### 3.2. Extraction of viral RNA

1. To remove any contaminating host DNA, incubate the purified virus for 2 h on ice with 25 μg/mL RNase-free DNase I in 1X DNase buffer. Stop the reaction by adding EDTA to 17 m*M*.
2. Incubate the virus at 37°C for 2 h with 200 μg/mL proteinase K in 1X proteinase K buffer to isolate RNA from the virus particles (*see* **Note 10**).
3. To extract the RNA, add 1 vol of phenol, and vortex vigorously. Separate the two phases by centrifugation at 10,000*g* for 5 min.
4. Remove the aqueous upper layer and re-extract it with an equal volume of phenol:chloroform-isoamylalcohol (1:1).
5. Pool the aqueous layers and precipitate the RNA with 0.1 vol of 3*M* sodium acetate, pH 5.5, and 2.5 vol of ethanol and collect the RNA by centrifugation.
6. Wash the pellet with 70% ethanol; then dry the RNA under vacuum and resuspend in sterile distilled water.
7. To determine the concentration of RNA measure the OD at 260 nm using an $OD_{260}$ of 25 = 1 mg/mL, or, alternatively, estimate the concentration after analysis by electrophoresis on an denaturing agarose gel (*see* **Note 11**).

## 4. Notes

1. Sap from a virus-infected plant or purified virus can be used as an inoculum. Sap is prepared by grinding virus-infected tissue in 30 m*M* sodium phosphate buffer, pH 7.5 (1 g tissue/1 mL buffer). The sap is then diluted 1:10 with 30 m*M* sodium phosphate buffer, pH 7.5, and 20 μL is inoculated onto each plant. The inoculum is stored at –20°C in an undiluted form. Alternatively, plants can be inoculated with purified virus at a concentration of approx 10 μg/mL.
2. Local lesions should appear on *Chenopodium* indicator plants 3–5 d postinoculation, confirming the infectivity of the inoculum.
3. Harvest whole plants, remove the roots, and homogenize the remaining plant material. 200–300 g of plant tissue should yield approx 20–30 mg of virus.
4. Tobraviruses can be purified using an alternative method that avoids the use of thioglycollic acid and solvents, and instead involves repeated cycles of low- and high-speed centrifugation. Omit thioglycollic acid from the extraction buffer and homogenate the tissue in 50 m*M* sodium phosphate buffer. pH 7.5. Freeze the homogenate at –20°C for approx 2 wk, thaw overnight, and then proceed with **steps 4–7** of **Subheading 3.1.** Obmit the solvent extraction **steps 8** and **9** of **Subheading 3.1.** and proceed with **step 10** of **Subheading 3.1.** Repeat the low- and high-speed centrifugations (**Subheading 3.1., steps 7** and **10**) until all traces of pigment and other contaminating materials are removed and the viral pellet is white. The purity of the virus prep can be assessed by determining the $OD_{260}/OD_{280}$ ratio. A ratio of 1.15 is expected for a pure tobravirus prep.
5. The solution should be stirred at room temperature until all the PEG dissolves, and then stirred in a cold room for 3 h.