developed a simple, effective, and inexpensive method for competent cells preparation from *E. coli*. Our procedure was tested by repeating the preparation, which has shown good stability and repetitiveness. The transformation efficiency of our competent cells is higher than the classic methods. There are four possible reasons for the high transformation efficiency: (1) Lower rotation speed in centrifugation (1000 g). (2) Lower concentration of Calcium Chloride solution (75 mmol/l); (3) Bacteria liquid OD_{600} value in 0.35 to 0.45 interval (4) Adding 15% glycerine in calcium chloride solution. All four reasons were proved to be essential for obtaining the high transformation efficiency of competent cells in this study.

In this study, we used the rotation speed at 1000 g instead of 2700 g as the traditional way during the preparation. High rotation speed in centrifugation probably will do harm to the cells and increase the dead cells in the sediments. We have also reduced the time of centrifugation in order to prevent the damage to cells, which could increase transformation efficiency of competent cells.

Concentration of $CaCl_2$ solution is an important factor effecting transformation efficiency of competent cells. In this report, we found that the transformation efficiency of competent cells increased together with the concentration of $CaCl_2$ solution and reached the maximum when the concentration was 75 mmol/l, then decreased rapidly when the concentration exceeded 100 mmol/l. The reason, we presumed, is that lipid array on cell membrane is destroyed by 75 mmol/l to 100 mmol/l Ca^{2+} and then a liquid crystal would be formed. The bacteria would be distended when incubated at 0℃, hypotonic calcium chloride solution, and DNA in the mixture can be formed into hydroxyapatite (anti-DNase) then stick to the surface of cells (Panja et al., 2006; Mahipal et al., 2010). The cellular absorption ability of exogenous DNA will be increased after heat shock at 42℃. This process may be inhibited and hence the transformation efficiency will be decreased when the concentration of calcium chloride solution exceeds 100 mmol/l.

Transformation efficiency is very important in molecular cloning experiments, and can be affected by many factors. The most important thing is that the bacterial should be in their early logarithmic growth period. The growth cycle of *E. coli* includes the retardation phase, logarithmic phase, stationary phases and decline phase. According to the growth curve of *E. coli*, the bacteria is under the best growth condition and easily be induced when the OD_{600} meets 0.35 to 0.45. Meanwhile, during this phase, the bacteria are in the best tolerance condition suffering the physical damage and this could increase the transformation efficiency. Tsen SD et al found out that the transformation efficiency of *E. coli* is the highest in its logarithmic phase (Tsen, 2002). It was only 0.71×10^6 in the stationary phases when OD meets 1.0. Finkel SE et al found out that the absorptivity of *E. coli* obviously decreased because of the mutation of hofQ during the stationary phases (Sambrook and Russell, 2001). Ryu and other authors have pointed out the importance of the early log phase for transformation (Ryu and Hartin, 1990).

The transformation efficiency of competent cells using our new method is about 10 to 30 times compared with the traditional method. One reason that we presumed is that the glycerine can react with the phosphoric acid and fatty acid, which change constituent of phospholipid from cell membrane and make lipid array on cell membrane be destroyed and allows exogenous DNA to easily permeate cell membrane. Another reason we presume is that glycerine could induce some special materies in the cell membrane, which makes cell membrane form tiny holes, the resistance of transports degrade, and thus exogenous DNA easily permeates cell membrane. Additionally, glycerine could easily permeate into cell membrane and make a static electricity reaction with phosphoric acid and fatty acid, enhance the membrane flowability, reduce the lipidic phase-transition temperature, and thus enhance the survivability of cell membrane towards the osmotic pressure and crenulation, thus maintaining cell membrane stability. In addition, it could reduce the possibility of contamination, since the competent cells could be directly freezed, whereas in the traditional method, there are more steps to preserve the cells in glycerine and hence cells have more chance to become contaminated.

According to our new method, the time spent on the preparation and transformation has been significantly reduced and the procedure is much simpler. Additionally, many bacterial strains, such as JM109, BL21 and TOP10,

could be used for preparation and transformation of Ultra-Competent Cells according to our protocol, and ours has potential applications in molecular cloning, and could soon be accepted in many laboratories.

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