**Tips for Primer Design**

**Overview**

1. Your sequence data will come from the *Chamaecrista* Genome Explorer. You will want it to be in FASTA format.
2. We will use the web-base application called Primer3 to design primers. Go to <http://frodo.wi.mit.edu/> and enter your FASTA sequence in the big box. Fill in the Sequence Id box with your sequence name and then click on the “Pick Primers” button. The first pair of forward and reverse primers is usually your best bet. More specific details are found below.
3. Enter your primers on the spreadsheet on the spreadsheet in lab. Be very careful to avoid typos.

# Polymerase Chain Reaction Primer Design

RT-PCR primers (also called oligos) are short, single stranded DNA molecules of known sequence that allow for amplification of a single region of the transcriptome. Your primers should hybridize with a sequence of DNA specific to the coding region of the targeted gene. Designing effective primers is an important first step towards cloning and sequencing because without working primers you will not be able to pull out the gene you wish to characterize. We will use the web-based computer program Primer3 to design our primers. Though you may use other applications well, this program is a particularly good primer design tool because it allows you to tweak an oligo sequences and easily test their hybridization ability. Whether or not you choose to test your primers in other ways, Primer3 is an excellent place to start.

Before you start working with a specific gene sequence, here are some general guidelines for designing primers that are likely to work well with the RT-PCR kit (Adopted from the *QIAGEN OneStep RT-PCR Handbook*):

**Length**: The primer should be 18 to 30 nucleotides in length. Generally primers are between 20 and 24 nucleotides.

**G/C Content**: 40% to 60% of the bases in an ideal primer should be either guanine or cytosine. G and C interact with three hydrogen bonds, as opposed to A and T, which bond with only two. Therefore, bonding between G and C is stronger and a high G and C content ensures that your primer will hybridize in a stable fashion with the region to be amplified.

**Tm (melting temperature) and Ta (annealing temperature)**: Melting temperature of the left and right primers, given by Primer3 and printed on the primer information sheet, should be similar. A melting temperature between about 55oC and 62oC is desirable. If Tm values for left and right primers are considerably different, it is difficult to be sure that each will bind to cDNA during the amplification steps of the PCR reaction.

**Primer3 Guidelines**:

<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi>

The cDNA sequence can then be pasted into the large box at the top of the Primer3 input screen. The program should not be particularly picky about sequence format, but only pasting in the nucleotides and deleting the tags at the 5’ end ensures that Primer3 will recognize your letters as a DNA sequence.

Hitting the “Pick Primers” button below the sequence will give you Primer3’s initial “best guess” oligos. There should be both a left and right primer displayed. If no primers are found, try the following

-Lower the minimum melting temperature from the default 57oC to about 54oC or 55oC and lower the minimum primer size by a nucleotide or two (from 18 to 16 bases). However, it is unlikely that Primer3 would not find oligos on its first try using the whole sequence for all product sizes.

-Once initial primers have been found, Primer3 will allow you save primers you like and check them together with other possible oligos. Note that Primer3 is only a website and your favorite primers can easily be lost by accident. Working with your journal or a Word document open and saving primers as you go may be a good idea.

-There are multiple ways to design effective primers, so absolutely don’t let this protocol stifle your creativity. However, below we’ve given guidelines about how to proceed from the initial primers.

-Primer3 will often find a good oligo at the extreme 5’ or 3’ end of the sequence you give. These are valuable regions to target because primers at the sequence ends will help your efforts to sequence a whole gene. One of these sequences can be pasted into the “Pick left primer or use left primer below” box (or the corresponding box for the right primer) on the Primer3 input site and used in the next search.

-After choosing one primer to work with, the product size ranges can be restricted to amplify a desired region. Choosing a primer at the 5’ or 3’ end to save and then restricting the product size range to the whole gene, or 601-700, 701-850, 851-1000 bp if your sequence is long, lets you amplify a large gene region. The program complains if you ask for products of a certain size independent of a range, but the range can easily be restricted in a way to allow you to amplify regions of desirable size.

-Warnings such as “High end self complementary” can be avoided by tweaking the end of your primers by a base or two. Primer3 will alert you if your sequence is likely to form a hairpin, and the program should avoid producing primers that will bind to each other in a PCR reaction. Too low a melting temperature can be alleviated by adding more bases to your primer on either side. The longer a primer, the higher its melting temperature.

-Once you find two acceptable primers that amplify a large portion of your gene and correspond relatively closely in their melting temperature values (±5oC) remember to print the Primer3 output.

# Checking for Single Gene Specificity

-Before ordering your primers, it is important to confirm that they do not bind to multiple coding regions in any species you will study. To check this, paste the primer sequence into the nucleotide-nucleotide BLAST on local site. Study all returned sequences with a high degree of homology to make sure that you are unlikely to pull out multiple gene regions in *Chamaecrista*. BLAST is not designed to work with such short reads so you may not always come up with good matches.

**Double Check for Self-Complementarity**

If your primer self-complements it won’t do a very good job amplifying the mRNA you are interested in. Primer3 checks for this, but you can explicitly check using the Oligo Calc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). This site at Northwestern University also provide information on the equations that are used to determine melting temperatures.