S07 Biol/Chem 100 basepair ladder
Part 2 (WEEK 8: Testing the primers that CT designed with Primer 3

Motto: Empiricism Rules

Info on primers we will be using for 100bp ladder (including sequence and Tms)
http://fire.biol.wwu.edu/trent/trent/Primerorder.htm

PCReaction parameters
http://www.mcb.uct.ac.za/pcroptim.htm

Week 8 PRELAB: Work up PCR Reaction in notebook (not to hand in)

- Each student should plan to set up 8 PCR reactions
- 6 separate reactions for ladder. (100, 200, 300, 400, 500 & 1000 bp reactions). Plus 2 controls --your choice. You might want to coordinate controls with your lab partner.
- Examine primer info and sort out primer partner for 400R, 300L 200L and 100L.
- Work up recipe for master mix using info on following pages; this mix should contain all reagents common to all of the reactions. Components not common to all reactions will be added separately to each tube. Your final volume after all components are added should be 20µl

Week 8 PRELAB:
Think about Cycling parameters -- See Rules of thumb in lecture
Annealing temp:

- Survey Tms of the primers (posted on web site):
- You will be running your reactions in the same thermocycler block as your lab partner, so consult with him/her and decide on annealing temperature (and extension time). Your TA will assign a block to you. NOTE: This will be a more interesting experiment if different annealing temps are used by different pairs of students.

Extension time:

- Decide on extension time. See guidelines on this site

PCR primer design and reaction optimization
http://www.mcb.uct.ac.za/pcroptim.htm

Week 8 BEFORE/DURING Week 8 lab

Go to Sequence Extractor

- Paste first ~4000-6000 bp of lambda sequence
- Paste primer sequences
- place primers on template sequence

Scan Primer 3 info for primer pairs (above link)

Go to ***Genewalke***r and/or ***idtdna*** and analyze

- Primer with highest 3' self value and analyze
- Primer with highest any value and analyze
- Primer pair with highest any
- Primer pair with highest 3' value
- Decide which primer pair might generate the most primer dimers

Calculate the number of starting template molecules in each of your PCR reactions

We will be using 1 ng of lambda genomic DNA in each PCR reaction:

- Calculate the number of copies that this amount corresponds to.
- See info in Problem Appendix

Calculate the primer-template ratio in your PCR reactions

- Calculate the μM concentration of lambda genome copies in the PCR reaction
- Compare this to the final concentration of each primer to determine the starting ratio of template to primer

Calculations for primer resuspension

The primers are shipped to us dried down in a 2ml tube. A solution of TE (10mM Tris pH~7.5 1mM EDTA) is added to the tube to make a concentrated stock of primer at 100μM

- If the total nmoles of primer in each tube is 30, how much TE is added?

NO unprotected pipeting – use barrier tips!

DNA Aerosols can be deadly to a PCR-based experiment

Total volume of PCR reaction should be 20µl

NOTE: think of a way to include template DNA in the final master mix, but to also do a no-template control

Reagent	Stock	Final Amt/Conc
Promega Taq buffer	10X	1X
Mg++	25 mM	2.5 mM
dNTPs	10 mM	0.25 mM
BSA	1 mg/ml	0.2 mg/ml
Lambda DNA	5 ng/µl	1 ng
CM Taq*		1 µl
Primer Left	10 µM	0.5 µM
Primer Right	10 µM	0.5 µM

10X PCR buffer is 10X *Promega* Taq buffer (Mg^{++} free)
[1X = 50mM KCl, 10mMTris(pH 9.0), 0.1% TritonX-100]

*the Taq polymerase we are using is a home brew of garden-variety Taq (not hot start) courtesy of Craig Moyer

The cycling conditions for your PCR reactions are as follows.

Step	Time	Temperature	
1	4 min.	94ºC	Denature DNA
2	1 min.	94ºC	denature
3	30 sec	**?? ºC**	Anneal primers
4	**?? min.**	72ºC	extend
5	Repeat 2-4 30 more times		
6	10 min	72ºC	
7	**Hold**	**8** ºC	

Appendix A
Some useful PCR factoids from the PCR Primer Book

To maximize efficiency of PCR, the reaction mixture must contain non-limiting amounts of primers and dNTPs

Primers: For a 100 µl reaction

- 0.3 µM to 3.0 µM (1.8×10^{13} - 1.8×10^{14} molecules for a 100 µl reaction) for each primer.
- Typically, the molar ratio of primers to the starting template is at least 10^8 to 1. Having a large excess of primers ensures that once the template DNA becomes denatured, it will anneal to primers rather than to itself.
- In a reaction that starts with 10^5 copies of the template, the final copy number of the target sequence is typically about 10^{12}. Therefore, the primers are always at least 10 times more abundant than the template sequence – even late in the cycling process.
- Too high of primer concentrations can result in mispriming. Optimal primer concentration may need to be empirically determined.

dNTPs:

- 37 µM to 1.5 mM (2.2×10^{15} to 9×10^{16} molecules for a 100 µl reaction).
- Amplifying longer sequences may require increasing dNTP concentration.
- Too high of dNTP concentration may result in misincorporation.
- Optimal dNTP concentration is empirically determined.

Magnesium chloride:

- Magnesium is required for Taq activity but is also bound up by dNTPs and primer.
- Optimal magnesium concentration is empirically determined between the range of 0.5 mM and 5.0 mM.

Appendix B

Potentially useful facts:

- The average molecular weight of a base pair is 660g/mole
- Avogadro's number is 6×10^{23}
- The haploid human genome size is 3×10^{9} base pairs (3000 megabases)
- The haploid Arabidopsis genome size is 125 Mb (megabases)
- The *E. coli* genome is 4.2×10^{6} base pairs
- Less than 10% of the human genome codes for protein
- The circumference of the earth is 25,000 miles.
- Our nuclear DNA is 98-99% identical to that of chimpanzees.

Calculating Template Numbers:

NOTE: This is a useful table, but you should be able to calculate template numbers just using Avogadro's number and the molecular weight of a base pair.

Avogadro's Number= 6.02×10^{23} molecules/mole
1 μg of 1 kb of DNA = 1.54 pmoles

mass	**DNA length**	**moles**	**number of molecules**
1 μg	1 kb	1.54 pico moles	9.3×10^{11}
10 μg	10 kb	1.54 pico moles	9.3×10^{11}
10 ng	10 kb	1.54 femto moles	9.3×10^{8}
10 pg	10 kb	1.54 x atto moles	9.3×10^{5}
1 pg	10 kb	0.154 atto moles	9.3×10^{4}

Remember:

milli	10^{-3}	micro	10^{-6}	nano	10^{-9}		
pico	10^{-12}	femto	10^{-15}	atto	10^{-18}	zepto	10^{-21}

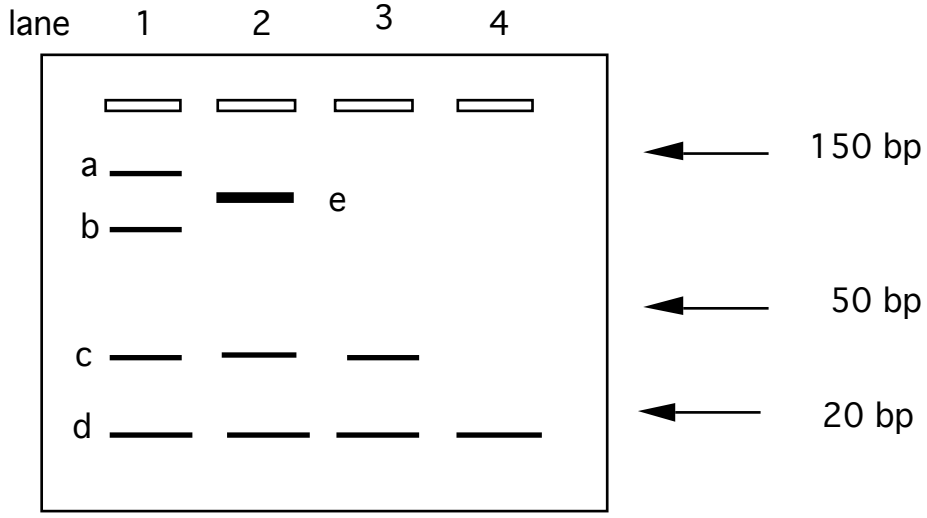
1. What is the molar ratio of primer to template (starting amount) in a 20 μl PCR reaction that has a final concentration of 0.5 μM for each primer and 1μg of human genomic DNA?

2. If a 250 bp target sequence is amplified 10^{6} times by the polymerase chain reaction, how many ng of 250 bp product do you expect to get in one reaction starting with 200ng of genomic human template DNA? of genomic *E. coli* template DNA? [This question assumes 100% efficiency, which is rarely achieved.]

3. Using PCR, you want to amplify a portion of a gene found in the human genome. You are going to borrow one of the primers (which is about 20 bases long) from the lab next door. The other primer will be custom synthesized by the local primer-supply company. You are going to use genomic DNA as the template and want a second primer that will not bind by chance to another portion of the genome. But, primers are expensive and each additional base increases the cost of the primer. If the genome size of the human is 3×10^{9} base pairs and the GC content is about 50%, how long should your primer be to make it unlikely that the primer will bind by chance to another region of the genome?

4. Recall that most DNA fingerprinting studies involve PCR amplification of polymorphic microsatellites. Here is a typical result for a microsatellite PCR run with the primer pair shown below. First analyze the primer pair with Genewalker and Integrated DNA Technologies
Primers:
5' GGGCTGTATCCTATAGC 3'
5' CCCGAACTCAAGGCTATA 3'

Lane 1: genomic DNA from Student #1
Lane 2: genomic DNA from Student #2
Lane 3: control reaction with no template DNA
Lane 4: control reaction with template DNA but no Taq polymerase



a. Which bands are amplification products?
b. What determines the size difference among bands a, b and e?
c. Why are there 4 bands in lane 1 and 3 in lane 2?
d. What is the band at the bottom of each lane?
f. Is there primer-dimer on this gel? Site two pieces of evidence supporting your conclusion.
e. Draw a picture showing how primer-dimers would be generated using the specific sequences of the primers.
f. **In a hotstart PCR reaction,** inappropriately annealed primers are denatured and no DNA synthesis occurs until the temperature is lowered later in the reaction, allowing the primers to anneal to the template DNA. In a hotstart PCR reaction, which bands should disappear?
[During a manual "hotstart" PCR reaction, the addition of Taq polymerase is delayed until the PCR mixture has been heated to 95°C. In a different hotstart method, the Taq polymerase is dispersed within a wax bead. During the initial 95° denaturation step, the Taq DNA polymerase is slowly released into the reaction mixture. Alternatively, Hotstart Taqs are only active after the initial incubation at 95.]