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Simple and Fail-safe Method to Transform Miniprep *Escherichia coli* Strain K12 Plasmid DNA Into Viable *Agrobacterium tumefaciens* EHA105 Cells for Plant Genetic Transformation

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Abstract

Agrobacterium-mediated gene transformation method is a vital molecular biology technique employed to develop transgenic plants. Plants are genetically engineered to develop disease-free varieties, knock out unsettling traits for crop improvement, or incorporate an antigenic protein to make the plant a green factory for edible vaccines. The method's robustness was validated through successful transformations, demonstrating its effectiveness as a standard approach for researchers working in plant biotechnology. It enables the introduction of foreign DNA into plant genomes. Conventionally, plant genetic transformation has relied on time-consuming, costly, and technically demanding procedures, such as electroporation and chimeric viruses or biolistic methods, which usually yield variable transformation efficiencies. This study presents a simple and fail-safe protocol that involves a modified freeze-thaw and heat-shock concoction method. This approach involves a streamlined plasmid miniprep procedure to isolate high-quality plasmid DNA from *Escherichia coli* K12 strain, followed by a target-specific transfer into *A. tumefaciens* EHA105 strain. The optimized method minimizes DNA degradation and maximizes uptake by *Agrobacterium* cells, making it a reproducible and accessible protocol for various genetic engineering applications. The transformation efficiency is consistently high, enhancing plasmid uptake while maintaining cell viability, requiring minimal specialized equipment and reagents. The proposed protocol offers significant advantages, including simplicity, reliability, and cost-effectiveness, positioning it as a valuable alternative to traditional techniques in the field of plant biotechnology.

Key features

- Uses liquid nitrogen as a proxy for freezing.
- Plasmid DNA from competent bacterial cells is extracted using a user-friendly high-copy isolation kit.
- A maximum of five consecutive days is sufficient to complete the procedures.

Keywords: *Agrobacterium tumefaciens*, Plasmid DNA, Freeze-thaw method, Heat-shock, Cell viability, Genetic transformation, Plant biotechnology, *Escherichia coli*, Potato

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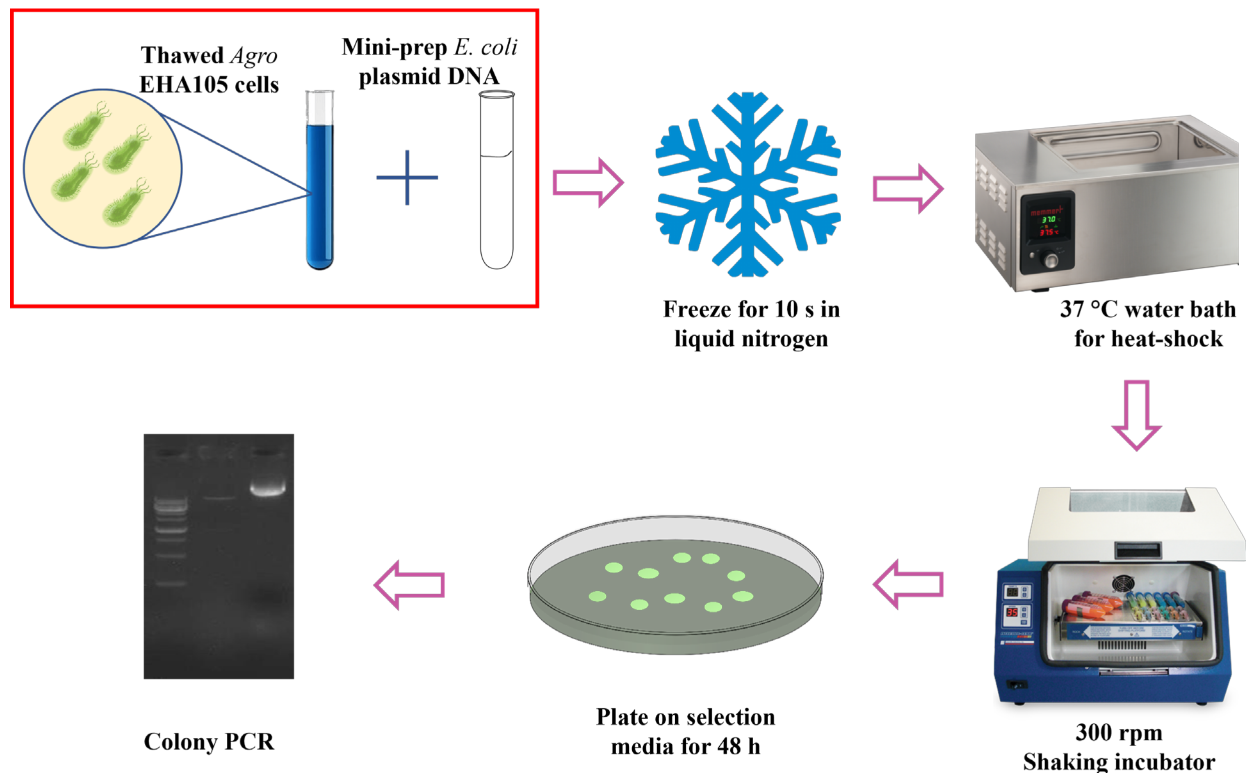
Graphical overview



Freeze-thaw *Agrobacterium* transformation method

Background

Agrobacterium tumefaciens is a bacterium found in soil that has a natural ability for transient and stable transfer of foreign DNA into plant cells, including dicotyledonous and monocotyledonous species. This makes it a dominant technique for creating transgenic plants [1]. In the field of plant biotechnology, *A. tumefaciens* is widely employed to genetically modify plant genomes with desirable traits, such as enhanced nutritional content [2], disease resistance [3], and improved yield, or to vehicle plant-derived edible vaccines [4]. Traditional transformation methodologies using *A. tumefaciens* include electroporation, direct DNA transfer using chemically competent protocols, and the triparental mating method. Electroporation is a widely used approach, involving the application of an electrical pulse in the bacterial membrane to form pores for penetration of the DNA [5]. Despite being extremely efficient, it needs accurately optimized conditions and specialized equipment, limiting its accessibility for routine use [6]. Chemically competent methods, such as polyethylene glycol (PEG)-mediated transformation, are less sophisticated [7]; however, they present lower transformation efficiency in comparison to electroporation. The triparental mating technique, discovered by Herrera-Estrella et al. in 1983, involves the use of three strains, namely a donor, a helper, and a recipient [8]. It is highly effective yet time-consuming and very labor-intensive.

The freeze-thaw method simplifies the transformation process by eliminating the need for complex electroporation steps or triparental mating setup, making it more adaptable and accessible for various laboratory investigations [9]. The use of miniprep plasmid DNA directly from *Escherichia coli* reduces turnaround time, allowing for simple and rapid plasmid DNA isolation [10], coupled with uncompromised transformation efficiency [11]. This streamlined approach minimizes procedural errors and contamination chances that may occur when performing transformation experiments with more complex methodologies [12]. Additionally, the protocol is cost-effective, as it does not rely on expensive reagents or equipment; hence, it is easily reproducible and suitable, especially for laboratories with limited resources [13]. Also, it reports a low copy number of integrated transgenes, together with the possibility to transfer larger fragments of DNA. Nevertheless, the protocol is not without its limitations, as the transformation efficiency may be lower than that of optimized electroporation, particularly when dealing with complex gene constructs or larger plasmids [14]. Furthermore, the freeze-

thaw method may need additional optimization when applied to different plasmid types or *Agrobacterium* strains, as variations in DNA uptake capabilities can influence transformation success [15]. Regardless of all these limitations, the protocol remains a practical alternative for routine transformation practices, especially in settings where simplicity and reliability are prioritized over maximal efficiency.

The protocol described in this article has broad utility and is a valuable tool in both basic and applied research across diverse areas of synthetic and microbial biotechnology [16]. It can be employed to introduce plasmids carrying reporter genes, selectable markers, and CRISPR/Cas9 components into *A. tumefaciens*, facilitating studies on gene expression, gene editing, and functional genomics in plants [17]. Similarly, the method can be extended to develop transgenic plants for biopharmaceutical production, environmental remediation, and biofortification.

Materials and reagents

The equipment, materials, and reagents listed below are appropriate for this protocol. Nonetheless, substitutes sourced elsewhere may be employed if they have shown comparable performance.

Biological materials

1. *A. tumefaciens* EHA105 strain (strains are preserved at -80 °C in our laboratory)
2. *E. coli* K12 strain (strains are preserved at -80 °C in our laboratory)
3. Plasmid DNA (stored at -80 °C in our laboratory)

Reagents

1. Lysogeny broth (LB) low salt (Sigma-Aldrich, catalog number: L3397-250G)
2. Agar powder, bacteriological grade (Himedia, catalog number: GRM026-500G)
3. Oligonucleotides (forward: AGGAAACAGCTATGACCATGATTACGAATTC, reverse: ACGTTGTAAAACGACGGCCAGTGCCAAGCTT)
4. OneTaq 2× Master Mix with standard buffer (New England Biolabs, catalog number: M0482)
5. 1 Kb Plus DNA marker (Invitrogen, catalog number: 10787-018)
6. SafeView gel stain (Applied Biological Materials, catalog number: G108)
7. DNA loading dye (Biolabs, catalog number: 10158560)
8. LE agarose (Cleaver Scientific, catalog number: 9012-36-6)
9. TAE buffer (Biological Industries, catalog number: 01-870-1A)
10. Nuclease-free water (BioConcept, catalog number: 3-07F04-H)
11. Rifampicin (GoldBio, catalog number: GB-R-120)
12. Kanamycin sulfate powder (Fisher Scientific, catalog number: BP 906-100)
13. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)

Solutions

1. LB low-salt liquid medium (see Recipes)
2. LB low-salt agar solid medium (see Recipes)
3. 1% gel (see Recipes)
4. Rifampicin (see Recipes)
5. Kanamycin (see Recipes)

Recipes

1. LB low-salt liquid medium, pH 7.0

Reagent	Final concentration	Quantity or Volume
Distilled water	n/a	100 mL
LB low salt	20 g/L	2 g

2. LB low-salt agar solid medium, pH 7.0

Reagent	Final concentration	Quantity or Volume
Distilled water	n/a	100 mL
LB low salt	20 g/L	2 g
Agar	1.12% (w/v)	1.12 g

3. 1% gel

Reagent	Final concentration	Quantity or Volume
LE agarose	n/a	0.5 g
TAE buffer	1×	50 mL
SafeView gel stain	n/a	2 μL

4. Rifampicin

Reagent	Final concentration	Quantity or Volume
Rifampicin	50 mg/mL	0.5 g
DMSO	99.9%	10 mL

5. Kanamycin

Reagent	Final concentration	Quantity or Volume
Kanamycin sulfate	50 mg/mL	5 g
Distilled water	n/a	100 mL

Laboratory supplies

1. Petri plates (Borosil, catalog number: 3160072)
2. 500 mL culture bottle (Beckman, catalog number: 356011)
3. Powder-free nitrile gloves (Starlab, article number: SG-N-M)
4. Parafilm (Amor, catalog number: PM-996)
5. PCR reaction strips (Simport, catalog number: 330180784)
6. 10 μL pipette tips (AHN Biotechnologie, catalog number: P-214782)
7. 1,000 μL pipette tips (Biologix group, catalog number: MO002462ZA1230-12)
8. P10 micropipette (Eppendorf, catalog number: 4861000-0005)
9. P1000 micropipette (Eppendorf, catalog number: 4861000-0001)
10. 50 mL Falcon tubes (Biologix group, catalog number: 10-9502)
11. 1.5 mL microcentrifuge safe-lock tubes (Eppendorf, catalog number: 022363204)
12. Cell spreaders (ISOLAB, catalog number: CPSA20000002)
13. Isolate II Plasmid DNA kit (Bioline Meridian Biosciences, catalog number: BIO-52056)
14. Laboratory forceps (Thermo Fisher Scientific, catalog number: 112805)
15. Spectrophotometer cuvettes (Thermo Fisher Scientific, catalog number: 14-955-127)
16. Syringe microfilters 0.22 μm (GE Healthcare, catalog number: 289320100)

Equipment

1. Water bath (Cole-Parmer, model: WB-200)
2. Gel doc imaging (UVITEC Cambridge, model: UVIOOC H06)
3. Gel electrophoresis (Cleaver Scientific, model: NANOPAC300P)
4. Clean bench (Haier Biomedical, model: HCB-1600H)
5. Growth chamber (Daihan LabTech, model: LG C-5301)
6. Laboratory freezer (Haier Biomedical, catalog number: 0270501824)
7. Laboratory refrigerator (Haier Biomedical, catalog number: 0270501219A)
8. Thermal cycler (Eppendorf AG, model: 22331 Hamburg)
9. Biological safety cabinet (Haier Biomedical, model: HR1200-IIA2-S)
10. Shaking incubator (Winpact, model: SI-200)

11. Refrigerated microcentrifuge (Eppendorf, catalog number: 5430)
12. 50 mL high-efficient centrifuge (Hermile, catalog number: Z 446 K)
13. BioSpectrometer (Eppendorf, catalog number: 6135K1605057)
14. Laboratory water distiller (Liston, model: A 1210)
15. NanoDrop One (Thermo Fisher Scientific, model: 58595 Intertek)
16. Vertical high-pressure sterilization pot (Labnics, model: NVA-104)
17. Weighing balance (Mettler Toledo, model: PB1302-S/FACT)
18. pH meter (HANNA Instruments, model: H12211)
19. Variable speed vortex mixer (Thermo Fisher Scientific, catalog number: 9501200)

Software and datasets

1. Primer3 (4.1.0, 11/04/2017)

Procedure

A. Plasmid DNA extraction

1. Isolation of plasmid DNA from *E. coli* K12 strain.

a. Grow *E. coli* K12 cells containing the desired plasmid.

NOTE: The gene construct in this experiment was cloned into the pCAMBIA1301 plasmid, which contains kanamycin resistance gene for bacterial selection [18] and hygromycin B resistance gene for plant selection [19].

CAUTION: The *E. coli* cells were grown from colony master plates for 16 h in LB low-salt liquid medium supplemented with 50 mg/L kanamycin at 37 °C and 200 rpm in the shaking incubator (Figure S1).

b. Perform a plasmid miniprep to extract high-copy DNA under the biological safety cabinet (Figure 1).

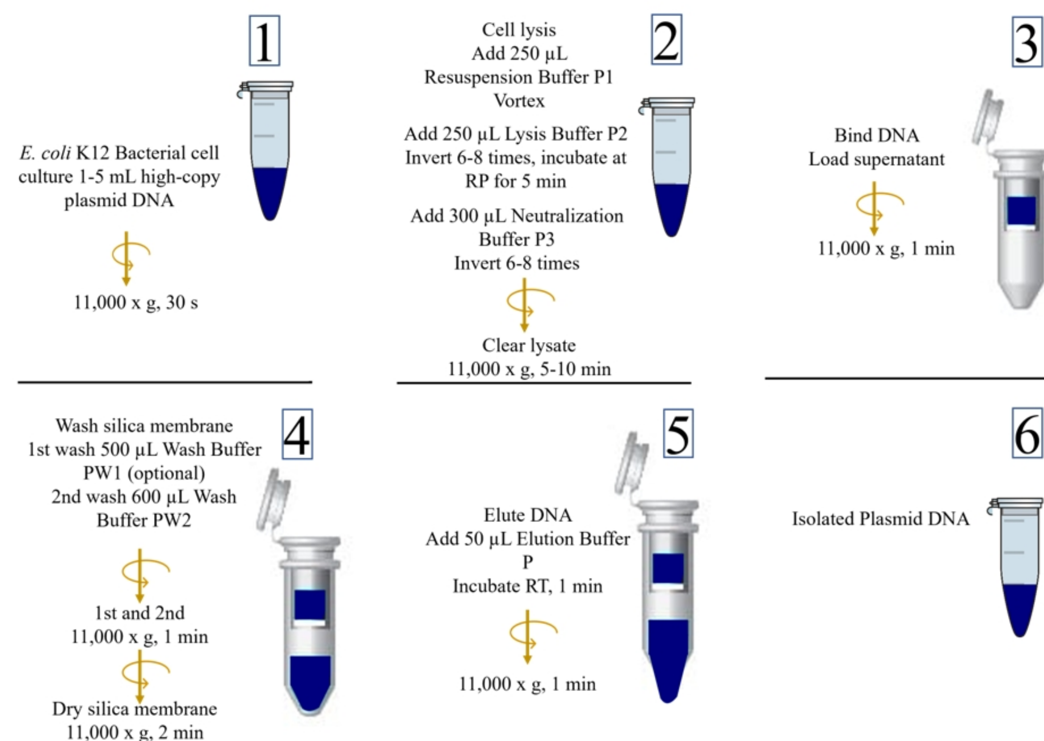


Figure 1. Isolation of high-copy plasmid DNA from *E. coli* K12 strain. The ISOLATE II Plasmid Mini kit was used to extract plasmid DNA from the bacterial cells containing the expression cassette of the gene of interest.

B. *Agrobacterium* setup

1. Preparation of *Agrobacterium tumefaciens* EHA105 cells.
a. Source for *A. tumefaciens* cells and test their viability.
b. Make 100 mL of LB low-salt agar solid medium (see Recipes) and sterilize it in a high-pressure sterilizing pot at 121 °C for 20 min.
c. Let it cool to 50 °C, then add 100 μL of rifampicin 50 mg/L and dispense 50 mL in two Petri plates, i.e., 25 mL each (Figure 2b).
NOTE: Agrobacterium tumefaciens *strain EHA105 contains rifampicin-resistance gene [20].*
d. Add 50 μL of kanamycin 50 mg/L to the remaining 50 mL of the media and dispense it in two separate Petri plates (Figure 2a).
e. Allow the media to jellify and then gently streak the *A. tumefaciens* colonies using sterile pipette tips.
f. Label and seal the plates with parafilm and incubate for 48 h at 28 °C.

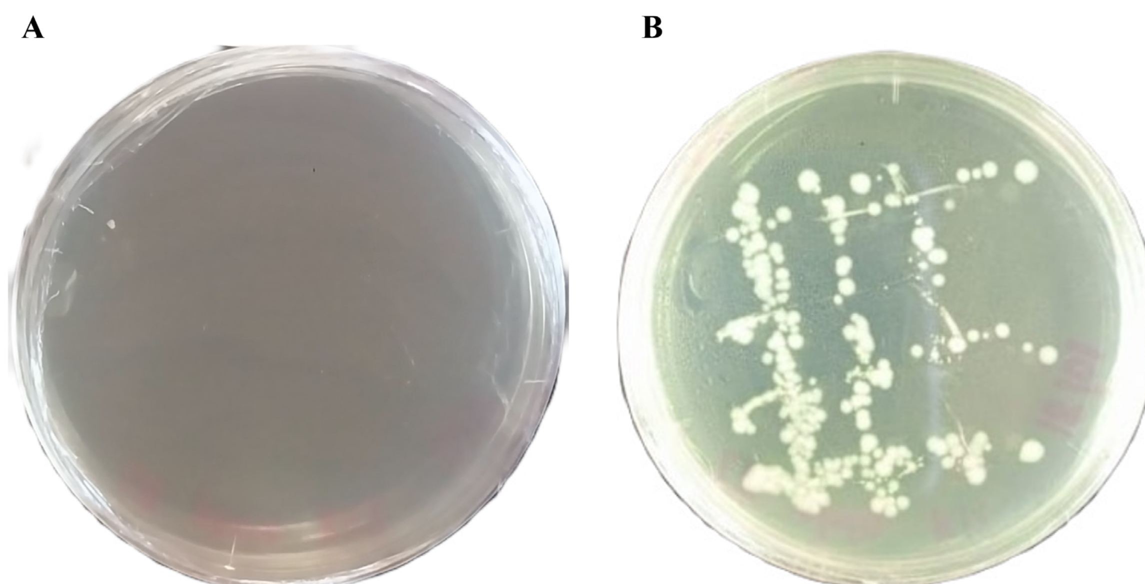


Figure 2. Prepared *A. tumefaciens* EHA105 cells. (A) No growth of colonies on LB solid media supplemented with rifampicin and kanamycin. The colonies were not expected to grow on this media before transformation, as they are devoid of the plasmid. Hence, the observation of no colony growth confirms the viability of this *Agrobacterium* strain. (B) Visible growth of incubated *A. tumefaciens* colony inoculated on LB solid media with rifampicin. Well-grown colonies are observed as a double confirmation that the cells are viable and suitable to proceed with the transformation.

2. Grow *A. tumefaciens* EHA105 cells to the mid-log phase.
a. Take a fresh, large, and distinct colony from the cells on the plate (Figure 2B), working under a clean bench.
b. Inoculate it in 250 mL of liquid LB media supplemented with only rifampicin in a 500 mL culture bottle.
c. Properly seal the culture bottle and incubate on a shaking incubator set at 300 rpm for 24 h at 28 °C.
d. Check for the optic density (OD_{600} 0.4) using the spectrophotometer.
CAUTION: Blank the spectrophotometer with plain LB liquid medium (without antibiotics) inside a sterile cuvette before loading the sample.

3. Making competent cells.
a. Pellet the cells at 4,500× *g* for 10 min.
b. Discard the supernatant.
c. Resuspend the pellet in 25 mL of fresh LB liquid media supplemented with rifampicin.
d. Aliquot 250 μL of cells in 1.5 mL Eppendorf tubes.
e. Freeze the tubes in liquid nitrogen for 10 s.
CAUTION: See general note 6. Follow safety guidelines when working with liquid nitrogen.
f. Store the tubes at -80 °C.

C. Bacterial transformation

1. Transformation of *E. coli* plasmid DNA into *A. tumefaciens.*
a. Thaw frozen competent cells on dry ice for approximately 3 min.
CAUTION: Do not let the last sliver of ice melt.
b. When the cells are close to complete thawing, place on ice immediately.
c. Split the 250 μL into two tubes, to have 125 μL of cells per tube.
d. Add 5 μL of standard mini-prepared binary plasmid DNA.
CRITICAL: A DNA concentration of 50–100 ng is enough to grow the number of colonies needed.
e. Gently mix by flicking the tube with your finger.
f. For precisely 10 s, freeze the tube in liquid nitrogen with the help of laboratory forceps.
g. Vertically immerse the tubes without the cap, submerging them in the liquid nitrogen.
NOTE: Have the water bath nearby or on the same working bench as the liquid nitrogen container.

2. Use heat-shock to facilitate the uptake of DNA by *A. tumefaciens* competent cells.
a. Place the tubes in a 37 °C water bath for exactly 5 min.
NOTE: Some tubes may pop out due to the change in temperature; this is expected. Beware of them bursting toward your face.

3. Grow the mixture on a shaker.
a. Lay the tubes horizontally in the shaking incubator.
b. Set the tubes to shake at 300 rpm for 60 min at 28 °C.
NOTE: The cells should have attained OD_{600} 0.4 to ascertain exponential growth.

4. Colony selection and confirmation.
a. Plate 50 μL of the transformed *A. tumefaciens* cells on a Petri plate containing agar LB media supplemented with rifampicin and kanamycin
b. Grow in a 28 °C incubator for 48 h.
NOTE: Between 20 and 500 colonies are expected (Figure 3).

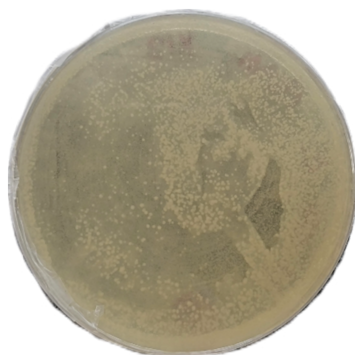


Figure 3. Colonies of transformed *A. tumefaciens*. Positive colonies following the successful transformation were grown on LB solid media with rifampicin and kanamycin for 48 h in a 28 °C incubator.

5. Run PCR to confirm successfully transformed colonies.
a. Design primers of pCAMBIA1301 binary vector targeting the gene of interest cloned in the expression cassette using Primer3 software (Table 1).
b. Synthesize the primers and dissolve them to 10μM.
CRITICAL: Make a 10× dilution to get 10μM. For 100 μL of 10μM working solution from the 100μM primer stock, add 10 μL of stock to 90 μL of nuclease-free water.

Table 1. Amplification primer sequences for binary plant vector pCAMBIA1301

	pCAMBIA1301	
Forward primer	AGGAAACAGCTATGACCATGA	TTACGAATTC
Reverse primer	ACGTTGTAAAACGACGGCCAG	TGCCAAGCTT

c. Get 1 µL of a distinct colony as DNA template, add primers (Table 2), and carry out the PCR procedure.

Table 2. PCR reaction master mix with standard buffer

Reagent	Amount
OneTaq2× master mix	12.5 µL
DNA template	1 µL
10 µM forward primer	0.5 µL
10 µM reverse primer	0.5 µL
Nuclease-free water	10.5 µL

d. Set the PCR conditions on the thermal cycler as shown in Table 3.

Table 3. Thermocycling conditions for the PCR reaction

Step	Temp. (°C)	Duration	No. of cycles
Initial denaturation	95	30 s	1
Denaturation	95	30 s	
Annealing	53	1 min	35
Extension	68	1 min per kb	
Final extension	68	5 min	1
Hold	4	∞	-

e. Prepare 50 mL of 1% LE agarose gel.
f. Heat it in a microwave until it is entirely dissolved (usually takes 1 min).
g. Let it cool down and then add 2 µL of SafeView gel staining solution.
CRITICAL: Be careful not to let the gel solidify before adding SafeView.
h. Mix gently and then pour the mixture into a gel tray with a gel comb to create wells.
i. Load 2 µL of the DNA marker in the first well.
j. Add 1 µL of DNA loading dye to each sample, then load them one after the other in the subsequent wells.
k. Set the gel electrophoresis apparatus to run at 160 V for 15 min.
l. Visualize the gel using the gel doc imaging system to assess band size.

6. Grow positive transformants on selection media.
a. Select one distinct positive colony.
b. Grow it on an LB solid plate containing selection media.
c. Seal the plate with parafilm and incubate at 28 °C for 48 h.
d. The positive colony from the master plate is expected to grow as shown in Figure 4.
NOTE: The bacterial culture is ready for plant genetic transformation.

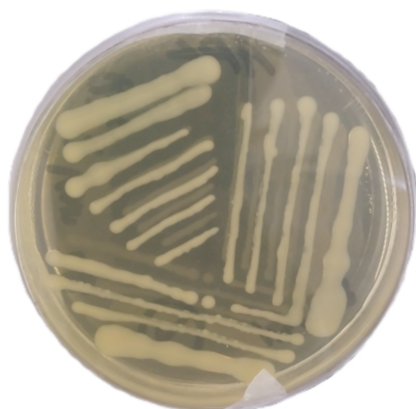


Figure 4. Positively transformed colony on LB medium supplemented with antibiotics. For plant genetic transformation, use a freshly grown positive colony for better results.

Data analysis

In this protocol, plasmid DNA requires stringent quality control measures and validation assessment. Thus, the first step is undertaken to measure and analyze the concentration and purity of the extracted plasmid DNA from the *E. coli* cells. This is a vital step in determining the quality of the isolation process. The PCR technique and gel electrophoresis are then performed to verify the size of the gene of interest. In this protocol, we present an analysis of the data collected and provide experimental references that align with Xu et al [21].
Data collected from the transformation experiments typically include the counted number of *A. tumefaciens* colonies that grew on selective media supplemented with correct antibiotics after the freeze-thaw method. Any notable features such as growth rate and colony morphology were observed and recorded. Only distinct colonies from clear LB solid media plates were included, as an indication of successful eyes-test transformation. Data from plates showing signs of contamination were excluded as well as those that appeared abnormal (possible false positives). Data from samples where the DNA quality was below acceptable standards, i.e., A_{260}/A_{280} ratio outside a 1.8–2.0 range, were disregarded.

A. Nanodrop analysis of plasmid DNA concentration and purity

Using NanoDrop One, the concentration and purity of the extracted plasmid DNA can be measured to assess the quality of the isolated *E. coli* K12 cells. The concentration is the amount of plasmid DNA in ng/μL, and a volume between 50 and 100 ng/μL is sufficient. The 260/280 purity ratio indicates protein contamination; it should be between 1.8 and 2.0 for pure DNA. Salt and contaminant presence are depicted by a 260/230 ratio, which should be greater than 2.0 for pure DNA. Results obtained show that the concentration, protein contamination, and salt presence of the double-stranded DNA are 88.8 ng/μL, 1.86, and 2.02, respectively (Figure 5).

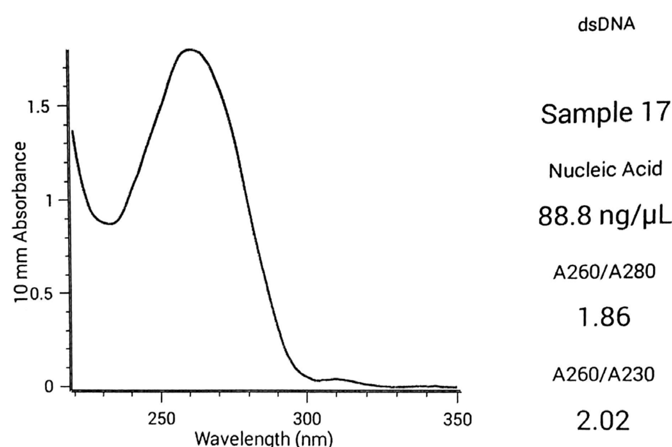


Figure 5. *E. coli* K12 plasmid DNA concentration and purity measured by the Nanodrop spectrophotometer method. Based on the Nanodrop software results and measurement analysis, we can deduce that the concentration of the measured sample is 88.8 ng/μL, while the 260/280 purity ratio is 1.86, and the 260/230 purity ratio is 2.02.

B. PCR verification of the positive transformants

In this experiment, we selected one distinct colony as an important indicator for detecting the presence of the gene of interest. PCR was conducted on the purified sample to amplify the gene sequence; we observed the characteristic band size (Figure 6) using gel electrophoresis, which further showed that the expected size was present in the transformed *A. tumefaciens* cells.

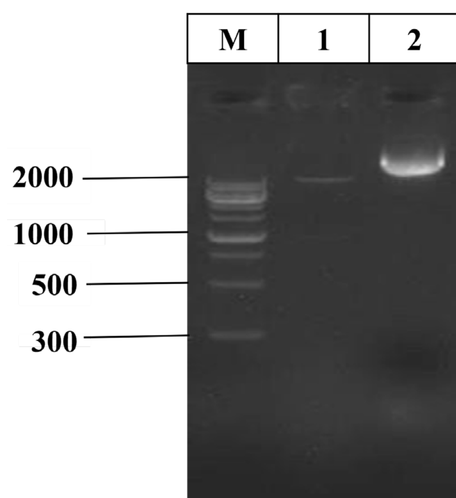


Figure 6. PCR verification of positive transformants. Lane M: DNA marker. The size of the gene of interest is 1,919 bp, and significant enrichment is visible around 2,000 bp in lanes 1 and 2. This ascertains the presence of the gene of interest in the transformed bacterial cells.

Validation of protocol

Parts of this protocol have been used and validated in the following research article:

• The methods described in this paper are widely accepted and have been used in the fields of plant biotechnology and genetic engineering. The protocol showed positive transgenic efficiency of up to 68.79% in Irish potato tetraploid recipient, Desiree cultivar [21]. Similarly, the procedure recorded a successful transformation of *Agrobacterium tumefaciens* EHA105 cells with plasmid DNA from *E. coli* strain K12, resulting in a DNA purity ratio A260/A230 of 2.02 (Figure 5). Furthermore, PCR analysis confirmed the presence of the transgene in the transformed Agrobacterium cells, with a 100% amplification rate (Figure 6).

General notes and troubleshooting

General notes

1. To prevent contamination, ensure that conditions are always sterile throughout the experiment. Autoclave all laboratory supplies, sterilize equipment, and do not use expired reagents. Perform the work within a clean bench or biological safety cabinet.
2. Ensure that the plasmid DNA extracted from *E. coli* cells is free from contaminants and of high quality. For best results, use freshly isolated plasmid DNA, as degradation can lower transformation efficiency.
3. To attain high competence levels, prepare *A. tumefaciens* cells appropriately. Confirm that cells are at mid-log phase before making them competent, as older or stationary-phase cells are less likely to take up DNA efficiently.
4. After transformation, use suitable selective media to correctly identify successfully transformed *A. tumefaciens* colonies. Include the right antibiotics corresponding to the plasmid's resistance markers.
5. Beware of the plasmid type and size; larger plasmids may need protocol modifications, such as increasing the amount of DNA to be eluted.
6. Handle liquid nitrogen with care as it is extremely cold (-196 °C) and can cause severe frostbite burns. Wear protective gear, use proper storage and handling containers with tight-fitting lids, and secure handles. Use in well-ventilated areas of the laboratory, label containers clearly, and store them upright and away from heat sources. When the experiment is completed, follow safety protocols for the disposal of liquid nitrogen.

Troubleshooting

Problem 1: Low transformation efficiencies.
Possible causes:
- Low-quality or degraded plasmid DNA.
- Improper preparation of competent *A. tumefaciens* cells (e.g., cells not at the correct growth phase).
- Insufficient DNA concentration or volume during the transformation step.
- Suboptimal heat shock conditions.
Solutions:
- Verify the quality and concentration of plasmid DNA using spectrophotometry.
- Confirm that *A. tumefaciens* cells are at mid-log phase (OD_{600} around 0.5–0.8) before making them competent.
- Optimize the amount of plasmid DNA used (generally 50–100 ng is sufficient).
- Adjust transformation conditions (e.g., duration, temperature) to fit the cell type and DNA used.

Problem 2: No growth on selective media.
Possible causes:
- Antibiotic concentration may be too high.
- Incorrect antibiotics or selection markers.
- Inefficient DNA uptake by *A. tumefaciens* cells.
Solutions:
- Check the antibiotic concentration and confirm it is compatible with the selection of transformed *A. tumefaciens.*
- Verify that correct antibiotics match the resistance markers present on the plasmid.
- Ensure that competent cells were prepared and stored correctly. Freshly prepared cells generally give better results.

Problem 3: Contamination of cultures.
Possible causes:
- Non-sterile technique during handling.
- Contaminated reagents or equipment.
Solutions:
- Perform all steps under sterile conditions and disinfect tools and surfaces regularly.
- Use fresh, sterile reagents and autoclaved consumables. Periodically check cultures for contaminants.

Problem 4: Inconsistent results.
Possible cause: Variations in cell competence, DNA quality, or environmental parameters.
Solutions:
- Maintain consistent growth conditions for bacterial cultures.
- Standardize procedures for DNA extraction and transformation.
- Repeat experiments to confirm reproducibility and refine any variable steps.

Supplementary information

The following supporting information can be downloaded here:
1. Figure S1. Making competent *E. coli* cells using $CaCl_2$ and the heat-shock method

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Competing interests

The authors declare no competing interests.

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