

Protocol for the High-quality Plasmid Isolation from Different Recalcitrant Bacterial Species: *Agrobacterium* spp., *Rhizobium* sp., and *Bacillus thuringiensis*

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Abstract

High yield of good quality plasmid DNA from gram -ve bacteria (*Agrobacterium tumefaciens*, *A. rhizogenes*, and *Rhizobium* sp.) and gram +ve bacterium (*Bacillus thuringiensis*) is difficult. The widely used plasmid extraction kits for *Escherichia coli* yield a low quantity of poor-quality plasmid DNA from these species. We have optimized an in-house modification of the QIAprep Spin Miniprep kit protocol of Qiagen, consisting of two extraction steps. In the first, the centrifugation after adding neutralization buffer is followed by ethanol (absolute) precipitation of plasmid DNA. In the second extraction step, the precipitated DNA is dissolved in Tris-EDTA (TE) buffer, followed by an addition of 0.5 volumes of 5 M sodium chloride and 0.1 volumes of 20% (w/v) sodium dodecyl sulfate. After incubation at 65 °C for 15 min, the plasmid DNA is extracted with an equal volume of chloroform:isoamyl alcohol (CIA). RNase (20 mg/mL) is added to the upper phase retrieved after centrifugation and is incubated at 37 °C for 15 min. The extraction of the plasmid DNA with an equal volume of CIA is followed by centrifugation and is precipitated from the retrieved upper phase by adding an equal volume of absolute ethanol. The pellet obtained after centrifugation is washed twice with 70% (v/v) ethanol, air dried, dissolved in TE buffer, and quantified. This easy-to-perform protocol is free from phenol extraction, density gradient steps, and DNA binding columns, and yields high-quality plasmid DNA. The protocol opens an easy scale up to yield a large amount of high-quality plasmid DNA, useful for high-throughput downstream applications.

Key features

- The protocol is free from density gradient steps and use of phenol.
- The protocol is an extension of the QIAprep Spin Miniprep kit (Qiagen) and is applicable for plasmid DNA isolation from difficult-to-extract bacterial species.
- The protocol facilitates the direct transformation of the ligation product into *Agrobacterium* by skipping the step of *E. coli* transformation.
- The plasmids isolated are of sequencing grade and the method is useful for extracting plasmids for metagenomic studies.

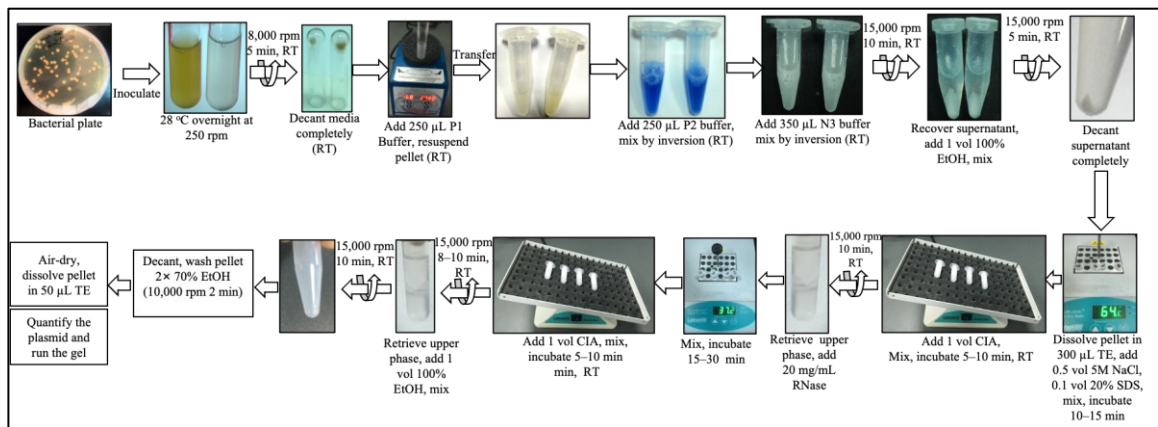
Keywords: *Agrobacterium*, *Bacillus thuringiensis*, Modified QIAprep Spin Miniprep kit protocol, Plasmid DNA, Low-copy number bacterial strains, *Rhizobium*, Sodium dodecyl sulfate

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Graphical overview



Overview of the plasmid isolation protocol (modified QIAprep Spin Miniprep kit) of the present study

Background

Plasmid isolation is an essential procedure in gene cloning, gene expression studies, sequencing, mutagenesis, and several downstream molecular processes. Easy-to-extract proprietary kits at different formats such as mini, midi, and maxi, and several published protocols are best suited for the plasmid extraction from widely used *Escherichia coli* strains. A simple and reliable method for isolating high-quality plasmid for downstream molecular applications from bacteria such as *Agrobacterium tumefaciens*, *A. rhizogenes*, *Rhizobium* sp., and *Bacillus thuringiensis* is lacking, due to the recalcitrance of the bacterial strains to cell lysis. Besides, lysozyme in the cell lysis solution is required to circumvent the non-lysis of the cell wall (Marmur, 1961). The *Agrobacterium* cells are not sensitive to the lysozyme-EDTA-detergent lysis procedures (Marmur, 1961; Clewell and Helinski, 1969), necessitating a relatively long treatment with proteolytic enzymes (Zaenen et al., 1974). Further, it is challenging to isolate Ti-plasmid free of chromosomal DNA (Ledebouer et al., 1976). In the case of agrobacteria, which is used to develop genetically modified and genome-edited plants, if there is no stock culture of *E. coli* with the desired plasmid, it necessitates retransformation of the plasmid into *E. coli* and subsequent extraction of plasmids for restriction digestion verification (Wise et al., 2006) and other downstream applications, e.g., biolistic transformation. There is no reliable protocol to extract high-quality plasmid DNA in large amounts from agrobacteria to accomplish downstream applications directly, i.e., without *E. coli* retransformation. The protocols described for the extraction of plasmid DNA from *Agrobacterium*, *Rhizobium*, and *Bacillus thuringiensis* are relatively lengthy and consist of sucrose gradients, CsCl-dye buoyant density gradients, or ethidium bromide treatment followed by phenol extraction (Ledebouer et al., 1976; Adachi and Iyer, 1980; Zhang and Kerr, 1993; Reyes-Ramírez and Ibarra, 2008). Our attempts to isolate plasmid from the gram -ve bacteria (*Agrobacterium tumefaciens*, *A. rhizogenes*, *Rhizobium* sp., and gram +ve bacterium (*Bacillus thuringiensis*) using kits (QIAprep Spin Miniprep kit from Qiagen and PureLink Quick Plasmid Miniprep kit from Invitrogen) and the user-modified protocol of QIAprep plasmid kit (Weber et al. 1998; <https://www.qiagen.com/no/resources/resource/detail?id=95083ccb-9583-489e-b215-99bd91c0604e&lang=en>) did not yield satisfactory results. Extracting and purifying plasmid DNA from these strains and diverse other bacterial strains, recalcitrant to quality plasmid isolation, warrant a simple, short, and reliable protocol. We believe that the present study's high-yielding, high-quality plasmid DNA protocol will be useful for other bacterial strains resistant to cell lysis, especially for low-copy number plasmid strains.

Materials and reagents

Biological materials

1. *Agrobacterium tumefaciens* strain EHA105, AGL1, GV3101, and LBA4404 [all containing binary plasmid pCAMBIA 1201 harboring *gusA* gene under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter and bacterial selection marker chloramphenicol]
2. *A. rhizogenes* strains ATCC15834 and A4, containing binary plasmid harboring green fluorescent protein (*GFP*) gene under the control of 35S promoter and bacterial selection marker kanamycin
3. *Rhizobium* sp. (isolated from root nodules of desert tree legume, *Prosopis cineraria*)
4. *Bacillus thuringiensis* (received as a gift from a colleague, collected from the United Arab Emirates)

Reagents

1. Luria-Bertani (LB) broth (LB Miller Modification; PhytoTech Labs, catalog number: L475)
2. Tryptone (PhytoTech Labs, catalog number: T832)
3. Yeast extract (PhytoTech Labs, catalog number: Y892)
4. Sodium chloride (NaCl) (Sigma, catalog number: S1679)
5. Potassium dihydrogen phosphate dibasic (KH₂PO₄) (Sigma, catalog number: P3786)
6. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (Sigma, catalog number: M2773)
7. Mannitol (Sigma, catalog number: M1902)
8. Bacto-agar (Himedia, catalog number: GRMO26)
9. Kanamycin sulfate (PhytoTech Labs, catalog number: K378)
10. Rifampicin (PhytoTech Labs, catalog number: R7382)
11. Formamide (Sigma, catalog number: 47671)
12. Chloramphenicol (PhytoTech Labs, catalog number: C1919)
13. QIAprep Spin Miniprep kit (Qiagen, catalog number: 27109)
14. PureLink Quick Plasmid Miniprep kit (Invitrogen, catalog number: K210011)
15. Resuspension solution P1 with RNase and Blue lysate (Qiagen, catalog number: 19051)
16. Resuspension buffer R3 (Invitrogen, catalog number: K2100-14)
17. Lysis solution P2 (Qiagen, catalog number: 19052)
18. Lysis buffer L7 (Invitrogen, catalog number: K2100-14)
19. Neutralizing solution N3 (Qiagen, catalog number: 19064)
20. Precipitation buffer N4 (Invitrogen, catalog number: K2100-14)
21. Sodium dodecyl sulfate (SDS) (Sigma, catalog number: L3771)
22. RNase (Thermo Scientific, catalog number: EN0531)
23. Ethanol absolute (Sigma, catalog number: NC1971050)
24. Disodium ethylene diamine tetraacetate (Na₂EDTA·2H₂O) (Sigma, catalog number: E6760)
25. Tris-base (Sigma, catalog number: T1503)
26. Hydrochloric acid (HCl) (Sigma, catalog number: 07102)
27. Sodium hydroxide (NaOH) (Sigma, catalog number: 06203)
28. Chloroform (Sigma-Aldrich, catalog number: C2432)
29. Glacial acetic acid (VWR, catalog number: VWRC20104.334)
30. Isoamyl alcohol (Sigma, catalog number: 19392)
31. Methanol Chromasolv™ (Sigma-Aldrich, catalog number: 34860-2.5L-R)
32. *Nco*I-HF (NEB, catalog number: R3193M), storage at -20 °C
33. *Bst*EII-HF (NEB, catalog number: R3162M), storage at -20 °C
34. HotStar Taq DNA Polymerase PCR kit (Qiagen, catalog number: 203205)
35. PCR primers (Macrogen, South Korea)
36. Agarose D1 Low CE (Conda Lab, catalog number: 8010.00)
37. Hydragreen™ Safe DNA Dye (20,000×) (ACTGene, catalog number: ACT-IDMG04)

38. 6× DNA loading dye (NEB, catalog number: B7024A)
39. GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific, catalog number: SM1333)
40. Lambda DNA/*EcoRI* Plus *HindIII* marker (Thermo Fisher Scientific, catalog number: SM0191)

Solutions

1. 1 N HCl
2. 1 N NaOH
3. LB *Agrobacterium* medium (see Recipes)
4. Yeast Mannitol medium (see Recipes)
5. Tris-EDTA (TE; see Recipes)
6. Tris (see Recipes)
7. Na₂EDTA (see Recipes)
8. Kanamycin (see Recipes)
9. Rifampicin (see Recipes)
10. Tris-acetate-EDTA (TAE, see Recipes)
11. Chloramphenicol (see Recipes)
12. 5 M Sodium chloride (see Recipes)
13. 20% (w/v) SDS (see Recipes)
14. Chloroform:isoamyl alcohol (CIA) (see Recipes)
15. Ethanol (70%, v/v) (see Recipes)
16. Media components (1 L) for the bacterial culture (see Recipes)
17. Antibiotics preparation (see Recipes)
18. Stock solutions (see Recipes)
19. Tris-EDTA (TE) buffer (100 mL) (see Recipes)
20. 10× Tris-acetate-EDTA (TAE) (see Recipes)
21. Agarose gel (0.8%, w/v) preparation (see Recipes)

Recipes

1. Media components (1 L) for the bacterial culture

Components	LB <i>Agrobacterium</i> (g/L)	LB (g/L)	YM (g/L)
Tryptone	10 (5 g for LBA4404 strain)	10	
Yeast extract	5	5	0.1
NaCl	5	10	0.2
KH ₂ PO ₄			10
MgCl ₂ ·7H ₂ O			0.2
Mannitol			10

Dissolve the components in 800 mL of MilliQ water and adjust to 1 L using MilliQ water. Transfer into a Duran bottle. Adjust the pH to 7.0 using 1 N HCl/1 N NaOH.

For solid media, add bacto-agar 15 g/L after adjusting the pH.

Autoclave at 121 °C (15 psi) for 15 min and store at room temperature (RT) for two months.

2. Antibiotics preparation

Antibiotics	Preparation and storage	Working concentration
Kanamycin	Weigh 50 mg in a 1.5 mL Eppendorf, add 1 mL of sterile water, dissolve, and store at 4 °C for one week.	50 mg/L
Chloramphenicol	Weigh 25 mg in a 1.5 mL Eppendorf, add 1 mL of absolute ethanol, dissolve, and store at 4 °C for one week.	25 mg/L
Rifampicin	Weigh 20 mg in a 1.5 mL Eppendorf, add 1 mL of formamide*, dissolve and wrap with aluminum foil, and store at 4 °C for one week.	20 mg/L

*Methanol is an alternative solvent.

3. Stock solutions

Stock	Preparation and storage
1 M Tris-HCl (pH 8.0)	Dissolve 121.1 g of Tris-base in 800 mL of MilliQ water. Adjust the pH to 8.0 by adding concentrated HCl. Adjust to 1 L using MilliQ water. Transfer into a 1 L Duran bottle. Autoclave at 121 °C (15 psi) for 15 min. Store at RT for six months.
0.5 M EDTA (pH 8.0)	Weigh 93 g of disodium EDTA·2H ₂ O in ~400 mL of MilliQ. Stir vigorously on a magnetic stirrer with a stir bar. Adjust the pH to 8.0 with NaOH (~10 g of NaOH pellets). Adjust to 500 mL using MilliQ water. Transfer into a 500 mL Duran bottle. Autoclave at 121 °C (15 psi) for 15 min. Store at RT for six months.
5 M NaCl	Dissolve 29.2 g of NaCl in 80 mL of MilliQ water and adjust to 100 mL using MilliQ water. Transfer into a 100 mL Duran bottle. Autoclave at 121 °C (15 psi) for 15 min. Store in a Duran bottle at RT for six months.
SDS (20%, w/v)	Weigh 20 g of SDS, dissolve it in MilliQ water, and adjust to 100 mL using MilliQ water. Transfer into a 100 mL Duran bottle. Autoclave at 121 °C (15 psi) for 15 min. Store at RT for six months. Caution: use face masks during preparation.
Chloroform:isoamyl alcohol (24:1) (CIA)	Add 2 mL of isoamyl alcohol in a 50 mL sterile Falcon tube and adjust to 50 mL by adding chloroform. Mix well and cover with aluminum foil. Store at RT for two weeks. Caution: prepare in a fume hood.
70% (v/v) ethanol	Measure 35 mL of absolute ethanol in a 50 mL sterile Falcon tube, add 15 mL MilliQ water, and mix well. Store at RT for six months.

4. Tris-EDTA (TE) buffer (100 mL)

Components	Volume (mL)	Final concentration
Tris-HCl (pH 8.0)	1.0	10 mM
Na ₂ EDTA (pH 8.0)	0.2	1 mM
MilliQ water	98.8	

Autoclave in a 100 mL Duran bottle at 121 °C (15 psi) for 15 min and store at RT for six months.

5. 10× Tris-acetate-EDTA (TAE)

Components	Amount	Concentration 1×	1× preparation
Tris-base	48.5 g	40 mM	Mix 10 mL of 10× with 90 mL
Na ₂ EDTA (pH 8.0)	20 mL	1 mM	MilliQ water
Glacial acetic acid	11.4 mL	20 mM	

Dissolve tris-base in approximately 800 mL of MilliQ water. Add acetic acid and EDTA. Adjust to 1 L. Transfer into 1 L Duran bottle. Store at RT for six months.

6. Agarose gel (0.8%, w/v) preparation

Weigh 0.48 g of agarose and add into 60 mL of 1× TAE buffer in a 250 mL Erlenmeyer flask. Microwave for 2 min, cool to 50 °C, add 6 µL of Hydragreen, and cast in the gel tray with 15 wells combs.

Laboratory supplies

- 90 mm Petri dish (Sangon Biotech, catalog number: F611001)
- Safe-lock tubes (2.0 and 1.5 mL) (Eppendorf, catalog number: 0030 120.094)
- Pipettes (1,000, 200, 100, 20, and 10 µL) (Rainin, catalog number: L1000XLS+, L200XLS+, L100XLS+, L20XLS+, and L10XLS+, respectively)
- Duran bottle [DURAN® ORIGINAL GL 45, catalog number: 10113399 (1,000 mL) and 10108298 (500 mL)]
- Beakers (0.6 and 1 L) (Nalgene, catalog number: 1201-0600 and 1201-1000, respectively)
- PYREX® 250 mL narrow mouth Erlenmeyer flask (Corning, catalog number: 4980-250)
- Graduated cylinders (0.5 and 1 L) (Nalgene, catalog number: 3662-0500 and 3662-1000, respectively)

8. Pipette tips (1,000, 250, and 20 μ L) (Rainin, catalog number: 30389294, 30389301, and 30389297, respectively)
9. Magnetic stirrer (Corning[®], model: PC-620D, catalog number: 6796-620D)
10. Stir bars (PhytoTech Labs, catalog number: B011)
11. Tube racks (Globe Scientific, catalog number: 456350B)
12. Kimwipes (KIMTECH, catalog number: 34120)
13. 14 mL sterile Falcon bacterial culture tubes (Falcon, catalog number: 352057)
14. Disposable gloves (Kimberly-Clark, catalog number: 52816)
15. Inoculation loops (VWR, catalog number: 10806-354)
16. Wide Mini-Sub Cell GT Horizontal Electrophoresis System, 15 cm \times 7 cm tray, with gel caster and casting gates (Bio-Rad Laboratories, catalog number: 1704469)
17. Weigh dish (Thomas Scientific, catalog number: 3846D19)
18. Weigh paper (Thomas Scientific, catalog number: 9885G50)
19. Spatula (PhytoTech Labs, KS, USA, catalog number: S798)
20. Face mask (Euromed, China, catalog number: EM1834)
21. Disposable cuvettes (BrandTech, catalog number: 759075D)

Equipment

1. Autoclave (Tomy, model number: SX-700)
2. Weighing scale (Mettler Toledo[®], model: MS603S/01)
3. Refrigerator (4 and -20 $^{\circ}$ C) (Evermed, model: BLCRF-370W)
4. pH meter (Thermo Fisher, model: Fisherbrand Accumet AB150, catalog number: 13636AB150B)
5. Laminar air flow cabinet (Esco, Horizontal Laminar Flow Cabinet, Gen 3, model: LHG-3AG-F8, catalog number: 2120387)
6. Bead sterilizer (PhytoTech Labs, model: ErgoSteri VT glass bead sterilizer, catalog number: S7520)
7. Shaker incubator (Eppendorf, model: New Brunswick Innova[®] 42, catalog number: M-1335-0004)
8. UV-Visible spectrophotometer (Thermo Scientific, model: Evolution 201, catalog number: 840-210600)
9. Centrifuge (Tomy, model: MX-307)
10. Vortexer (Scientific industries, model: Vortex-Genie[™] 2T mixer, catalog number: SI-T266)
11. Heating block (Labnet International, AccuBlock, Digital dry bath, model: Labnet International D1100, catalog number: D1100-230V)
12. Fume hood (Esco, Ascent[™] Max Ductless Fume Hood, model: ADC-4BI, catalog number: 2040042)
13. Nutating Mixer (Labnet International, model: Labnet GyroMini[™] Nutating 3-D Mixer with dimpled mat, 120 V, catalog number: S0500)
14. NanoDrop[™] (Thermo Scientific, catalog number: ND2000C)
15. Microwave (Samsung, model: ME733K)
16. MilliQ water purification system (MilliQ, model: Elix integral 10)
17. Gel Doc[™] EZ imager (Bio-Rad Laboratories, catalog number: 170-8270)
18. PowerPac[™] Basic (Bio-Rad Laboratories, catalog number: 164-5050)
19. Electrophoresis unit (Bio-Rad Laboratories, catalog number: 1704405)
20. Desktop (Dell, model: Optoplex 9020)
21. Thermal cycler (Bio-Rad Laboratories, model: T100)

Software and datasets

1. NanoDrop 2000 (version 1.6, free, Thermo Fisher Scientific)
2. Image Lab 6.0 (Bio-Rad Laboratories)
3. Insight2 (Thermo Fisher Scientific, catalog number: 837-002700)
4. Microsoft Excel for Mac (v16.69.1, 2022)

Procedure

Note: All steps are carried out at room temperature (RT) unless otherwise stated.

1. Streak the bacterial species: EHA105, AGL1, GV3101, and LBA4404 strains on solid LB *Agrobacterium* media supplemented with 20 mg/L rifampicin and 25 mg/L chloramphenicol; *Bacillus thuringiensis* on LB solid medium; *A. rhizogenes* strains ATCC15834 and A4 on YM solid medium with 50 mg/L kanamycin; and *Rhizobium* sp. on YM solid medium (see Recipes). Incubate for two days at 28 °C in an incubator.
2. Transfer bacterial culture from step 1 into 5 mL of liquid media in 14 mL bacterial culture tubes with appropriate antibiotics as above specified to each strain and incubate at 28 °C overnight on an orbital shaker at 250 rpm.

Note: Transfer a single colony for A. tumefaciens, Rhizobium, and Bacillus, and 2–3 colonies of A. rhizogenes from the solid medium to 5 mL liquid medium. Check the OD of the culture using the spectrophotometer; use culture media with no bacteria as blank. Cultures of A. tumefaciens with OD 0.5–0.7 and an OD of 0.8–1.0 for other strains are used for plasmid extraction. A 10 mL culture of slow-growing or low-copy number plasmid results in a high yield.

3. Centrifuge the cultures at 9,000× g (8,000 rpm) for 5 min.
4. Decant the supernatant. Remove the liquid media completely by pipetting out or by keeping the tube upside-down on a Kimwipe for 2–5 min. (Caution: do not disturb the pellet.)
5. Add 250 µL of resuspension solution (P1 buffer containing RNase and Blue lysate of QIAprep Spin Miniprep kit) into each tube and vortex to resuspend the cell pellet completely.
6. Transfer the resuspended bacterial pellet into 1.5 mL Eppendorf tubes using a pipette.
7. Add 250 µL of lysis solution (P2 buffer of QIAprep Spin Miniprep kit) and mix by gently inverting the tubes 5–6 times. The solution quickly turns blue and viscous, indicating bacterial lysis.
8. Add 350 µL of neutralizing solution (N3 buffer of QIAprep Spin Miniprep kit) and mix by inverting the tubes 5–6 times. The blue solution turns white, and the bacterial chromosomal DNA appears as a white precipitate.
9. Centrifuge the tubes at 20,000× g (15,000 rpm) for 10 min.
10. Carefully transfer the supernatant into new 1.5 mL Eppendorf tubes using a pipette. (Caution: do not disturb the white precipitate.)
11. Add an equal volume of absolute ethanol into each tube and mix by inverting the tubes a few times.
12. Centrifuge the tubes at 20,000× g (15,000 rpm) for 5 min.
13. Decant the supernatant and carefully remove the liquid completely using a pipette or by leaving the tube upside-down on a Kimwipe for 1–3 min.
14. Add 300 µL of TE buffer (pH 8.0) (see Recipes), 0.5 vol (150 µL) of 5 M NaCl, and 0.1 vol (45 µL) of 20% SDS. Mix by inversion and incubate at 65 °C in a heating block for at least 10 min.
Note: Make sure the DNA is completely dissolved in TE. If not, increase the volume of TE. If using 10% SDS, add 0.2 vol.
15. Add an equal volume (500 µL) of CIA (see Recipes) and keep shaking on a Nutating Mixer for at least 5 min.
16. Centrifuge at 20,000× g (15,000 rpm) for 7–10 min.
17. Carefully transfer the upper phase (~400 µL) into new 1.5 mL Eppendorf tubes, add 16 µL (20 mg/mL) of RNase, mix well by inversion, and incubate at 37 °C in a heated block for at least 15 min. Add an equal volume of CIA and keep shaking on a Nutating Mixer for at least 5 min.
Note: Take the heat block and keep it at RT after step 14 for a few minutes. Incubation of the vial after adding RNase in the block will also work.
18. Centrifuge at 20,000× g (15,000 rpm) for 7–10 min.
19. Carefully transfer the upper phase (300 µL) into new 1.5 mL Eppendorf tubes, add an equal volume of absolute ethanol, and mix well by inversion.
20. Centrifuge at 20,000× g (15,000 rpm) for 7–10 min.
21. Carefully decant or pipette out the liquid phase and add 500 µL of 70% ethanol. Tap the tube to release the pellet.
22. Centrifuge at 20,000× g (15,000 rpm) for 1–2 min and repeat step 21.
23. Centrifuge as above, pipette out the liquid completely, and air dry by keeping the tube open at RT for 2–5 min.

24. Add 50 μL (if the pellet is large, add more volume) of TE. Completely dissolve the pellet by finger tapping.
25. Measure the quantity of the plasmid DNA using NanoDrop (Figure 1A–1D; Table 1).

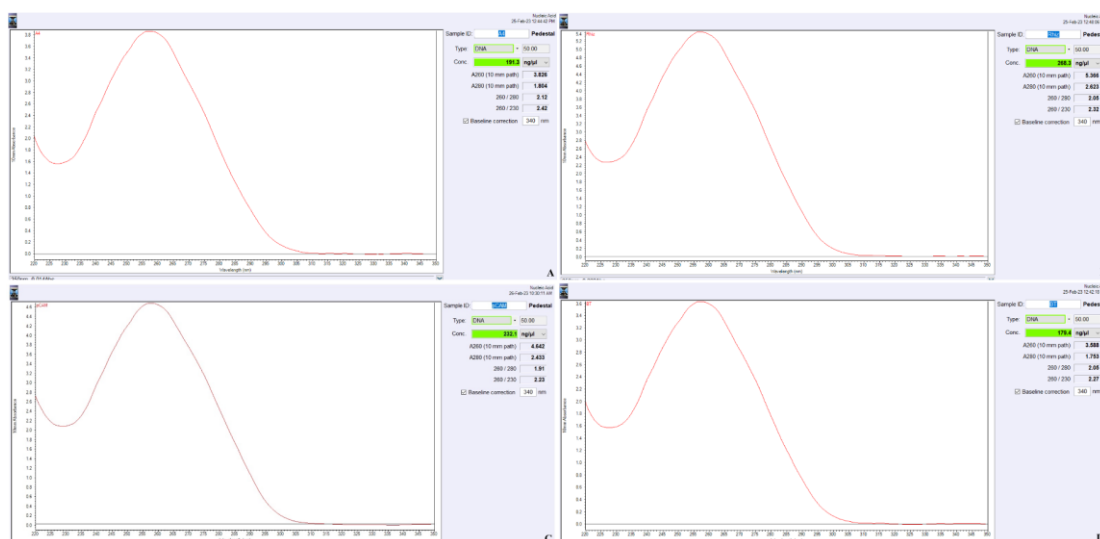


Figure 1. NanoDrop quantitation of the isolated plasmids. A. A4; B. *Rhizobium*; C. EHA105; and D. *Bacillus thuringiensis*. The x-axis represents the wavelength (nm), and the y-axis represents the 10 mm absorbance.

Table 1. Purity and quantity of the plasmid isolated from different bacterial strains using Qiagen protocol for *Agrobacterium* (Weber et al., 1998) and the optimized protocol

Bacterial strain	A _{260/280} ratio		A _{260/230} ratio		Yield (ng/μL)	
	Control (Qiagen)	Optimized protocol	Control (Qiagen)	Optimized protocol	Control (Qiagen)	Optimized protocol
<i>A. tumefaciens</i> EHA105	1.61 ± 0.03	1.91 ± 0.08	1.60 ± 0.04	2.10 ± 0.05	32 ± 1.8	232.4 ± 10.5
<i>A. tumefaciens</i> AGL1	1.40 ± 0.19	2.0 ± 0.05	1.52 ± 0.10	2.21 ± 0.1	24 ± 4.8	185 ± 8.6
<i>A. tumefaciens</i> GV3101	1.51 ± 0.07	1.93 ± 0.06	1.51 ± 0.07	2.09 ± 0.08	34 ± 1.4	212 ± 9.2
<i>A. tumefaciens</i> LBA4404	1.38 ± 0.14	2.05 ± 0.07	1.40 ± 0.10	1.98 ± 0.07	26 ± 3.4	176 ± 7.2
<i>A. rhizogenes</i> A4	1.49 ± 0.05	1.97 ± 0.08	1.49 ± 0.05	2.28 ± 0.1	22 ± 2.0	192.2 ± 9.8
<i>A. rhizogenes</i> ATCC15834	1.36 ± 0.11	1.99 ± 0.05	1.21 ± 0.08	2.15 ± 0.09	20 ± 3.1	183.1 ± 9.1
<i>Rhizobium</i> sp.	1.59 ± 0.03	1.89 ± 0.08	1.53 ± 0.04	2.11 ± 0.07	37 ± 4.3	372.0 ± 11.2
<i>Bacillus thuringiensis</i>	1.47 ± 0.12	1.91 ± 0.04	1.38 ± 0.11	2.10 ± 0.09	31 ± 3.5	211.7 ± 10.8
<i>E. coli</i> *	1.94 ± 0.06	-	2.09 ± 0.05	-	245 ± 8.1	-

Data represent the mean ± SE of five replicates. The purity and quantity of plasmid isolated using PureLink Quick Plasmid Miniprep kit, which also served as the control, are not significantly different from that of the Qiagen protocol; hence, it is not shown. The same plasmid of that in *A. tumefaciens* strains in *E. coli* served as the positive control*.

26. Load 100 ng of plasmid DNA mixed with 6× loading dye (1× final) on a 0.8% agarose gel and run at 100 V for 25 min (Figure 2).
27. Document the gel using the Gel Doc™ EZ Imager with the Image Lab 6.0 program.

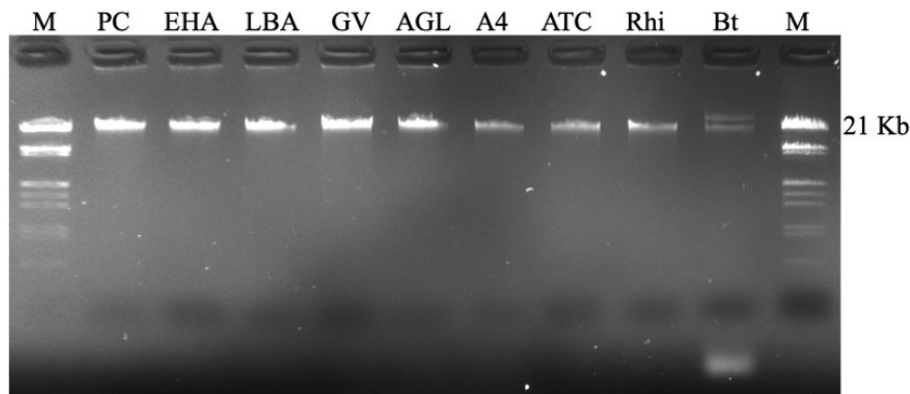


Figure 2. Gel electrophoresis of the isolated plasmids from different bacterial strains. EHA: EHA105, LBA: LBA4404, GV: GV3101, and AGL: AGL1 (all are *A. tumefaciens*); A4: *A. rhizogenes* A4; ATC: *A. rhizogenes* ATCC15834; Rhi: *Rhizobium*; Bt: *Bacillus thuringiensis* (shows two bands). PC: positive control of the same plasmid from *E. coli* using QIAprep Spin Miniprep kit; M: Lambda DNA/*EcoRI* Plus *HindIII* marker.

Data analysis

The quantity of the isolated plasmid DNA is determined using NanoDrop 2000 (Table 1; Figure 1A–1D). The data analyzed using Microsoft Excel represents the mean \pm SE of five replicates (Table 1).

Validation of protocol

The protocol is repeated five times for each sample, once with a 10 mL culture of each bacterial strain, and control for each sample is carried out. Figure 2 shows the electrophoresis of the isolated plasmid DNA in an agarose (0.8%, w/v) gel in TAE containing Hydragreen (a noncarcinogenic nucleic acid stain), carried out in a Bio-Rad electrophoresis system, documented using the Gel Doc™ EZ imager with the Image Lab 6.0 program. The quality of the plasmid DNA isolated in the present protocol is validated by gel electrophoresis (Figure 2) and restriction digestion (Figure 3): double digestion of 1 μ g of plasmid DNA (pCAMBIA 1201) using *NcoI*-HF and *BstEII*-HF following the manufacturer's double-digest protocol (NEB, USA). The digestion resulted in the *gusA* gene fragment of 2,060 bp (Figure 3). PCR is carried out using isolated plasmids with specific primers (Macrogen, Korea). Amplification of the *gusA* gene (Figure 4) of the extracted plasmid DNA from *A. tumefaciens* strains is carried out as per Kodackattumannil et al. (2023). PCR of the extracted *Rhizobium* plasmid with the primer pairs fD1 – rP2 for 16S rRNA (Weisburg et al., 1991) and R16-1 – R23-3R for ITS region (Figure 4) is performed as described by Kwon et al. (2005). Amplification of the *GFP* gene in the isolated plasmid DNA from *A. rhizogenes* strains (Figure 4) is accomplished according to Dutta et al. (2013). PCR of the *Cry3a* gene using the primers (forward: ATGAATCCGAACAATCGAAG and reverse: TTAATTCACCTGGAATAAATTCAATTTTG) is carried out with an initial denaturation of 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 2 min, and followed by a final extension at 72 °C for 10 min. In all cases, the PCR is carried out using HotStar Taq polymerase kit following the manual. The transient expression of *GFP* (plasmid isolated from ATCC15834) in *indica* rice IR5 callus (Figure 5), checked using particle bombardment (PDS-1000/Bio-Rad), validated the downstream application following the protocol of Sarangi et al. (2019).

Note: The isolated plasmid with the GFP gene (from ATCC15834) using the Qiagen protocol is not of good quality, and the quantity is very low (Table 1), which is insufficient to perform the particle bombardment.

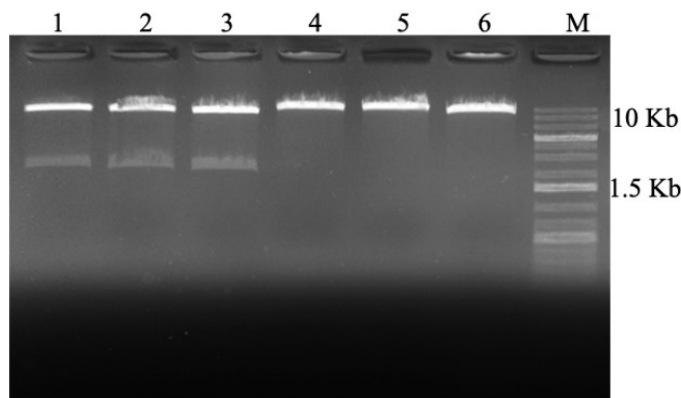


Figure 3. Restriction digestion of the plasmids isolated from *A. tumefaciens* strains EHA105, GV3101, and *E. coli* (positive control) containing the binary plasmid pCAMBIA 1201 harboring *gusA* (2,060 bp) gene under the control of 35S promoter (digestion of *gusA* gene using the restriction enzymes *NcoI* and *BstEII* (NEB)). 1. EHA105; 2. GV3101; 3. *E. coli*; 4, 5, and 6 are undigested. M—1 Kb Plus DNA ladder.

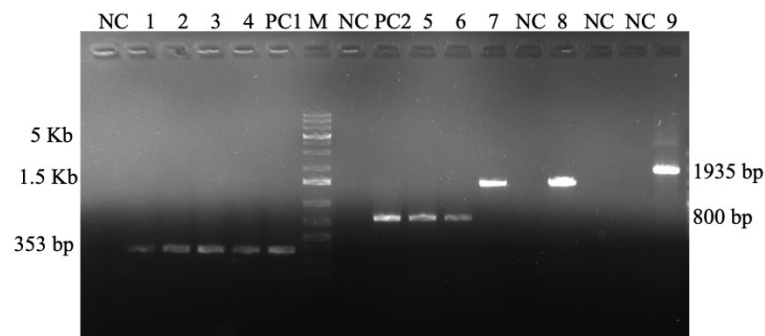


Figure 4. PCR analysis of the isolated plasmids from different bacterial strains. 1, 2, 3, and 4: *gusA* (353 bp), *A. tumefaciens* strain EHA105, GV3101, LBA4404, AGL1, and *E. coli* (PC1); 5 and 6: *GFP* (800 bp) from *A. rhizogenes* A4 and ATCC15834, respectively; 7 and 8: *Rhizobium* with R16-1–R23-3R, and fD1–rP2 primer pairs (1,500 bp); 9: amplification of *cry3A* gene (1,935 bp) from *Bacillus thuringiensis*. PC2: positive control of *GFP*; NC: negative control of the respective primers; M: 1 Kb Plus DNA ladder.

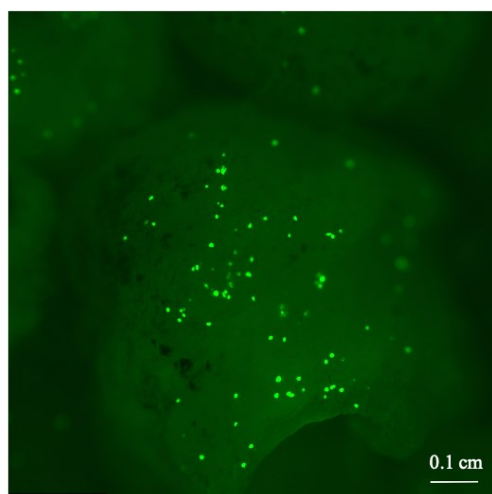


Figure 5. Green fluorescent protein expression on *indica* rice IR5 callus bombarded (using PDS-1000/Bio-

Rad) as per the protocol of Sarangi et al. (2019) with the plasmid (harboring *GFP* gene under the control of 35S promoter) isolated from *A. rhizogenes* ATCC15834. Image is captured using Leica Thunder Model Organism Microscope with *GFP* filters (excitation 488 nm, emission 507 nm). Scale bar: 0.1 cm.

General notes and troubleshooting

Troubleshooting

Problems	Troubleshooting
The quality of plasmid DNA is not good	a. Completely remove the bacterial culture medium before the addition of the P1 buffer. b. Completely remove the ethanol before the addition of TE in the second extraction.
SDS carry over	Do not use more than 0.1 vol of 20% SDS.
RNA contamination	Incubate at 37 °C for 30 min.

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Author contributions statement: MK designed the experiment. PK and SS optimized the protocol. SK and GL validated the protocol. MK and PK drafted the manuscript. KA supervised the project. All the authors reviewed the manuscript.

Competing interests

The authors declare no conflict of interest by any means.

Ethical considerations

The protocol has no animal or human subjects.

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