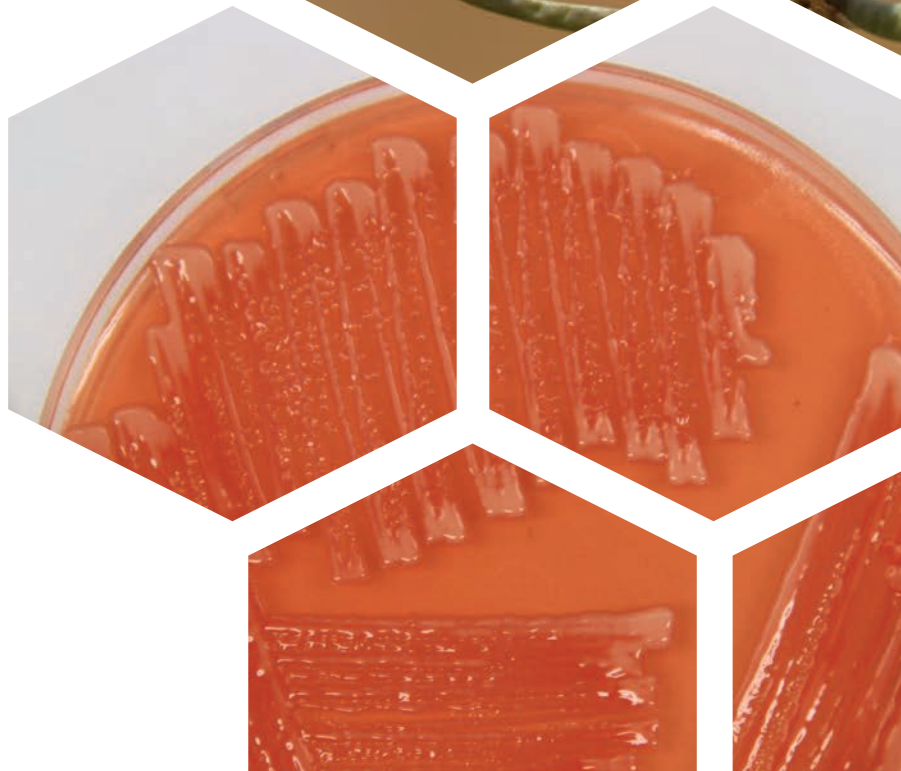
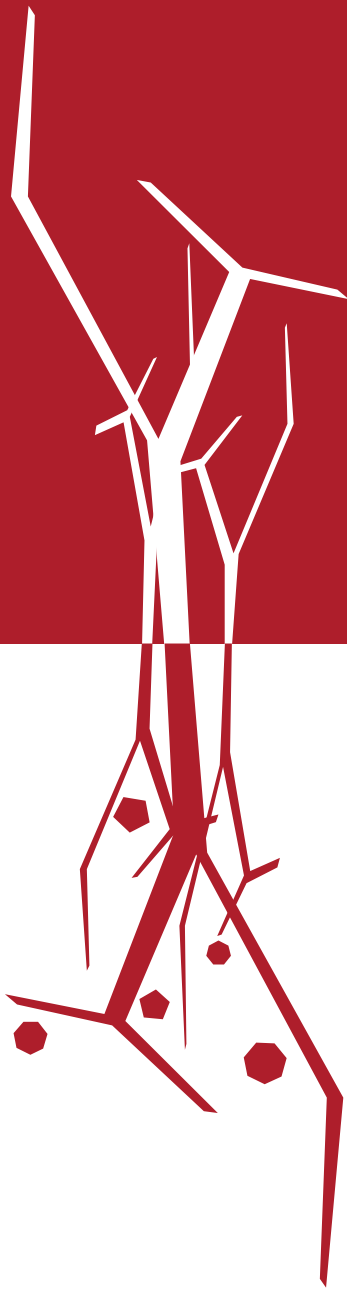


# Working with rhizobia

*J.G. Howieson and M.J. Dilworth (Eds.)*



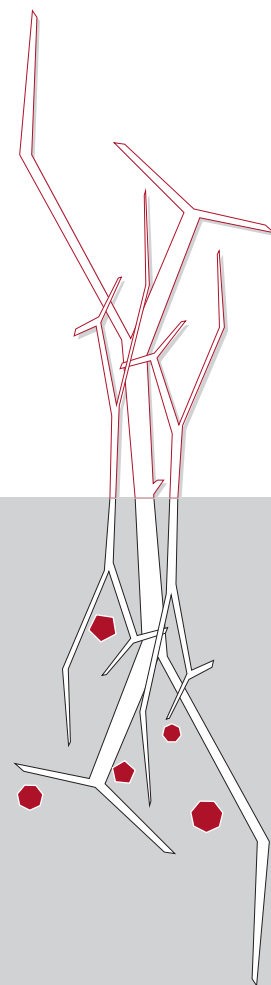
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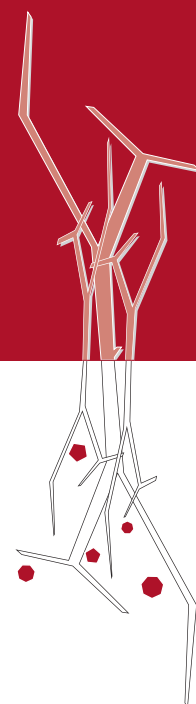
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## CHAPTER 11

# Fundamental molecular techniques for rhizobia

*W.G. Reeve, R.P. Tiwari, V. Melino and P.S. Poole*



### 11.1 Introduction to nucleic acid purification

Working with DNA is now a fundamental skill in working with rhizobia. It is necessary for typing strains using PCR methods and for sequencing activities applied to understanding genomes; their structure, how they function, and their taxonomic position.

Nucleic acid purification is the separation of nucleic acids from proteins, cell wall debris and polysaccharide after lysis of cells. For rhizobia, we provide here a number of commonly used methods for the extraction of genomic and plasmid DNA. Methods for extraction of total RNA are presented in Chapter 13. The CTAB method (Protocol 11.1.1) has been used extensively for extraction of total genomic DNA for DNA sequencing while Protocol 11.1.2 gives higher yields but generally with slightly lower purity. Plasmid DNA can be differentially displayed using Protocol 11.2.1 for determination of replicon number. This method allows localisation of genes to replicons, confirmation of genome assemblies and identification of genetic changes. The plasmids can subsequently be purified from low melting point gels using GELase (Epicentre, <http://www.epibio.com/item.asp?id=297>). Protocol 11.2.2 presents a method to recover introduced plasmids from rhizobia (*i.e.* complementing plasmids) for transformation into *Escherichia coli* prior to restriction analysis. Protocol 11.2.3 provides an alternative method to the GELase procedure for purifying plasmids but has not been tested as extensively.

### 11.1.1 Protocol for bacterial genomic DNA isolation using CTAB

This CTAB protocol comes from the DOE Joint Genome Institute Standard Operating Procedures (<http://my.jgi.doe.gov/general/protocols.html>) and has been successfully used by the authors of this chapter for the isolation of genomic DNA from 130 rhizobial strains to date.

#### 11.1.1.1 Materials and reagents

Materials and reagents are listed in Table 11.1

**Table 11.1** Materials and reagents for isolation of genomic DNA

Disposables	Vendor	Stock number
1.5 mL microcentrifuge tube	Eppendorf	22 36 320-4
50 mL Nalgene Oak Ridge polypropylene centrifuge tube	VWR	21010-568
10 mL pipette	Falcon	357551
1 mL pipette tips	MBP	3781
Reagents:	Vendor	Stock number
CTAB (cetyltrimethylammonium bromide)	Sigma	H-6269
NaCl	Sigma	S-3014
TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0)	Ambion	9858
Lysozyme	Sigma	L-6876
Proteinase K	Qiagen	19131
5M NaCl	Ambion	9759
10% (w/v) SDS (Sodium dodecyl sulfate)	Sigma	L-4522
Chloroform	Sigma	C-2432
<i>iso</i> -Amyl alcohol	Sigma	I-9392
Phenol	Sigma	P-4557
Isopropanol	VWR	PX-1835-14
Ethanol	AAPER	
RNase A (100 mg mL <sup>-1</sup> )	Qiagen	19101

#### Equipment

- ▶ Hot plate
- ▶ 250 mL glass beaker
- ▶ Magnetic stirring bar
- ▶ Thermometer
- ▶ Automatic pipette dispenser
- ▶ Sorvall 500 Plus centrifuge (DuPont, Newtown, CT)
- ▶ 65°C water bath
- ▶ 37°C incubator

**11.1.1.2 Reagent/stock preparation****CTAB/NaCl (cetyltrimethylammonium bromide)**

Dissolve 4.1 g NaCl in 80 mL of water and slowly add 10 g CTAB while heating (~65°C) and stirring. This takes more than three hours to dissolve CTAB. Adjust final volume to 100 mL and sterilise by filtering or autoclaving.

**Procedure***Cell preparation and extraction techniques*

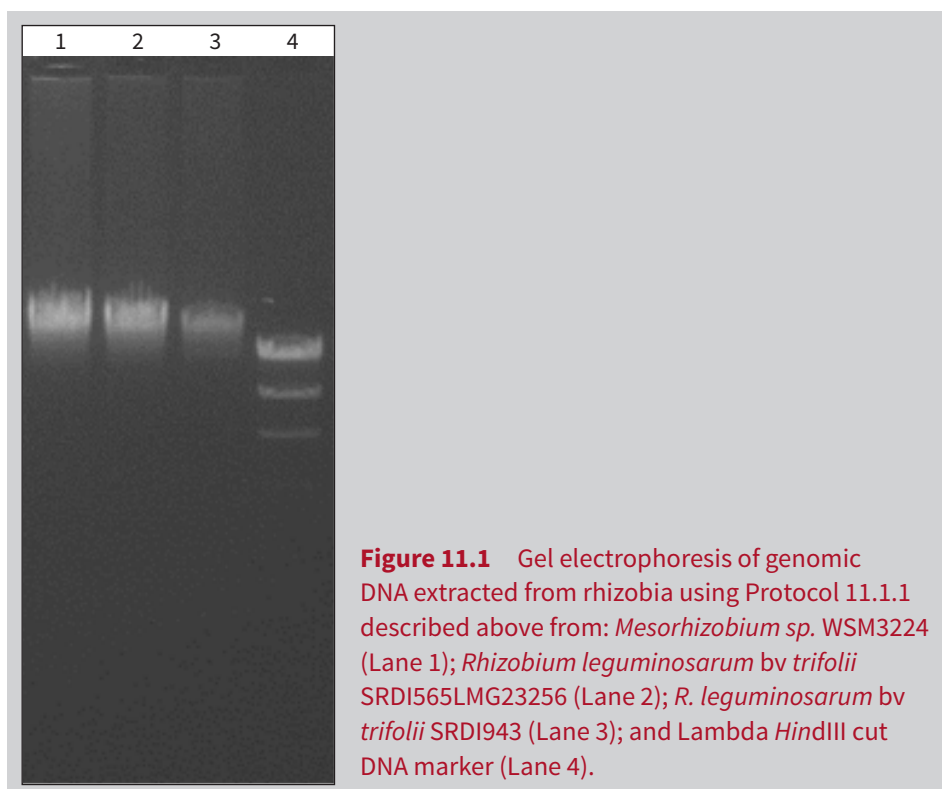
## Notes

- ▶ In step 1 below, do not use too many bacterial cells ( $OD_{600nm}$  of not more than 1 is recommended) or DNA will not separate well from the protein.
- ▶ Most of the time, inverting several times is sufficient to mix well. Avoid vigorous shaking which will shear the DNA.
- ▶ Use any standard protocol for DNA precipitation (Ausubel *et al.* 2013).

**Total volume** ..... **1.5 mL**    **30 mL**

1. Grow cells in broth and pellet for five minutes or scrape from plate.
2. Transfer bacterial suspension to the appropriate centrifuge tube.
3. Pellet cells for five minutes.
4. Discard the supernatant.
5. Re-suspend cells in TE buffer (10 mM Tris; 1mM EDTA; pH 8.0) to an  $OD_{600nm} \sim 1.0$ .
6. Transfer given amount of cell suspension to a clean centrifuge tube..... **740  $\mu$ L**    **14.8mL**
7. Add lysozyme (conc. 100 mg mL<sup>-1</sup>). Mix well. .... **20  $\mu$ L**    **400  $\mu$ L**  
*This step is necessary for hard-to-lyse bacteria.*
8. Incubate for five minutes at room temperature.
9. Add 10% (w/v) SDS. Mix well. .... **40  $\mu$ L**    **800  $\mu$ L**
10. Add Proteinase K (10 mg mL<sup>-1</sup>). Mix well..... **8  $\mu$ L**    **160  $\mu$ L**
11. Incubate for one hour at 37°C
12. Add 5 M NaCl. Mix well. .... **100  $\mu$ L**    **2 mL**
13. Add CTAB/NaCl (heated to 65°C). Mix well. .... **100  $\mu$ L**    **2 mL**
14. Incubate at 65°C for 10 minutes.
15. Add chloroform:isoamyl alcohol (24:1). Mix well.....**0.5 mL**    **10 mL**

16. Spin at maximum speed for 10 minutes at room temperature.
17. Transfer aqueous phase to clean microfuge tube (should not be viscous).
18. Add phenol:chloroform:isoamyl alcohol (25:24:1).  
Mix well.....**0.5 mL 10 mL**
19. Spin at maximum speed for 10 minutes at room temperature.
20. Transfer aqueous phase and add 0.6 volumes (vol.) of isopropanol ( $-20^{\circ}\text{C}$ ).  
▷ E.g. if 400  $\mu\text{L}$  of aqueous phase is transferred, add 240  $\mu\text{L}$  of isopropanol
21. Incubate at room temp for 30 minutes.
22. Spin at maximum speed for 15 minutes.
23. Wash pellet with 70% (v/v) ethanol, spin at maximum speed for five minutes.
24. Discard the supernatant and let pellet dry for five to 10 minutes at room temperature.
25. Re-suspend in TE buffer plus RNase (99  $\mu\text{L}$  TE + 1  $\mu\text{L}$  RNase ( $10\text{ mg mL}^{-1}$ ).... **20  $\mu\text{L}$  400  $\mu\text{L}$**
26. Transfer to sterile micro-centrifuge tubes.
27. Incubate at  $37^{\circ}\text{C}$  for 20 minutes.
28. Run 1  $\mu\text{L}$  in a 1% (w/v) agarose gel with concentration standards (Figure 11.1).



## 11.1.2 Protocol for genomic DNA preparation using phenol-chloroform

### 11.1.2.1 Precautionary measures

- ▶ All pipette tips should be filter barrier tips.
- ▶ Benches should be scrupulously clean (wipe with 70% (v/v) ethanol before use) and gloves must be worn at all times to prevent contamination.
- ▶ Containers should be disposable plastic ware, and glassware needs to be soaked in 0.1 N HCl and then 0.1 N NaOH prior to rinsing with RODI (reverse-osmosis, deionised) water.
- ▶ Genomic DNA should be prepared using pipette tips with the tip end cut off to prevent shearing of the DNA.

### *General preparation*

#### *TY broth*

- ▶ Tryptone            5 g
- ▶ Yeast-extract     3 g
- ▶ CaCl<sub>2</sub>·2H<sub>2</sub>O     0.75 g (reduced from original recipe to avoid precipitation)
- ▶ Distilled H<sub>2</sub>O     to 1 L
- ▶ Adjust pH to neutral

### *Procedure*

1. Inoculate a 10 mL TY broth in a 50 mL conical flask with a loopful of cells and grow to late log phase (for root nodule bacteria, it usually requires two to three days at 28°C). Subculture the cells if necessary to ensure the cells are in the log phase.
2. Transfer the log phase culture into a centrifuge tube.
3. Pellet cells at 7,500 g for five minutes at room temperature.
4. Remove the supernatant (last drops can be removed using a pipette).
5. Re-suspend pellet in 1 mL TES buffer (30 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA) by vortexing for one to two minutes.
6. Repeat steps 3 and 4.
7. Re-suspend cell pellet in 1 mL TES buffer.
8. Add 0.2 mL lysozyme solution (10 mg mL<sup>-1</sup> in TES buffer).
9. Incubate at 37°C for 30 minutes.



10. Add 0.15 mL 10% (w/v) SDS and 60  $\mu$ l of proteinase K (6 mg mL<sup>-1</sup> in TES).
11. Incubate at 45°C for one hour.
12. Add another aliquot of 60  $\mu$ l proteinase K and incubate at 55°C for one hour.
13. Extract DNA twice using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).
14. Add 0.1 volume of 3 M sodium acetate (pH 5.2) to the extracted DNA.
15. Add an equal volume of isopropanol and mix gently.
16. Centrifuge at room temperature at 7,500 g for 10 minutes.
17. Remove supernatant carefully without losing the pellet.
18. Add 0.5 mL of 70% (v/v) ethanol, close the lid and invert the tube carefully a few times.
19. Centrifuge at 7,500 g at room temperature for five minutes and remove supernatant carefully without losing the pellet.
20. Invert tubes on a sterile paper towel and drain the last drops of liquid.
21. Leave the tubes to air dry for 15 to 20 minutes.

*There is no need to vacuum dry DNA; it makes resuspension of the DNA difficult.*

22. Re-suspend pellet in sterile RODI water.
23. Treat with RNase (final concentration 20  $\mu$ g mL<sup>-1</sup>) for one hour at 37°C to remove RNA.

### 11.1.3 Protocol for nucleic acid extraction from soil

This method is modified from Griffiths *et al.* (2000).

#### General preparation

- ▶ 5% CTAB/phosphate buffer (120 mM, pH 8)
  - ▷ mix equal volumes of 10% (w/v) CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer (pH 8.0) and autoclave
- ▶ phenol:chloroform:isoamyl alcohol (pH 8.0) (25:24:1)

**Danger—never autoclave**

- ▶ chloroform:isoamyl alcohol (24:1)

**Danger—never autoclave**

- ▶ 30% (w/v) PEG (Polyethylene glycol) purchased from Fluka, SIGMA)

**Don't autoclave PEG**

- ▶ 70% (v/v) ethanol. Keep in the freezer.

**No need to autoclave**

- ▶ Molecular grade, sterile water
- ▶ 100 mL of 240 mM potassium phosphate buffer (pH 8)
  - ▷ 1 M  $\text{K}_2\text{HPO}_4$  : 22.56 mL
  - ▷ 1 M  $\text{KH}_2\text{PO}_4$  : 1.44 mL
  - ▷ Dilute to 100 mL
- ▶ Elution Buffer (EB)
  - ▷ 10 mM Tris-Cl, pH 8.5
- ▶ Cell homogenisation/lysis equipment (*i.e.* Fast-Prep beadbeater)
- ▶ Bead tubes
- ▶ 0.1 mm zirconia/silica beads, 0.5 mm glass beads (fill this bead mixture up to 20% of the tube's volume and add one of a 2 mm glass ball).

**Procedure**

1. Weigh out 1 g of soil.
2. Add 0.5 mL CTAB buffer and 0.5 mL phenol:chloroform:isoamyl alcohol (25:24:1) into ready-made 2 mL bead tubes, tighten lids and keep on ice.
3. Place tubes in Fast-Prep beadbeater and lyse cells for 30 seconds at speed 5.5 (repeat three times with a five minute break in between lysing treatments).
4. Centrifuge at full speed (16,000 g) for five minutes at 4°C.
5. Extract the top aqueous layer and transfer to a new microfuge tube.
6. Add 0.5 mL of chloroform:isoamyl alcohol (24:1) and vortex for five seconds to form an emulsion.
7. Centrifuge at full speed (16, 000 g) for five minutes (4°C).
8. Extract the top layer again and precipitate nucleic acids by adding 1 mL of PEG solution. Mix well.
9. Leave samples for one to two hours at room temperature.
10. Centrifuge at 16,000 g for 10 minutes (room temperature).

11. Pour off supernatant and wash pellet with 70% (v/v) ethanol (200  $\mu$ L). Vortex, centrifuge for 10 minutes (16,000 g) and discard alcohol.
12. Repeat step 11, removing all traces of liquid.
13. Re-suspend pellet in 50  $\mu$ L of EB buffer.

### Removal of humic acids from DNA samples

One-step<sup>TM</sup> PCR inhibitor removal kit (ZYMO RESEARCH cat. No D6030). The protocol is provided here.

1. Remove the cap and snap off the base of the supplied filter tubes. Place them into collection tube (provided).
2. Spin down at 8,000 g for exactly three minutes.
3. Place the filter into 1.5 mL microfuge tube and add 100 to 200  $\mu$ L of DNA sample.
4. Spin down at 8,000 g for exactly one minute. The eluate should be free of humic acid.

## 11.2 Plasmid purification

### 11.2.1 Protocol for plasmid profiling using a modified Eckhardt procedure

The original method (Eckhardt 1978) has been subsequently modified (Priefer, 1984; Hynes *et al.*, 1985; and Hynes and McGregor, 1990).

#### Recipes (stocks and media)

The components of TBE buffer and HP media are given in Tables 11.2 and 11.3.

**Table 11.2** 10 $\times$  TBE buffer

Component	Quantity
Tris	108 g L <sup>-1</sup>
Boric acid	55 g L <sup>-1</sup>
EDTA (0.5 M, pH 8.0)	40 mL L <sup>-1</sup>

Make up to 1 L with RODI water, pH to 8.0 with HCl, autoclave.

#### 1 $\times$ TBE buffer

- ▶ Dilute from 10 $\times$  TBE buffer using RODI water. Also maintain a stock of sterile TBE buffer for preparing the agarose gels, Sarcosyl and lysis solution.

**10% SDS in 1× TBE buffer (200 mL)**

- ▶ Dissolve 20 g of SDS in 160 mL of pre-warmed 1× TBE buffer.
- ▶ Adjust to pH 8.0.
- ▶ Make up to 200 mL with 1× TBE buffer.
- ▶ Autoclave.

NB: if the SDS precipitates, re-dissolve it at 50°C in a water bath.

**0.3% (w/v) Sarcosyl in 1× TBE buffer**

- ▶ Dissolve 0.15 g Sarcosyl (*n*-lauroylsarcosine) in 50 mL of 1× sterile TBE buffer.
- ▶ Chill at 4°C for at least one hour prior to use.

**E1 Solution—10% (w/v) sucrose in 1× TBE buffer + 10 µg mL<sup>-1</sup> RNase**

- ▶ Dissolve 1 g sucrose in 10 mL 1× sterile TBE buffer.
- ▶ Store at 4°C.

**Lysozyme Stock Solution 100×**

- ▶ 100 mg mL<sup>-1</sup> lysozyme dissolved in 1× TBE
- ▶ Store at -20°C.

**Lysis Solution (100 µg mL<sup>-1</sup> lysozyme in E1 Solution)**

- ▶ Prepare fresh on the day.
- ▶ Mix 986 µL of E1 solution with 4 µL RNAase stock solution (10 mg mL<sup>-1</sup>).
- ▶ Add 10 µL of 100× lysozyme stock solution .

**Table 11.3** HP media

Component	Quantity
Peptone	4.0 g L <sup>-1</sup>
Yeast extract	0.5 g L <sup>-1</sup>
Tryptone	0.5 g L <sup>-1</sup>
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.2 g L <sup>-1</sup>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2 g L <sup>-1</sup>

**Procedure****Step 1**

Inoculate strains into prepared 5 mL broths (add antibiotics if selection pressure is required) and grow at 28°C with shaking at 200 rpm.

*NB:* selection of the broth media depends on which genera of root nodule bacteria (RNB) are used. *Rhizobium leguminosarum* lyses well when grown in TY while *Sinorhizobium medicae* lyses better when grown in TY with half the normal  $\text{CaCl}_2$  or in HP media (when using HP media, omit the antibiotics). *Agrobacterium sp.* may be grown in LB. Grow overnight (28°C, 200 rpm) to an  $\text{OD}_{600\text{nm}}$  of 0.3. (OD is important; try not to grow over 0.3). It is best to do a range of subcultures to ensure the correct OD; *i.e.* subculture 2, 5, 10 and 20  $\mu\text{L}$  into 5 mL broths. Faster growing strains can be further subcultured.

### Step 2

Prepare and pour the 0.75% (w/v) agarose plus 1% (w/v) SDS gel:

1. Add 7.5 g agarose to 900 mL 1× TBE (pH 8.0) buffer and microwave (stirring occasionally) until clear.
2. Cool the agarose to 55°C, pour 90 mL into a conical flask and add 10 mL of 10% (w/v) SDS solution. Mix by gentle swirling and pour into the gel tray.

*This quantity is sufficient for a 15 cm × 15 cm tray; the gel should be as thin as possible (3 to 4 mm) to give the best results.*

3. When the gel is set, place in the gel tank and cover with 1× TBE buffer.

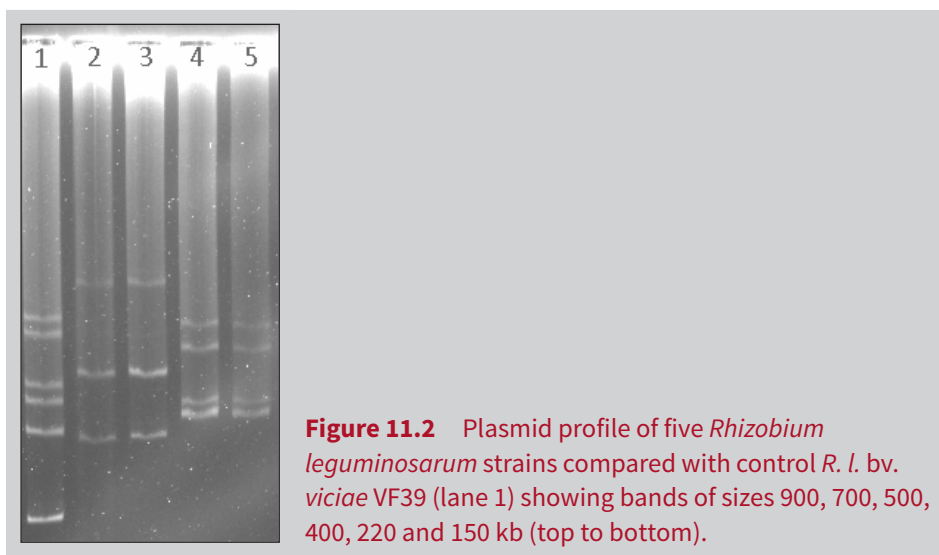
### Preparation of the cultures

1. Set the centrifuge to 4°C (or place centrifuge in a refrigerated room).
2. Pipette 200  $\mu\text{L}$  of culture ( $\text{OD}_{600\text{nm}} \leq 0.3$ —this is the best cell density but it can be up to 0.5) into a 1.5 mL micro-centrifuge tube and place on ice.

*You can vary the volume of culture used in this step according to the  $\text{OD}_{600\text{nm}}$  *i.e.* 300  $\mu\text{L}$  at OD 0.2. Additionally, a series of aliquots *i.e.* 200, 400, and 800  $\mu\text{L}$  may be used. Exopolysaccharide may be removed by pelleting cells and re-suspending them in sterile RODI water.*

3. Add 1.0 mL of cold 0.3% (w/v) Sarcosyl and mix by inversion twice.
4. Place on ice for 10 minutes.
5. Prepare the lysis solution and place on ice.
6. Centrifuge the Sarcosyl-culture mix (4°C, 16,000 rpm; five minutes).
7. Carefully pour off the supernatant and place the tube back on ice. Using a P200 pipette, carefully remove the remaining supernatant, leaving the pellet as dry as possible.
8. Place the tubes in a rack at room temperature, add 20  $\mu\text{L}$  of lysis solution and re-suspend the pellet by carefully pipetting up and down twice. Avoid creating bubbles in the solution.

9. Immediately load 20  $\mu$ L of sample into the gel well. The sample should be evenly re-suspended and slightly cloudy. The loading dye can be added to one well as a marker.
10. When loading of all samples is complete, initiate electrophoresis at 5 to 10 V for 15 to 30 minutes (maximum) until the samples have lysed and are clear. Then run the gel electrophoresis at 70 to 80 V overnight. LEAVE THE GEL TANK LID OFF to avoid over-heating the gel tank. DANGER: Indicate with appropriate signage that the lid off means exposed live wires. Alternatively, run in the cold room or in an air-conditioned laboratory leaving the gel tank lid on.
11. After staining with ethidium bromide (for 30 minutes to one hour), de-stain in sterile RODI water for 30 minutes (longer de-stain times may improve the resolution of the bands; see Figure 11.2).



### 11.2.2 Protocol for plasmid preparation by alkaline lysis

This alkaline lysis method is recommended for rapid extraction and purification of small (<50 kb) plasmids that have been introduced into rhizobia (*i.e.* via conjugation; see Protocol 11.2.1). The following protocol (Ausubel *et al.* 2013) was adapted specifically for the isolation of rhizobial megaplasmids. It is important to mention that although plasmids may be isolated from RNB using this method, the profile after separation by gel electrophoresis will appear smeared and the authors have noted inhibition of restriction enzyme digest reactions. Plasmids prepared from RNB using this method will need to be transformed back into *E. coli* prior to extraction (using the original version of this protocol) and restriction enzyme digestion.

**General preparation**

- ▶ Prepare Solution I from standard stocks in batches of approximately 100 mL; sterilise by autoclaving.
- ▶ Prepare Solution II fresh using sterile distilled H<sub>2</sub>O and use at room temperature. Warm solution II if a precipitate is observed.
- ▶ Store Solution III at 4°C and use at 4°C.
- ▶ 100% (v/v) ethanol at room temperature.
- ▶ 70% (v/v) ethanol at room temperature.
- ▶ Molecular grade H<sub>2</sub>O.

**Recipes*****Alkaline Lysis Solution I***

- ▶ 25 mM Tris-Cl (pH 8.0)
- ▶ 10 mM EDTA (pH 8.0)

***Alkaline Lysis Solution II***

- ▶ 0.2 N NaOH (freshly diluted from a 10 N stock)
- ▶ 1% (w/v) SDS (diluted on the day of use from a 10% (v/v) SDS stock)

***Alkaline Lysis Solution III***

*The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Prepare 100 mL total volume.*

- ▶ 5 M potassium acetate, pH 4.8      60.0 mL
- ▶ glacial acetic acid                      11.5 mL
- ▶ molecular grade H<sub>2</sub>O                      28.5 mL

**Procedure**

1. Inoculate 2 mL of TY containing the appropriate antibiotic with a single colony of bacteria. Incubate the culture overnight at 28°C with vigorous shaking.
2. Pour 1.5 mL of the culture into a micro-centrifuge tube. Centrifuge at maximum speed for 30 seconds in a micro-centrifuge at room temperature. Store the unused portion of the original culture at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Re-suspend the bacterial pellet in 100  $\mu\text{L}$  of Alkaline Lysis Solution I at room temperature by vigorous vortexing.
5. Add 200  $\mu\text{L}$  of freshly prepared Alkaline Lysis Solution II at room temperature to each bacterial suspension. Close the tube tightly and mix the contents by inverting the tube rapidly several times. Do not vortex and rapidly proceed to Step 6.
6. Add 150  $\mu\text{L}$  of ice-cold Alkaline Lysis Solution III. Close the tube and disperse Alkaline Lysis Solution III through the viscous bacterial lysate by inverting the tube several times. Transfer tube to  $-20^{\circ}\text{C}$  for 15 minutes to promote precipitation of SDS.
7. Centrifuge the bacterial lysate at maximum speed for five minutes at room temperature in a micro-centrifuge. Transfer the supernatant to a fresh tube and warm to  $>15^{\circ}\text{C}$  to solubilise any remaining SDS.
8. Precipitate nucleic acids from the supernatant by adding two volumes of ethanol at room temperature (cold ethanol is best avoided as this promotes precipitation of residual SDS). Mix the solution by inverting several times.
9. Collect the precipitated nucleic acids by centrifugation at maximum speed for five minutes at room temperature in a micro-centrifuge.
10. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
11. Add 1 mL of room temperature 70% (v/v) ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for two minutes at room temperature in a micro-centrifuge.
12. Remove all of the supernatant by gentle aspiration as described in Step 3. Take care with this step as the pellet sometimes does not adhere tightly to the tube.
13. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (five to 10 minutes).  
  
*NB:* residual ethanol in the prepared DNA will cause the DNA (visualised with loading dye) to rise out of the agarose gel well when setting up for gel electrophoresis.
14. Dissolve the nucleic acids in 50  $\mu\text{L}$  of sterile  $\text{H}_2\text{O}$ . Gently mix by repetitive pipetting. Warm solution to  $55^{\circ}\text{C}$  for 10 minutes.
15. Store the DNA solution at  $-20^{\circ}\text{C}$ .



### 11.2.3 Protocol for plasmid preparation by cesium chloride

#### General preparation

- ▶ Cesium chloride (CsCl) preparation.
  - ▷ Dissolve 11 g CsCl in 5 mL TE buffer, pH 8.0.
  - ▷ Because CsCl will not dissolve at low temperatures, a water bath may be required. All solutions and equipment must be maintained at >20°C during the procedure to ensure CsCl remains in solution.
- ▶ Ethidium bromide (EtBr) is a carcinogen and appropriate protective wear (gloves, lab coat and glasses) should be worn at all times during this procedure. Consult the MSDS for EtBr prior to use.
- ▶ Preparing salt-saturated isopropanol (flammable solution, avoid contact with open flame).
  - ▷ Prepare 500 mL of a 5 M NaCl solution (dissolve the NaCl in TE, pH 8.0). Autoclave in a 1 L Schott bottle.
  - ▷ Add 500 mL isopropanol to the solution above, mix and allow the phases to separate.
  - ▷ On the day of use, dispense the top layer of the salt-saturated isopropanol into 30 mL aliquots (1 aliquot per DNA sample).
- ▶ Prepare the bent needle for the isopropanol extraction steps; the 90° bend helps to maintain phase separation.
  - ▷ To bend the needle, hold the needle firmly with a sterile implement either side (*i.e.* flame-sterilised pliers).
  - ▷ Pass the needle through a flame to bend it into a loop.
  - ▷ Avoid touching the needle tip to minimise DNA contamination.
- ▶ TE buffer.
  - ▷ 10 mM Tris and 1 mM EDTA, pH 8.0.
- ▶ TY broth (see Protocol 11.1.2).

#### Procedure

1. Grow overnight culture of bacteria in 400 mL TY broth.
2. Spin cultures in sterile 500 mL tubes.
3. Remove all growth media; use vacuum if required.
4. Re-suspend pellet in 30 mL TE buffer ensuring there are no lumps.

5. Prepare lysate using genomic method in 15 mL quick-seal (Beckman) centrifuge tubes.

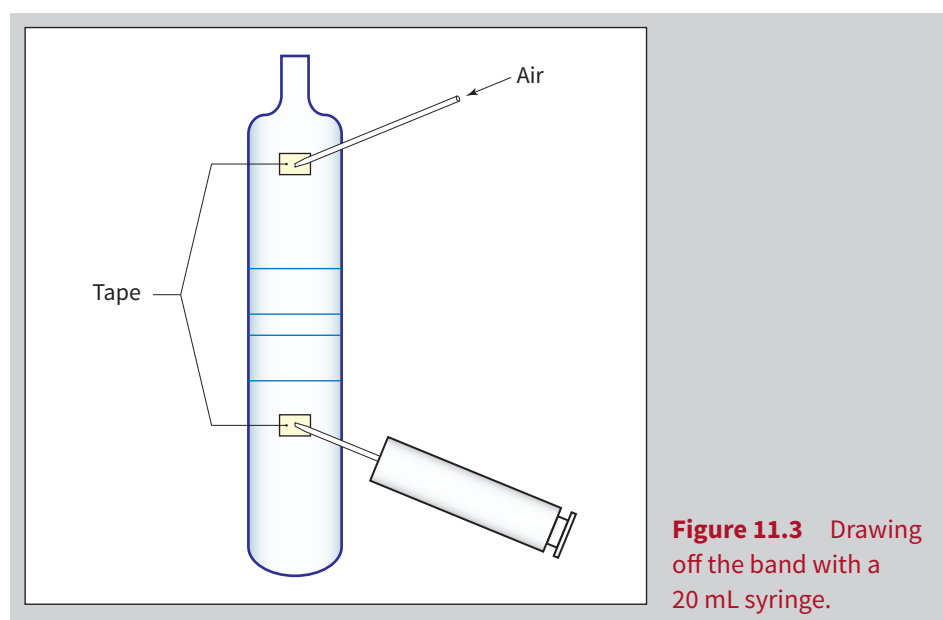
*Steps 6–13 must be carried out in dim light to prevent UV/EtBr interaction that can damage the DNA.*

6. Add 5 mL of CsCl solution to the cleared lysate in the quick seal tube. Take up 0.2 mL pre-warmed EtBr ( $10 \text{ mg mL}^{-1}$ ) into a syringe with needle attached and dispense into the quick-seal centrifuge tubes containing the DNA/cesium chloride solution.
7. Inject the DNA/EtBr solution and mix thoroughly but gently.

*There must be no bubbles in the gradient tubes.*

8. Seal the tubes with the heat sealer and place in a fixed angle rotor which has been pre-warmed to  $28^\circ\text{C}$ .
9. Centrifuge at  $45,000 \text{ g}$  for 48 hours to enhance separation of the forms of DNA.
10. Remove the desired DNA from the gradient by drawing off the band (intensely stained red) with a 20 mL syringe (Figure 11.3), beveled edge of needle uppermost. (NB: supercoiled DNA will migrate faster and be located at the bottom of the gradient.)

*The procedure is easier if a needle is inserted in the top of the tube to allow air in, and a piece of tape is applied to the side of the tube at the point where the second needle is inserted to prevent blocking up the needle (Figure 11.3).*



**Figure 11.3** Drawing off the band with a 20 mL syringe.

11. Replace the straight needle with a needle pre-bent at  $90^\circ$  to allow ease of isopropanol uptake. Using the syringe containing the DNA, take up an equal volume of salt-saturated isopropanol. Mix gently and allow phases to separate while keeping the syringe upright. Expel the top phase with the syringe upright.

*Steps 11 and 12 must be carried out in dim light to prevent UV/EtBr interaction that can damage the DNA.*

12. Repeat the above step six times, or as required, until the ethidium bromide is no longer visible.
13. Add the washed DNA to sterile 50 mL tubes then add two volumes of sterile water and six volumes of 100% (v/v) EtOH (volumes are proportional to the original volume of DNA solution).
14. Spin for 10 minutes at maximum speed in a micro-centrifuge at 25°C. Dry pellet under vacuum.
15. Re-suspend pellet in 0.45 mL TE buffer and transfer solution into a micro-centrifuge tube.
16. Add 50 µL of 3 M Na acetate (pH 5.2) then 1 mL of 100% EtOH; mix well.
17. Spin for 10 minutes at 10,000 g.
18. Wash pellet with 70% EtOH, then dry under vacuum.
19. Re-suspend pellet in 0.5 mL (high copy number) or 0.1 mL (low copy number) TE buffer, pH 8.0 (adjust volume according to pellet size).
20. Determine the concentration using the Dot method (Ausubel *et al.* 2013).

## 11.3 PCR techniques

Polymerase Chain Reaction (PCR) is a common molecular biology technique used to amplify DNA from a starting template (*i.e.* cells, genomic DNA or plasmids). Methods for cell preparation are provided in 11.3.1 and can be used in both single and multi-amplicon generation (11.3.2 and 11.3.3 respectively). Genomic DNA preparations (Protocol 11.1.1 and 11.1.2) may be used in PCR when problems arise from the use of cell templates. The amplified product, known as the amplicon, can then be used for a variety of purposes, including sequencing, cloning, probe design. Multi-amplicons can be used for fingerprinting RNB.

### 11.3.1 Protocol for preparation of cell template

#### A. Basic cell preparation

This procedure is modified from Schneider and deBruijn (1996).

- ▶ Prepare a pure rhizobial culture by dilution streaking on to ½ LA or TY agar plates.
- ▶ Incubate at 28°C until growth is visible on final dilution streak.
- ▶ Re-suspend a loopful of culture in 1 mL of 0.89% (w/v) saline.

1. Centrifuge the culture at 10,000 g for 30 seconds and remove the supernatant.

*NB:* cultures producing large amounts of EPS may need to be washed with 0.89% (w/v) saline for a second time.

2. Re-suspend the cell pellet in sterile (DNA-free) water to an OD<sub>600nm</sub> of 6.0 (*NB:* an OD<sub>600nm</sub> of 10 has been used reproducibly for *Rhizobium leguminosarum*).
3. Store at 4°C for up until one month or use immediately in the PCR reaction below.

*NB:* it is important that fresh cultures of rhizobia, free of contaminants, are used to prepare the template.

## B. Cell preparation using PEG

Method (Chomczynski and Rymaszewski 2006).

### Reagents

1. Alkaline PEG reagent preparation
  - a. Combine 60 g PEG 200 (Sigma-Aldrich or equivalent) with 0.93 mL 2 M KOH and 39 mL water. If desired, NaOH can substitute for KOH in the reagent. *Note that PEG 200 is measured by mass rather than volume because of the viscosity of the liquid.*
  - b. Confirm that the pH is 13.3 to 13.5. Due to storage, some batches of PEG 200 have an acidic rather than neutral pH. In this case, add an additional amount of alkali to reach the target pH range.
2. Scoop a loopful of a RNB colony containing your gene of interest into 500 µL of sterile molecular- grade water in a sterile microfuge tube.
3. Spin down at 6000 rpm for four minutes.
4. Leave 10 µL of water in the microfuge tube.
5. Add 100 µL of alkaline PEG reagent and mix well.
6. Leave at room temperature for 15 minutes.
7. Take 1.0 to 1.5 µL of the mixture and add into 20 µL of PCR reaction.

### 11.3.2 Protocol for single amplicon PCR

Targeted single amplicons can be generated and sequenced for the purposes of typing and taxonomic analysis (see Protocol 12.7.1 Building phylogenetic trees). A selection of commonly used primers for single amplicon generation is provided in Tables 11.4 and 11.5. Products used for phylogenetic analysis should be fully

sequenced on both DNA strands for the largest fragment possible. Cells can be prepared as described in Protocol 11.3.1A but if the desired amplicon cannot be generated, an alternative cell preparation method is provided in Protocol 11.3.1.B.

**Table 11.4** Oligonucleotide primers used for PCR amplification of specific loci (chromosomal and pSYM localised) from rhizobia

Target gene	Primer Name	Primer sequence (5'-3')*	Reference
<i>nodC</i>	<i>nodC</i> -251F	AYGTHGTYGAYGACGGTTC	(Laguerre <i>et al.</i> 2001)
	<i>nodCI</i> -1160R	CGYGACAGCCANTCKCTATTG	(Laguerre <i>et al.</i> 2001)
<i>nodA</i>	<i>nodAF</i>	TGCRGTGGAARNTRNNCTGGGAAA	(Haukka <i>et al.</i> 1998)
	<i>nodAR</i>	GNCCGTCTRCRAAWGTCARGTA	(Haukka <i>et al.</i> 1998)
<i>nifH</i>	<i>nifH1</i>	AAGTGCCTGGAGTCCGGTGG	(Eardly <i>et al.</i> 1992)
	<i>nifH2</i>	GTTCGGCAAGCATCTGCTCG	(Eardly <i>et al.</i> 1992)
<i>dnaK</i>	DnaK1468F	AAG GAGCAGCAGATCCGCATCCA	(Stepkowski <i>et al.</i> 2003)
	DnaK1772R	GTACATGGCCTCGCCGAGCTTCA	(Stepkowski <i>et al.</i> 2003)
<i>recA</i>	<i>recAF</i>	ATCGAGCGGTCTGTCGGCAAGGG	(Gaunt <i>et al.</i> 2001)
	<i>recAR</i>	TTGCGCAGCGCCTGGCTCAT	(Gaunt <i>et al.</i> 2001)
16s rRNA <sup>1</sup>	16S rRNA Universal forward (fD1)	AGAGTTTGATCCTGGCTCAG	(Weisburg <i>et al.</i> 1991)
	16S rRNA Universal reverse (rP3)	ACGATACCTTGTACGACTT	(Weisburg <i>et al.</i> 1991)
	FGPS1490	TGCGGCTGGATCACCTCCTT	(Navarro <i>et al.</i> 1992)
	FGPS6	GGAGAGTTAGATCTTGGCTCAG	(Laguerre <i>et al.</i> 1996)
	FGPS132'	CCGGGTTTCCCCATTCCGG	(Ponsonnet and Nesme 1994)
<i>gyrB</i>	<i>gyrB</i> 343F	TTCGACCAGAAAYCTCTAYAAGG	(Martens <i>et al.</i> 2008)
	<i>gyrB</i> 1043	AGCTTGTCTTSGTCTGCG	(Martens <i>et al.</i> 2008)
<i>rpoB</i>	<i>rpoB</i> 83F	CCTSATCGAGGTTACAGAAGGC	(Martens <i>et al.</i> 2008)
	<i>rpoB</i> 1061R	AGCGTGTGCGGATATAGGCG	(Martens <i>et al.</i> 2008)
<i>atpD</i>	<i>atpDF</i>	ATCGGCGAGCCGGTTCGACGA	(Gaunt <i>et al.</i> 2001)
	<i>atpDR</i>	GCCGACACTTCCGAACCNGCCTG	(Gaunt <i>et al.</i> 2001)
<i>glnA</i>	<i>glnA</i> 532F (GSI-1)	AAG GGC GGC TAY TTC CCG GT	(Turner and Young, 2000)
	<i>glnA</i> 1124R (GSI-2)	GTC GAG ACC GGC CAT CAG CA	(Turner and Young, 2000)
<i>dnaJ</i>	<i>dnaJF</i>	CAGATCGAGGTSACCTTCGAC	(Alexandre <i>et al.</i> 2008)
	<i>dnaJR</i>	CGTCRYCATMGAGATCGGCAC	(Alexandre <i>et al.</i> 2008)

\* Abbreviations: H = Adenine, Cytosine or Thymine; I = Inosine; N= Adenine, Cytosine, Guanine or Thymine; Y = Cytosine or Thymine.

<sup>1</sup> A single primer pair needs to be selected from the collection provided here

**Table 11.5** Common reference genes selected for qRT-PCR amplification from prokaryotic RNA

Gene	Bact.*	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
Recombinase A ( <i>recA</i> )	<i>Pa</i>	GGTGAGCTGGTTGATCTGGG	GCATTGCTTTACCTGACC	Takle <i>et al.</i> (2007)
Malate dehydrogenase ( <i>mdh</i> )	<i>Ec</i>	CTGCGTAACATCCAGGACACTAACG	CGACGGTTGGGTATAAATAACAGG	Wang <i>et al.</i> (2009)
Malate dehydrogenase ( <i>mdh</i> )	<i>Rlv</i>	GCGATGACCTTCTCGGCATCA	CATGGCGTCGAGCGGATTG	Karunakaran <i>et al.</i> (2009)
Sigma factor $\sigma_{54}$ , ( <i>rpoN</i> )	<i>Pp</i>	GTAAAGGCTTTGCACCAG	GATTTTCATCGACCTGCTC	Chang <i>et al.</i> (2009)
sigma factor $\sigma_{70}$ ( <i>rpoD</i> )	<i>Pp</i>	CGATGGAAATCACCAGAC	GCTGATCGACCTTGAGAC	Chang <i>et al.</i> (2009)
16S ribosomal RNA (16s rRNA)	<i>Pp</i>	CCGTGTCTCAGTTCCAGT	TGAGCCTAGGTCGGATTA	Chang <i>et al.</i> (2009)
Cation transporting ATPase ( <i>atkA</i> )	<i>Pp</i>	GTAGTCGGCAAAGGTCTG	CAACTTCTGGGTCGACAT	Chang <i>et al.</i> (2009)

\* *Pa*, *Pectobacterium atrosepticum*; *Ec*, *Escherichia coli*; *Rlv*, *Rhizobium leguminosarum* bv. *viciae*; *Pp*, *Pseudomonas putida*

The products of those reactions can be purified from the reaction components (*i.e.* salts, nucleotides and enzymes) or from agarose gel using either a commercially available silica-column based kit or a non-commercial method (Ausubel *et al.* 2013). A number of the latter methods have been used by the current authors, including isolation from low melting temperature agarose gels followed by phenol extraction or purification of DNA fragments using a Sephacryl S-300 column. However, commercially available kits are selected when rapid sample processing is required. The factors to consider before purchasing these kits include both the DNA yield/recovery, the size of the DNA fragment and the elution buffer volume and type (for example Tris-EDTA buffer may interfere with downstream reactions such as ligations). The authors frequently use both the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and the QIAquick Gel Extraction Kit (QIAGEN, Germany). If the final concentration of your purified DNA sample is low, then a simple ethanol-salt precipitation of that DNA followed by re-suspension in a smaller volume is an easy solution. Alternatively, the MinElute PCR purification kit (Qiagen) enables re-suspension of the purified DNA in a small volume.

### 11.3.3 Protocol for multi-amplicon profiling (fingerprinting)

Identification and phylogenetic analysis of rhizobia can be performed using PCR and gel electrophoresis techniques. Oligonucleotide primers (Table 11.6) targeting naturally occurring interspersed repetitive DNA elements enable amplification of multiple DNA fragments of different sizes simultaneously. The unique banding pattern (DNA fingerprint) is visualised using gel electrophoresis to type rhizobia. Primer pairs have been designed from the repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) (Versalovic

*et al.* 1991) and BOX sequences. The complete protocols for preparation of cell templates and repetitive-PCR (see Table 11.7 for PCR reaction conditions) are provided here, and have frequently been used by the authors for the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Information and protocols for additional repetitive-PCR types are available from <http://www.msu.edu/~debruijn/> (Rademaker and De Bruijn 1997).

**Table 11.6** Oligonucleotide primers used for PCR fingerprint profiling of rhizobia

Primer	Sequence (5'-3')*	Reference
ERIC 1R	ATGTAAGCTCCTGGGGATTAC	Versalovic <i>et al.</i> (1991)
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	Versalovic <i>et al.</i> (1991)
REP1R-I	IIICGICGICATCIGGC	Versalovic <i>et al.</i> (1991)
REP2-I	ICGICTTATCIGGCCTAC	Versalovic <i>et al.</i> (1991)
BOXAIR	CTACGGCAAGGCGACGCTGACG	Versalovic <i>et al.</i> (1994)
RPO1	AATTTTCAAGCGTCGTGCCA	Richardson <i>et al.</i> (1995)
PucFor	GTAACACGACGGCCAGT	Laguerre <i>et al.</i> (1996)

\* Abbreviations: I = Inosine.

**Table 11.7** PCR reactions for ERIC, BOX and rep

Reagent (stock concentration)	Volume ( $\mu\text{L}$ ) for 1 reaction
Template cells ( $\text{OD}_{600\text{nm}}$ 6.0)	1
Gitschier buffer (5 $\times$ ) (See Table 11.8)	5
Bovine Serum Albumin (BSA at 20 mg mL <sup>-1</sup> )	0.2
Dimethyl sulfoxide (100% DMSO)	2.5
Primer 1 (50 $\mu\text{M}$ )	1
Primer 2 (50 $\mu\text{M}$ )*	1*
dNTP mix (25 mM)	1.25
<i>Taq</i> Polymerase (5 U/ $\mu\text{L}$ )	0.4
DNase-free water	to 25

\* Not relevant for BOX, RPO1, pUCF as they are the only primer required.

*NB:* the authors use *Taq* Polymerase (Invitrogen).

Use DMSO at room temperature and mix stock if precipitates form.

Use filter-tips when preparing the PCR mixture and all stock solutions.

### PCR Cycle

Temperature ( $^{\circ}\text{C}$ )	Time	Cycles
95	7 minutes	1
94	1 minutes	
52*	1 minutes	35
65	8 minutes	
65	16 minutes	1

\* 53 $^{\circ}\text{C}$  for BOX, 40 $^{\circ}\text{C}$  for REP

*NB:* store the reaction tubes at  $-20^{\circ}\text{C}$  until ready to visualise by gel electrophoresis.

### 11.3.4 Gel electrophoresis

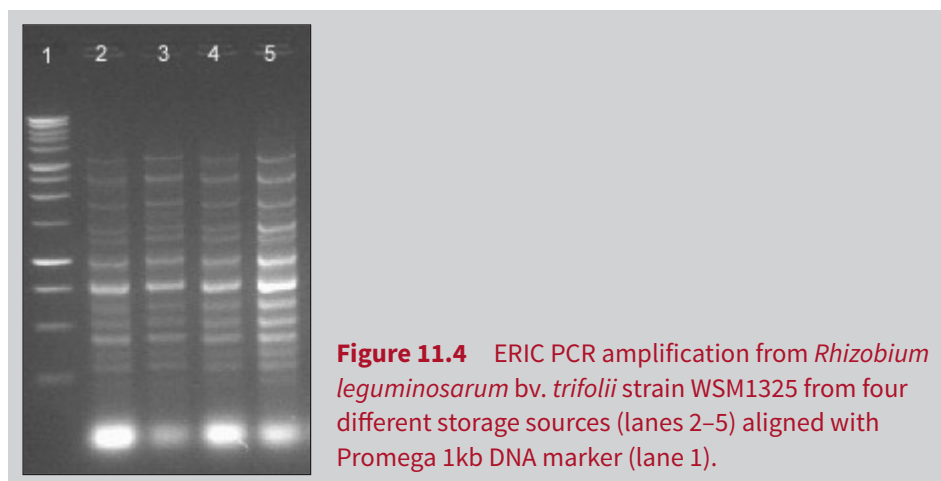
1. Prepare a 1.5% (w/v) agarose gel in TAE buffer (0.75 g of molecular-grade agarose in 50 mL of  $1\times$  TAE buffer).

Melt mixture in a microwave or water bath until clear. Cool to  $50^{\circ}\text{C}$  and add SYBR- safe DNA gel stain (Invitrogen; use at a final concentration of  $1\times$ ) or Ethidium Bromide (final concentration of  $0.5\ \mu\text{g mL}^{-1}$ ) and mix by swirling. Pour the gel into a gel tray, slot in the appropriate size comb and allow to set.

2. Mix loading buffer to  $1\times$  final concentration with PCR amplified sample.
3. Place the set gel inside the gel tank filled with  $1\times$  TAE buffer.
4. Load PCR-dye mix prepared in Step 2 into a well. Also load a commercial 1 kb DNA ladder with size ranges from 250 to 10,000 bp in a well.
5. Connect the electrodes to the power source and run the gel at 80 V (time is dependent on gel size but approximately three to six hours; reproducible results require this to be standardised between runs).

### 11.3.5 Analysis

Unique banding patterns (e.g. Figure 11.4) can be obtained by these repetitive-PCR methods to assist with rhizobia typing. Banding patterns are analysed by determination of amplicon size relative to a 1 kb ladder. The presence of a control (mother-culture-isolate) is necessary to validate your samples. Reproducibility may be enhanced by performing duplicate PCR reactions in independent cycling runs. The authors also use the *Phoretix*<sup>™</sup> 1D Advanced software analysis package (Nonlinear Dynamics, UK) for analysis of banding patterns from uploaded gel images. Alternative software packages such as AMBIS and GelCompar are mentioned at <http://www.msu.edu/~debruijn/> (Rademaker and De Bruijn 1997).



**Figure 11.4** ERIC PCR amplification from *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 from four different storage sources (lanes 2–5) aligned with Promega 1kb DNA marker (lane 1).



## Reagent stock preparation

**Table 11.8** 5× Gitschier Buffer

Reagent	Volume (mL)
Tris (1 M, pH 8.8)	6.7
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (1 M)	1.66
0.005 M EDTA (pH 8.8)	0.13
MgCl <sub>2</sub> (1 M)	0.67
2-Mercaptoethanol (14.4 M)	0.21
DNase-free water	10.63
Total Volume	20

NB: prepare 5× Gitschier buffer and store 1 mL aliquots at –20°C.

6× Loading Dye: 0.4% (w/v) bromophenol blue in 50% (v/v) glycerol.

TAE Buffer (50×): 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), distilled water to 1 L final volume.

## 11.4 References

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