9. 3*M* Sodium acetate, pH 5.5.
10. 70 and 100% Ethanol.

3. Methods

3.1. Purification of Virus

The virus purification method described in this chapter has been developed to purify PEBV from *Nicotiana benthamiana* or *Nicotiana clevelandii*. This method has proved equally successful for purifying TRV from *Nicotiana tabacum*. An alternative method is also included (*see* **Note 4**). *Chenopodium amaranticolor* is a local lesion host for both viruses and can therefore be used as an indicator plant for infection.

1. Take plants at the 4–5 leaf stage, dust one leaf with Celite and mechanically inoculate with virus (*see* **Notes 1** and **2**).
2. Harvest plants 10–12 d postinoculation and, using a blender, homogenize the tissue in approx 2 vol of 50 m*M* sodium phosphate buffer, pH 7.5, containing 0.15% (v/v) thioglvcollic acid, adjusted to pH 7.5, with NaOH (*see* **Notes 3** and **4**).
3. Freeze the homogenate at –20°C for 1–2 wk, and then allow it to thaw slowly overnight to room temperature.
4. Clarify the homogenate by filtering through two layers of muslin, followed by centrifugation at 10,000*g* for 5 min at 4°C.
5. To precipitate virus particles from the supernatant, add PEG 6000 to a final concentration of 10% (w/v) and NaCl to 2% (w/v), at room temperature, then stir slowly at 4°C for 3 h (*see* **Note 5**).
6. Pellet the virus by centrifugation at 15,000*g* for 15 min at 4°C, and resuspend in 30 m*M* sodium phosphate buffer, pH 7.5 (*see* **Note 6**).
7. Clarify the resuspended virus by centrifugation at 10,000*g* for 5 min.
8. To remove any remaining plant material, extract the virus suspension with an equal volume of chloroform:butan-1-ol 1:1. Collect the upper layer containing the virus by centrifugation at 2500*g* for 5 min.
9. Re-extract the chloroform:butanol lower layer with distilled water, then pool the aqueous upper layers and re-extract with chloroform:butanol (*see* **Note 7**).
10. Pellet the virus by centrifugation for 4 h at 85,000*g* and 18°C and resuspend in 10 m*M* Tris-HCl, pH 7.5 (*see* **Note 8**).
11. If the virus is to be further purified, resuspend the viral pellet in 30 m*M* sodium phosphate buffer, pH 7.5, and layer the suspension onto a 30% sucrose cushion. Sediment the virus by centrifugation for 2–3 h at 140,000*g* and 15°C in a swing-bucket rotor and resuspend the pellet in 10 m*M* Tris-HCl, pH 7.5 (*see* **Note 9**).
12. To determine the concentration of the virus measure the optical density (OD) at 260 nm. An OD_{260} of 3 = 1 mg/mL for TRV and PEBV. An OD_{260}/OD_{280} ratio of 1.15 is expected for a pure tobravirus prep.
13. Store virus at 4°C.