Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory
Lab 3: Oligonucleotide Primer Design for the Polymerase Chain Reaction

(note: Please bring an electronic copy of your *formatted* sequence file from the *Database Searching* Lab)

The Polymerase Chain Reaction (PCR) technique has had a profound influence on biochemistry and molecular biology since its development in the mid-1980's. Using a temperature-stable enzyme and oligonucleotide "primers", which are complementary to a known DNA sequence, it is now possible to make many copies of DNA from a target sequence. The amplified DNA can be made in sufficient quantity for further analytical techniques such as sequencing ("reading" the order of bases in a gene), restriction enzyme analysis or cloning. We will learn more about these and other techniques as the semester progresses, but for right now we'll concentrate on the PCR technique and primer design.

In order to design primers for amplification of a DNA target, we must know something about its nucleotide sequence. In our case, the DNA sequence of the green fluorescent protein gene is already known. We will use the sequence we obtained last week to design primers for a PCR reaction. As mentioned, DNA primers are short (15-30 bases), oligonucleotide polymers designed to base pair to a particular target sequence in a complementary fashion (A pairs with T, C pairs with G) (figure 1). Once bound to the target sequence, the double-stranded complex is recognized by the enzyme *DNA polymerase*, which uses it as a starting point for the synthesis of a new strand of DNA.

If we have the DNA sequence for a target gene, the process of designing these oligonucleotide primers is relatively easy. Many primer design programs are available on the internet for free. We will be using one known as "Web primer". These programs automate the design process by prompting the user to enter relevant information such as the DNA target sequence to be amplified, the approximate region on the target sequence where the primers are desired to anneal, minimum, optimum and maximum annealing or "melting" temperatures (T_m) for the primers (the temperature above which the the oligonucleotides have trouble hydrogen bonding to their complementary target DNA sequence) and other, important parameters such as the desired guanidine and cytosine base content (higher content increases the strength of annealing).

Procedure

1. **DNA sequence**

Go to the *Web Primer* web site (www.yeastgenome.org/cgi-bin/web-primer). Copy and paste the GFP DNA sequence from the file you created last week into the DNA sequence text box. DO NOT include text information associated with the sequence (name, genbank ID, etc.), although it is OK to keep the numbers from the beginning of each line of sequence. DO NOT Enter the name for the gene in the "locus" text box above. Select "PCR" as the purpose of the primer design task and submit.

2. **Location**

Web Primer will bring you to a page where you will enter information about the design of the primers. The first option, "Location" determines where on the GFP sequence the primers will anneal (bind to a complementary sequence). We need to have some flexibility here, so check the "NO" reply to "EXACT endpoints". We would like a region of 50 bases at each end of the

sequence for designing workable primers approximately 20 bases long, so enter “50” for the length of DNA in which to search for valid primers.

3. **Melting temperature**

Melting temperature is a parameter used to dtermine the strength of binding between a primer and its target sequence. A higher melting temperature ensures that nonspecific regions, that may resemble the desired primer binding site, are not amplified. However, if the melting temperature is too high the primer will have trouble binding to even the correct target site. In general, primer design usually calls for an optimum temperature of 60˚C, a minimum temperature of 45˚C, and a maximum temperature of 65˚C.

A primer length of approximately 20 bases is also preferred. Enter 18 for the minimum and 22 for the maximum number of bases.

4. **Primer composition**

Primer composition refers to the number of deoxyadenylate (A), deoxyguanylate (G), deoxycytidylate (C) and deoxythymidylate (T) residues in the primer. The higher the number of G + C residues present, the higher the temperature that will be required to “melt” (unbind) the primer from its target DNA. This occurs because a G/C base pair in DNA consists of three hydrogen bonds, while an A/T base pair consists of only 2. In general, it is desirable for a primer to have between 40 and 50% G and C content. You may leave these parameters set to their default values.

5. **Primer annealing**

Because DNA primers, like other DNA molecules, hydrogen bond to complementary DNA sequences, we must take care to design our primers so that they are not *too* complementary to each other. Otherwise, the primers will anneal to each other rather than their target DNA site. Leave these parameters set to their default values.

6. Submit the primer design information.

If the entered parameters for your sequence worked, the primer design program will list information regarding how many primer possibilities met the entered criteria.

If it did *not* work, the program should indicate *why* your parameters did not work. In this case, you will need to go back and make some adjustments.

Once you’ve found a workable set of parameters, you will also see a link that says “*This is the best pair of primers*”. This link provides a table of relevant information on the best primers for your PCR reaction, including the sequence of the primers, their length, G/C% and melting temperature (T_m). Note that the output also includes the sequence of DNA which will be amplified when these primers are used.

7. Complete the worksheet.

Answer the relevant questions on your worksheet. You may use internet sources to answer questions related to PCR enzymes. Turn in your completed worksheet at your next laboratory session, along with the modified copy of your sequence results.

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Please perform assignment during your laboratory section

1. Answer the following questions pertaining to your primers. (3 pt)

	Forward Primer	Reverse Primer
a. Sequence (5'-3')		
b. Length		
c. % GC		
d. Melting Temp (Tm)		

2. Determine the *reverse* and *complementary sequence* for your reverse primer. To do this, write down the complementary sequence of the reverse primer directly beneath it (recall, 'A' bases are complementary to 'T' and 'C' bases are complementary to 'G'). Now reverse the order of the bases in this complementary sequence so that the last base is first and the first base is last. You now have a sequence which is the *reverse* and *complement* of your reverse primer, in a 5'-3' orientation.

   Use Microsoft Word to open the electronic copy of the GFP DNA sequence file you created last week. Use the *highlight* command to find the sequence of your forward primer on the GFP DNA sequence. Because the forward primer binds to the *non-coding* DNA strand (not shown in your sequence file) it's sequence will be identical to a region found on the *coding strand* (our GFP DNA sequence).

   Now use the *reverse* and *complementary* sequence of your reverse primer to find the primer binding site for your reverse primer. Normally, the reverse primer binds to a sequence on the *coding* strand. To find this sequence we had to produce the *reverse* and *complementary* sequence of the reverse primer. Highlight this sequence on your GFP DNA. Turn in a new copy of your GFP DNA sequence file, with the appropriate primer binding site highlighted (2 pt)

3. What would happen to the melting temperature (T_m) of the primers if their % GC content was increased to 50%? What about if the length of the primers were increased to 30 bases? Why? (3 pt)

4. Use internet explorer to go to the web site for the *Dolan DNA Learning Center* (http://www.dnalc.org/ddnalc/resources/spotlight/index.html). Scroll down and select the "2D animation of PCR" and "3D animation of PCR" links to get a visual feel for how PCR works. Work through the 2D tutorial to answer the following questions:

a. Look at the graph for the production of PCR amplification products. How many DNA copies will be made after 10 rounds of amplification? After 20? (2 pt).

b. A typical PCR reaction consists of 30 cycles of denaturation, primer annealing, and extension. Theoretically, how many copies of a target sequence would be produced? (1 pt)

5. We know from our studies of enzymes and proteins in Biochemistry I that they are rather sensitive to environmental conditions of temperature, pH, etc. What is it about the enzymes used for polymerization of DNA in a PCR reaction (e.g. *Taq* polymerase) that distinguishes them from other DNA polymerases? (1 pt)