5.36 Biochemistry Laboratory
Spring 2009

5.36 Lecture Summary #2 Tuesday, February 10, 2009
Next Laboratory Session: #3

Topics: **Molecular Cloning and Site-Directed Mutagenesis**

I. Overview of Molecular Cloning
II. Ligation (Step 1 of Cloning)
- **A.** Polymerase chain reaction (PCR)
- **B.** Restriction enzymes and gene insertion
- **C.** Session 3: digestion to check for the Abl(229-511) insert

III. DNA Site-Directed Mutagenesis
- **A.** PCR primer design
- **B.** Overview of the Quickchange strategy (preview of sessions 9-11)

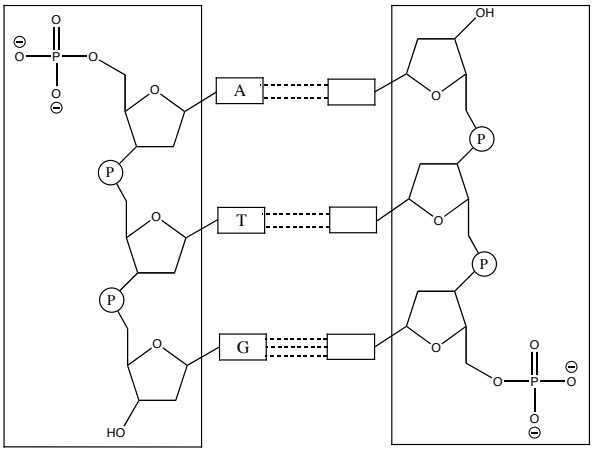
I. OVERVIEW OF MOLECULAR CLONING

REVIEW OF DNA

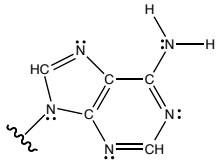
The central dogma of biology:

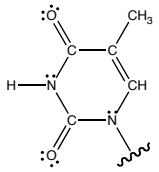
DNA → RNA → protein (→ protein folding and post-translational modification)

We are interested in expressing and studying proteins, so we need to start with the correct DNA or alter the DNA to make desired protein mutants.

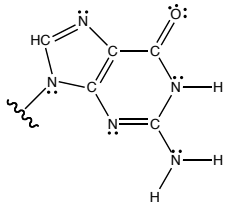


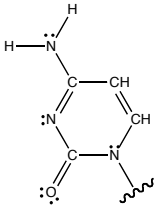
_____ backbone





Adenine (a) _____ (___)





Guanine (g) _____ (___)

The 5′ end of a DNA strand terminates with a _____ group. The 3′ end of a DNA strand terminates with a _____ group. By convention, we write a DNA sequence ____′ to ____′.

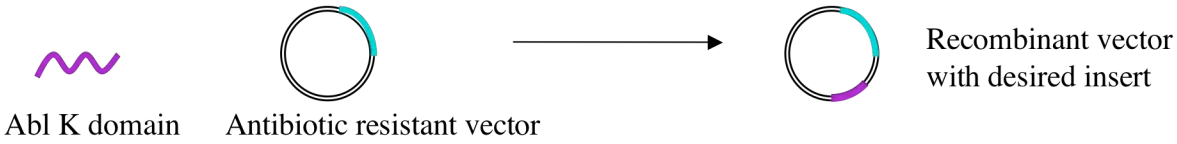
A DNA single strand is defined as a _____ strand if the mRNA version of the identical sequence can be translated to a protein.

The compliment DNA sequence (the opposite strand) is called the _____ strand. Hydrogen bonding binds together complementary strands of DNA to form a double helix.

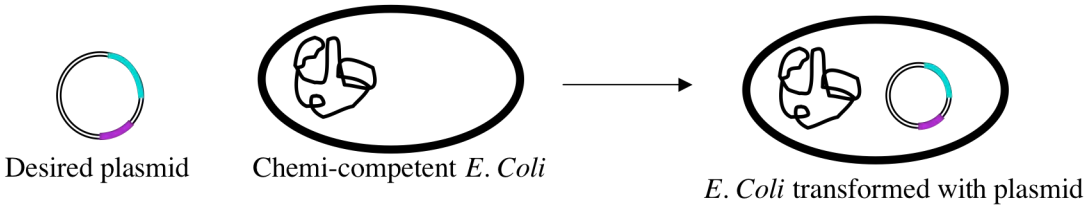
The lower bond enthalpies of hydrogen bonds compared to covalent bonds facilitate the separation of DNA strands during DNA replication.

DNA CLONING: IN-VIVO AMPLIFICATION OF DNA

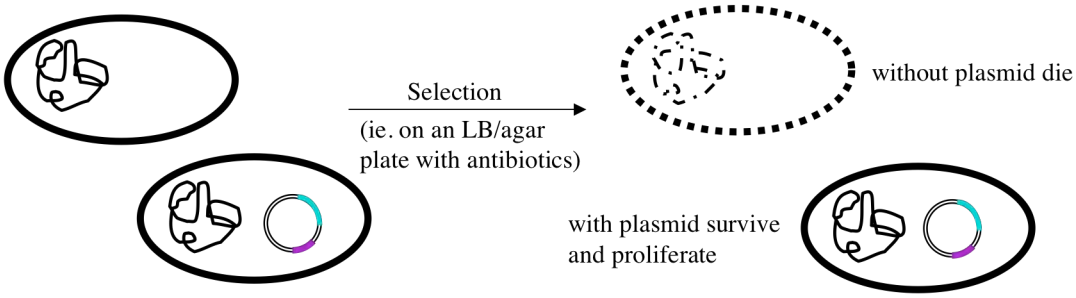
1. _____



2. _____



3. _____



In Modules 4 and 5, we are using *E. coli* cells for storage and expression. _____ for storage. _____ for protein expression. For lab Session 2, you were provided with:

- BL21(DE3) cells transformed with an H396P Abl(229-511)-encoding vector for protein expression.
- DH5α cells transformed with a wt Abl(229-511)-encoding vector for isolation of the wt vector DNA (by doing a miniprep).

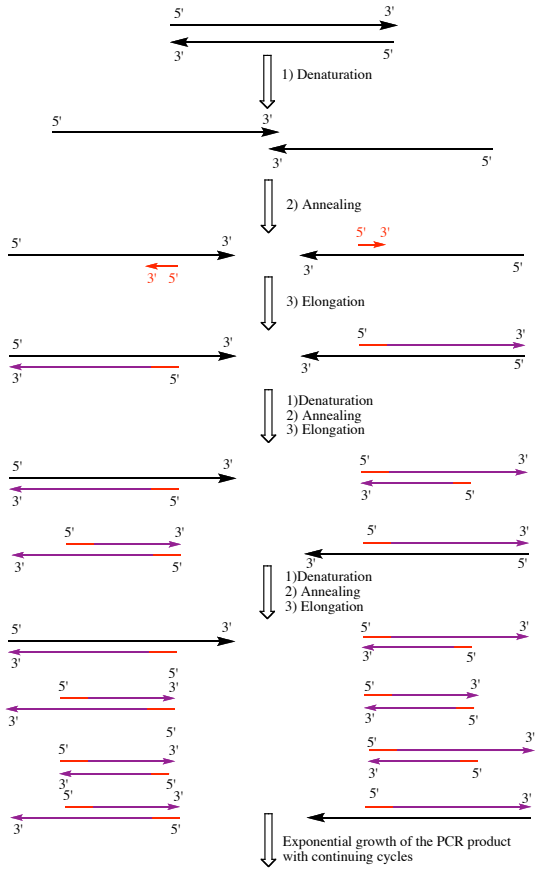
II. LIGATION (step 1 in molecular cloning)

A) POLYMERASE CHAIN REACTION (PCR)

How do we get enough of the desired DNA insert to work with for the ligation?
How can we introduce RE cut sites into the insert DNA?
Answer: PCR

- Allows you to amplify desired regions of DNA
- Utilizes *in vitro* enzymatic replication by a polymerase (such as Taq or Pfu)

polymerase: an enzyme that catalyzes the polymerization of deoxyribonuclotides (dATP, dGTP, dTTP, and dCTP) into a strand of DNA.



General components of any PCR reaction:

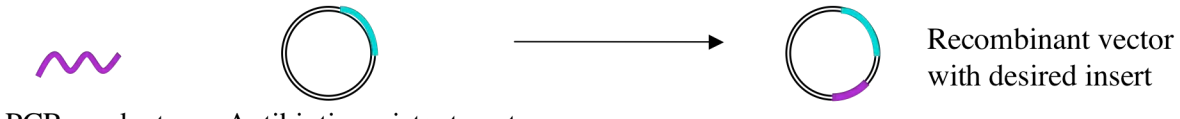
- Template DNA. DNA that includes the desired sequence to be amplified.
- Nucleotides (_____s). The building blocks to build new DNA strands.
- Primers. Complimentary _____ to the start and end of target sequence.
- A _____ polymerase (such as Taq or Pfu)
- A buffer compatible with the polymerase
- Thermal cycler

General PCR protocol for thermal cycling:

- Initialization Step (92 ºC for 2 min): Activates the heat-stable polymerase
- 25-30 cycles of
  1) Denaturation Step (___ ºC): denatures template DNA
  2) Annealing Step (___ ºC): allows _____ to anneal to target sequences
  3) Elongation Step (72 ºC): elongation of the annealed primers
- Final Elongation Step (72ºC for 10 min)

For a video of pcr in action, see http://www.dnalc.org/ddnalc/resources/animations.html.

PCR yields enough of the target DNA insert for subsequent ligation.



B) RESTRICTION ENZYMES AND GENE INSERTION

Question: How do you get the desired DNA insert into the vector?

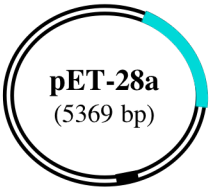
Restriction Enzymes (also called _____) selectively cut DNA within a specific sequence (called a _____ site) by cleaving a _____ bond within the DNA backbone.

For restriction enzymes that cleave double-stranded DNA, some cut straight across the DNA molecule producing _____ ends. Others cut in an offset fashion producing _____ ends.

Examples of restriction enzymes and their corresponding recognition sites:

"blunt end" REs		*"sticky end" RE's*	
Sma1	5'...CCCGGG...3' 3'...GGGCCC...5'	_____	5'...CATATG...3' 3'...GTATAC...5'
Alu1	5'...AGCT...3' 3'...TCGA...5'	_____	5'...CTCGAG...3' 3'...GAGCTC...5'
Sca1	5'...AGTACT...3' 3'...TCATGA...5'	EcoR1	5'...GAATTC...3' 3'...CTTAAG...5'
EcoRV	5'...GATATC...3' 3'...CTATAG...5'	BamH1	5'...GGATCC...3' 3'...CCTAGG...5'
		Sac1	5'...GAGCTC...3' 3'...CTCGAG...5'

Circle the RE sites above that are found in the cloning region of the pET-28a vector:

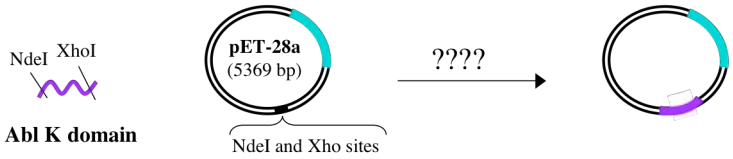


5 CCATATGGCTAG…GGATCCGAATTCGAGCTCCGTCGACAAGCTGCGGCCGC ACTCGAG **3'**

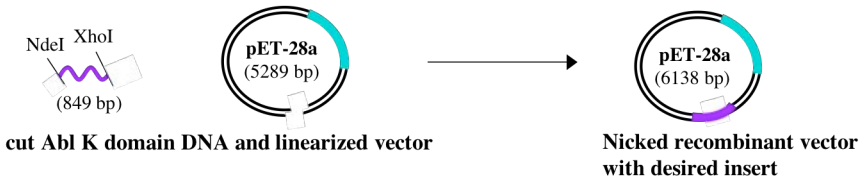
Resources for visualizing/identifying RE cut sites

- The information sheet that comes with commercial vectors. (The pET-28a vector information sheet will be available in the lab for session #3.)
- Vector visualization software:
  Ape (free) http://www.biology.utah.edu/jorgensen/wayned/ape/
  Vector NTI (free if you provide an academic e-mail address)
  https://catalog.invitrogen.com/index.cfm?fuseaction=userGroup.home

So how was the wt Abl(229-511)-containing vector DNA (isolated in Session 2) constructed?



Cut both desired DNA fragment and vector with the same restriction enzymes (a digestion) then incubate together.



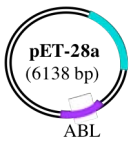
Use a DNA Ligase to connect 5′ and 3′ sticky ends and create a continuous plasmid.

C) LAB SESSION 3: DIGESTION TO CHECK FOR THE ABL(229-511) INSERT

It is common to receive cloning/expression vectors containing a DNA insert of interest from another laboratory…and it is wise to check that these vectors contain the DNA you are expecting. You can check your vector by:

- DNA _____ (This is the most thorough method.)
- Restriction digestion (to confirm the DNA insert size and location)

Session 3 will entail restriction digestion of the wt Abl(229-511)-encoding vector.



The 849-bp ABL DNA insert should be between the Xho1 and Nde1 restriction sites of the pET-28a vector.

You will set up four digestion reactions, with the following expected results:

- no-enzyme "digestion" → 6138-bp _____ vector DNA
- Xho1-only digestion → 6138-bp _____ vector DNA
- Nde1-only digestion → 6138-bp linear vector DNA
- Xho1 / Nde1 digestion → _____-bp linear vector and 849-bp insert

(See page 13 of you lab manual for a sample gel.)

III. DNA SITE-DIRECTED MUTAGENESIS

What you have: a wt Abl(229-511)-encoding plasmid (isolated in Session #2)

What you want: a plasmid encoding an Abl (229-511) mutant.

You will use the Quickchange strategy to generate mutant DNA that encodes the corresponding Bcr-Abl protein mutant of your choice.

The Bcr-Abl kinase domain aa sequence:

```
                                                    (229)SP NYDKWEMERT
              HF V
   V   V E   RH K  260         270       GA280          A          300
DITMKHKLGG GQYGEVYEGV WKKYSLTVAV KTLKEDTMEV EEFLKEAAVM KEIKHPNLVQ
              N                                                    A
       310 L   I L320         330         340   T    350 T   G   V
LLGVCTREPP FYIITEFMTY GNLLDYLREC NRQEVNAVVL LYMATQISSA MEYLEKKNFI
                                    R
       370          I   L    F390       PP400         410        Y420
HRDLAARNCL VGENHLVKVA DFGLSRLMTG DTYTAHAGAK FPIKWTAPES LAYNKFSIKS

       430         440         450          K          470         480
DVWAFGVLLW EIATYGMSPY PGIDLSQVYE LLEKDYRMER PEGCPEKVYE LMRACWQWNP

     S 490         500         510
SDRPSFAEIH QAFETMFQES SISDEVEKEL G
```

A) PCR PRIMER DESIGN FOR QUIKCHANGE MUTAGENESIS

After selecting your target amino acid mutation, you will design primers to create the corresponding DNA mutation. Each group should turn in a primer design worksheet (p. 55 of the lab manual) to their TA during lab session #4.

Abl(229-511) nucleotide sequence (Appendix B of your lab manual):

```
                        (688)tcc cccaactacg acaagtggga gatggaacgc 721
acggacatca ccatgaagca caagctgggc gggggccagt acggggaggt gtacgagggc 781
gtgtggaaga aatacagcct gacggtggcc gtgaagacct tgaaggagga caccatggag 841
gtggaagagt tcttgaaaga agctgcagtc atgaaagaga tcaaacaccc taacctggtg 901
cagctccttg gggtctgcac ccgggagccc ccgttctata tcatcactga gttcatgacc 961
tacgggaacc tcctggacta cctgagggag tgcaaccggc aggaggtgaa cgccgtggtg 1021
ctgctgtaca tggccactca gatctcgtca gccatggagt acctggagaa gaaaaacttc 1081
atccacagag atcttgctgc ccgaaactgc ctggtagggg agaaccactt ggtgaaggta 1141
gctgattttg gcctgagcag gttgatgaca ggggacacct acacagccca tgctggagcc 1201
aagttcccca tcaaatggac tgcacccgag agcctggcct acaacaagtt ctccatcaag 1261
tccgacgtct gggcatttgg agtattgctt tgggaaattg ctacctatgg catgtcccct 1321
tacccgggaa ttgacctgtc ccaggtgtat gagctgctag agaaggacta ccgcatggag 1381
cgcccagaag gctgcccaga gaaggtctat gaactcatgc gagcatgttg gcagtggaat 1441
ccctctgacc ggccctcctt tgctgaaatc caccaagcct ttgaaacaat gttccaggaa 1501
tccagtatct cagacgaagt ggaaaaggag ctgggg
```

Note: amino acid 229 corresponds to nucleotides 688-690 (not 685-687 as might be expected) because there are 3 bases prior to the start of the open reading frame.

For example, let's design primers to make the T315I mutant. Note that complete instructions on primer design can be found on page 54 in Appendix C of your lab manual.

The Thr315 amino acid corresponds to bases _____.

Original nucleotide sequence including the 12 bases on either side of the mutant codon:
5′ ccc ccg ttc tat atc atc *act* gag ttc atg acc tac ggg 3′

Ala/A	**GCU, GCC, GCA, GCG**	**Leu/L**	**UUA, UUG, CUU, CUC, CUA, CUG**
Arg/R	**CGU, CGC, CGA, CGG, AGA, AGG**	**Lys/K**	**AAA, AAG**
Asn/N	**AAU, AAC**	**Met/M**	**AUG**
Asp/D	**GAU, GAC**	**Phe/F**	**UUU, UUC**
Cys/C	**UGU, UGC**	**Pro/P**	**CCU, CCC, CCA, CCG**
Gln/Q	**CAA, CAG**	**Ser/S**	**UCU, UCC, UCA, UCG, AGU, AGC**
Glu/E	**GAA, GAG**	**Thr/T**	**ACU, ACC, ACA, ACG**
Gly/G	**GGU, GGC, GGA, GGG**	**Trp/W**	**UGG**
His/H	**CAU, CAC**	**Tyr/Y**	**UAU, UAC**
Ile/I	**AUU, AUC, AUA**	**Val/V**	**GUU, GUC, GUA, GUG**
START	**AUG**	**STOP**	**UAG, UGA, UAA**

(You can alternatively use a DNA-to-protein translation program to check your DNA (http://www.expasy.ch/tools/dna.html).

T315→I is a _____→___ nucleotide point mutation

Forward primer:
5′ ccc ccg ttc tat atc atc *att* gag ttc atg acc tac ggg 3′

Reverse primer (the reverse compliment):

3′ ggg ggc aag ata tag tag taa ctc aag tac tgg atg ccc 5′

5′ ccc gta ggt cat gaa ctc aat gat gat ata gaa cgg ggg 3′

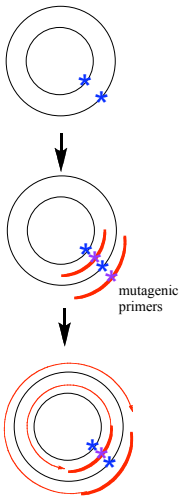
Check that your primers have a minimum GC content of 40%.

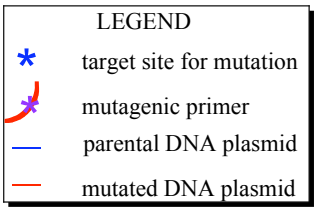
B) OVERVIEW OF QUIKCHANGE STRATEGY (Preview of Sessions 9-11)

Modified from the QuikChange handbook (http://www.stratagene.com/manuals/200518.pdf).

Step 1: plasmid preparation

Step 2: thermal cycling (_____)
(session 9)

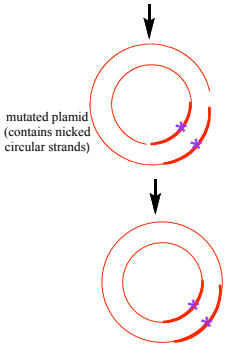




Note: for PCR using mutagenic primers

- Instead of replicating just a desired fragment, replicate the entire plasmid
- Need a much more powerful polymerase that has higher fidelity than Taq
- Use Pfu Turbo (isolated from *pyrococcus furiosus*)
- _____ gives Pfu Turbo higher fidelity.

Step 3: Digest the methylated, non-mutant DNA template with Dpn 1. (*session 10*)



Step 4: Transformation
(session 10)

Isolate mutant DNA (session 11) and send for sequencing.

Quikchange allows you to make point mutants in one day (or several days when limited to 4-hour lab session). Less than 25 years ago it took months to make point mutants.

PCR, Quikchange, and similar cloning techniques have been instrumental in advancing recombinant technologies and making molecular biology methods much more efficient