2. The concentration of primers is estimated by measuring the absorbance of an aliquot at 260 nm, since a 33-μg/mL solution of ssDNA equals 1 A_{260} unit. For example, 10 μL of the adaptor primer was diluted with 990 μL of SDW and the A_{260} was 0.19 U. Since the sample was diluted 100×, the concentration of the stock solution is 0.19 × 100 × 33 μg/mL. i.e., 627 μg/mL. Often, it is necessary to calculate the molarity of primers; a rule of thumb is: 1 μg of primer = 1.52 × (2000/*n*) pmol, where *n* = the number of bases. For example, the dT_{17} adaptor primer is 35 bases long and is at 627 μg/mL. Therefore, 1 μg of primer contains approx 87 pmol, 1.15 μg of primer contains 100 pmol, and so 1.8 μL of the 0.627 μg/μL stock is required for the cDNA reaction.

3. The T_m of a primer is the temperature at which 50% of the primer is annealed to its complementary target sequence; this figure is used as a starting point in determining the optimum annealing temperature. A rule of thumb for calculating the T_m of a primer is: T_m = (4 × number of G + C residues) + (2 × number of A + T residues) For example, the adaptor primer contains 10 G + C bases and 8 A + T bases, and so the T_m is approx 56°C, i.e., the sum of (4 × 10) + (2 × 8). It is desirable to construct primers with roughly equal T_ms, but in practice this is not always possible. The annealing temperature for a PCR reaction is usually 2–5°C below the T_m, but can be increased above the T_m, to increase the specifity of the reaction if required. At normal primer concentrations (0.2–1.0 μm), the annealing step requires only a few seconds.

4. In practice, it is more convenient and accurate to make up a cocktail containing all reaction components except the template DNA. The cocktail is then aliquoted and the template added separately to each tube to minimize the opportunity for crosscontamination from sample to sample.

5. A wide range of DNA thermocyclers are now available; and the best machines have good uniformity of temperature over the block, reproducible heating, and cooling rates, and are set up to ensure that the contents of the tube (rather than just the block itself) reach the programmed temperature. Specially designed thin-wall PCR tubes can also be used for fast heat transfer. Most PCR reactions are performed as step programs in which the machine moves from one temperature to the next at the maximum rate, but with many machines it also possible to control the rate of heating and cooling, as required. The temperatures and length of time required for each part of a PCR cycle will vary, depending on the nature of the template, T_m of the primers, and length of the product. A temperature of 94°C is adequate for most templates but some GC rich DNA or complex genomic DNA samples may require higher temperatures or longer incubation times. The annealing temperature depends on the T_m of the primers (**Note 3**); the elongation time at 72°C varies according to the length of the target being amplified, but most reactions allow 1 min for each kb of sequence. The elongation time can be increased toward the end of a PCR reaction when the product concentration is high. In addition, a 5- to 10-min incubation at 72°C is attached to the end of most PCR programs to ensure complete elongation of products in the final cycle to maximize the final yield. The number of cycles required will vary with the amount