4. Add test antigen diluted in sample buffer. Any convenient volume between 50 and 200 μL is suitable, but it should not be greater than that used in coating the plate. For assay of virus capsid protein in infected sap, the optimum dilution of sap may range from 1:5 to 1:1000 and has to be determined by a preliminary assay (*see* **Notes 3** and **8**). The layout of test samples and calibration samples on a plate is discussed in **Note 4**. Preferably, incubate dilute sap overnight at 4°C. Alternative incubation periods are 3–4 h at 25°C or 2–3 h at 37°C.
5. Wash thoroughly with PBS-Tween to remove all traces of nonspecifically bound antigen. Wells should be filled and emptied at least three times.
6. Add alkaline phosphatase-conjugated antigen-specific antibody diluted in conjugate buffer warmed to the intended incubation temperature. Use at a dilution previously determined to give maximum discrimination between positive and negative samples. Volume per well is that used for antigen samples. Incubate for 3–4 h at 25–37°C, or overnight at 4°C (*see* **Notes 6** and **7**).
7. Wash thoroughly with PBS-Tween.
8. Add freshly prepared *p*-nitrophenyl phosphate substrate, 1 mg/mL (preferably the same volume/well as in **step 6**), diluted in substrate buffer warmed to room temperature. Incubate at room temperature, away from direct sunlight, and record absorbance at 405 nm, when color has developed, usually after 15–60 min (*see* **Note 5**). If necessary, development of color can be halted by the addition of 50 μL 3*N* NaOH/well.

3.3. Calculation of Virus Protein Concentration in Test Samples

1. Prepare a calibration curve for purified virus protein based on mean absorbance values obtained for each concentration of purified viral protein.
2. Calculate mean absorbance values for each virus-inoculated sample and subtract the absorbance value obtained for the noninoculated (virus-free) control.
3. Using the calibration graph, convert absorbance values into ng virus protein per well.
4. Allowing for dilution of sap and volume per well, calculate weight of viral protein per unit weight of plant tissue or unit volume of sap. For example, in **Fig. 1A**, the 1:1000 dilution of sap yielded an absorbance value of 0.6, which **Fig. 1B** shows is equivalent to 62 ng viral protein. Because each well contained 50 μL of diluted sap, the calculation for concentration of virus protein is: 62 ng × 1000 (dilution factor) × 1000 μL/50 μL = ng virions/mL of undiluted sap = 1.24 mg PVX virions/mL of undiluted sap.

   A portion of the leaves from which sap was extracted was weighed, dried, reweighed, and calculated to have 14.4% dry matter. The concentration of virions therefore can be recalculated as: 1.24 × (100 – 14.4)/100 = 1.06 mg PVX virions/g fresh wt of leaf.

4. Notes

1. At all stages of ELISA, thorough washing of wells is essential to minimize background color. Flaws in the performance of mechanical/automatic washers are a