PRACTICE EXERCISE
PRIMER DESIGN USING CONSED AND PRIMER3

The following exercises will use Consed. Please move to the appropriate directory by executing (from your home directory):

```
cd AssemblyData/BAC/scratch/edit_dir/
```

Generate the needed consed file (.ace):

```
ta2ace –i bac.asm –o bac.ace –s bac.seq
```

Designing primers for sequencing gaps in Consed

Try to design primers to close sequencing gap 11 (between contigs 1 and 2) from the example closure BAC scaffolds.

1. In Consed, open Contig 2.
2. Go to the end of Contig 2 using the >>> button.
3. Find the 3'-most area of double sequence coverage and count back about 100 bases from that area to the left in Contig2.
4. Find a region of high quality sequence. To select a sequencing primer, highlight a portion of the sequence by holding down the left mouse button. Make sure that the length of the primer is between 18 and 21 nucleotides and that the melting temperature is 56-58°C. Make sure that the 3' end of the primer terminates in one or two G's or C's.
5. Using the emacs text editor, create a fasta file, copy your primer sequence to the file and save the file. Remember, a fasta file has the following format:
   ```
   >My_sequence_name
   ATGCATGCATGCATGCATGC
   ```
6. Close Contig 2.
7. Open Contig 1.
8. To design a primer from the beginning of the contig, Contig 1 has to be reverse complemented by clicking on the 'Comp Con' button in the 'Aligned Reads' window. Now the beginning of the contig becomes the end. Follow steps 3 to 5. Be sure to name your fasta primer file differently from your primer file for Contig 2.
9. Now, align your primers to the contigs to ensure that they are locally unique in the regions where you are likely to choose subclones to sequence. For example: `fasta34 –H my_primer.seq bac.fasta | more`
10. Are your primers locally unique around sequencing gap 11?
11. Next, select two gap-spanning subclones to use as sequencing template for your new primers.
12. In Consed, open the Assembly View.
13. Use the 'What to Show' button to ensure that gap-spanning mates are displayed.

14. Find the gap between Contigs 1 and 2 and mouse-over some of the spanning mates.
15. Click to display coordinate information of the mate pairs. Check the coordinates to ensure that your primers fall inside the subclone.

If you have time, try designing primers and selecting spanning subclones for gap 10.

Designing PCR primers for physical ends

Let's assume that we have information suggesting that two physical ends are adjacent, for example, the end of Contig 9 and the beginning of Contig 6. Try to design PCR primers to amplify a product between these two physical ends.

1. In Consed, open Contig 9.
2. Go to the end of Contig 9 using the >>> button.
3. Find an area of double sequence coverage and count 200 bases to the left.
4. Find a region of high quality sequence. Select a primer as above.
5. In a web browser (Netscape or Internet Explorer), go to the following web site: http://www.basic.northwestern.edu/biotools/oligocalc.html.
6. Copy and paste the selected primer into the window of the 'Oligonucleotide Properties Calculator' and click on the 'Calculate' button. The selected primer's length and Tm will be calculated. Make sure that the length of the primer is between 24 and 28 nucleotides and the melting temperature is 62-64°C. All PCR primers should be designed with similar melting temperatures (+/- 2 ^{o}C).
7. Check your primer for potential hairpin formation and self-complementarity by selecting the 'Self-Complementarity' button.
8. Next, the PCR primer has to be tested for uniqueness. Using emacs as above, create a fasta file containing your new primer sequence.
9. Align your primer against all the contigs in your project. Remember that these primers must be globally unique to the BAC, rather than simply locally unique as for sequencing gap primers.
   ```
   fasta34 –H PCR_primer bac.fasta | more
   ```
10. Open Contig 6.
11. To design a primer from the beginning of Contig 6, the contig has to be reverse complemented by clicking on the 'Comp Con' button in the 'Aligned Reads' window. Now the beginning of the contig becomes the end.
12. Follow steps 3 to 9 to design your second primer. Be sure to name the primer fasta file differently from your first primer.

Designing primers using MIT's Web-based Primer3

Try to design primers for sequencing gap 10 (between Contigs 1 and 5) using Primer3. Remember that Primer3 is designed to find primers flanking the input sequence for PCR amplification of that sequence.

1. Using a web browser, navigate to the Primer3 website: http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
2. Back in your UNIX terminal, split the bac.fasta file into individual fasta files:
   `seqretsplit bac.fasta`
3. Display approximately the last 600bp of Contig 1 by:
   `tail asm_1.fasta`
4. Copy and paste this sequence into the Primer3 input window.
5. In the Primer3 input window, pad the gap with N's and mark the region (with [] brackets) to be flanked by primers by typing [NNNNNNNNNNNNNNN].
6. Now, display the first 600bp of Contig5 by:
   `head asm_5.fasta`
7. Not including the def-line (>asm_5), copy and paste the seuqnece into the Primer3 input window after the run of N's.
8. Click 'Pick Primers' to find primer pairs.

How are the primers? How is the location? Tm? Etc.

Pretend the primers are for physical ends. Align them to the BAC contigs to check uniqueness.

Now, try to go back and adjust some parameters to find new primers. For instance:

- Change the target Tm values.
- Move your marking [] brackets wider by bracketing more sequence to push the primers back farther from the gap.
- Change the 'Product Size Range' value to accomplish the same thing.
- Exclude some sequence, such as runs of identical nucleotides from consideration using <> brackets.