An Improved Method of Preparing High Efficiency Transformation *Escherichia coli* with Both Plasmids and Larger DNA Fragments

Jingjing Liu² · Wenwen Chang² · Lei Pan² · Xiaoyun Liu¹ · Lufang Su¹ · Weiyang Zhang¹ · Qin Li³ · Yu Zheng¹

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Abstract The high-throughput, cost-efficient transformation systems determine the success of gene cloning and functional analysis. Among various factors that affect this transformation systems, the competence ability of target cells is one of the most important factors. We found antimicrobial peptides LFein-B can increase the permeability of the cell membrane, and their lethal antibacterial properties can be inhibited by moderately high concentrations of Ca^{2+} and Mn^{2+} . In this study, we established a convenient and rapid method (CRM) by adding small concentrations of (0.35 mg/L) and moderately high concentrations of MnCl_2 (50 mM) and CaCl_2 (30 mM) in transformation buffer. The transformation efficiency of *E. coli* cells (DH5 α , JM109 and TOP10) prepared by CRM were comparable with electroporation for plasmid transformation ($3.1 \pm 0.3 \times 10^9$ cfu/ μg). Unlike competent cells prepared using other chemical methods, those obtained using CRM method are extremely competent for receiving larger size DNA fragments (> 5000 bp) into plasmid vectors. The competent *E. coli* cells prepared by CRM method are particularly useful for most high-

efficiency transformation experiments under normal laboratory conditions.

Keywords Biotransformation · Competent cells · High transformation efficiency · Molecular cloning

Introduction

Transformation involves introducing exogenous DNA into receptor bacteria to produce new genetic traits. It is the most basic and important technique in molecular cloning, which is the process of isolating a target DNA fragment and making multiple copies of it [1]. There are two types of transformation methods: chemical and physical [2, 3]. The physical transformation method is electroporation. It has high transformation efficiency of up to 10^9 – 10^{10} transformants/ μg DNA in *E. coli* [2]. Several other studies have also reported high-efficiency transformation via electroporation in *Agrobacterium tumefaciens/rhizobium* and *Bacillus brevis* [4–7]. However, expensive specialized equipment is required for electroporation, and not all laboratories can provide it. Chemical transformation is very welcome in molecular cloning experiments because it is simple and inexpensive. The classical chemical method is the calcium chloride method (CaCl_2 method) published by Mandel and Higa, which is still a common choice for many laboratories [8]. The role of Ca^{2+} in this method is to destroy the lipid array on the cell membrane, and forming a complex with poly-hydroxybutrate and poly-inorganic phosphate on cell membrane to facilitate the infiltration of exogenous DNA, and the transformation efficiencies was 10^5 – 10^6 transformants/ μg DNA which is proved to be sufficient for the majority of cloning purpose [9, 10]. Although the calcium chloride method is convenient and

Jingjing Liu, Wenwen Chang and Lei Pan have contributed equally to this study.

✉ Yu Zheng
zhengyu320@126.com

¹ Institute for Interdisciplinary Research, Jiangnan University, Wuhan 430056, People's Republic of China

² Hubei Province Engineering Research Centre of Legume Plants, College of Life Sciences, Jiangnan University, Wuhan 430056, People's Republic of China

³ College of Chemistry and Environment Sciences, Jiangnan University, Wuhan 430056, People's Republic of China

repeatable, the preparation of competent cells is relatively time-consuming and requires considerable power. More important is that the transformation efficiency is much lower than that of electroporation, which renders it unsuitable for several molecular cloning experiments such as the construction of high-complexity cDNA libraries with a minimum expenditure of mRNA [11]. A great number of studies have made efforts toward establishing a quick and efficient method for the preparation of such competent *E. coli* cells with an extremely high transforming frequency, which meets the requirements of modern molecular cloning experiments [3, 12–16]. There are two avenues to improving the preparation protocol, the first is simplifying the steps and shorting preparation time and the second is increasing the transforming frequency of chemically competent cells [11, 17–21]. However, none of the methods cited above can provide competent *E. coli* cells that are particularly useful for receiving large target DNA fragments into plasmid vector. Such cells are the basis for almost all the molecular experiments such as subcellular localization analysis, yeast two-hybrid test, fluorescence immunoassay analysis, and similar factors.

More and more studies have reported that antimicrobial peptide can kill microorganisms by destroying microorganisms' cell membrane, and the effect of some antimicrobial peptides, such as increasing the permeability of cell membrane, is not lethal to *E. coli* [22–24]. For this reason, we improved the protocol described by Inoue [11], which is one of the best chemical methods presently available, by adding small concentrations of LFCin-B, an antimicrobial peptide found in mammals [25] to establish a method for the preparation of such cells both efficient in plasmid and large target DNA transformation.

Materials and Methods

Bacterial Strains, Plasmids and Chemicals

Three strains of *E. coli* (DH5 α , TOP10 and JM109) were used in this study to prepare competent cells. The commercial competent cells DH5 α and JM109 were purchased from Takara Biomedical Technology (Beijing) Co., Ltd, commercial competent cells TOP10 were purchased from TIANGEN BIOTECH (Beijing) Co., Ltd. The *pUC19* plasmids and restriction enzymes used in our laboratory were purchased from New England Biolabs (USA). The *pGEM-Teasy* vector were purchased from Promega Corporation, an affiliate of Promega (Beijing) Biotech Co., Ltd. The other chemicals used in this study were purchased from Sigma-Aldrich Co. LLC or Sinopharm Chemical Reagent Co., Ltd.

Media for Bacterial Growth

For LB fluid medium, 1% Bacto-Tryptone (10 g/L), 0.5% Bacto-Yeast Extract (5 g/L), 0.5% NaCl (5 g/L), adjust the pH to 7.5, autoclave to sterilize. For LB plates, 1.5% Bacto-agar (15 g/L) was added prior to autoclaving. For S.O.C. fluid medium, 2% Bacto-Tryptone (20 g/L), 0.5% Bacto-Yeast Extract (5 g/L), 0.05% NaCl (0.5 g/L), 2.5 mM KCl (0.186 g/L), 1 mM MgCl₂ (0.95 g/L), 10 mM MgSO₄ (1.2 g/L), 75 mM (13.6 g/L) glucose, adjust the pH to 7.0, autoclave to sterilize. For S.O.B fluid medium, 2% Bacto-Tryptone (20 g/L), 0.5% Bacto-Yeast Extract (5 g/L), 0.05% NaCl (0.5 g/L), 2.5 mM KCl (0.186 g/L), 1 mM MgCl₂ (0.95 g/L), adjust the pH to 7.0, autoclave to sterilize.

The Competent Cell Preparation Methods and Transformation Methods

There were three methods for competent cell preparation in this study. One was CaCl₂ method [8, 26], the second was Inoue method [11], and the last one was our improve method (CRM). All these three competent cells were used for heat shock transformation, the details for preparation and transformation were described as follow:

CaCl₂ Method [8, 26]

The CaCl₂ method using to prepare competent cells and transformation were both followed the protocols described by Sambrook [26].

Inoue Method [11]

The transformation buffer (TB) was made of 10 mM Pipes, 55 mM MnCl₂, 15 mM CaCl₂ and 250 mM KCl, the pH was adjusted to 6.7 with KOH. And then, the solution was sterilized by filtration through a pre-ripped 0.45 μ m filter unit and stored at 4 °C. All salts were added as solids. Frozen stock *E. coli* competent cells were thawed, streaked on an LB agar plate, and cultured overnight at 37 °C. The large (diameter 2–3 mm) colonies were isolated and inoculated to 250 mL of SOB medium in a 2-L flask, and grown to an OD₆₀₀ of 0.6 at 18 °C, with vigorous shaking (200–250 rpm). The flask was removed from the incubator and placed on ice for 10 min. The culture was transferred to a 500 mL erlenmeyer flasks and centrifuged at 3000 rpm for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 80 mL of ice-cold TB, and then incubated in an ice bath for 10 min, and then centrifuge as above. Discard the supernatant and resuspend the pellet gently in 20 mL of DMSO-TB buffer [final concentration of DMSO (m/v) was 7%]. After incubating in an ice bath for 10 min,

the cell suspension was dispensed by 1–2 mL into tissue-culture cell-freezing tubes and immediately chilled by immersion in liquid nitrogen. The frozen competent cells were stored in -78°C or used for transformation immediately.

Usually, 1–5 μL plasmid (ligation product) was added into the competent cells for transformation, and then the cells were incubated in an ice bath for 30 min. They were then heat-shocked without agitation at 42°C for 30 s and transferred to an ice bath. After 0.8 mL of SOC was added, the tubes were placed in a 37°C incubator and shaken vigorously for 1 h. A desired portion of the mixture was poured on the LB plate with ampicillin (final concentration 100 $\mu\text{g}/\text{mL}$). Colonies were counted after overnight incubation at 37°C .

500 μL LB liquid medium were added into the mix, they incubate at 37°C with shaking at 200 rpm for 1 h. A desired portion of the mixture was poured on the LB plate with ampicillin (final concentration 100 $\mu\text{g}/\text{mL}$). Colonies were counted after overnight incubation at 37°C .

The electroporation experiment, blue-white spot screening and individual bacterial PCR was performed according to the protocol described by Sambrook [26].

The Calculation of Transformation Efficiency

Transformation efficiency (TE) is defined as the number of cfu (colony forming units) produced by 1 μg of plasmid DNA, the equation for calculating the number of transformant cfu (TC) is as follow:

$$\text{TC (cfu)} = \frac{\text{The number of bacteria colonies} \times \text{dilution ratio} \times \text{original transformation volume}}{\text{Plated volume}}$$

CRM Method (Our Improved Method)

The transformation buffer (TB) was made of 10 mM Pipes, 50 mM MnCl_2 , 30 mM CaCl_2 , 250 mM KCl and 0.35 mg/L LFCin-B, the pH was adjusted to 6.7 with KOH. The solution was also sterilized by filtration through a 0.45 μm filter unit and stored at 4°C .

Streak a LB agar plate with *E. coli* cells from a frozen stock. Incubate the plate upside down at 37°C until colonies appear. Inoculate one colony into 500 mL S.O.C. liquid medium in a 1 L flasks, incubate at 18°C with shaking at 100 rpm overnight until the OD_{600} reaches 0.6. Incubate cells at ice for 10 min, and then centrifuge the cells at 2500 rpm for 10 min at 4°C . Discard the supernatant and resuspend the pellet in 16 mL TB. Incubate cells at ice for 10 min, and then centrifuge as above. Discard the supernatant and resuspend the pellet in 8 mL TB, and then centrifuge the cells at 2500 rpm for 10 min at 4°C . Discard the supernatant and resuspend the pellet in 4 mL DMSO-TB buffer [final concentration of DMSO (m/v) was 7%]. Incubate the cells at ice for 30 min. Aliquot 100 μL into individual 1.5 mL tubes. Frozen in liquid nitrogen immediately and stored at -78°C or used for transformation immediately.

For transformation, the first step is gently mix 1–5 μL plasmid (ligation product) with competent cells. Secondly incubate the mix on ice for 30 min immediately. The mix were then heat-shocked without agitation at 42°C for 30 s and transferred to an ice for 2 min immediately. After

And then the transformation efficiency (TE) is calculated according to the following equation:

$$\text{TE (cfu}/\mu\text{g)} = \frac{\text{TC}}{\text{Plasmid DNA } (\mu\text{g})}$$

In this study according to the methods, the dilution ratio for Inoue and CRM method was 5×10^6 times, for CaCl_2 method was 1×10^6 times. The dilution ratio for commercial competent cells was also calculated as 500,000 times in this study. For calculation of transformation efficiency, 2 μL (500 ng/ μL) plasmid DNA was added in 100 μL competent cells, the plated volume was 50 μL . There are three repeats for each assay.

Analysis the Permeability of Cell Membrane

NPN assay was used to detect the outer membrane permeability of *E. coli* [27]. The *E. coli* cell in logarithmic phase in LB medium (to an optical density of OD_{600} of 0.4) was collected and resuspended with normal saline. To 1 mL volume of bacteria in a quartz cuvette, NPN (*N*-phenyl-1-naphthylamine) was added (final concentration: 10 μM). Different concentrations of LFCin-B are added to make the concentration of them 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L. A fluorescence spectrophotometer (PE LS45) was used to record fluorescence every 30 min, the excitation and emission wavelengths were set at 350 and 429 nm respectively. Control tests were performed to verify

that the enhanced fluorescence was due to NPN uptake by bacteria.

Inner membrane permeability of *E. coli* was determined by measuring the release of β -galactosidase activity into the culture medium using ONPG (*ortho*-nitrophenyl- β -galactoside) as a substrate [27]. β -galactosidase is a typical *E. coli*-inducible enzyme located within the bacterial cell membrane and is capable of hydrolysis Lactose into glucose and galactose. ONPG can be used to detect β -galactosidase activity because it was a chromogenic substrate for β -galactosidase. If the cell membrane permeability changes, ONPG can enter into cytoplasm and catalyze β -galactosidase to produce yellow products. The maximum absorption peak of this yellow product was 415 nm [27]. Therefore, ONPG was chosen as the substrate for the reaction, and the inner membrane permeability of *E. coli* was determined using β -galactosidase activity. In this study, the *E. coli* cells in logarithmic phase in LB medium which containing 2% lactose (to an optical density of OD₆₀₀ of 0.4) was collected and resuspended with normal saline too. *E. coli* cell suspension (200 μ L) was pipetted into the wells of a standard microtiter plate followed by adding ONPG (final concentration: 1.5 μ M). Different concentrations of LFcIn-B are added in each well to make the concentration of them 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L. Slight vibration at 37 °C and the production of o-nitrophenol over time was monitored with a spectrophotometer at 415 nm.

Prokaryotic Expression and Purification of Antimicrobial Peptide Lactoferricin B (Lfci B)

The Lfci B used in this study were obtained by prokaryotic expression and purification in our laboratory according to the protocol of Feng [28]. The purity and antimicrobial activity of Lfci B produced in our laboratory was the same as a purified Lfci B from pepsin digestion of bovine by Central Laboratory of Food Science & Technology (Huazhong Agricultural University, Wuhan, People's Republic of China).

Results

High Concentrations of MnCl₂ and CaCl₂ Accompanied by Small Concentrations of LFcIn-B Increase the Competence of DH5 α

Competent cells are those that have had their cell membranes altered to render it easier to bring foreign DNA inside. Therefore, the permeability of cell membranes is the most important and fundamental condition for competent cell with high transformation efficiency. To improve the

transformation efficiency of competent cells, we began to optimize the Inoue method by increasing the permeability of cell membranes. Because the *E. coli* DH5 α strain is the most common competent cell used in DNA cloning experiments, we selected this strain to examine the permeability of the inner and outer layers of the cell membrane. According to the research on antimicrobial peptides which can affect the cell membranes of microorganism such as *E. coli* [22, 23], we first added three common antimicrobial peptides in LB medium at small concentrations (0.5 mg/mL) to assess their effects on the survival of *E. coli* DH5 α strains. The results showed that the *E. coli* DH5 α clones only grew LB medium and in LB with LFcIn-B, but there were significant differences ($P < 0.05$) between the number of clones. LB medium had more than twice as many as LB-LFcIn-B⁺ (195–213) versus 525–567, Fig. 1a). This indicates that the *E. coli* DH5 α strain could grow on small concentrations (< 0.5 mg/L) of LB-LFcIn-B⁺ medium, which means that LFcIn-B can be added to the transformation buffer used to prepare competent cells to obtain cells with strong competence and high permeability. We then examined the variations in permeability of cell membrane caused by different concentrations of LFcIn-B. The results of inner and outer cell membrane permeability both showed that the more LFcIn-B present, the greater the permeability of the inner and outer cell membrane. We also found the influence on permeability of different concentration can be divided into three groups based on their differences from controls (without LFcIn-B): the small effect ($P > 0.05$) of trace concentrations of LFcIn-B (0.1–0.2 mg/L), the significant effect ($P < 0.05$) caused by small concentrations (0.3–0.4 mg/L) and the huge effect ($P < 0.01$) caused by normal concentrations (> 0.5 mg/L, Fig. 1b). To establish the influence of LFcIn-B on the competence of *E. coli* DH5 α strain, we added the same gradient concentration of LFcIn-B into Inoue transformation buffer, and we found that the transformation efficiency of DH5 α decreased as the concentration of LFcIn-B increased (Fig. 1c). The low transformation efficiency may have been caused by the antibacterial properties of LFcIn-B [25]. In the course of next investigation, we found that moderately high concentration of MnCl₂ (50 mM) and CaCl₂ (30 mM) with slight concentration of LFcIn-B (0.35 mg/L) can be stimulatory to transformation by increasing the permeability of cell membrane (Fig. 1d). This may be due to the antibacterial property of LFcIn-B can be inhibitory by high concentration of Ca²⁺ and Mn²⁺ without losing the increase influence of LFcIn-B on DH5 α cell membrane, as the mechanism about the antibacterial property of LFcIn-B is complex and there remain many unresolved issues [22, 25]. The above observation about the increase in the competence of *E. coli* in the presence of LFcIn-B, Ca²⁺, and Mn²⁺ at moderate concentrations suggested that it may improve the preparation of competent cells.

Fig. 1 The effect of LFcIn-B on the competence of DH5α. **a** The survival of DH5α grow on LB medium plate with different imicrobial peptides. Three common imicrobial peptides Cecropin A, PR-39 and LFcIn-B were added in LB medium plate in normal concentration (0.5 mg/mL). LB medium plate was as control. DH5α strains were grow in these plates overnight at 37°C and then observed, calculated and photographed. **b** LFcIn-B increase the permeability of inner and outer cell membrane determined by NPN assay and ONPG assay.

The CRM Method Can Produce Better-Quality Competent Cells than Previously Reported Methods

In molecular cloning experiments, transformation efficiency is commonly used as an index to assess the competence of competent cells. CaCl₂ and Inoue methods were chosen to

compare with CRM method in terms of the quality of competent cells because CaCl₂ method was the most widely used chemical method of preparing competent cells in laboratories and the Inoue method can produce stable competent cells in a highly efficient manner [16]. In the first place, the transformation efficiency for *pUC19* of three common competent

E. coli strains (DH5 α , JM109, and TOP10) separately prepared by CaCl₂ method, Inoue method and CRM method were calculated and compared. Results showed that the transformation efficiency of all three *E. coli* competent strains (DH5 α , JM109 and TOP10) in this study were similar. The transformation efficiency of CRM method was $3.2\text{--}3.5 \times 10^9$ cfu/ μ g for DH5 α , $1.8\text{--}2.2 \times 10^9$ cfu/ μ g for JM109 and $3.4\text{--}3.7 \times 10^9$ cfu/ μ g for TOP10, which were all significantly higher ($P < 0.05$) than those of the Inoue method ($1.9\text{--}2.8 \times 10^9$ cfu/ μ g for DH5 α , $0.8\text{--}1.2 \times 10^9$ cfu/ μ g for JM109 and $2.7\text{--}3.1 \times 10^9$ cfu/ μ g for TOP10), and very significantly higher ($P < 0.01$) than those associated with the CaCl₂ method ($3.0\text{--}3.7 \times 10^7$ cfu/ μ g for DH5 α , $3.9\text{--}4.3 \times 10^7$ cfu/ μ g for JM109 and $1.6\text{--}2.3 \times 10^7$ cfu/ μ g for TOP10). These results indicated that our improved method (CRM method) can be used to make at least three kinds of *E. coli* competent strains with higher transformation efficiency than the other widely used chemical method (Inoue method and CaCl₂ method), and this may be true of other *E. coli* strains. Electro-transformation is a non-chemical method widely used in molecular cloning experiments due to its high transformation efficiency [2]. We also compared these methods. It gave a transformation efficiency of ($2.9\text{--}3.6 \times 10^9$ cfu/ μ g for DH5 α , $1.7\text{--}2.3 \times 10^9$ cfu/ μ g for JM109 and $3.1\text{--}3.6 \times 10^9$ cfu/ μ g for TOP10) of *pUC19*, a value comparable to the frequency ($P > 0.5$) of CRM method (data not shown). However, electroporation requires special equipment that many laboratories cannot provide. These results indicated that the CRM method can produce competent *E. coli* cells with high transformation efficiency ($1.8\text{--}3.7 \times 10^9$ cfu/ μ g), much higher than other chemical transformation and comparable to electro-transformation, indicating that it is sufficient for the genetic transformation necessary to create high-quality competent cells (Fig. 2).

Fig. 2 The transformation efficiency (TE) of three *E. coli* competent cells (DH5 α , TOP10 and JM109) made by different methods. Bars represent standard error (n = 3). One and double asterisks above the columns indicated significant difference at $p < 0.05$ and $p < 0.01$ separately

In modern molecular biology, increasing numbers of experiments require insertion of target DNA fragments into specific plasmids using PCR, restriction enzyme digestion, ligation reactions, and transformation. The larger the target DNA fragment, the fewer positive clones are obtained. To investigate the competent cells prepared by CRM method has better ability to transfer large target DNA fragments than other methods. A 8105 bp ligation product which connected a 5100 bp gene fragment (AT3G52250.1) with a 3015 bp double digestion *pGEM-Teasy* vector fragment was transferred using different competent cells prepared using different methods. The transformants were selected on LB agar containing IPTG, X-gal, and ampicillin. Blue and white colonies were observed after overnight incubation. White colonies indicate positive transformed colonies with target DNA fragments and blue colonies indicate negative colonies that do not contain the target DNA fragment. Results show that, among three DH5 α strains prepared using different methods, only the CRM method gave positive results. No transformants appeared in CaCl₂ method plate, and there were only negative colonies (blue) in Inoue method plate (Fig. 3a). As no positive colonies were observed on the Inoue and CaCl₂ method plates, we were not able to perform statistical analysis of these data. Two white transformants from CRM method plate and two blue transformants were randomly chosen to extract plasmids. These plasmids were subjected to PCR and enzyme digestion for confirmation of target DNA insertion in plasmids. The result of PCR and enzyme digestion both confirmed that the target DNA were successfully inserted into plasmid only by competent cell using CRM method (Fig. 3b, c). Further sequence reaction also proved this (data not shown). These results indicated that the competent cells prepared using the CRM method was best for inserting larger (> 5000 bp) target DNA fragments into plasmid vector than other competent cells were. This may be because of the increased cell membrane permeability attributable to addition of LFCin-B. Results indicate that the CRM method can not only produce competent *E. coli* cells with better transformation efficiency than other chemical methods, comparable to the electro-transformation method, and it can also produce highly competent *E. coli* cells, which are extremely efficient for incorporating larger DNA fragments into plasmid vectors.

Discussion

Cloning PCR products into plasmid vectors is a common and necessary upstream application for most modern molecular cloning experiments. Transformation efficiency is very important because it can directly affect the success of all the follow-up assays, and it can be affected by factors

Fig. 3 Inserting larger size of target DNA fragment into plasmids by different *E. coli* competent cells. **a** Blue-white spot screening experiment of a 8105 bp ligation product using different *E. coli* competent cells. White colonies indicate positive transformed colonies with target DNA fragment and blue one indicate negative colonies without target DNA fragment. **b** PCR assay for confirmation of target DNA insertion in plasmid. Line 1 and 2 were the

gel electrophoresis results of CRM method PCR products, line 3 was the marker, line 4 and 5 were the gel electrophoresis results of Inoue method PCR products. **c** Enzyme digestion assay for confirmation of target DNA insertion in plasmid. Line 1 and 6 were marker. Line 2 and 3 were the gel electrophoresis results of CRM method enzyme digestion products, line 4 and 5 were the gel electrophoresis results of Inoue method enzyme digestion products

such as the quality of competent cells. Electroporation, which has high transformation efficiency requires special and expensive equipment [9, 10], so the establishment of a convenient and rapid chemical method of transformation for all *E. coli* bacterial strains and large DNA fragments is necessary and important to the development of modern molecular biology. As the increasing reports on the non-lethal effect of some antimicrobial peptide by increasing the permeability of cell membrane [22, 23], we here improved upon the Inoue method by adding various common antimicrobial peptides found in mammals [22]. We found one antimicrobial peptide, LFcIn-B, to have a non-lethal effect on *E. coli* DH5 α strains. Through repeated attempts, we found that the low transformation efficiency caused by LFcIn-B's antibacterial property [25] can be increased by adding moderate high concentration of MnCl₂ (50 mM) and CaCl₂ (30 mM). This may be because the antibacterial properties of LFcIn-B can be inhibited by high concentrations of Ca²⁺ and Mn²⁺ without losing the increasing permeability of the DH5 α cell membrane [22, 25]. Although the mechanism underlying the antibacterial properties of LFcIn-B is complex and still

remain unclear, the observation can utilized to obtain competent cells with high competence which is urgently necessary in modern molecular cloning experiments because the permeability of the cell membrane directly influences the competence of *E. coli* cells. We are the first team to prepare highly competent cells by adding moderate concentrations of LFcIn-B. Further assays indicated that CRM method with its small concentration of LFcIn-B (0.35 mg/L) with moderately high concentrations of MnCl₂ (50 mM) and CaCl₂ (30 mM) can also increase the transformation efficiency of other two common *E. coli* strains, JMI09 and TOP10. These results indicated that CRM method can applied to produce high quality competent cells of most common *E. coli* cells which means this method can be widely used. Compared with other chemical methods (CaCl₂ and Inoue), CRM can produce competent *E. coli* cells with much higher transformation efficiency than those produced with other methods ($1.8\text{--}3.7 \times 10^9$ cfu/ μ g), comparable to electro-transformation. The best-known protocol for preparing competent *E. coli* cells and chemical transformation is the CaCl₂ method published by Mandel and Higa [8]. This method is still widely used in many

laboratories due to its convenience. Another good chemical methods was described by Inoue [11] because its transformation efficiency has been carefully optimized through a great number of studies [3, 15, 29–31]. The basic steps of this technique have undergone a few modifications for optimizing the efficiency of transformation, the majority of these efforts were indeed able to enhance the transformation efficiencies but the increase depended on suitable smaller DNA and differences among *E. coli* bacterial strains [10, 32, 33]. Unlike this methods, CRM method can increase the transformation efficiencies at least three common strains of *E. coli* cells, which strongly suggests it may be applied in most *E. coli* cells, and the huge increased transformation efficiencies exceed the maximum previous report. Moreover, the competent cells prepared by CRM method not only have high transformation efficiency, which is sufficient for modern molecular experiments but also have an especially good ability to insert larger DNA fragments into plasmid vectors, which Inoue and other transformation methods do not tend to offer. With the increasing development of genetic recombination technology, the most convenient and effective pathway to producing LFcIn-B protein has been expression recombinant target protein in vitro [34]. At present, there are four major systems for production recombinant protein in vitro. These are the prokaryotic protein expression system, mammalian cell protein expression system, yeast protein expression system, and insect cell protein expression system [35]. According to previous reports, it is possible to produce good-quality LFcIn-B protein via prokaryotic protein expression system and yeast protein expression system [28, 36–39]. Producing recombinant LFcIn-B protein through prokaryotic protein expression systems is more welcome both in research and industrial production because *E. coli* has a clear genetic background and strong tolerance to many proteins, which means high levels of expression of the target proteins can be induced under appropriate conditions [28, 38]. Producing LFcIn-B protein using the prokaryotic expression system offers convenient operation, low cost, fast speed, and large-scale, high-quality expression. Adding small concentration of LFcIn-B is not expensive or troublesome, and the cost of CRM method in both time and money are similar to those of other chemical methods, but the transformation efficiencies and level of competence of competent cells can be significantly increased by the CRM method.

In conclusion, we have described a protocol for preparing the most suitable competent *E. coli* cells both for efficient plasmid transformation and insertion of larger DNA fragments into plasmid vectors in this study. Cloning PCR products into plasmid vectors is a common upstream application of many modern molecular research such as CO-IP and BiFc [26]. Our method is simple, reliable, and

highly reproducible. It works for at least three *E. coli* strains (DH5 α , JM109, and TOP10), and possibly other *E. coli* strains. The results reported here could be useful to various biological studies.

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