

Designing Dual Labelled Probes

ABgene® Ltd do not manufacture hybridisation probes. For detailed protocols and/or troubleshooting please follow the manufacturer's guidelines. However, the points below offer a general guide:

Probes

- Probe should be 20–30 bases in length.
- Avoid including long sequences of identical nucleotides.
- The 5' end of a probe cannot be a guanosine residue. A guanosine residue adjacent to the reporter dye will quench some of the reporter fluorescence even after cleavage.
- The melting temperature (T_m) of the primers should be 65 to 67°C. The probe T_m should be approximately 10°C higher than the primer T_m .
- The reporter dye should be located on the 5' end and the quencher dye should be on 3' end.
- For allelic discrimination use VIC™ and FAM™ reporter dyes to label the probes.
- For allelic discrimination the polymorphic site should be positioned near the centre of the probe.

Primers

- Primers should be 20–24 bases in length.
- Short amplicons work best. Amplicon size should not exceed 300bp.
- Keep the GC content in the range of 20–80% (ideally 40–60%).
- Avoid including long sequences of identical nucleotides.
- T_m should be 55 to 60°C.
- Place the forward and reverse primers as close as possible to the probe without overlapping it.
- Ideally, primers should end in a G or C. This is called a GC clamp. However a run of 3 or more Gs or Cs is not recommended.

Tips

Simplified formula for estimating melting temperature:

$$T_m = 2^\circ\text{C} \times (\text{number of [A+T]}) + 4^\circ\text{C} \times (\text{number of [C+G]})$$

Optimal annealing temperatures may be just above or below the estimated T_m . As a starting point, use an annealing temperature 5°C below T_m and increase upwards in 2°C increments.

Avoid complementary sequences within primer sequences and between primer pairs. Commercially available computer software (e.g. Primer Express) and web-based software (Primer 3 www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) can be used for primer design. Use the software to minimise the likelihood of forming stable primer-dimers.

Special considerations are required for the design of RT-PCR primers and probes to avoid the amplification of genomic DNA. Primers should be designed so that they hybridise to half the 3' end of one exon and to the 5' end of the next exon along. Therefore primers will anneal to cDNA synthesised from spliced mRNAs only. Alternatively primers can be designed to span at least one intron-exon boundary so that amplified genomic DNA will produce small products to which the probe cannot bind. Amplified cDNA will produce the full length amplicon.