Rapid and Efficient Method to Make Competent Bacterial Cells for Genetic Transformation

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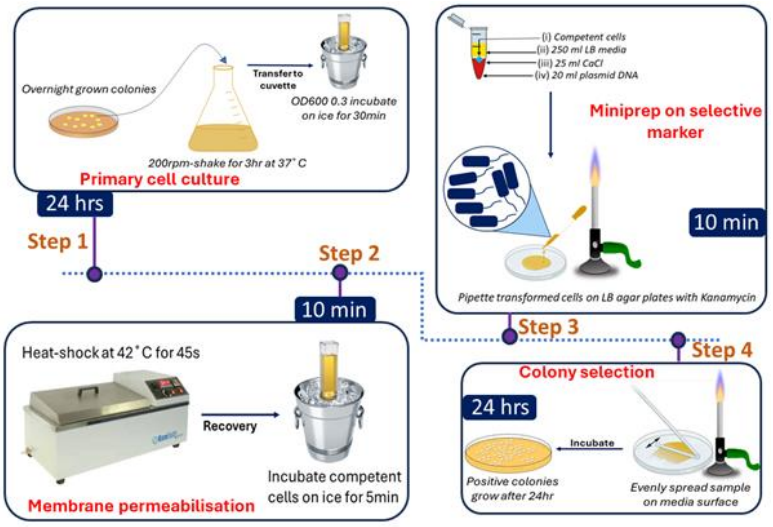
Abstract: There is absence of natural occurrence of genetic transformation in many bacteria due to the negative charges of DNA and bacteria, hence the DNA must be forced into the bacterial cells. *Escherichia coli* is mainly the host of choice because of its simplistic genetic mechanism and readily available molecular biological tools to inexpensively cultivate it. After the bacterial cells are made competent, it is anticipated that they contain and express the gene of interest that is cloned in the plasmid they take up during transformation. The Polymerase Chain Reaction method is then used to determine the presence of the gene and identify the band size, thereof. This paper will tabulate the step-by-step protocol of making competent *E. coli* cells strain K12 using calcium chloride solution and the heat-shock method and outline the procedure to confirm the positive transformants.

Keywords: $CaCl_2$ solution, plasmid DNA, genetic transformation, competent cell, *E. coli*, LB media.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Genetic transformation involves the alteration of genes when cells take up uncoded DNA from the extracellular environment. Calcium chloride can render competency to the untransformed cells [1], while the heat-shock method perforates the cell membrane to make it permeable for the foreign DNA to penetrate easily [2], allowing them to be transformed. The use of divalent cations such as calcium has over time emerged as the most efficient mechanism

to make competent cells, topping other chemical methods and electroporation transfer [3]. Transforming bacteria is a pivotal stage in the production of multiple copies of recombinant DNA molecule, this process is known as cloning [4]. *Escherichia coli* cells strain K12 were employed in this study, a gene cassette carrying the gene of interest was designed and synthesized into the plant binary vector pCAMBIA1301. However, it should be noted that not all bacteria can take in exogenous DNA from their environment [7]. The pCAMBIA1301 plasmid is kanamycin resistant for bacterial selection and hygromycin resistant for plant selection – it was inserted into the competent bacterial cells for replication of the desired sequence, in sufficient numbers for future use in diverse molecular analyses, such as plant genetic transformation via *Agrobacterium tumefaciens* mediated transgenic system [5].

2. MATERIALS AND METHODS

2.1. MATERIALS

1. Ice
2. Microcentrifuge tubes: D200 Gilson Diamond
3. Sterile pipette tips: MO002462ZA1230-12 Biologix group
4. Sterile falcon tubes: 10-9502 Biologix group
5. Parafilm: PM-996 Amor, USA
6. Sterile petri dishes: 3160072 Borosil, India
7. Cell spreaders: CPSA20000002 ISOLAB Laborgerate GmbH, Germany
8. Isolate II plasmid DNA kit: BIO-52056 Bioline Meridian Biosciences
9. Micropipette 20-200µL: QK644592 Lab Basics
10. PCR reaction strips: 330180784 Simport, Canada
11. Gloves: E2022-07-06 AXG industries, Malaysia

2.1.1. Reagents

1. Ice-cold 100mM $CaCl_2$ solution – recipe for stock solution in Appendix
2. Ice-cold 85% (v/v) 100mM $CaCl_2$ and 15% (v/v) glycerol solution
3. 100mL of fresh Lysogeny Broth (LB) liquid media
4. 5mL of overnight *Escherichia coli* (*E. coli*) strain K12 culture
5. 4 Petri plates containing LB agar supplemented with kanamycin
6. 1 Petri plate containing LB agar without kanamycin
7. 1pg – 100ng plasmid DNA (1-5 µL)
8. Primers: 231123-027_A10 mgEU_CES Macrogen Europe
9. Accocheck Taq buffer: M1200 SolarBio Life Sciences

10. 1kb ladder/DNA marker: 10787-018 Invitrogen

11. Stain – SafeView Classic: G108 abmGood, Canada

12. DNA loading dye: 10158560 Biolabs

13. LE Agarose: 9012-36-6 Cleaver Scientific

14. TAE buffer: 01-870-1A Biological Industries

15. Nuclease free water: 3-07F04-H BioConcept

16. 70% ethanol: ET00052500 Scharlab

17. Sodium hypochlorite: SM#29015 JIK

2.1.2. Equipment

1. Water bath: 04653 Kottermann Labortechnik

2. Shaking incubator: SI-200 Winpact

3. Refrigerated microcentrifuge: 5430 Eppendorf

4. Biomedical freezer: MDF-U5412H-PE Panasonic

5. PCR thermal cycler: GTC96S/GTC96S-230 Cleaver Scientific Ltd

6. Gel electrophoresis: NANOPAC300P Cleaver Scientific Ltd

7. Gel doc imaging: X20-88075 Vilber

8. Spectrophotometer: 6135KI605057 Eppendorf

9. Tissue culture biosafety cabinet: AH-100 Telstar

10. Water distiller: TT-98-III HS.Z11.20L

11. Ice maker: AF-80 Scotsmann

12. Nanodrop: Intertek 58595 Thermo fisher

13. Autoclave: MAN205-0503

14. Weighing balance: PB1302-S/FACT Mettler Toledo

15. pH/ORP meter: HI2211 HANNA instruments

2.2. METHODS

Caution: This is a temperature sensitive protocol; thus, it is imperative to keep all plastic ware such as tubes on ice throughout the procedure. The use of a refrigerated microcentrifuge is highly advised. Additionally, it is cardinal to conduct all the steps in this experiment in a very aseptic environment – sterilize working surfaces using 70% ethanol.

1. Source for viable *E. coli* cells, preferably strain K12 and grow them on fresh LB agar plates for reactivation. Incubate overnight at 37°C.

NOTE: One plate should be supplemented with the appropriate antibiotics for bacterial selection (for instance, kanamycin in this case study), while the other plate should be plain LB agar, without any antibiotics. The first plate acts as the control, to ascertain that the *E. coli* cells to be used are untransformed, at least for the plasmid's selectable marker. Hence no colonies should be seen on the kanamycin supplemented plate, after the 18-hour incubation period. Whereas, the second plate, on the other hand – without any antibiotics is the working plate, on it, colonies are expected to grow, see supplementary figures (a) and (b), respectively.

2. The next morning, fill a 50mL falcon tube with 25mL of plain LB liquid media and use a sterile micropipette tip or toothpick to inoculate some bacteria from the overnight grown colonies in plate two.

3. Then wrap the tube with parafilm and loosely seal it with aluminium foil, thereafter, put it in a shaking incubator or water bath at 37°C and 200 rpm.

4. Observe the growth of the cells hourly, after 3 hours check for the optical density of the culture.

5. Use a spectrometer to determine whether the exponential growth phase has been attained. Blank it with sterile plain LB liquid medium before use.

6. Take 2mL of the culture and put them into a cuvette; OD_{600} 0.3 should have been obtained.

7. Put 1mL bacterial culture into two separate sterile Eppendorf tubes and centrifuge for 4 minutes at 8,000 x rpm and 4°C.

8. Carefully discard the supernatant from both tubes, leaving only the pellets.

9. To increase the number of harvested cells, repeat steps 6 and 7 using the same tubes.

10. Flick the tubes containing the pellets and resuspend them in 1.5mL of ice-cold 100Mm calcium chloride ($CaCl_2$).

NOTE: Avoid touching the bottom part of the tubes, to prevent transfer of heat to the soon-to-be competent cells, from your hands.

11. Leave the tubes on ice for 30 minutes.

12. To pellet cells, centrifuge tubes for 4 minutes at 8,000 x rpm and 4°C and discard the supernatant.

13. Resuspend the pellets in 40µL of ice-cold 85% v/v 100 Mm $CaCl_2$ and 15% v/v glycerol (for long shelf-life preservation).

NOTE 1: The cells may be utilized right away, alternatively they may be stored in the freezer at -80°C.

NOTE 2: This method will yield approximately 15 tubes of 50µL competent cells.

NOTE 3: Discard all used tips and tubes in 10% sodium hypochlorite to get rid of residual bacteria, and dispose of all other materials employed, appropriately.

2.2.1. Transformation

1. Get 10µL of prepared competent cells, add 1µL of plasmid DNA and mix gently by inverting tube 8 – 10 times.

2. Place tubes on ice for 30 minutes.

3. Heat-shock by carefully immersing the tubes in 42°C water bath for exactly 45 seconds.

4. Incubate for 5 minutes on ice.

5. Add 1mL antibiotic free LB liquid media.

6. To let the kanamycin resistance gene express, shake incubate at 37°C, 200 rpm for 60 minutes.

7. Centrifuge for 5 minutes at 9,000 x rpm and discard the supernatant.

8. Add 100µL pre-warmed LB liquid media to resuspend the pellet.

9. Spread 1:50 dilutions on warmly fresh LB agar plates with the selective marker – Kanamycin (recipe in Appendix).

10. Seal the plates with parafilm and incubate them upside down at 37°C for 12 – 16 hours (usually overnight).

3. RESULTS AND DISCUSSIONS

3.1. Results

The next morning, inspect the plates for isolated colonies, which should have the assembled plasmid embedded in them. Incubation more than 16 hours should not be considered, as the antibiotic is likely to have degraded, hence causing growth of satellite colonies. Each plate is expected to have a countable number of colonies (50 – 500) as in Figure. 1 below. Plasmid DNA isolation of the transformed *E. coli* colonies then follows, for this experiment.

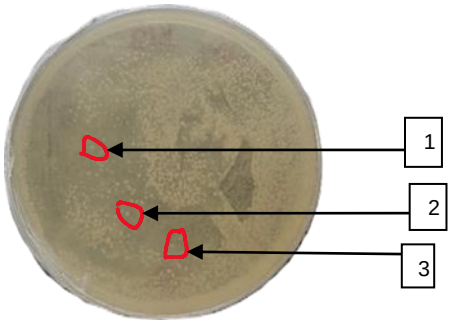


Figure. 1: The transformed *E. coli* K12 colonies on LB agar media supplemented with kanamycin.

3.2. Transformation efficiency

Transformation efficiency denotes the number of Colony Forming Units (CFU) likely to be produced by 1µg of supercoiled plasmid DNA employed in the transformation process of bacterial cells.

1. Count all overnight grown colonies appearing on the kanamycin supplemented LB agar plate

2. Calculate the transformation efficiency (TE) using the formula shown below:

   TE = [number of colonies on plate / amount of plated DNA (ng)] x 1000 (cfu/ng)

3.3. Selection of colonies

NOTE: Avoid prolonged incubation as these may lead to larger colonies merging as well as false positive colonies

[6]

1. Select several distinct well grown colonies by labeling them at the bottom of the plate.

2. Pick the colonies one after the other using a separate sterile pipette tip.

3. Inoculate in 5mL LB liquid media with kanamycin from the stock solution.

4. Grow cells overnight at 37°C in a shaking incubator at 200 rpm.

5. The following day, isolate the plasmid DNA using a high-copy commercial kit for small-scale purification.

3.4. Confirmation of transformants

Further analysis for each colony is carried out, to confirm the presence of the DNA construct and screen for the appropriate gene sequence.

1. Polymerase chain reaction (PCR) using specific primers and trusted Taq buffers alongside the extracted plasmid DNA as the template.

2. Optimal PCR conditions are set before setting the Thermocycler to run.

3. Run gel electrophoresis of the PCR products and a DNA marker to aid determine the expected length of the DNA fragment.

4. Visualize the bands using the imaging gel doc and save the picture for future use (Figure. 2)

5. Single out the positive colonies and store them in glycerol stock for further applications in various biological applications, such as plant genetic transformation and protein expression, among others.

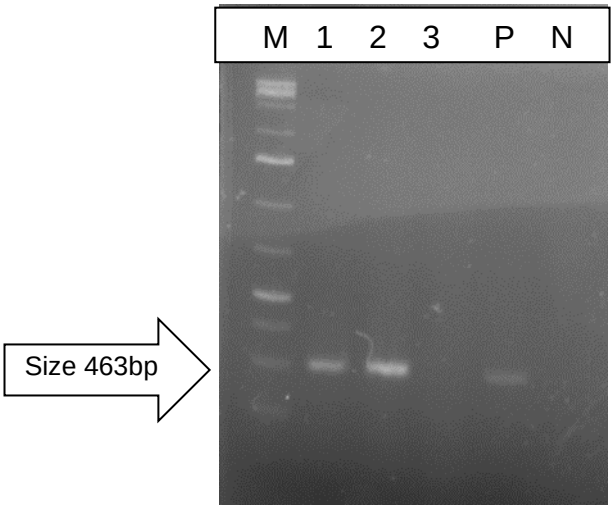


Figure. 2: PCR method identified: M = 1kb DNA marker, 1 = first colony selected, 2 = second colony selected, 3 = third colony selected, P = positive control, and N = negative control.

APPENDIX

Recipes

LB growth media

- 1 Liter distilled water
- 10g tryptone
- 5g yeast extract
- 10g sodium chloride
- 15g agar (uniquely for LB agar plates): (GRM026-500G) HIMEDIA
- pH adjusted to 7.0

Calcium chloride ($CaCl_2$) 1M: (GB227509) LOBA Chemie

- 14.7g $CaCl_2$: (GB227509) LOBA Chemie
- 100mL nuclease free water: (3-07F04-H) BioConcept

Glycerol – 50% (v/v)

- 50mL Glycerol 99.5% AR: (0015602500) LOBA Chemie
- 50mL distilled sterile water

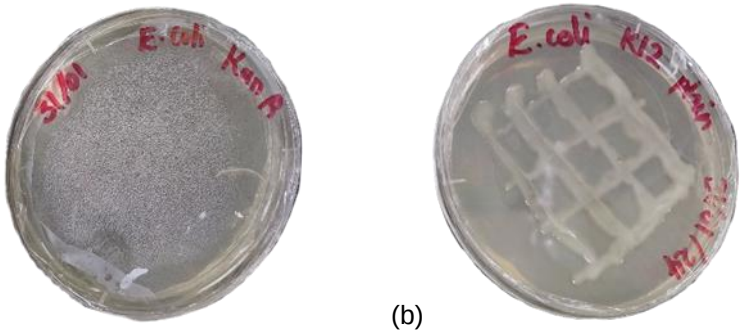
Kanamycin: (010297.08) Duchefa Biochemie

- 100mg/mL (1000x concentrated)

Gel (2%)

- 1g LE agarose: (9012-36-6) Cleaver Scientific
- 50mL 1X TAE buffer

SUPPLEMANTARY FIGURES



(a) (b)

The viability test efficiency of *E. coli* cells as competent, pre-transformation by the plant binary vector, pCAMABIA1301

(a) The *E. coli* colonies, strain K12 grown on LB agar media supplemented with kanamycin

(b) The *E. coli* colonies, strain K12 grown on plain LB agar media without antibiotics

ABBREVIATIONS

DNA: Deoxyribose nucleic acid; ***E. coli***: *Escherichia coli*; **LB**: Lysogeny Broth; $CaCl_2$: Calcium chloride

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Conflicts of Interest

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Beenzu Siamalube: Writing – original draft, Formal analysis, Methodology. **Emmanuel Ehinmitan**: Data curation, Software, Writing – original draft. **Maina Ngotho**: Validation, Writing – review and editing, Supervision. **Justus Onguso**: Conceptualization, Project administration, Supervision. **Steven Runo**: Investigation, Resources, Supervision.

TROUBLESHOOTING

Complication	Clarification	Conclusion
No colonies present or very few transformants seen	Misuse/underuse/overuse of the antibiotic	Ensure that the ideal antibiotic is used with precise concentration
	Employed an inappropriate heat-shock procedure	Adhere to the transformation protocol keenly, as instructed by the producer
	Incompetency of bacterial cells	Work out the transformation efficiency of the competent cells. If not high enough, it will be imperative to make a fresh cluster of competent cells
Absence of plasmid in grown colonies	Usage old plates with probably degraded antibiotic	Use freshly warm lb agar plates with newly prepared antibiotic stock
	Selection of satellite colonies	Select distinctly large, confirmed colonies for analysis
Formation of a mat or field of colonies on agar plates	Addition of antibiotic to hot agar causes degradation of the antibiotic and it becomes less effective	Prior to adding the antibiotic, see to it that the agar is cool enough but not gel-like yet. That is, be able to comfortably touch the agar container when adding the antibiotic
	Utilization of excess dilution ratio of transformed cells	Plate 1:50ul dilution of cells in subsequent experiments, for even spread and fair distribution of individual colonies on the agar plate.

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