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.

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 complement 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. _______________

```
Abl K domain Antibiotic resistant vector
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2. _______________

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Desired plasmid Chemi-competent E. Coli
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3. _______________

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Selection (ie. on an LB/agar plate with antibiotics) without plasmid die
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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 deoxyribonucleotides (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 °C for 2 min): Activates the heat-stable polymerase
- 25-30 cycles of
  1) Denaturation Step (___ °C): denatures template DNA
  2) Annealing Step (____ °C): allows ______ to anneal to target sequences
  3) Elongation Step (72 °C): elongation of the annealed primers
- Final Elongation Step (72°C 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:

<table>
<thead>
<tr>
<th>“blunt end” REs</th>
<th>“sticky end” RE’s</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sma1</td>
<td>5′...CCCGGG...3′</td>
<td>3′...GGGCCC...5′</td>
</tr>
<tr>
<td></td>
<td>5′...CATATG...3′</td>
<td>3′...GTATAC...5′</td>
</tr>
<tr>
<td>Alu1</td>
<td>5′...AGCT...3′</td>
<td>3′...TCGA...5′</td>
</tr>
<tr>
<td></td>
<td>5′...CTCGAG...3′</td>
<td>3′...GAGCTC...5′</td>
</tr>
<tr>
<td>Sca1</td>
<td>5′...GATATC...3′</td>
<td>3′...CTAGA...5′</td>
</tr>
<tr>
<td>EcoR1</td>
<td>5′...GATTAC...3′</td>
<td>3′...CTAACG...5′</td>
</tr>
<tr>
<td>EcoRV</td>
<td>5′...GATATC...3′</td>
<td>3′...CTAGA...5′</td>
</tr>
<tr>
<td>BamH1</td>
<td>5′...GATATC...3′</td>
<td>3′...CTAGA...5′</td>
</tr>
<tr>
<td>Sac1</td>
<td>5′...GATATC...3′</td>
<td>3′...CTAGA...5′</td>
</tr>
</tbody>
</table>

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

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/](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](https://catalog.invitrogen.com/index.cfm?fuseaction=userGroup.home)
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 your 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
```

DITM KH K LG QQ GEVY EG WKKY STLVA V KT KLEDT MEV EEFLKEAAVM KEIKHPNLVQ

```
310 L I L320 330 340 T 350 T G V
LLGVT REPP FYIITE FMTY GNLDDY LREC N RQEVNA VVL LYM ATQISSA MEYLEKKNFI
```

```
370 I L P390 PP400 410 Y420
HRDLA ARNRCL VGENHLVKVA DFGLSR LM TGA DTYTA HAGA R PPIKWTAPES LAYNKFISKS
```

```
430 440 450 K 470 480
D VWAFG VLL V EIATYGMSPY PGIDLS QVYE LLEKDYR ME R PEGCPEK VY E LMRACQW QN P
```

```
S 490 500 510
SDRPS FAEI H QAFTEM PQE S 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 cccaactg c acaagtgg a gatggaacgc
721 acggacatca ccatgaagca caagctgggc gggggccagt
781 acggggaggt gtacgagggc
841 gttatgggaaga aatacagcct gcaggtggcc
gttgaagacct tgaaggagga caccatggag
gttgaagagt tcttgaaaga agctgcagtc atgaaagaga
901 tcaaacaccc taacctggtg cagctccttg gggtctgcac
961 ccggagccc cgggtctata tcatcactga gttcatgacc
tacgggaacc tcctggacta cctgagggag
tgcaaccggc aggaggtgaa cgccgtggtg
tctgctgtaca tggccactca gatctcgtca gccatggagt
tacctggagaa gaaaaacctc taacccgagct
tgggagggag
1021 gctctgcttg gcagttttgc
ttgggagggag
1081 gctgattttg gcctgagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1141 gagctagtct gctggagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1201 gagctagtct gctggagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1261 gagctagtct gctggagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1321 gagctagtct gctggagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1381 gagctagtct gctggagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1441 gagctagtct gctggagcag
ttgatgaca ggggacacct acacagcctcc tgtgaggag
1501 gagctagtct gctggagcag
```

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’

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

(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 act 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)


Step 1: plasmid preparation

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

LEGEND
* target site for mutation
mutagenic primer
—— parental DNA plasmid
—— mutated DNA plasmid
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.