15. The concentration of $MgCl_2$ should generally be in the range of 0.5–2.5 m*M*, and, if the DNA solution contains EDTA, this should be taken into consideration, because it will chelate out the magnesium. Primer concentrations are generally in the range 0.1–1 μ*M* (10–100 pmol/50-μL reaction), and annealing should occur within a few seconds. It is best to use the minimum amount of primer that does not limit amplification, to avoid nonspecific priming.
16. The choice of polymerase depends on the properties desired; e.g., Vent polymerase has 3'–5' proofreading exonuclease activity, thus resulting in a higher fidelity. *Taq* polymerase has historically been the most commonly used enzyme, and is generally added at 1–2 U/50-μL reaction, but the units needed are highly dependent on the commercial source. Use of too much enzyme can result in nonspecific amplification. The specificity and sensitivity can also be improved by adding the chosen polymerase enzyme once the initial denaturation step has been completed (termed hot-start PCR).
17. The duration of each cycle should also be optimized. It is important that complete denaturation of the starter template is achieved, without causing excessive denaturation of the enzyme (*Taq* polymerase has a half life of ~40 min at 95ºC). Increasing the duration of the annealing step will decrease specificity. *Taq* polymerase can incorporate about 150 bases/s/enzyme molecule, under optimum conditions. A safe rule is to allow 1 min at 72ºC for every 1 kb of desired product.
18. 3:1% NuSieve:SeaKem (w/w) agarose (FMC, Rockland, ME) gives better resolution for experiments in which the additional cost can be justified.
19. Ethidium bromide staining is the most common method, but, if greater sensitivity is required, it can be coupled with blotting and a hybridization step. An alternative is to include a fluorescent-labeled nucleotide during PCR. The greatest sensitivity of detection of fluorescence is by gel electrophoresis on a fluorescent DNA sequencer, which is 100-fold more sensitive than detecting fluorescence on a solid phase ***(23)***. The amount of PCR product can also be quantified by incorporating a radiolabel (e.g., 50 μCi/mL [–^{32}P]dCTP). In this case, measurement of particle emission can be carried out over a 2-log larger range than light transmittance, and hence may be preferred ***(24)***.
20. Synthesizing an internal RNA standard: Alternatively, clone a section of DNA containing primer sequences into a vector containing T3, T7, or SP6 polymerase recognition sequence, and perform in vitro transcription according to manufacturer's instructions. Because it is often desirable to be able to compare the results from one RT-PCR run with another, ideally, the standard used should be from the same preparation. To avoid RNA degradation between experiments, precipitate the RNA in the presence of carrier RNA, and keep suitable aliquots at –80ºC. When needed, pellet, redissolve, and quantify the amount by UV spectroscopy.
21. Our experience with this technique is with PCR from insects, rather than plants. For PCR from DNA, primers against any single copy genomic sequence are worth trying. For RT-PCR in aphids, we have used actin as a control; sequences are highly conserved, and expression is essentially constitutive. The primers