**A simple and effective method for isolating rhizobia from fresh, -80 oC-frozen or dry nodules on agar plates and the generation glycerol socks of isolates obtained[[1]](#footnote-1)**

1. Take a series of 5 Petri dishes and into each add 20 ml of either: 1) 70 % ethanol; 2) 2.5 % Na hypochlorite (NaClO)[[2]](#footnote-2); 3), 4) and 5) sterile distilled water. For dry nodules soak over-night in sterile distilled water prior to embarking on this step.
2. Allow the nodule to thaw out (if necessary) then add to each container with 5 min/EtOH, 1 min/NaClO, and then wash through each water bath, *ca.* 20 s each.
3. Place onto yeast mannitol agar (**YM[[3]](#footnote-3)A[[4]](#footnote-4)**) plates. Squash the nodule against the side of the plate using sterile tweezers and use a plate spreader to disperse the nodule juices across the plate. Incubate upside down (agar side upper-most), overnight at 28 oC. Do not enclose/seal the plates in any way box.[[5]](#footnote-5)
4. Check for growth of colonies, and select individual white/pale and mucilaginous looking colonies for further growth. NB - avoid dark red colonies/growths, or those that look ‘dry’ or fungal. Aim to select one colony and streak this onto a fresh Tryptone-yeast agar (**TYA),** plate. Again, incubate overnight at 28 oC.
5. Once re-grown, take a single colony using a pipette with 200 µl (yellow tip), from the pure isolate on TYA. Inoculate a sterile 5 ml **TYB**roth[[6]](#footnote-6) by washing the tip in the liquid (the tip may be ejected into the culture too).
6. Grow the TYB over night at 28 oC (with shaking). After 24h (at log phase), take 0.9 ml of culture and combine with 0.9 ml of sterile 50% glycerol. Mix and freeze in liquid-N2. Store the glycerol stock at -20 oC.
7. The stock may now be used directly for PCR or infection tests.

1. All operations should take place in a sterile flow hood. [↑](#footnote-ref-1)
2. Household bleach is generally *ca.* 12.5 % chlorine, therefore dilute this x5. [↑](#footnote-ref-2)
3. **YM -** For 1 L:10 g, mannitol; 0.5 g, glutamate; 0.5g, K2HPO4; 0.1 g, MgSO4.7H2O; 0.05 g, NaCl; 1 ml (40 g L-1) CaCl2; 1 ml (4 g L-1) FeCl3; 1g, yeast extract. Add distilled water to almost 1L, pH to 6.8, make volume to 1L. Dispense into 25 ml medical (universal) sample bottles as 5 ml aliquots. Autoclave, or add agar and Congo Red first (see below). [↑](#footnote-ref-3)
4. **Agar Plates**: (1 L) add 15 g (1/5 % [w/v]) technical grade agar, and 10 ml L-1 of **Congo Red Stock Solution** (sterile; 0.25 % [w/v]). Autoclave, and whilst still warm/liquid dispense 20 ml *per* 9 cm diameter Petri dish. [↑](#footnote-ref-4)
5. Fresh nodule extracts will yield cultures that generally grow well over-night. However, isolation from the other nodules types (dry or frozen), may take up to 3-4 days to appear as colonies that are large enough to discern their phenotype. [↑](#footnote-ref-5)
6. **TY -** For 1 L: 5 g, tryptone; 3 g, yeast extract; 0.913 g, CaCl2.2H2O. Add distilled water to almost 1L, pH to 6.8, make volume to 1L. Dispense into 25 ml medical (universal) sample bottles as 5 or 10 ml aliquots. Autoclave, or add agar and Congo red first (see below). [↑](#footnote-ref-6)