to 19-fold as compared to the sequence-specific primer. For approximately 40% RT-PCR assays, oligo-dT primer is used, which yields more reliable cDNA from the RNA pool. Oligo-dT is best when amplification of mRNA is required from a limited RNA source. Full-length RNA is required for oligo-dT, which limits the transcription of RNA likely to be fragmented. If the primary binding site is at the extreme 5′-end of long mRNA, the RT may fail to reach the upstream primer binding site. Almost 20% of RT-PCR uses target specific primers. Target specific primers provide high sensitivity for quantitative assays and synthesize most specific cDNA. However, this method requires careful experimentation, as it is a wasteful technique. This method also requires separate priming reactions, which limits its reproducibility at a later stage. Almost 10% of real-time PCR uses oligo-dT and random primers in combination. Every method of generating cDNA differs from each other depending on target availability, synthesis techniques, and cDNA yield. The commonly used RTs are Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and avian myeloblastosis virus reverse transcriptase (AMV-RT). MMLV-RT possesses less RNAse activity as compared to AMV-RT. AMV-RT can retain polymerization activity up to 55°C, thereby eliminating any problem with the secondary structure of RNA.

6.5.2 Polymerase Chain Reaction

Polymerase chain reaction is a method of obtaining DNA replication, that is, many copies of a DNA sequence from a small DNA sequence are obtained. Hence, the single gene/DNA sequence amplification is obtained even for a small tissue piece (Fig. 6.7). PCR is widely used in criminal forensics, biomedical research, and molecular biology. The aim of the experiment determines the DNA polymerase choice. Taq DNA polymerase is the most commonly used enzyme, which does not have a 3′–5′ proofreading exonuclease activity but exhibits a 5′–3′ nucleus activity. Vent/deep vent or Pfu can be used as exonucleases, with almost two orders of magnitude difference for the Michaelis constant from the Taq DNA polymerase. For every enzyme, different conditions and primer length must be used. Primer degradation is a concern from the 3′ end during the proofreading process. Primers with a longer size are degraded at a faster rate than those with a small size. If phosphorothioate linkages are present on 3′ bases, then the degradability issues can be counteracted. Fig. 6.8 demonstrates the nucleic acid quantification by real-time PCR.

During the process of cycling in RT-PCR, the product accumulation is quite high to increase the fluorescence signal above the background noise. The point at which the product accumulation is high defines the crossing point (C_p) or threshold cycle (Ct) and is given by (Ståhlberg et al., 2005)

$$N_{Ct} = N_0 \left(1 + E\right)^{Ct} \tag{1.1}$$