- primers should be 20-30 nts in length

- Tm = 2*(#AT) +4*(#GC)

- Aim for a Tm of 75 or greater by the above calculation. This allows you to to the PCR step with an annealing temp of 70. If calculated Tm is lower, do PCR with annealing temp 5 degrees lower than calculated Tm

- Ideal is 15 GCs with 10-15 ATs

- Avoid extensive stretches of A/T or of G/C in the primer.

- Tm for each primer should be within 10 degrees of each other, or one may bind more promiscuously than the other

- Final 1-3 nts at <u>3' end of primer</u> MUST be Gs or Cs. Minimum of 2 is best. NEVER end in an A or T. This is where the polymerase extends from, so it needs to be pinned down.

- Length of exon included product should be within ~2x length of excluded product. i.e. don't put primers so close to the junction that you have excluded = 60 and included = 300 nts. This will increase bias in amplification. Sometimes this means putting a primer 2 exons away from the variable exon. Also, think about resolution of the gel. Optimal sizes for products are 200-500 nts. You can distinguish 200 from 250, but harder to resolve 450 from 500. If differential will be small (i.e. variable exon is small) plan primers to give a total product closer to ~ 200 nts.

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