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IMPROVEMENT OF A SIMPLE AND EFFECTIVE TRANSFORMATION ASSAY FOR *E COLI*

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Abstract– Searching for efficient means by which bacteria uptake plasmid DNA is of a great practical importance in molecular biology and genetic engineering. We have described in this study five assays for preparing transformants *E. coli* cells (strain JM109) which are extremely efficient for plasmid uptake that typically yielded transformation efficiencies between 7.2×10^5–1.9×10^7 colony-forming units per microgram (CFU/μg) of plasmid DNA. Assay 05 with transformation efficiency 1.9×10^7 CFU/μg is the simplest and most effective. This assay was considered easy if compared with the original rapid colony transformation protocol described by Hanahan *et al.,* 1991, in which they used transformation buffer (TFB) as transformation solution and the super-optimal broth with catabolite repression (SOC) media, these two are more complex if compared with $CaCl_2$ solution and Luria Bertani (LB) media. One more point in favor of this protocol is its low time consumption. Transformation efficiency between 10^6 and 10^7 is not optimal but it is above average for molecular experiments. Therefore, it is possible that these assays may be widely applicable for bacterial transformations with plasmids and can be used as an inexpensive and reliable alternative to existing methods.

INTRODUCTION

Bacterial transformation is a phenomenon of horizontal gene transfer, it was first discovered by Griffith for *Streptococcus pneumoniae* Griffith (1928). More than 70 species distributed in different taxonomic groups have been found to be naturally transformable. These include *H. influenzae, Neisseria gonorrhoeae* and *Vibrio cholerae* belonging to proteobacteria β and γ, *Bacillus subtilis* and *Streptococcus pneumoniae* from Firmicutes Sun (2011).

During natural transformation, large multiprotein polar complexes take up the exogenous DNA into cells by which allows a single-stranded DNA (ssDNA) from the donor double-stranded DNA (dsDNA) to enter into the cytoplasm, these complexes are assembled from components resembling those that form surface appendages;type IV pili (T4P) and transport systems as diverse as type II secretion systems T2SS, T4SS and ABC transporters Chen and Dubnau (2004).

Such a phenomenon is uncommon in *Escherichia coli*, although artificial transformation of *E. coli* has been widely used Sun (2011). DNA transfer into *Escherichia coli* was first demonstrated in 1970 by Mandel and Higawho showed that treated bacteria with ice cold solution of $CaCl_2$ followed by a brief heat choc could integrate bacteriophage λ DNA. This was referred to as calcium chloride method Cohen, Chang and Hsu (1972).

In 1983, Douglas Hanahan proposed a new method that yielded a transformation efficiency of 1-5 × 10^8cfu/μg across many *E. coli* strains Hanahan (1983); Hanahan *et al.* (1991). However, his method was slightly complex. Other highly efficient chemical transformation protocols are complex, time consuming using complex solution such as transformation buffer (TFB), frozen storage buffer (FSB)…, and require relatively dangerous chemical compounds such as polyethylene glycol (PEG).

The introduction of plasmids into *E. coli* is an essential step for molecular cloning experiments, and a number of different procedures have been described for this purpose. The most widely used transformation methods include chemical/ mechanical treatments, electroporation, and virus-/

nanocarrier-mediated approaches Choi and Lee, *et al. (*2013). Advantages of chemical transformation include ease, relative efficiency, and lack of need for a specialized apparatus such as an electroporator Bonnick (2010).

Consequently,the effect of various factors has been studied. Such factors include prolonged incubation of bacteria with $CaCl_2$, addition of multiple cations into the transformation mixture and treatment of bacteria with dimethyl sulfoxide (DMSO), hexamine cobalt, and dithiothreitol in the presence of both Monovalent and divalent cations Chung, Niemela and Miller (1989).

All these procedures have an objective to achieve the highest transformation efficiencies with the most survival rate of transformants in short time.

Unfortunately, in developing countries research laboratories have insufficient budgets, so there is a need to use simple, effective and inexpensive methods. Therefore,we attempt in this study to simplifying a number of procedures from existing methods and chose the one who is rapid and gives the highest transformation efficiencies.

MATERIALS AND METHODS

Chemicals and Media

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) and calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$) were purchased from Sigma–Aldrich (Germany). Luria–Bertani (LB) agar (LBA) medium and LB broth media were supplied from Liofilchem Company (Italy). LBA with 100 μg/ml ampicillin was used to select for transformants of *E. coli* JM109

Bacterial Strain and Plasmid DNA

E. coli JM109 was used as recipients of plasmid. Bacteria were grown in LB broth at 37 °C with agitation. Antibiotic ampicillin (100 μg/ml) was used for selection of transformants, when pMD2 VSV-G envelope expressing plasmid of 5.822 kb (Addgene plasmid # 12259; http://n2t.net/addgene :12259; RRID: Addgene_12259) was used for bacterial transformation. *E. coli* JM109 and pMD2 VSV-G envelope expressing plasmid were a gift from Professor Yahia Cheboune.

Plasmid DNA was purified from overnight cultures of *E. coli* JM109 Amp+ using a MACHERY-NAGEL NucleoSpin® Plasmid Transfection-grade extraction kit. DNA concentration was determined using an agarose gel electrophoresis. For transformation purposes we diluted the initial extract by 100 folds, a 1μl of the extraction product was added to 99μl of ultrapure water. The extraction product and the diluted suspension were stored at -20°C.

Preparation of Competent Bacteria

A single bacterial colony of *E. coli* JM109 Amp- (ampicillin sensitive) was picked from an LB agar plate that has been incubated for 20 hours at 37 °C. Transfer this colony into 50 ml of LB broth in a 500 ml flask. Incubate the culture at 37 °C with vigorous agitation until an OD_{600}=0.4~0.5 was reached. This logarithmic-phase culture was divided into three portions. Each portion was then chilled for 10 min in ice, and the cells were harvested by centrifugation at 5000xg for 10 min.

To one portion of the harvested cells add 2.5 ml of $CaCl_2$ (0.05 M) and incubate in ice for 30 minutes, then centrifuge at 5000xg for 10 min at + 4 °C. Discard the supernatant and resuspend the pellet in 0.5 ml of $CaCl_2$ (0.05 M). Disperse the final volume in aliquots of 100μl and store them at +4°C for 20 hours (this portion was used for assay 01).

To one of the remaining two portions of harvested cells add 5 ml of $CaCl_2$ (0.1 M) and incubate in ice for 10 minutes then centrifuge at 5000xg for 10 min at + 4 °C. Discard the supernatant and resuspend the pellet in 2 ml of $CaCl_2$ (0.1 M). Disperse the final volume in aliquots of 200μl and store them at +4 °C for 20 hours (this portion was used for assay 02).

To the remaining portion of harvested cells add 5 ml of Calcium Magnesium Chloride (CMC) solution ($CaCl_2$ 0.8M and $MgCl_2$ 0.2M) and incubate in ice for 10 minutes then centrifuge at 5000xg for 10 min at + 4 °C. Discard the supernatant and resuspend the pellet in 2 ml of CMC solution ($CaCl_2$ 0.8M and $MgCl_2$ 0.2M). Disperse the final volume in aliquots of 200 μl and store them at +4°C for 20 hours (this portion was used for assay 03).

Transformation Assays

Transformation was done by a variation of the procedure of Mandel and Higa and a little modification to Hanahan's method.

Assay 01

To the first portion of competent cells add 1μl of diluted plasmid (1/100) to an aliquot of 100 μl, and incubate the transformation mixture in ice for 30 minutes. Apply a heat shock in 42 °C water bath and agitate gently for 2 minutes, then transfer the sample immediately to ice for 5 minutes. Add 400μl LB

broth preheated at 37 °C and incubate the mixture at 37 °C for 30 minutes, then concentrate the transformants by centrifuging the Eppendorf tubes at 5000xg for 5 minutes, discard 400µl of the supernatant and resuspend the cells in the remain 100µl. finally plate the LB Agar Amp+ with this volume.

Assay 02

(Classic calcium chloride method)

Add 2µl of diluted plasmid (1/100) to an aliquot of 200µl and incubate the transformation mixture in ice for 30 minutes. Heat shock at 42 °C for 30 seconds in a water bath, then transfer the sample immediately to ice for 5 minutes. Add 400µl LB broth preheated at 37 °C and incubate the mixture at 37 °C for 1 hour 49 minutes then at 4°C for 1 hour. Plate the LB Agar Amp+ with 100 µl.

Assay 03

This assay follows steps as the preceding, it differs from it by two steps, it uses a heat shock of 42°C for 45 seconds and a CMC solution ($CaCl_2$ 0.8M and $MgCl_2$ 0.2M) instead of $CaCl_2$ solely.

Assays 04 and 05

The assays 04 and 05 are based on a rapid colony transformation protocol described by Hanahan *et al.*, 1991, but they differ in three points, first we used $CaCl_2$ (0.05M) as competence solution instead of transformation buffer (TFB) or frozen storage buffer (FSB), second, in the recovery phase we replaced the media super optimal broth with catabolite repression (SOC) with LB broth, third, a step of centrifugation was added before plating in the assay 05 which make it different from assay 04.

With an inoculation loop select few colonies of *E. coli* JM109 Amp- from a 24-hour culture on LB agar media and disperse them in 200 µl of $CaCl_2$ (0.05M) cold solution. Incubate in ice for 10 minutes. Add 2µl of diluted plasmid sample (1/100) that was stored at -20 °C, then agitate for a brief moment with a vortex shaker. Heat shock at 42 °C for 90 seconds in a water bath. Add 600 µl of LB broth preheated at 37 °C and incubate the transformation mixture at 37 °C for 1 hour. Plate 100µl in the selective media LB Agar Amp+.

In the assay 05 follows the same steps as the assay 04 until the fifth step, a centrifugation at 5000xg for 5 minutes was performed before plating, this to concentrate the cells and explore the effect of centrifugation on the transformation efficiencies. Discard the supernatant and resuspend the cells in 200µl of LB broth. finally plate the LB Agar Amp+ with 100 µl.

Note: for all experiments we plated an LB Agar Amp- as a control with 100 µl of each assay.

In table 1 we put in a nutshell different parameter that vary between all assays.

RESULTS

Cell suspension purity

The macroscopic aspect of colonies is uniform, all colonies present the same characteristic of the reference strain. After wet mount technic bacterial cells appeared dispersed and mobile, gram stain revealed pink rod-shaped bacteria. These results are consisting with *E. coli* strains and the uniformity of macroscopic and microscopic characteristics confirm the purity of the cell suspension.

Plasmid Extraction and Electrophoresis

Under UV light the extraction product appears as only one band suggesting the absence of nucleic contaminant of degradation of the extracted plasmid. The size of the extracted plasmid is the same as the reference sample (pMD2a) although the

Table 1. Summary of the experimental conditions used in the study

Assays	Time of incubation recovery at 37°C	$CaCl_2$ Concentration (M)	Heat shock T°C	Time (sec)	Ice incubation time after heat shock
A01	30 min	0.05	42 °C	120	5 min
A02	1h49min	0.1	42 °C	30	5 min
A03	1h49min	CMC[a]	42 °C	45	5 min
A04	1h	0.05	42 °C	90	No
A05	1h	0.05	42 °C	90	No

[a]: Was used a CMC solution ($CaCl_2$ 0.8M and $MgCl_2$ 0.2M) instead of $CaCl_2$ solely

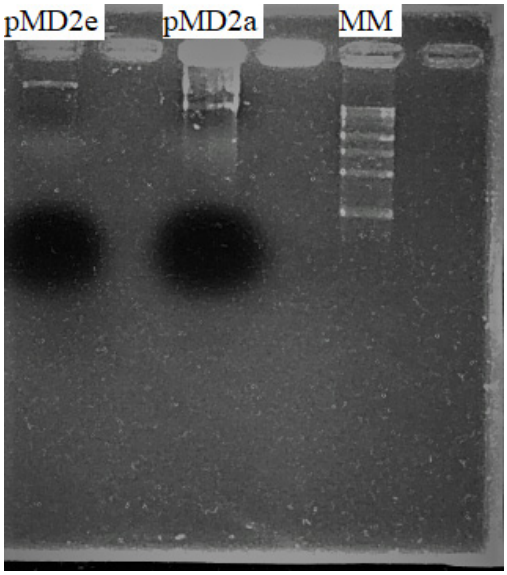

Fig. 1. Visualization of the plasmid pMD2e after extraction (pMD2e: extracted plasmid; pMD2a: control plasmid; MM: 100 pb molecular marker).

latter appears as a smear indicating a partial degradation due to repeated freezing and thawing.

Chemical transformation protocols

The transformants of *E. coli* JM109 (Amp+) are counted using a bacterial colony counter after 24 hours of incubation at 37 °C, and the transformation efficiency (TE) per microgram plasmid DNA of each assay was calculated as the number of colony-forming units (CFU) produced by 1 μg of DNA and is measured by performing a control set of transformation reaction using a known quantity of DNA and then calculating the number of CFU formed per μg of DNA.

$$\text{Transformation efficiency} = \frac{[\text{No. of transformants (colonies)} \times \text{Final volume at recovery (ml)}]}{(\mu\text{g of plasmid DNA} \times \text{Volume plated (ml)})}$$

The highest yield of transformation efficiencies in our study 1.9×10^7cfu/μg was obtained with assay A05. This assay was considered easy if compared with the original rapid colony transformation protocol described by Hanahan *et al*., 1991, in which they used TFB as transformation solution and the SOC media, these two are more complex if compared with $CaCl_2$ solution and LB media. One more point in favor of this protocol is its low time consumption.

DISCUSSION

The introduction of exogenous DNA into *E. coli* was first demonstrated by Mandel and Higa (1970), who observed that the incubation of a suspension of *E. coli* cells and bacteriophage λDNA in a solution of $CaCl_2$ at 0° C resulted in cellular transformation Yoshida and Sato (2009).

In molecular cloning experiments transformation efficiency is most important, and can be affected by many factors. Chemical method involved treating the bacteria with DNA in the presence of Ca^{2+} ions, followed by a brief heat shock. This was referred to as the 'calcium chloride' ($CaCl_2$) method Chan and Verma, *et al. (*2013).

We attempt in this study to get a simple and optimized method by modifying some procedures of the chemical methods of bacterial transformation, we chose the method of Mandel and Higa and that of Hanahan. These resulted in five assays of the original $CaCl_2$ method that typically yielded transformation efficiencies of 7.2×10^5–1.9×10^7 colony-forming units per microgram (CFU/μg) of DNA (Table 2).

In 1983, Douglas Hanahan proposed a new method that yielded competency levels of 1–5×10^8cfu/μg across many *E. coli* strains Hanahan, Jessee and Bloom (1991). However, his method was quite complex, combining several parameters such as the nature of chemicals, plasmids and labware Chan and Verma *et al. (*2013).

The highest yield of TrE transformation efficiencies in our study 1.9×10^7cfu/μg was obtained with assay A05. This assaywas considered easy if compared with the original rapid colony

Table 2. Results of transformations assays and calculation of efficiency

Procedures	Plated volume (μl)	Final volume (μl)	Plasmid mass (ng)	The result of growth (CFU) after 24 h at 37°C	TE (x10^7CFU/μg)
A01	100	500	0,1	27	0.135
A02	100	600	0,1	68	0.408
A03	100	600	0,1	48	0.288
A04	100	800	0,1	9	0.072
A05	100	800	0,1	238	1.904

transformation protocol described by Hanahan and *al*., 1991, in which they used TFB as transformation solution and the SOC media, these two are more complex if compared with $CaCl_2$ solution and LB media. One more point in favor of this protocol is its low time consumption.

The second-best assay is A02 with TrE of 4.08 x 10^6cfu/µg. Liu *et al.* (2014) mentioned that the transformation efficiency of the JM109 strain treated by Ca^{+2} is lower than 2×10^4cfu/µg. JM109 by itself is originally doesn't yield high transformation efficiencies when treated with Ca^{+2}. On the other hand, Inoue, Hiroshi and Hiroto, (1990), assessed their novel method by testing JM109 and they recorded a competence (1.44 ±0.65) x 10^9CFU for this strain Inoue *et al.* (1990).

Other basic factors can be the origin of the relatively low transformation efficiencies

The plasmid sample was stored at -20 ^{0}C for 5 months after the extraction, also the repeated freezing and thawing of the sample could damage the plasmid DNA and change its spatial configuration (nicked or relaxed). The use of freshly extracted, carefully stored, and supercoiled plasmids yield the best transformation efficiencies Casali and Preston (2003). The concentration of the plasmid suspension was not measured by a proper quantitative technic like the spectrophotometry at 260 nm, although it was only estimated by comparing the densities of the extracted plasmid DNA and the reference sample. The band of the extracted plasmid DNA (Figure 1) was a little low in density than the reference sample, given that the concentration of the reference sample is 1.5µg/µl the concentration of the extracted plasmid DNA sample was estimated to be 1µg/µl. The plasmid's

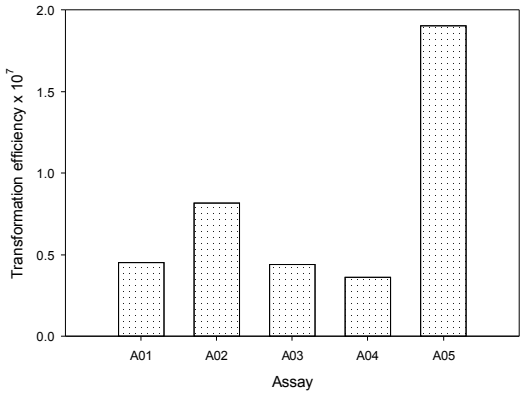

Fig. 2. Transformation efficiency after modifying protocols with different procedures

concentration is a must-know factor to calculate the transformation efficiency and it was only given as an estimated number so the real transformation efficiencies can either lower or higher than the numbers mentioned above.

If the transformed cells were not given enough time to recover and restore their integrality after the osmotic choc (recovery phase), the centrifugation can generate high sheer force that could destroy the weak cells which did not recover properly, thus reducing the number of viable transformants and by consequence the transformation efficiencies. There is a correlation between the recovery phase duration and transformation efficiency. Therefore, the transformation efficiencies of assay 01 can be optimized if the duration of the recovery phase is augmented.

There is a distinct correlation between the plated volume and transformation efficiency. In theory, if the final resuspension of transformants is well homogenized there should be a distinct and clear proportional correlation between the plated volume and the transformation efficiency, this is what we found in our study, in assays 04 and 05 we used the same steps except for plated volume, assay 05 is five folds greater than the assay 04 (Table 2).

Another important factor is the concentration of $CaCl_2$. Although 50-100 mM calcium chloride can be used, Tu and *al*. 2005 used 75 mM $CaCl_2$ in TB solution it was found to be the optimum concentration (Tu and He *et al.*, 2005).

It is known that the effect of calcium chloride treatment can be enhanced if followed by a heating step, in this study we fixed the heating temperature to 42 °C, but the time ranged from 30 second to 120 seconds, we found that 30 and 90 seconds gives the best results.

Discovering efficient means by which to introduce DNA into bacteria is of great practical importance in genetic engineering and molecular biology (Yoshida and Sato, 2009). We have described in this study five assays for preparing competent *E. coli* cells (strain JM 109) which are extremely efficient for plasmid uptake, assay 05 (TrE 1.9×10^7cfu/µg) is the simplest and most effective and time saving.

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Declaration of interests

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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