3. The primers should be 18–30 nucleotides long, with a T_m of 55°C or above. The T_m can be approximated by assuming each A or T contributes 2°C and each G or C contributes 4°C, and adding up the values. Where Inosine is used (as a totally redundant base), it will not contribute to the T_m. Where redundant primers are used, calculate the lowest T_m and start from there.

4. As far as possible, design primers with the same T_m.

5. Primers should be approx 200–2000 bases apart; if less, the product can be more easily confused with primer-dimers, and, if larger, the product may not be efficiently amplified.

6. Primers forming hairpin loops, or pairs having complementarity at 3' ends should be avoided.

7. Do not use oligo(dT) on its own: Have this anchored, by using a mix of 3 oligo(dT) primers with A, C, or G at 3' end (*see* **Note 8**).

8. Mismatching of T bases in target sequence or primer occurs at a higher rate than for other bases, so it is best to avoid a T at the 3' end of the primer for discriminating similar DNAs *(8)*. In most cases, try to ensure that the 3' end has a GC clamp.

9. Avoid runs of three or more of the same base.

Notwithstanding these comments, primer unpredictability is such that, if there is no other choice, try it anyway. A final comment: Check the primer sequences against the nucleic acid databases to make sure you have not inadvertently designed a primer that would amplify some other known sequence.

3.3. Reverse Transcription

1. Prepare a master mix of all the components listed below, except the RNA template. Make sufficient mix for all samples, plus available positive and negative controls, plus one spare reaction to allow for pipeting errors.

    For 1 × 25-μL reaction (15 μL mix, 10 μL RNA): 4 μL sterile ddH$_2$O (DEPC-treated), 5 μL AMV RT buffer (5X), 2.5 μL dNTP mix (10 m*M*), 2 μL downstream primer (50 μ*M*); or 2 μL random hexamers (100 μ*M*), 0.5 μL RNasin (40 U/μL), 1 μL AMV RT (20 U/μL) (*see* **Note 9**).

2. Heat RNA samples (0.05–1 μg in 10 μL vol) at 70°C for 3 min and chill on ice (*see* **Note 10**). Spin samples for a few seconds to remove condensation before adding 15 μL master mix. Perform RT at 42°C (37°C for random hexamers) for 30–60 min.

3. Because reverse transcriptase activity inhibits *Taq* polymerase, heat-inactivate samples (95°C, 5 min) after RT, then chill on ice.

3.4. Polymerase Chain Reaction (**see** *Note 11*)

The great sensitivity of PCR results in crosscontamination being a common occurrence. Extreme care should be taken with template preparation and setting up of RT and PCR, particularly at steps where aerosols can be formed, e.g., opening of tubes and ejecting tips used for pipeting PCR products (*see* **Note 12**).