Appendix A. Robertson and Phillips
Primer Design Instructions

The advent of the Polymerase Chain Reaction (PCR) brought about the ability to rapidly make many copies of a segment of DNA. The PCR reaction depends on short pieces of DNA, called primers, to bind to the denatured DNA strands and act as a template for replication. Primers are usually 17-24 nucleotides in length and must be specific to the target DNA. Listed below are some rules and helpful tools that scientists use in designing primers. Finally, there are instructions on how to perform a nucleotide BLAST to check the specificity of each primer. Read through the information and then use these instructions to complete the Primer Design Exercise.

Rules for primer sequence design (adapted from Sambrook and Russell, 2001[1])

1. *To increase specificity, primers should range from 17-28 nucleotides in length and the forward and reverse primers should not differ by more than 3 base pairs in length from each other.* If you consider that there is a ¼ (4^{-1}) probability of finding an A, T, C, or G in any given DNA sequence then there is a 1/16 or 4^{-2} chance of finding any two nucleotide sequence such as AC. Therefore, a specific 16 base pair sequence will statistically occur once in every 4^{16} or approximately 4 billion bases. Thus complementary binding of a sequence that is greater than 16 base pairs is an extremely sequence-specific process.
2. *The base composition should have 40-60% (G+C) content*. GC content is used to determine the annealing temperature of the reaction. A GC content of 40-60% will ensure that the melting temperature is above 50ºC.
3. *Melting temperatures (Tm) between 52-62º C are preferred and it is best that the Tm, of the two primers, is within 5º.* Melting temperature indicates the stability of the double helix. Tm above 65ºC may form secondary annealing structures and should therefore be avoided.
4. *The 3'-ends of primers should not be complementary to any other part of the other primer, otherwise the ends will pair and create a primer dimer preferentially to any other product.* If primer dimers are formed early in the PCR, they can reduce the amplification of the target DNA by competing for the DNA polymerase, nucleotides, and primers.
   i.e. 5'-ACGTCCAAGAGG**AAGC**G-3'
   3'-ACTTACGACT**TTCG**AATGTAA-5'
5. *Primer self-complementarity should be avoided so that secondary structures are not formed or are very weak.* The example below shows how the underlined portions of the primer could pair with another primer molecule.
   i.e. 5'-AGTCCTA**GTC**CGAATGTTCT**GAC**

[1] Sambrook, S. and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.

Calculating Melting Temperatures of primers and target DNA
(adapted from Sambrook and Russell, 2001[1])

1. "The Wallace Rule"
   T_m (in ^oC) = 2(A+T) + 4(C+G)
   (A+T) = sum of A and T residues in the primer
   (C+G) = sum of C and G residues in the primer

2. DNA calculator
   This website will allow you to fill in your primer sequence to determine the length, Tm, and molecular weight for your primers and if there will be any secondary structures or possibilities of primer dimers.
   http://www.sigma-genosys.com/calc/DNACalc.asp

Determining the uniqueness of the primer sequence:

Specificity to the target gene is one of the most important properties of a primer. One way to determine the uniqueness of the primer sequence is to compare the primer to all other sequence data using a bioinformatics tool that is a Basic Local Alignment Search Tool, otherwise known as BLAST. The BLAST tool is maintained by the National Center for Biotechnology Information (NCBI) and can be found at the following link: www.ncbi.nlm.nih.gov/BLAST/ . There are many types of BLASTs, but we will only be dealing with the nucleotide BLAST (blastn) to determine the specificity of the primers.

1. Go to the above website. Under the heading "Basic BLAST" click on the link titled "nucleotide BLAST".
2. Enter the query sequence by typing the primer sequence into the text box.
3. In the "Choose Search Set" box select "nucleotide collection (nr/nt)" from the Database category.
4. Next type in the organism of interest, in this case we are looking for Bacteria (*P. putida*) or Viruses (Cauliflower Mosaic Virus).
5. In the next box, "Program Selection", select "blastn" as the type of algorithm.
6. Click on the blue BLAST Icon near the bottom of the page.
7. A new page will appear entitled, "Job Title". This page will update until the results are available.
8. The first figure on the results page provides an overview of the database sequences that are aligned to the query sequence, which is your primer sequence in this case. Following the figure is a list of sequences that are significantly similar to the query sequence. Each matched sequence contains a link to view the entire sequence, the name of the sequence, a score and an E value. The Expectation value (E) is a number that describes the number of matches a particular sequence would get by random chance. The lower the E value, the less likely the match would happen by chance and thus making the similarity of the query sequence to another sequence more significant.

   When working with short sequences, such as primers, the best way to determine how well the sequences align is to look at the alignment and the length of homology between the primer sequence and the hits from the genome database.

As long as there are not any matches in the species you are working in, the primers should amplify only your gene of interest.

9. Scroll down and see how the base pairs align with the sequence matches of the same species. If more than 5 matches are found, the primer is not specific enough.

After designing the primers and checking the quality, the primer sequence is sent to a DNA synthesis library such as Sigma-Genosys or Integrated DNA Technologies. Primers are synthesized, desiccated, and returned to you. Once you have received the primers, you need to re-hydrate the primers in sterile water or Tris- EDTA buffer.