Chapter 7
Analysis and Troubleshooting

Contents

Bibliography ................................... 246
7.1 How to Design Primers for Real-Time PCR Applications ............ 247
  7.1.1 TaqMan® Probes and Primer Design .................. 249
  7.1.2 Storage of Primers and TaqMan® Probes ............... 250
  7.1.3 SYBR® Green Assays .................................. 250
  7.1.4 Optimisation of Primer Concentration .................. 252
  7.1.5 Multiple Bands on Gel or Multiple Peaks in the Melting Curve .... 253
  7.1.6 Effect of Magnesium Chloride and Primer Concentration .......... 254
  7.1.7 Molecular Beacons Assays .......................... 254
7.2 Assay Performance Evaluation Using Standard Curves ............. 254
  7.2.1 Threshold Selection .......................... 255
  7.2.2 Quantification of Gene Targets with the
  Quantitative Real Time PCR: Absolute and Relative
  Gene Quantification .................................. 256
  7.2.3 Relative Quantification .................................. 256
7.3 Most Common Problems When Performing Real-Time PCR ........... 257
  7.3.1 PCR Amplification Problems .......................... 257
  7.3.2 Control Samples .................................. 258
  7.3.3 Signal Problems in Real Time PCR ....................... 258
  7.3.4 Amplification Plots .................................. 259
7.4 Summary: Optimised Real-Time PCR Assay .......................... 260

7.1 How to Design Primers for Real-Time PCR Applications

When designing primers, it is of initial importance to define the target area,
and secondly the type of application. The BLAST function from the National
Center for Biotechnology Information (NCBI) will help to identify the most suit-
able gene sequence to be used. There are many software programs; some free on
websites/pages on the internet, dedicated to primer design and primer optimisation. In this chapter the most important factors that need to be taken into consideration when designing and optimising primers are highlighted.

As a rule of thumb, the following guidelines should be followed when designing primers:

- Primers and probes should be selected in a region with a GC content lower than 80%.
- The amplicon size recommended for real-time PCR applications is between 50 and 150 bp. Do not exceed the amplicon size of 300 bp when designing primers as time efficiency is paramount as it will have a delayed effect on the \( C_T \).

When dealing with cDNA or mRNA amplification procedures – it is advisable to design a primer or probe which crosses one exon junction, thus in the case of genomic DNA contamination the latter will not be amplified. (Fig. 7.1 and Fig. 7.2)

- Primers should be 15–30 mer in length.
- Avoid the presence of secondary structures or primer-dimer formation, as they can interfere with the amplification, particularly in SYBR® green applications.

**Fig. 7.1** Primers aligning to exons flanking an intron. Any product amplifying gDNA will be much larger than a product amplified from intronless mRNA

**Fig. 7.2** Primers that bridge an exon-exon junction on mRNA. No amplification of gDNA