

Biol/Chem 475 Primer Design Spring 2007

Generating a 100 bp ladder using phage lambda DNA as template

FAQS

What are we doing in lab for the next couple of weeks?

- Week 7: design primers for a 100 bp ladder of PCR products
- Week 8: try primers out; assess PCR results and work up strategy to optimize yield and specificity
- Week 9: each student reruns PCR varying one or two parameters of the reaction

What's the Point?

- The goal of this exercise is to introduce you to the the basics of primer design and troubleshooting PCR reactions – where artifacts and suboptimal reactions can kill an experiment or, worse yet, provide misleading results

Where should I go & What should I do? In this handout

What do I need to hand in? See extra sheet

Appendix A: I thought molecular biologists don't usually care about details like T_m

Appendices B: Jeff Young's general guidelines for primer design

Appendix C: Downloading sequence from NCBI in Fasta Format

Where should I go?

NCBI: Obtain template DNA sequence

<http://www.ncbi.nlm.nih.gov/>

Primer-3: Design Primers

<http://frodo.wi.mit.edu/>

Sequence Extractor: Display primers on DNA sequence

<http://bioinformatics.org/seqext/>

Sequence Extractor features

<http://bioinformatics.org/seqext/features.html>

GeneWalker: analyze potential for primer artifacts/interactions

<http://www.cybergene.se/primerdesign/genewalker/genewalker11.html>

GeneWalker Manual

<http://www.cybergene.se/primerdesign/help.html>

IDT-DNA Another site to analyze primer-primer interactions – gives ΔG 's!

<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx>

Other sites of interest Online PCR TOOLS

<http://molbiol-tools.ca/PCR.htm>

Tips on primer design

http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html

What's the point?

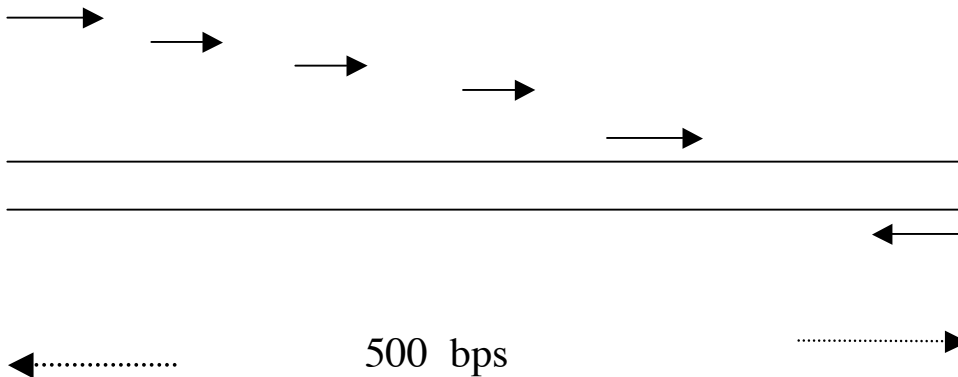
DNA size standards are expensive, so we are going to make our own.

Inexpensive Reagents: TAQ polymerase (buffers, dNTPs),
PCR primers,
DNA template (*lambda bacteriophage*)

Invaluable: Your Time. You will be designing primers to amplify, from a single template, a 1000 bp product & a nested set of products 100-500 bps in length in 100 base pair increments &. In other words we want (using 7 or more primers) products of 100, 200, 300, 400, 500 bp, & 1000 [+/- as few bases as possible]. The primers will be used in six different PCR reactions; the products of the reactions will then be combined to make a 100 base pair ladder mix.

Suggested strategy:

- Note that you can pair one primer with more than one partner to generate different sized PCR products and to reduce the number of primers needed
- Start with largest product and generate primers
- Pick one of these primers and look for nested sets (see drawing below)
- if that primer isn't working out, pick the other outside primer and look for nested sets



FIRST: GET TEMPLATE SEQUENCE

Go to National Center for Biotechnology Information (NCBI)

<http://www.ncbi.nlm.nih.gov/>

Choose **Genome** in the pull down menu

Type in Enterobacteria phage lambda

Entrez Genome

Search Genome for Enterobacteria phage lambda

Display Summary Show 20 Send to

All: 1

1: **NC_001416**
 Enterobacteria phage lambda, complete genome
 dsDNA; linear; Length: 48,502 nt
 Created: 1993/04/28

Entrez Genome

Search Genome for

Display Overview Show 20 Send to

All: 1

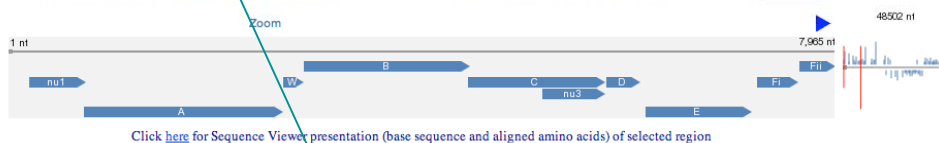
Genome > Viruses > Enterobacteria phage lambda, complete genome

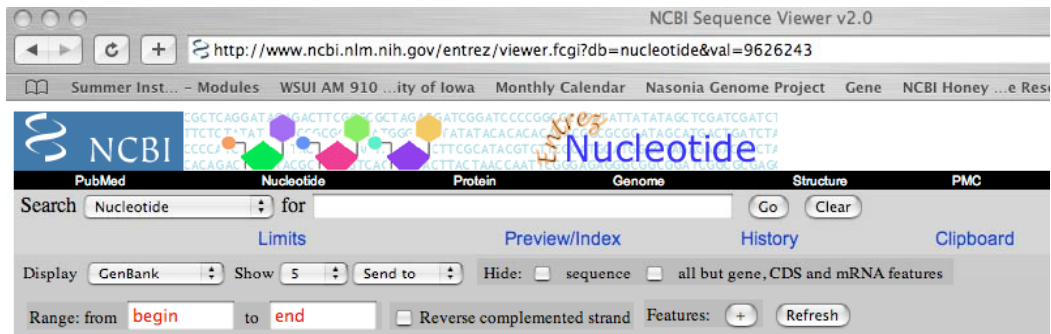
Lineage: [Viruses](#) ; [dsDNA viruses, no RNA stage](#) ; [Caudovirales](#) ; [Siphoviridae](#) ; [Lambda-like viruses](#) ; [Enterobacteria phage lambda](#)

Genome Info:	Features:	BLAST homologs:	Links:	Review Info:
Refseq: NC_001416	Genes: 90	COG	Genome Project	Publications: [13]
GenBank: J02459	Protein coding: 71	3D Structure	Refseq FTP	Refseq Status: Reviewed
Length: 48,502 nt	Structural RNAs: None	TaxMap	GenBank FTP	Seq Status: Completed
GC Content: 49%	Pseudo genes: None	TaxPlot	BLAST	Sequencing center:
% Coding: 87%	Others: 94	GenePlot	TraceAssembly	Completed: 1993/04/28
Topology: linear	Contigs: 1	gMap	CDD	Organism Group
Molecule: dsDNA			Other genomes for species	

Gene Classification based on [COG functional categories](#)

Search gene, GeneID or locus_tag:





1: NC_001416. Reports Enterobacteria ph...[gi:9626243]

[Comment](#) [Features](#) [Sequence](#)

LOCUS NC_001416 48502 bp DNA linear PHG 30-MAR-2006
 DEFINITION Enterobacteria phage lambda, complete genome.
 ACCESSION NC_001416
 VERSION NC_001416.1 GI:9626243
 PROJECT GenomeProject:14204
 KEYWORDS .
 SOURCE Enterobacteria phage lambda
 ORGANISM [Enterobacteria phage lambda](#)
 Viruses; dsDNA viruses, no RNA stage; Caudovirales; Siphoviridae;
 Lambda-like viruses.
 REFERENCE 1 (sites)
 AUTHORS Chen,C.Y. and Richardson,J.P.
 TITLE Sequence elements essential for rho-dependent transcription
 termination at lambda tR1
 JOURNAL J Biol Chem 263 (1988) 11282-11286 (1988)

Scroll (way) down to nucleotide sequence
 Select and copy your assigned sequence block next page)

ORIGIN
 AT KALEAALVVAHDNNMMDIIDLVD
 1 gggcggcgac ctgcggggtt ttogctatth atgaaaatth tcoggttaa ggcgtttcog
 61 ttcttcttcg tcataactta atgtttttat ttaaaatacc ctctgaaaag aaaggaaaacg
 121 acaggtgctg aaagcgagcg tttttggcct ctgtcgtttc ctttctctgt ttttgtccgt
 181 ggaatgaaca atggaagtca acaaaaagca gctggctgac attttcggtg cgagtatccg
 241 taccattcag aactggcagg aacaggggat gcccgttctg cgaggcggtg gcaagggtaa
 301 tgaggtgctt tatgactctg ccgocgtcat aaaatggtat gccgaaaagg atgctgaaat
 361 tgagaacgaa aagctgcgcc gggaggttga agaactcggc caggccagcg aggcagatct
 421 ccagccagga actattgagt acgaaagcca tcgacttacg cgtgcgcagg ccgacgcaca
 481 ggaactgaag aatgccagag actccgctga agtgggtggaa accgcattct gtactttcgt
 541 gctgtcgcgg atcgcaggtg aaattgccag tattctcgac gggctcccc tgtcggtgca
 601 gcggcgthtt ccggaactgg aaaaccgaca tgttgatttc ctgaaacggg atatcatcaa
 661 agccatgaac aaagcagccg cgctggatga actgataccg gggttgctga gtgaatatat
 721 cgaacagtca ggttaacagg ctgcggcatt ttgtccgcgc cgggcttcgc tcaactgtta
 781 gggggggggg gggggggggg gggggggggg gggggggggg gggggggggg gggggggggg

➔ You can directly copy the Genbank sequence (numbers and all) and paste it into the Primers3 sequence window. The program will ignore the numbers.
 [also see appendix C for alternative way to download sequence]

OPEN PRIMER 3

<http://frodo.wi.mit.edu/>

PASTE LAMBDA SEQUENCE in to source sequence box

Primer3 (v. 0.3.0) Pick primers from a DNA sequence.	Checks for mispriming in template.	New/beta (0.4.0) interface
---	--	--

Paste source sequence below (5'→3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA undesirable sequence (vector, ALUs, LINES, etc.) or use a [Mispriming Library \(repeat library\)](#):

```
1 gggcgggcag ctcgcggtt ttcgctatt atgaaaatt tccggttaa ggcgtttccg
61 ttcttctc tcataacta atgtttat ttaaaatcc ctctgaaaag aaaggaaacg
121 acagggtcct aaagcgaggc ttttggcct ctgctgttc cttctctgt tttgtccgt
181 ggaatgaaca atggaatca acaaaaagca gctggctgac atttccggtg cgagtatccg
241 taccattcag aactggcagg aacaggaat gcccttctg cgaggcgggtg gcaagggtaa
301 tgagggtcct tatgactctg ccgcccgtcat aaaatggat gccgaaaggg atgctgaaat
```

- Tell program to pick right and left primers and indicate product size range
- Keep the size range narrow at first - open only if the program can't find primers in the range that you have set
- Leave defaults setting for other parameters

<input checked="" type="checkbox"/> Pick left primer, or use left primer below:	<input type="checkbox"/> Pick hybridization probe (internal oligo), or use oligo below:	<input checked="" type="checkbox"/> Pick right primer, or use right primer below (5' to 3' on opposite strand):
---	---	---

Sequence ID: A string to identify your output.

Targets: E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the [source sequence](#) with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.

Excluded Regions: E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the [source sequence](#) with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.

Product Size Ranges:

[Click here to specify the min, opt, and max product sizes only if you absolutely must. Using them is too slow \(and too computationally intensive for our server\).](#)

Number To Return	<input type="text" value="5"/>	Max 3' Stability	<input type="text" value="9.0"/>
Max Repeat Mispriming	<input type="text" value="12.00"/>	Pair Max Repeat Mispriming	<input type="text" value="24.00"/>
Max Template Mispriming	<input type="text" value="12.00"/>	Pair Max Template Mispriming	<input type="text" value="24.00"/>

General Primer Picking Conditions:

The specificity of amplification of target sequences in a PCR reaction is controlled by the length and sequence of the primer (which affects T_m) and the annealing temperature used in the PCR run. The sequence of the 3' end of the primer is also important.

- Click on each parameter to view a description – just browse through the info here -- we'll come back and explore a couple of these parameters more closely
- Start out using the default values for parameters in this section
- See appendix D for a definition of T_m
- Ignore Other Per-Sequence Inputs and what follows...

General Primer Picking Conditions

Primer Size Min: 18 Opt: 20 Max: 27
Primer T_m Min: 57.0 Opt: 60.0 Max: 63.0 Max T_m Difference: 100.0
Product T_m Min: Opt: Max:
Primer GC% Min: 20.0 Opt: Max: 80.0
Max Self Complementarity: 8.00 Max 3' Self Complementarity: 3.00
Max #N's: 0 Max Poly-X: 5
Inside Target Penalty: Outside Target Penalty: 0 Note: you can set Inside Target Penalty to allow primers inside a target.
First Base Index: 1 CG Clamp: 0
Salt Concentration: 50.0 Annealing Oligo Concentration: 50.0 (Not the concentration of oligos in the reaction mix but of those annealing to template.)
 Liberal Base Show Debugging Info Do not treat ambiguity codes in libraries as consensus

Pick Primers Reset Form

→ Tell program to pick primers and then carefully inspect the primer-3 output – be sure to scroll down to the bottom of the page

Primer3 Output

WARNING: Numbers in input sequence were deleted.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO      start  len  tm    gc%  any  3'  seq
LEFT PRIMER 2260  20  59.73 55.00 4.00 0.00 gtacggataccgcgaaagag
RIGHT PRIMER 2457  20  60.30 50.00 5.00 3.00 gctttttgctgtcccacagt
SEQUENCE SIZE: 4080
INCLUDED REGION SIZE: 4080
```

PRODUCT SIZE: 198, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

NOTE info a bottom of output page

ADDITIONAL OLIGOS

	<u>start</u>	<u>len</u>	<u>tm</u>	<u>gc%</u>	<u>any</u>	<u>3'</u>	<u>seq</u>
1 LEFT PRIMER	1556	20	60.53	55.00	6.00	2.00	ccagcaggagctggacttta
RIGHT PRIMER	1760	20	59.93	45.00	2.00	1.00	cgtatccccctttcgttttca
PRODUCT SIZE: 205, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00							
2 LEFT PRIMER	625	20	59.50	45.00	6.00	2.00	cgcacatggttgatttctga
RIGHT PRIMER	823	20	59.89	45.00	6.00	2.00	taattagcatccgcccattc
PRODUCT SIZE: 199, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00							
3 LEFT PRIMER	1230	20	59.82	50.00	5.00	3.00	gtggcgggttatgatgaact
RIGHT PRIMER	1427	20	59.57	40.00	4.00	1.00	ggcaacatgaaaacgcataa
PRODUCT SIZE: 198, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00							
4 LEFT PRIMER	1032	20	60.08	50.00	3.00	3.00	gatggtgatgccgagaactt
RIGHT PRIMER	1227	20	60.54	55.00	4.00	1.00	ccaccgacttttcacggtag
PRODUCT SIZE: 196, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00							

Statistics

	con	too	in	in	no	tm	tm	high	high	high	high	high	high
	sid	many	tar	excl	bad	GC	too	too	any	3'	poly	end	end
	ered	Ns	get	reg	GC%	clamp	low	high	compl	compl	X	stab	ok
Left	31766	0	0	0	319	0	3614	22272	0	16	105	760	4680
Right	32185	0	0	0	188	0	3243	23274	0	4	103	721	4652

Pair Stats:

considered 20667, unacceptable product size 20643, high end compl 2, ok 22
primer3 release 1.1.0

- What do **any**, **3'**, **pair any compl** and **pair 3' comple** refer to?
- How do the alternate primer picks (additional oligos) differ from the "chosen" primer?
- Note the statistics which indicates the number of primer candidates examined and why the primer was rejected

→ If you are OK with the primer pick, copy the info shown on previous page and paste it into a word file – keep Courier 10 font so the collums will stay aligned.

→ Pick your second primer pair

This time enter your left or right primer from above, and tell program to find the right or left primer

→ Don't forget to enter a new product size

→ Repeat instructions from above –don't forget to copy the primer 3 output info for each primer pair.

What if Primer 3 can't find acceptable an acceptable primer or primer pair?

- First examine the statistics to get clues as to what the limitations are
- Look at general primer picking parameters and make some conservative adjustments
- You can open the size range limits, but too big a size window will make the 100bp ladder less useful
- Try the other outside primer
- NOTE: exert a little effort here to generate nested PCR products -- the fewer primers you need for your ladder, the better

→ Repeat until you have primer pairs that will generate 100,200,300,400,500 and 1000bp products

→ In your word document, name your primers and make a list of each unique primer. In Appendix B, there are primer design rules put together by Jeff Young. Do your primer sequences meet the standards suggested in this appendix? Briefly summarize your assessment

Go to Sequence Extractor and align primers on sequence:

Sequence Extractor: Display primers on DNA sequence

<http://bioinformatics.org/seqext/>

Sequence Extractor features

<http://bioinformatics.org/seqext/features.html>

- First **clear** primer and sequence box
- Paste in your sequence and your primer list as instructed
- Tell program that you don't want the sequence translated
- Don't allow any mismatches

Advanced Options

Use the following options to alter the output of Sequence Extractor. For more details about individual options, see the [help](#).

- Genetic code:
- Restriction set:
- Translate reading frame:
- Topology:
- Allow primers to have mismatched: 5' tails, 3' tails.
Matching bases required when mismatching bases allowed:
- Bases per line:
- Show reverse strand, number line, spacer line.
- Return restriction summary, primer summary, help information, coding sequence links, translation links, options selected.

Carefully examine output:

```

ggaggctcacggagcgaagaacaggcagcgctggcagaaaccccggtatgaccgtgaaaacggcccgcgcattc
      ^5530      ^5540      ^5550      ^5560      ^5570      ^5580      ^5590
cctccgagtgccctgcgctctctgtccgtgcgcaagcagcgtctcttgggggccatactggcacttttggcggcgcgtaag
-----
5' CAGCACCCACAGAGTGCACA 3' 200L

      BssHII      MboI      HpaII
      NdeII      MspI
tggccgcagcaaccacagagtgcacagggcgcgagtgacactgcgctggatcgtctgatgcagggggcaccggcaccgctg
      ^5610      ^5620      ^5630      ^5640      ^5650      ^5660      ^5670
accggctcgtggtgtctctcaagtgcccgcgctcaactgtgaacggacotagcagactacgtcccccggtggcgtggcgac
-----
3' TCCATTGGCCGTAGACTA 5' 400R      Position=5712 100L >>> direction >>>
      PstI      HpaII      HincII
      MspI      MspI      HpaI
gctgcaggtaacccggcactctgatgcggttaacgatttgcgtaaacaccagtgtaagggatgttatgacgagcaaga
      ^5690      ^5700      ^5710      ^5720      ^5730      ^5740      ^5750
cgacgtccattggcggtagactacggcaattgctaaacgacttgtgtgtcacattccctacaaatactgctggtttt
-----
3' TTGTCACTGGGCCGAGTATG 5' 500R
      HpaII      HpaII
      MspI      MspI
aacctttaccattaccagccgagggcaacagtgaccggctcataccgcaaccggcggcggttgagtgcgaaag

```

Inspect alignments

- Capture desktop picture of part of alignment (as above) and paste into word file
- Capture primer summary (below) and past into word document

Primer Summary	
100L	ACGATTTGCTGAACACACCA: 5712.
200L	CAGCACCCACAGAGTGCACA: 5607.
300L	TTATCGGGATCCTCAACTGT: 5499.
400R	ATCAGATGCCGGTTACCT: -5705.
500L	GATGCACGTAATCCCGTCT: 5309.
500R	GTATGAGCCGGTCACTGTT: -5809.

sequence Extractor copyright © 2006 Paul Stothard
mail: stothard@ualberta.ca

An ideal set of primer should anneal efficiently with the target template sequences and not with other template sequences or with each other. The Primer 3 program assesses the ability of primer pairs to hydrogen bond with each other at the 3' end (serving potentially as templates for DNA pol) or generally with each other at any position.

Max Complementarity

The maximum allowable local alignment score when testing a single primer for (local) self-complementarity and the maximum allowable local alignment score when testing for complementarity between left and right primers. Local self-complementarity is taken to predict the tendency of primers to anneal to each other without necessarily causing self-priming in the PCR. The scoring system gives 1.00 for complementary bases, -0.25 for a match of any base (or N) with an N, -1.00 for a mismatch, and -2.00 for a gap. Only single-base-pair gaps are allowed. For example, the alignment

```
5' ATCGNA 3'
   |||
3' TA-CGT 5'
```

is allowed (and yields a score of 1.75), but the alignment

```
5' ATCCGNA 3'
   |||
3' TA--CGT 5'
```

is not considered. Scores are non-negative, and a score of 0.00 indicates that there is no reasonable local alignment between two oligos.

Max 3' Complementarity

The maximum allowable 3'-anchored global alignment score when testing a single primer for self-complementarity, and the maximum allowable 3'-anchored global alignment score when testing for complementarity between left and right primers. The 3'-anchored global alignment score is taken to predict the likelihood of PCR-priming primer-dimers, for example

```
5' ATGCCCTAGCTTCCGGATG 3'
      ||| ||| ||| |||
3' AAGTCCTACATTTAGCCTAGT 5'
```

or

```
5' AGGCTATGGCCTCGCGA 3'
      ||| ||| ||| |||
3' AGCGCTCCGGGTATCGGA 5'
```

The scoring system is as for the Max Complementarity argument. In the examples above the scores are 7.00 and 6.00 respectively. Scores are non-negative, and a score of 0.00 indicates that there is no reasonable 3'-anchored global alignment between two oligos. In order to estimate 3'-anchored global alignments for candidate primers and primer pairs, Primer assumes that the sequence from which to choose primers is presented 5'->3'. It is nonsensical to provide a larger value for this parameter than for the Maximum (local) Complementarity parameter because the score of a local alignment will always be at least as great as the score of a global alignment.

Max 3' Compl

Go to GeneWalker and analyze one pair of primers in detail

GeneWalker: analyze potential for primer artifacts/interactions

<http://www.cybergene.se/primerdesign/genewalker/genewalker11.html>

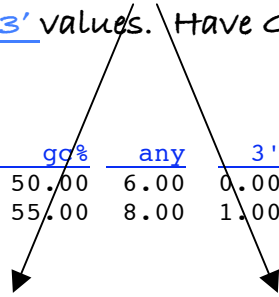
GeneWalker Manual

<http://www.cybergene.se/primerdesign/help.html>

Pick a primer pair that with non-zero values here and with primers that have different any and 3' values. Have GeneWalker show you primer dimer interactions.

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	5309	20	59.96	50.00	6.00	0.00	GATGCACGTAAATCCCCTCT
RIGHT PRIMER	5808	20	60.00	55.00	8.00	1.00	GTATGAGCCGGGTCACTGTT

SEQUENCE SIZE: 48502
INCLUDED REGION SIZE: 8000



PRODUCT SIZE: 500, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

LEFT Primer annealing with itself -- ask for primer dimer

Primer 1 sequenc
GATGCACGTA AATCCCCTCT

Primer 2 sequenc
GATGCACGTA AATCCCCTCT

Primer/Target alignn

```

5'----- Align comparative: -----3'
1      10      20      30      40      50      60
TCTGCCCTAAATGCACGTAG
  ||| ||| |||
  GATGCACGTA AATCCCCTCT      26
  
```

GeneWalker CyberGene DNA analysis and bioinformatics

For each indicate the 5' and 3' ends
Which interaction would amplify in a PCR reaction?
Repeat with RIGHT Primer annealing with itself

Left and right primers with each other

Primer 1 sequenc
GATGCACGTA AATCCCCTCT

Primer 2 sequenc
GTATGACCCG GTCAC--TGT

Primer/Target alignn

```

5'----- Align comparative: -----3'
1      10      20      30      40      50      60
TCTGCCCTAAATGCACGTAG
  ||| ||| |||
  GTATGACCCGGG--TCAC--TGTT      23
  
```

GeneWalker CyberGene DNA analysis and bioinformatics

PRIMER intrastrand STRUCTURE

Paste in your primer sequence and ask for primer structure

The structure will be coded as shown

Go to genewalker help and draw out the structural meannig of this code – first two lines

The screenshot shows a web interface for primer analysis. It includes a 'Target sequence' input field, a 'Primer 1 sequenc' input field containing 'GATGCACGTA AATCCCGTCT', and a 'Primer structure' output field. The primer structure is displayed as a sequence of characters in brackets, representing secondary structure. A central column of buttons includes 'Clear all', 'Clear target', 'Sequence', 'Format', 'Order oligo', 'HELP', 'Clear results', 'Primer dimer', 'Clear primer', 'Rev compl 1', and '2:ary struct 1'. An arrow points from the text 'first two lines' to the first two lines of the primer structure output.

Target sequence

Primer 1 sequenc
GATGCACGTA AATCCCGTCT

Primer structure

```
[GA]GC[ACGT]AA[ATC]CCGTCT  
GATGC[ACG]TA[A]A[T]CC[CGT]CT  
GAT[G]C[A]CG[TA]AA[T]C[C]CGTCT  
GA[ TGCA]CGTAAATCCCGTCT  
[GA]T[G]CACG[T]A[A]ATCC[C]G[TC]T  
GAT[G]CAG[G]T[A]AA[T]C[C]CGT[C]T  
GA[T]G[C]AC[G]T[A]AATCCCGTCT  
G[A]T[G]CA[C]G[T]AAATCCCGTCT  
GATGCACGTAATCC[CG]TCT  
GATGCACGTAA[AT]CCCGTCT
```

Primer/Target alignn

OPTIONAL for those who want to do more

Go to IDT-DNA Another site to analyze primer-primer interactions

Hey, this site will gives the ΔG 's of the interactions!

<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx>

Appendix A What is T_m and why is it important?

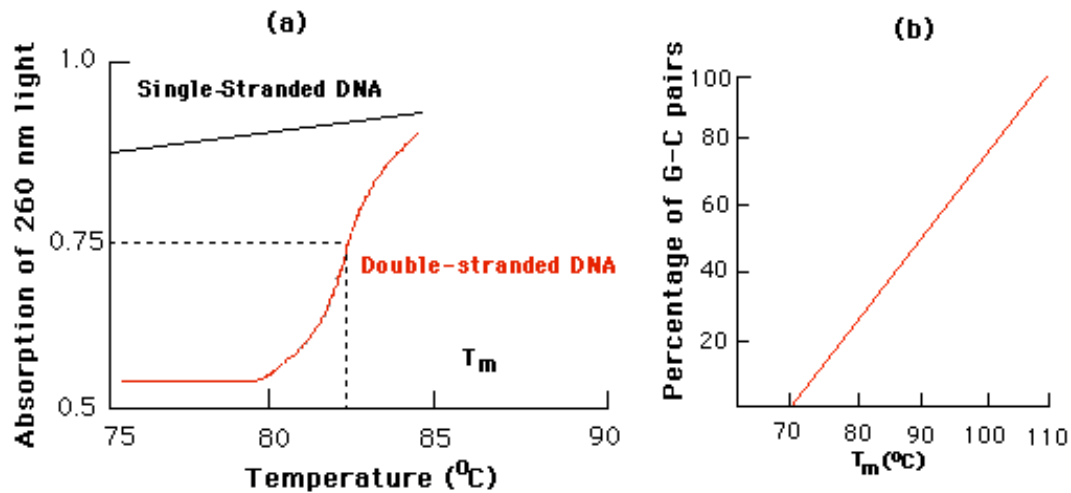


Fig. (a) The absorption of ultraviolet light of 260-nm wavelength by solutions of single-stranded and double stranded DNA. As regions of ds-DNA unpair, the absorption of light by those regions increases almost twofold. The temperature at which half the bases in a ds-DNA sample have denatured is denoted T_m (for temperature melting). Light absorption by single-stranded DNA changes much less as the temperature is increased. **(b)** The T_m is a function of the G + C content of the DNA; the higher the G + C percentage, the greater the T_m .

For primers shorter than 20 bases, T_m can be calculated as

$$T_m = 4(G + C) + 2(A + T).$$

- Single base mismatches can significantly lower the T_m of the primer-template.
- Mismatch tolerance between template and primer -- is the 5' or 3' end the most critical?

Read about annealing temp and primer design at this site

<http://www.mcb.uct.ac.za/pcroptim.htm>

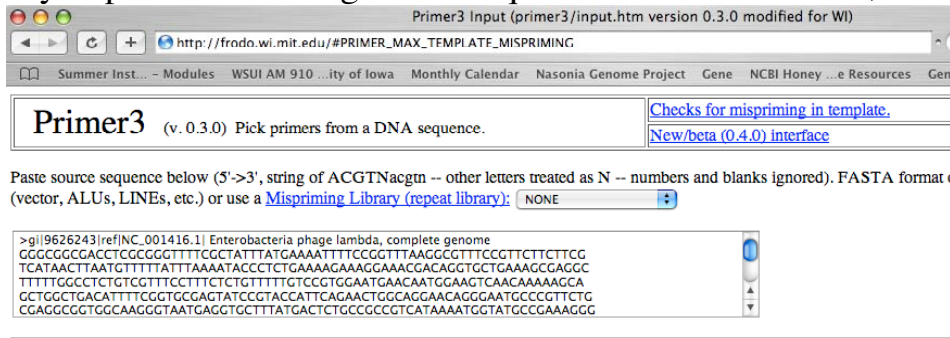
Appendix C

Downloading sequence from NCBI OR select FASTA format and send to TEXT



```
>gi|9626243|ref|NC_001416.1| Enterobacteria phage lambda, complete genome
GGCGCGGACCTCGCGGGTTTCGCTATTTATGAAAATTTCCGGTTAAGGCCTTCCGTTCTTCTTCG
TCATAACTTAATGTTTTTATTTAAAATACCCCTCGAAAAGAAAGGAAACGACAGGTGCTGAAAGCGAGGC
TTTTTGGCCTCTGTCGTTTCTCTCTGTTTTGTCCGTGGAATGAAACAATGGAAGTCAACAAAAGCA
GCTGGCTGACATTTTCGGTCCGATATCCCTACCATTTCAGAACTGGCAGGAACAGGGAATGCCGTTCTG
CGAGCGGTGGCAAGGGTAATGAGGTGCTTTATGACTCTGCCCGCTCATAAAATGGTATGCCGAAAGGG
ATGCTGAAATTCAGAACGAAAAGCTGCCCGGGAGGTTGAAGAACTGGCGCAGGCCAGCGAGGCAGATCT
CCAGCCAGGAATTCAGTACGAAACGCCATCGACTTACGCGTGGCGAGCCGACGACAGGAATGAAG
AATGCCAGAGACTCCGCTGAAGTGGTGGAAACCGCATTCGTACTTTTCGTGCTGTCGGGGATCGCAGGTG
AATTCGCAATTTTCGACGGGTCCCGCTGTCGGTGCAGCGGGCTTTCCGGAATCGAAAACCGACA
TGTTGATTTCCGAAACGGGATATCATCAAAGCCATGAACAAAGCAGCCCGCTGGATGAATGATACCC
GGTTGCTGAGTGAATATATCGAACAGTCAGGTTAACAGGCTGCCGCAATTTGTCGGCGCCGGCTTCGC
TCACTGTTCCAGGCGGAGCCACAGACCGCGTTGAATGGGCGGATGCTAATTAATCTCCCGAAAGAAAT
CCGCATACCAGGAAGGGCGCTGGGAAACACTGCCCTTTCAGCGGGCCATCATGAATGGATGGGCGGCA
TCACATCCGCTGAGTGAATGAGTGGTGAAGTCTGCCCGTTCGGTTATTCCAAATGCTGCTGGGTGTTAT
GCCTACTTTATAGACATAAAGCAGCGCAACCCCTTATCTGGTTGCCGACGGATGGTATGCCGAGAACT
TTATGAAAACCCACGTTGAGCCGACTATTCGTGATATTCGCTCGCTGCGCGCTGGCCCGTGGTATGG
CAAAAAGCACCGGGATAACACGCTCACCATGAAGCGTTTCACTAATGGGCGTGGCTTCTGGTGCCTGGGC
GGTAAAGCGGCAAAAATACCCGTGAAAACCTCCGTTGGATGTGGCGGGTATGATGAATTCGCTGTTTTG
ATGATGATATTTGAACAGGAAGGCTCTCCGACGTTCCGGGTGACAAGCGTATTTGAAGGCTCGGCTCGGCC
```

If you paste the entire genome sequence in IN PRIMER 3,



Primer3 (v. 0.3.0) Pick primers from a DNA sequence. [Checks for mispriming in template.](#)
[New/beta \(0.4.0\) interface](#)

Paste source sequence below (5'→3', string of ACGTnacgtm -- other letters treated as N -- numbers and blanks ignored). FASTA format or (vector, ALUs, LINES, etc.) or use a [Mispriming Library \(repeat library\)](#): NONE

```
>gi|9626243|ref|NC_001416.1| Enterobacteria phage lambda, complete genome
GGCGCGGACCTCGCGGGTTTCGCTATTTATGAAAATTTCCGGTTAAGGCCTTCCGTTCTTCTTCG
TCATAACTTAATGTTTTTATTTAAAATACCCCTCGAAAAGAAAGGAAACGACAGGTGCTGAAAGCGAGGC
TTTTTGGCCTCTGTCGTTTCTCTCTGTTTTGTCCGTGGAATGAAACAATGGAAGTCAACAAAAGCA
GCTGGCTGACATTTTCGGTCCGATATCCCTACCATTTCAGAACTGGCAGGAACAGGGAATGCCGTTCTG
CGAGCGGTGGCAAGGGTAATGAGGTGCTTTATGACTCTGCCCGCTCATAAAATGGTATGCCGAAAGGG
```

then you will need to limit the primer sequence to your assigned sequence block as follows:

Scroll down to other sequence input

Other Per-Sequence Inputs

Included Region: E.g. 20,400: only pick primers in the 400 base region starting at position 20. Or use { and } in the [source sequence](#) to mark the beginning and end of the included region: e.g. in ATC{TTC...TCT}AT the included region is TTC...TCT.

Start Codon Position: