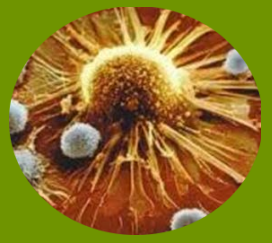
Cryphonectria parasitica tendrils on chestnut tree bark (Photo: Ministry of Agriculture and Regional Development Archive, Ministry of Agriculture and Regional Development, Bugwood.org)



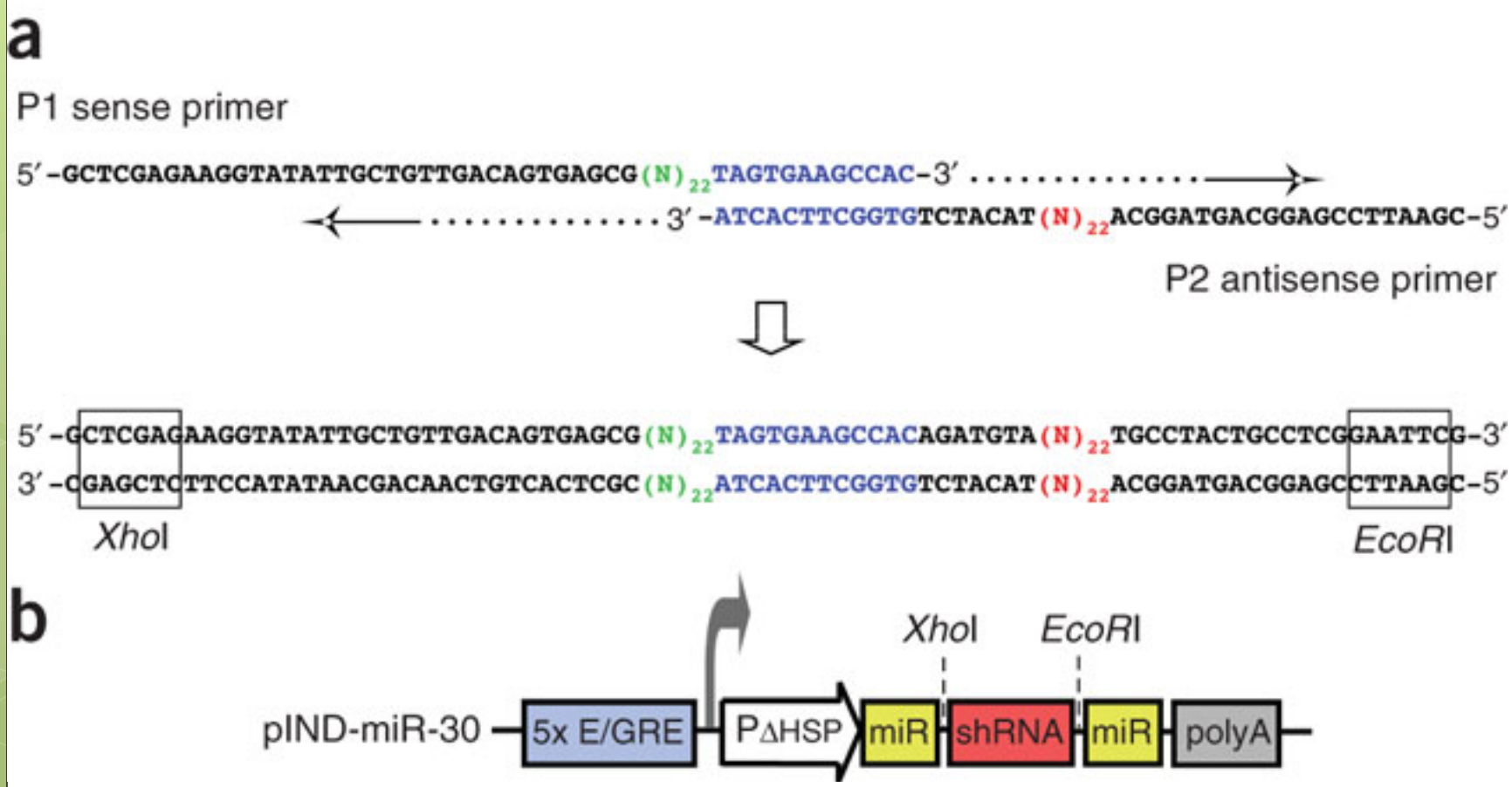
6/22/2014

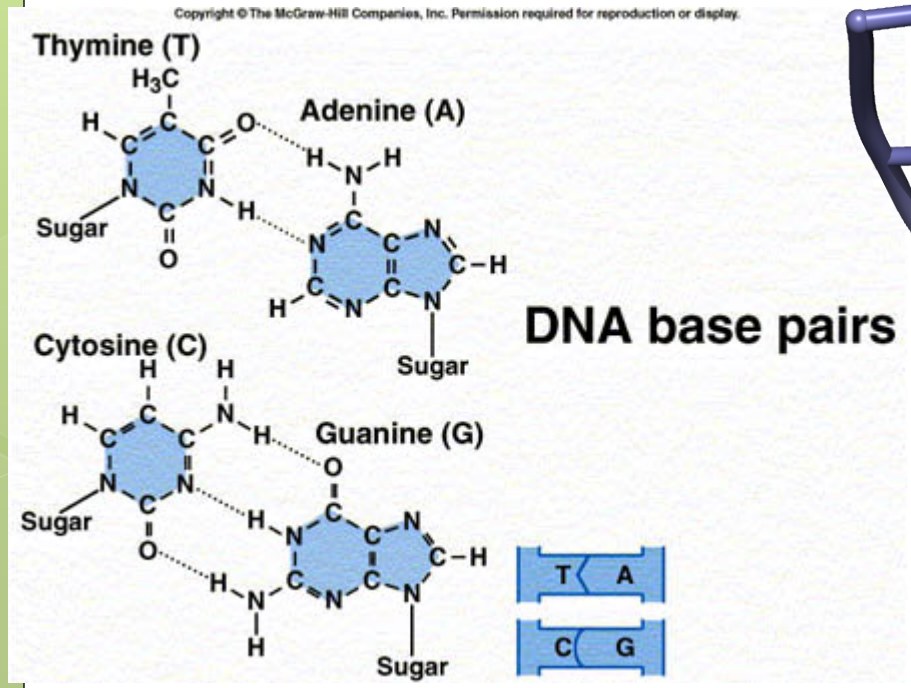
How/Why to Design PCR Primers

B3 Summer Science Camp at Olympic High School

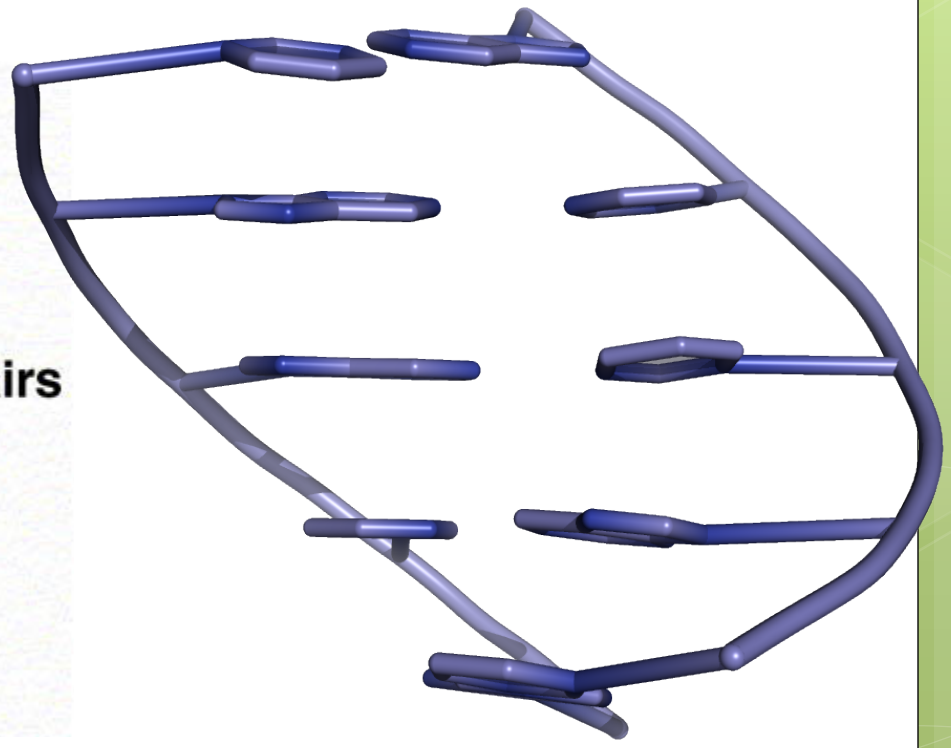
Dr. Jennifer Weller

How to Design PCR Primers

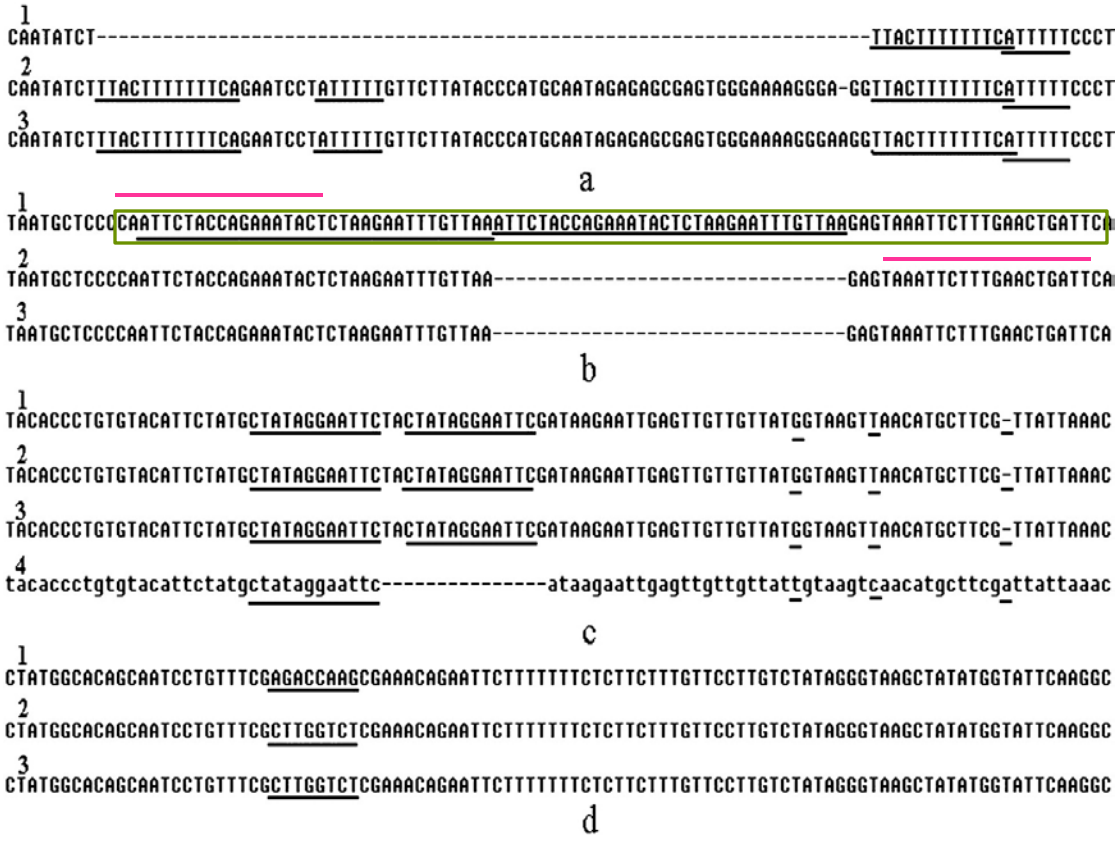




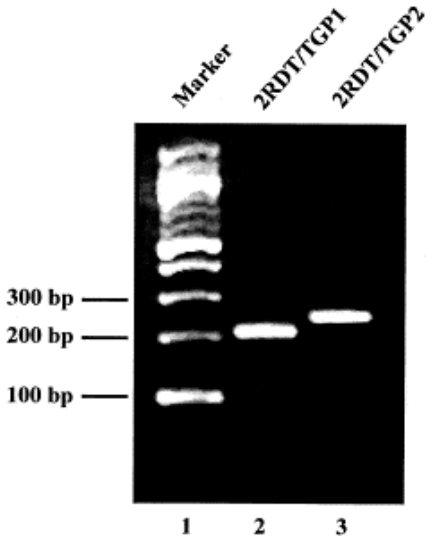
DNA base pairs



When we design the chloroplast primers – what are we trying to do?



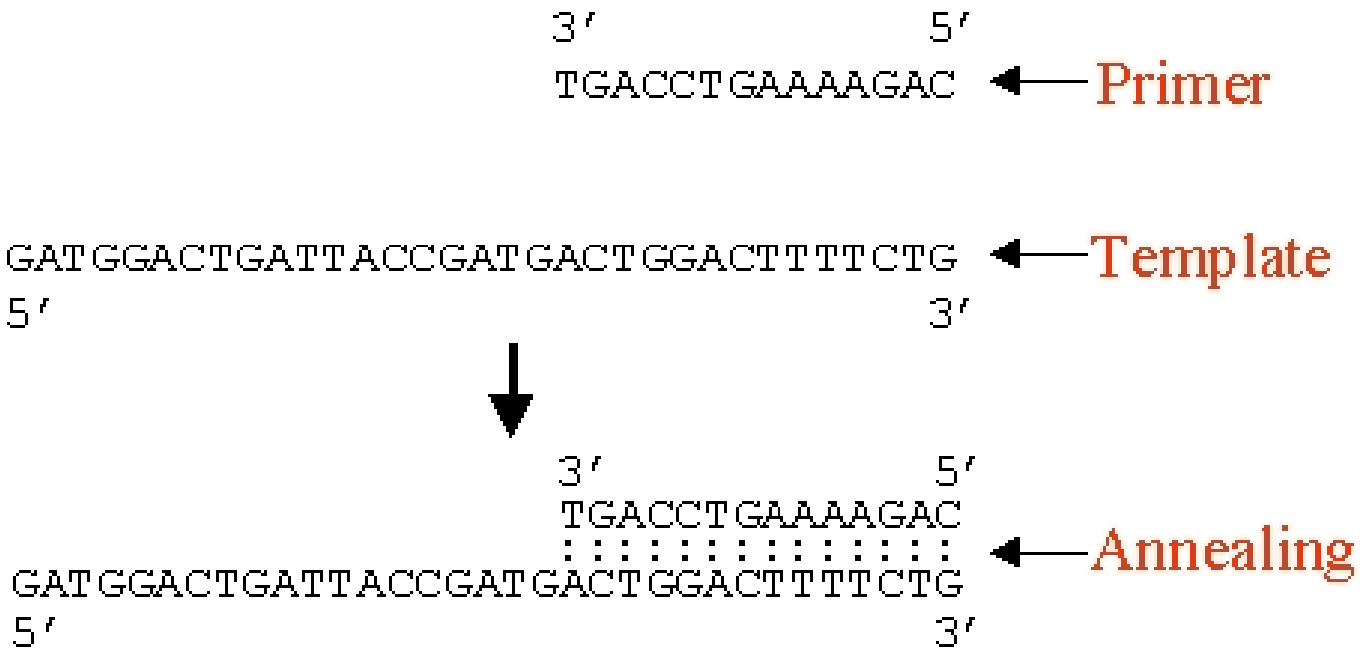
One sequence is 32 bases shorter than the other.



A. General Rules for PCR Primer Design

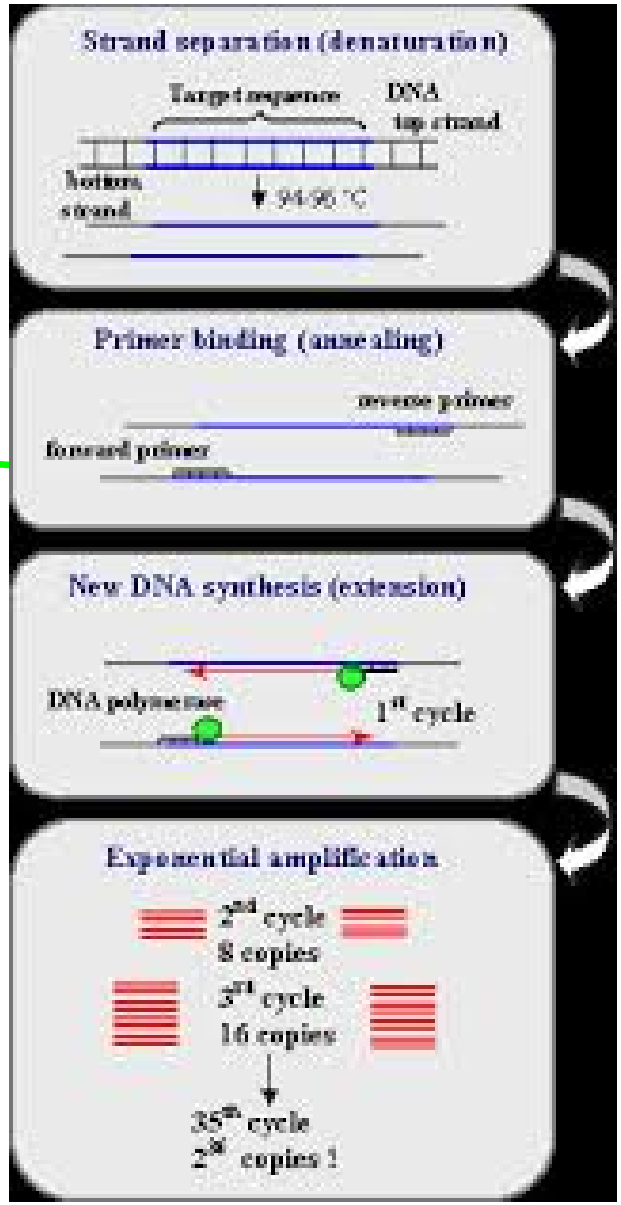
B. Tools to help you design PCR Primers

C. Checking how good your primers really are.



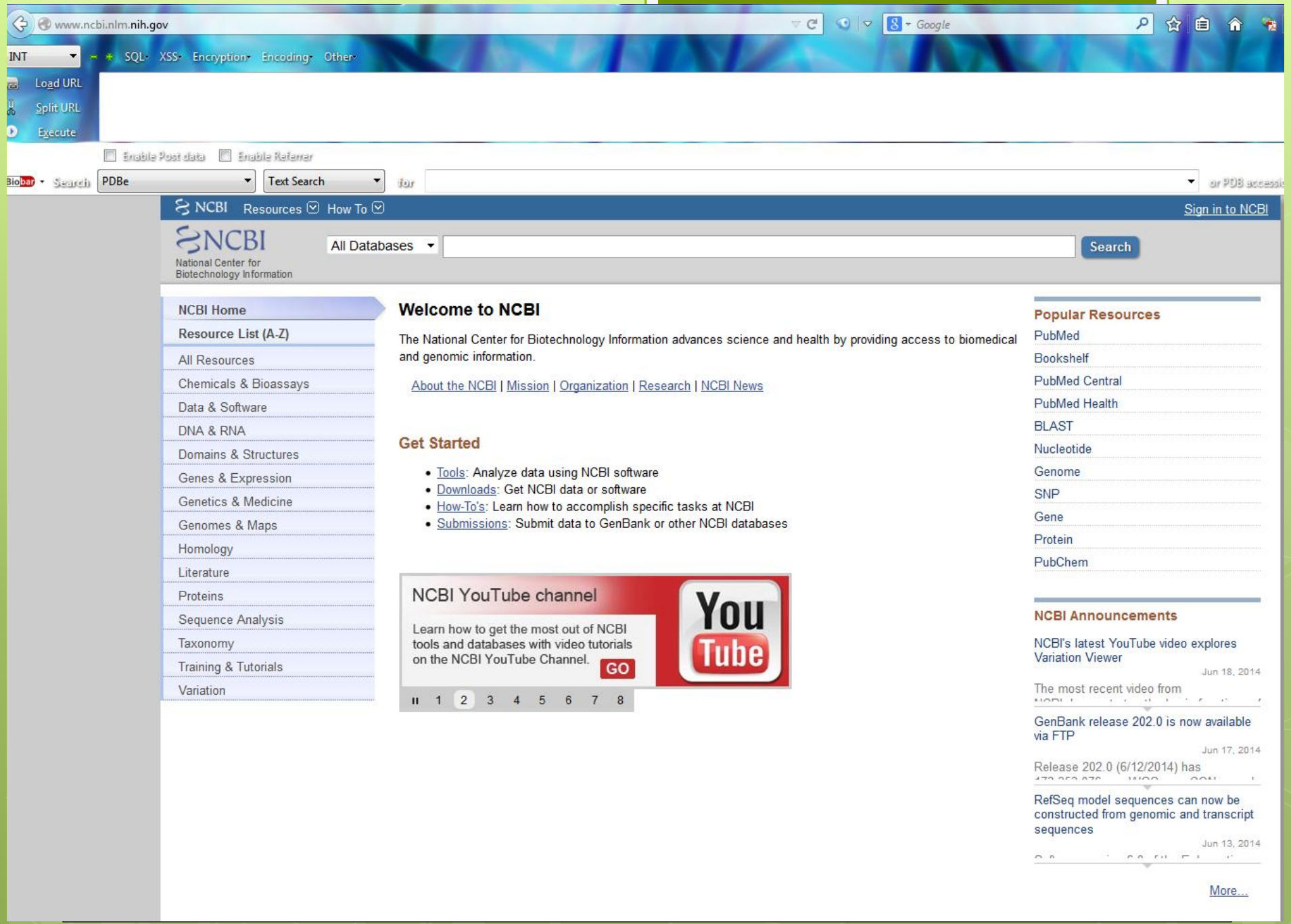
Primers determine whether PCR produces the product you want

Specificity



What type of PCR are you doing?

- Usually a single gene or part of a gene is the target, but sometimes more than one gene is desired.
  - Single-gene amplification?
  - Multiple-site amplification?
- Design depends on what information you have when you start:
  - Single DNA sequence?
  - Multiple DNA sequences?
  - Database record (GenBank)?


www.ncbi.nlm.nih.gov
Google
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National Center for Biotechnology Information
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Chemicals & Bioassays
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DNA & RNA
Domains & Structures
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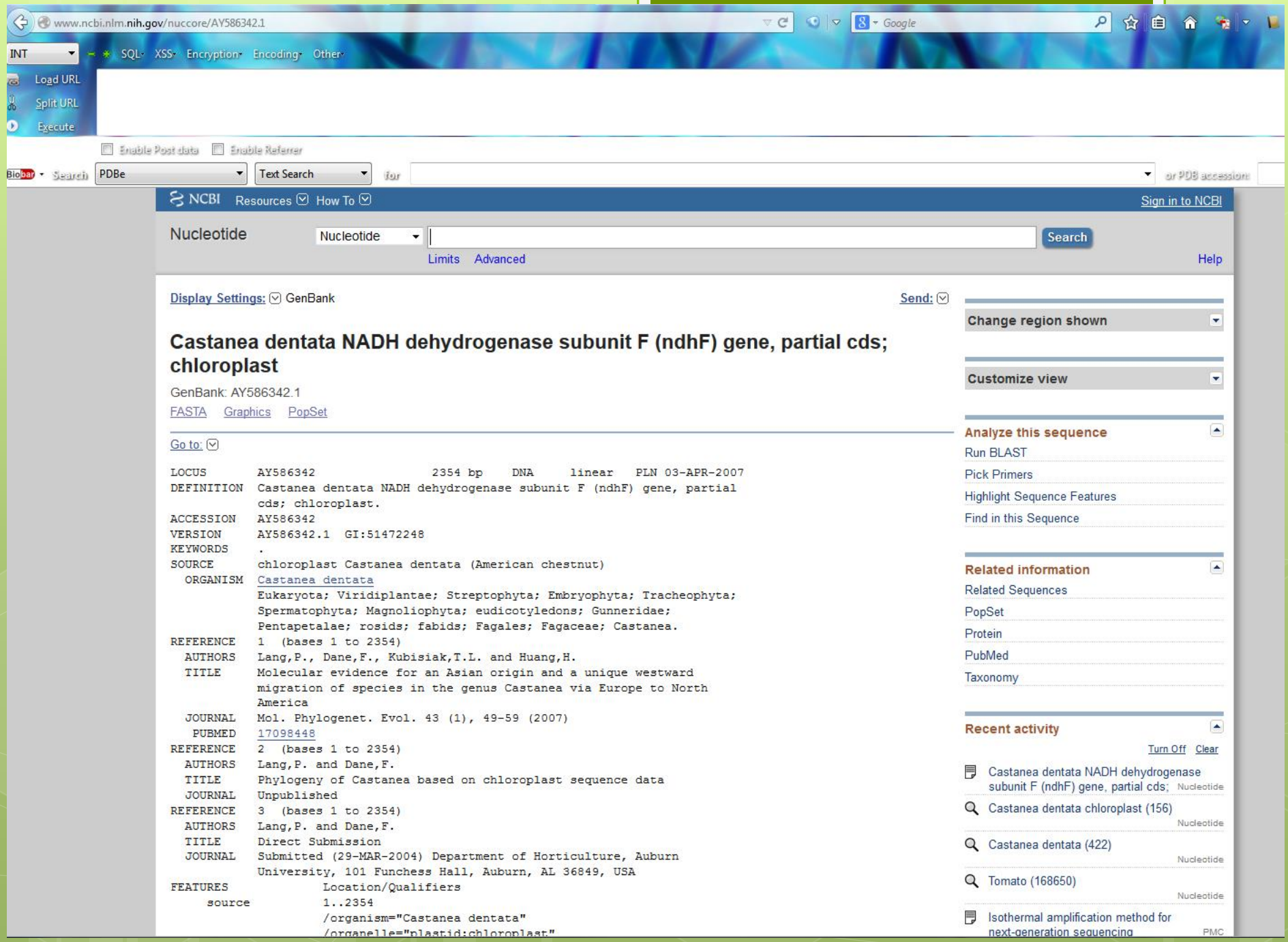
NCBI Announcements
NCBI's latest YouTube video explores Variation Viewer
Jun 18, 2014
The most recent video from
GenBank release 202.0 is now available via FTP
Jun 17, 2014
Release 202.0 (6/12/2014) has
RefSeq model sequences can now be constructed from genomic and transcript sequences
Jun 13, 2014
More...


www.ncbi.nlm.nih.gov/nuccore/AY586342.1
INT
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Castanea dentata NADH dehydrogenase subunit F (ndhF) gene, partial cds; chloroplast
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Go to:
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VERSION AY586342.1 GI:51472248
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SOURCE chloroplast Castanea dentata (American chestnut)
ORGANISM Castanea dentata
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fagales; Fagaceae; Castanea.
REFERENCE 1 (bases 1 to 2354)
AUTHORS Lang,P., Dane,F., Kubisiak,T.L. and Huang,H.
TITLE Molecular evidence for an Asian origin and a unique westward migration of species in the genus Castanea via Europe to North America
JOURNAL Mol. Phylogenet. Evol. 43 (1), 49-59 (2007)
PUBMED 17098448
REFERENCE 2 (bases 1 to 2354)
AUTHORS Lang,P. and Dane,F.
TITLE Phylogeny of Castanea based on chloroplast sequence data
JOURNAL Unpublished
REFERENCE 3 (bases 1 to 2354)
AUTHORS Lang,P. and Dane,F.
TITLE Direct Submission
JOURNAL Submitted (29-MAR-2004) Department of Horticulture, Auburn University, 101 Funchess Hall, Auburn, AL 36849, USA
FEATURES Location/Qualifiers
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/organelle="plastid:chloroplast"
Change region shown
Customize view
Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
Find in this Sequence
Related information
Related Sequences
PopSet
Protein
PubMed
Taxonomy
Recent activity
Turn Off
Clear
Castanea dentata NADH dehydrogenase subunit F (ndhF) gene, partial cds; Nucleotide
Castanea dentata chloroplast (156) Nucleotide
Castanea dentata (422) Nucleotide
Tomato (168650) Nucleotide
Isothermal amplification method for next-generation sequencing PMC

Primer and PCR product length rules

- Primer length determines the specificity
  - Too short – binds many places → non-specific amplification
  - Too long – likely to have internal structure and binds to itself
- Optimal primer length

$$T_m(^{\circ}C) = 64.9 + \frac{41(G + C - 16.4)}{L}$$

  - 18-24 bp for single-gene products
  - 30-35 bp for multiple gene products
- Optimal product (amplicon) size
  - 300-1000 bp for most purposes (> 3000bp takes special conditions)

Parameters are values for variables, like the temperature.

- T_m is the temperature at which 50% of the DNA duplex dissociates to become single stranded
  - Determined by primer length, base composition, and concentration of the primer, template (input DNA) and salt (monovalent cation means Na^+)
- Optimal melting temperature
  - Generally reactions are aimed at 52°C- 65°C
  - Higher T_m (75°C-- 80°C) is recommended for amplifying high GC content targets.
- Matching Primer T_m - you need two primers
  - If their T_m s are too different you will get single-sided PCR and a poor yield.
  - Desirable T_m difference < 5°C between the primer pair

Avoiding multiple products when you DON'T want them.

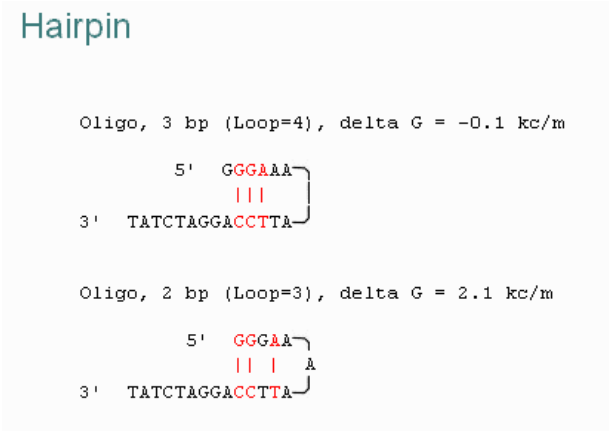
- Cross matches (homology)
  - If some regions of genes are very similar in sequence and your primers bind there, you will get products from all matching regions.
  - Primers containing highly repetitive sequence are very likely to generate multiple products.
- Checking for cross-matching
  - Use a pattern matching program, like BLAST, with the sequence of your PCR primers as the query and the NCBI nr (non-redundant) sequence database as the target – it will return all matches and you can see if you have a problem.

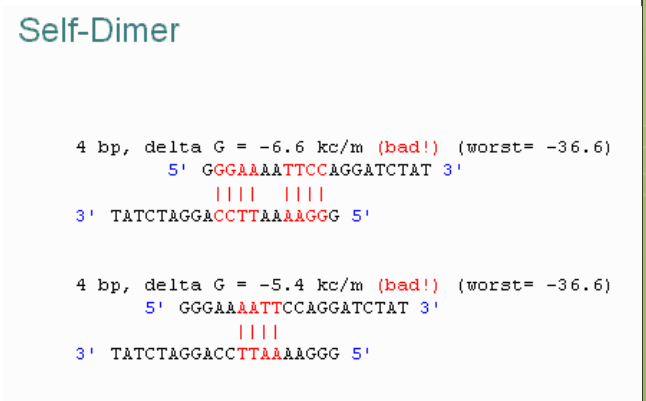
General rules for primer design -- GC content; repeats and runs

- Primer G/C content
  - Optimal G/C content: 45-55%
  - Common G/C content range: 40-60%
- Runs (single base stretches)
  - Long runs increases mis-priming (non-specific annealing) potential
  - The maximum acceptable number of runs is 4 bp
- Repeats (consecutive di-nucleotide)
  - Repeats increases mis-priming potential
  - The maximum acceptable number of repeats is 4 di-nucleotide

General rules for primer design -- Primer secondary structures

- Hairpins
  - Formed via intra-molecular interactions
  - Negatively affect primer-template binding, leading to poor or no amplification
  - Acceptable ΔG (free energy required to break the structure): >-2 kcal/mol for 3'end hairpin; >-3 kcal/mol for internal hairpin;
- Self-Dimer (homodimer)
  - Formed by inter-molecular interactions between the two same primers
  - Acceptable ΔG: >-5 kcal/mol for 3'end self-dimer; >-6 kcal/mol for internal self-dimer;
- Cross-Dimer (heterodimer)
  - Formed by inter-molecular interactions between the sense and antisense primers
  - Acceptable ΔG: >-5 kcal/mol for 3'end cross-dimer; >-6 kcal/mol for internal cross-dimer;





General rules for primer design -- GC clamp and max 3' end stability

- GC clamp
  - Refers to the presence of G or C within the last 4 bases from the 3' end of primers
  - Essential for preventing mis-priming and enhancing specific primer-template binding
  - Avoid >3 G's or C's near the 3' end
- Max 3'end stability
  - Refers to the maximum ΔG of the 5 bases from the 3'end of primers.
  - While higher 3'end stability improves priming efficiency, too higher stability could negatively affect specificity because of 3'-terminal partial hybridization induced non-specific extension.
  - Avoid $\Delta G < -9$.

General rules for primer design -- Annealing temperatures and other considerations

- T_a (Annealing temperature) vs. T_m
  - T_a is determined by the T_m of both primers and amplicons: optimal T_a=0.3 x T_m(primer)+0.7 x T_m(product)-25
  - General rule: T_a is 5°C lower than T_m
  - Higher T_a enhances specific amplification but may lower yields
  - Crucial in detecting polymorphisms
- Primer location on template
  - Dictated by the purpose of the experiment
  - For detection purpose, section towards 3' end may be preferred.
- When using composite primers
  - Initial calculations and considerations should emphasize on the template-specific part of the primers
  - Consider nested PCR

Resources for General Purpose PCR Primer Design

- Primer3
- Primer3Plus
- PrimerZ
- FAstPCR

General Purpose PCR Primer Design Tool– Primer3

Name	**Primer3 -- an online tool for PCR primer design**
Type	Web-based software
Key Functions	Design PCR primers and hybridization probes.
Publication Info	Methods Mol Biol 2000
Times Cited	823
Pros	The original and most widely used PCR primer design program; uses sequence as input; a huge number of options for customizing primer design;
Cons	busy interface;
Note	In OBRC; the program has been widely adopted by many primer design software.
YiBu's Rating	**4 out of 5**

Web Site:

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

More Info:

http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr_oligos/URL1043858198/info

General Purpose PCR Primer Design Tool– Primer3Plus

Name	**Primer3Plus, an enhanced web interface to Primer3**
Type	Web-based software
Key Functions	Design PCR primers for a given DNA sequence.
Publication Info	NAR 2007
Times Cited	N/A
Pros	Uses sequences or sequence file as input; a huge number of configuration options; automates specific tasks such as designing primers for cloning or step-wise sequencing; primers can be sent to an order form; clean, intuitive and well organized interface;
Cons	
Note	in OBRC. It is an updated, task-oriented web-interface to the original Primer3.
YiBu's Rating	**4.5 out of 5**

Web Site:

http://www.bioinformatics.nl/primer3plus

More Info:
http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr_oligos/URL1191263055/info

General Purpose PCR Primer Design Tool– PrimerZ

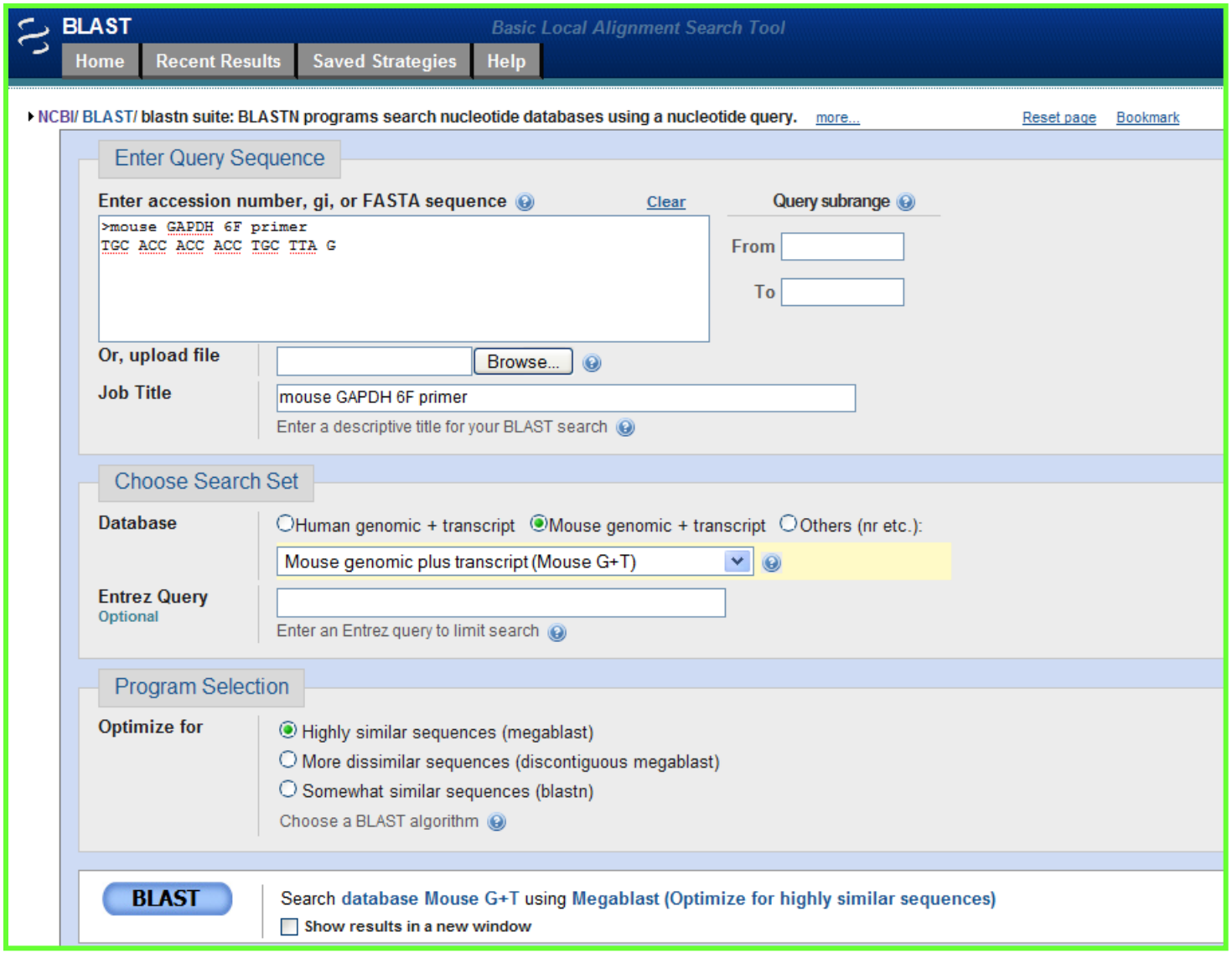
Name	**PrimerZ -- streamlined primer design for promoters, exons and human/mouse SNPs**
Type	Web-based software
Key Functions	Design PCR primers for promoters, exons, and human/mouse SNPs
Publication Info	Nucleic Acid Research 2007
Times Cited	0 (too new)
Pros	Uses gene name, Ensembl ID, rs# as input; settings for amplicon region and length, as well as PCR product sizes; allow batch rsSNP processing; frequently updated; offers many advance design settings; interactive results output
Cons	reported successful rate over 70%; only for human and mouse
Note	built on Primer3; in OBRC.
YiBu's Rating	**4.5 out of 5**

Web Site:

http://genepipe.ngc.sinica.edu.tw/primerz/beginDesign.do

More Info:
http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr_oligos/URL1190992855/info

Resources for PCR Primer Specificity Analysis – NCBI BLAST



http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on

Resources for PCR Primer Mapping – UCSC In-Silico PCR

Home Genomes Blat Tables Gene Sorter Session FAQ Help

UCSC In-Silico PCR

Genome: Mouse | Assembly: Jul. 2007 | Forward Primer: TGCACCACCAaCTGCTT | Reverse Primer: GGATGCAGGGATGATG | submit

Max Product Size: 50000 | Min Perfect Match: 18 | Min Good Match: 18 | Flip Reverse Primer: ☐

About In-Silico PCR

In-Silico PCR searches a sequence database with a pair of PCR primers, using an indexing strategy for fast performance.

Configuration Options

Genome and Assembly - The sequence database to search.
Forward Primer - Must be at least 15 bases in length.
Reverse Primer - On the opposite strand from the forward primer. Minimum length of 15 bases.
Max Product Size - Maximum size of amplified region.
Min Perfect Match - Number of bases that match exactly on 3' end of primers. Minimum match size is 15.
Min Good Match - Number of bases on 3' end of primers where at least 2 out of 3 bases match.
Flip Reverse Primer - Invert the sequence order of the reverse primer and complement it.

Output

When successful, the search returns a sequence output file in fasta format containing all sequence in the database that lie between and include the primer pair. The fasta header describes the region in the database and the primers. The fasta body is capitalized in areas where the primer sequence matches the database sequence and in lower-case elsewhere. Here is an example:

```
>chr22:31000551+31001000  TAACAGATTGATGATGCATGAAATGGG CCCATGAGTGGCTCCTAAAGCAGCTGC
TtACAGATTGATGATGCATGAAATGGGgggtggccaggggtgggggggtga
gactgcagagaaaggcagggctggttcataacaagctttgtgcgtcccaa
tatgacagctgaagttttccaggggctgatggtgagccagtgagggtaag
tacacagaacatcctagagaaaccctcattccttaaagattaaaaataaa
```

http://genome.ucsc.edu/cgi-bin/hgPcr?db=mm9