amount of flanking sequence, necessitated by the absence of restriction enzyme sites at the N- and C-termini of the gene. If cloning of only the CP gene sequence is required, it will usually be necessary to engineer appropriate restriction sites, either by mutagenesis ***(24)*** or by PCR amplification of the required fragment with mutagenic primers, in which case it is advisable to sequence the PCR fragment. Such mutagenesis should not be necessary if the aim is to engineer resistance, rather than identify the resistance mechanism; but always ensure that a translation initiation codon is not produced between the promoter and the CP initiation codon. For genes that are normally expressed as a polyprotein (e.g., potyvirus genes), an AUG initiation codon within a Kozak consensus may need to be added for CP expression ***(25)***; for those CP genes that are not the 3'-terminal gene (e.g., in comoviruses), a translational terminator should be inserted at the appropriate site.

The amount of DNA to be digested will also vary, depending on how large a proportion of the construct the CP gene occupies. For example, a 1-kb CP gene cloned into a pUC-based plasmid (of approx 3-kb size) represents about 25% of the total DNA concentration, thus a maximum of 2.5 μg of CP gene DNA may be released from 10 μg of the construct. It is wise to assume that up to 50% of the total gene fragment may be lost during the extraction procedure and always to start with more construct DNA than is strictly necessary.

6. A 1% agarose gel is suitable for most CP gene fragments; the buffer used will depend on the gel purification procedure used. Many commercial kits are available for gel purification, in addition to the techniques described in Chapters 27, 33, and 43. **Note:** Ethidium bromide is carcinogenic; use gloves when handling it and limit exposure by purchasing preweighed tablets or solutions (Sigma, Poole, Dorset UK). To avoid breakage of the CP DNA, expose it to UV irradiation for the minimum time, and if possible use the preparatory option on the transilluminator, rather than the analytical setting. **Note:** Ultraviolet light may burn exposed skin and damage eyes; always wear appropriate protective gloves and glasses and/or face visors.
7. The restriction enzyme sites used will depend on the sequence of the CP gene being used. Gel purification of the cut vector will minimize the inclusion of any uncut vector and remove the small *Bam*HI-*Sac*I fragment. If the vector is digested with a single enzyme, remove the 5' phosphate using calf intestinal phosphatase (*see* Chapter 27). It is useful to do this dephosphorylation step, even when two different enzymes have been used, as it will prevent religation if one of the enzymes has not completely digested the vector DNA. Dephosphorylation is especially advisable when the vector does not have color selection to facilitate identification of insert-containing colonies.
8. Estimate the DNA concentration of the vector and CP fragments by comparing their fluorescence following gel electrophoresis and ethidium bromide staining with that of a marker of known concentration. For the ligation, include two tubes: one containing 100 ng vector, the other 100 ng vector plus ligase, to monitor the success of the vector digestion and dephosphorylation.