The goal of this experiment:
Starting with a previously generated cDNA clone (in the vector pCR2.1 TOPO) from *Nasonia vitripennis* male pupae, *reclone part of the cDNA into a different vector: pGEM 7Z.*

**WEEK 6: 5/4 & 5/6 & 5/7**
- Restriction digest of pGEM vector
- Quantitation of plasmid DNA and Ligations reactions

**WEEK 7: 5/11 & 5/13 & 5/14**
- Generation of competent *E. coli* (XL1-Blue) cells
- Transformation of competent cells with ligated DNA mix
- Analysis of results: blue/white screen

**WEEK 8: 5/18 & 5/20 & 5/21**
- PCR CloneCheck analysis OR Total Nucleic Acids analysis
- Preparation of plasmid DNA from clones with confirmed inserts
- Analysis of clones by restriction enzyme digests

- EST sequence analysis

**WEEK 10: 6/1 & 6/3 & 6/4**
- Finish up experiments
- Lab clean-up (absolutely required)
- Lab Final
Week 6 Prelab Assignment:
Read through this handout

• Work out the recipe for your restriction digest of pGEM DNA. Assume that the stock of vector DNA is 500ng/µl and that the concentration of the restriction enzyme (BAM HI 20 units/µl)
• In your lab notebook, write out all steps through the ligation reaction.
• See pg 3-4 for assignment on generating a restriction map of the pCT704 clone.

Week 6 protocols

### 1. Restriction digest of the plasmid vector  pGEM 7Z

All of the following steps should be carried out in 1.5 ml Eppendorf tubes.

a) Check the tubes of vector DNA and enzyme to doublecheck the concentrations of each. Work out a protocol for digesting 3 µg of pGEM vector DNA with the restriction enzyme Bam HI. The total volume of your digest should be about 100µl. You should use excess enzyme: 5-10 fold more units than necessary. For this reaction you should add BSA to final concentration of 0.1 mg/ml. The BSA stock is 10mg/ml.

<table>
<thead>
<tr>
<th>A unit of restriction enzyme activity is the amount of enzyme that cuts 1 µg of DNA in 1 hour at 37 degrees.</th>
</tr>
</thead>
</table>

b) After incubating the digest for about 1 1/2 hours at 37ºC, remove an aliquot containing ~120 ng and set aside.

c) Obtain a tube with phase divider gel and centrifuge at high speed for 20-30 seconds to collect gel at the bottom of the tube. Transfer your digest to this tube.

d) Add one volume of a 1:1 mixture of phenol/chloroform to the digest. [*One volume means an equivalent volume.*] Thoroughly mix the aqueous and organic phase to form a homogeneous suspension. The gel will not become part of the suspension.

e) Centrifuge at 12,000g for 2 minutes to separate the phases.

f) The phase divider gel should form a barrier between the aqueous and organic phases. Carefully remove the top (aqueous) phase to a fresh tube. Estimate the volume recovered.

g) Add linear acrylamide (STOCK: 5 mg/ml) to a final concentration of 20 µg/ml and mix well. The acrylamide acts as an inert carrier to increase the efficiency recover of the DNA after ethanol precipitation (next step).

h) Ethanol precipitate the digested DNA by adding 1/10 volume of 3M Na acetate and 2 volumes of ice-cold 95% or 100% ethanol. Place on ice for 15-30 minutes.
i) Pellet the vector DNA by centrifuging at top speed for about 10 minutes. Carefully remove the ethanol and let the pellet dry completely. The pellet is likely to be to one side of the bottom of the tube.

j) Resuspend the pellet in ~20 µl of TE buffer: 10mM Tris pH8, 1mM EDTA.

k) Store on ice or in the refrigerator (4º C). Be sure to label the tube appropriately.

### (2) Quantitation of pGEM plasmid DNA recovered after ethanol precipitation.

Since you are working with small quantities of DNA, absorbance at A_{260} cannot be used to quantitate the vector DNA recovered after ethanol precipitation. You will run a small amount of your vector (recovered after ethanol precipitation) on a minigel and compare the brightness of the band to known quantities of Bam digested pGEM (aliquot set aside in step c)

**MINIGEL:** What % agarose should you use?

**NOTE:** We may try out a new agarose buffer system that increases band migration rate

Run at least two different quantities of your vector DNA (such as 1µl of undiluted and 1 µl of a 1/5 or 1/10 dilution).

1. From the aliquot set aside in step c, run 100 ng of Bam digested pGEM in one lane and 10 ng in another lane.
2. Run about 100 ng of uncut vector DNA
3. Don’t forget HiLo markers.
4. Also include a sample of the purified fragment DNA from pCT704

### (3) Gel Purification of a 1.0kb Bam HI-Bgl II DNA fragment from pCT 704 by Monique

This step will be done for you. Each pair of students will be given a tube containing gel-purified fragment from a Bgl II -Bam HI double-digest of the pCT704 clone. [The identity of this clone will be withheld for the time being.]

**Prelab Assignment**

- Generate a restriction enzyme map of the pCT704 cDNA insert and of the pCR2.1 vector-- use this spiffy web site or another of your choice:
  
  
  The DNA sequence of pCT704 cDNA insert is at this link:
  
  http://fire.biol.wwu.edu/trent/trent/pCT704sequence.htm
  
  The DNA sequence of pCR2.1TOPO vector is at this link:
  
  http://www.invitrogen.com/content/sfs/vectors/pcr2_1topo_seq.txt

- Be sure to take note of the recognition site sequences for Bam HI, Bgl II and EcoRI
**Prelab Assignment continued..**

Draw a cartoon of the pCT704 clone (cDNA insert plus vector) showing Eco RI, Bgl II and Bam H I sites. Since the cDNA can insert into the vector in two different orientations, there will be two possible maps.

**During LAB**

A file of the gel will be placed on various lab computers - along with NIH image. During lab, determine the sizes of the bands shown in the gel below after digestion with the various enyzmes. Which digestion will differentiate between the two orientations?

<table>
<thead>
<tr>
<th>Lane#</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hi LO size standards</td>
</tr>
<tr>
<td>2</td>
<td>pCT704 no restriction enzyme</td>
</tr>
<tr>
<td>3</td>
<td>pCT704  Bam HI digest</td>
</tr>
<tr>
<td>4</td>
<td>pCT704  Eco RI digest</td>
</tr>
<tr>
<td>5</td>
<td>pCT704  Bgl II digest</td>
</tr>
<tr>
<td>6</td>
<td>pCT704  Bam HI/Bgl II digest</td>
</tr>
<tr>
<td>7</td>
<td>pCT704  Bam HI/EcoRI digest</td>
</tr>
</tbody>
</table>

1.5% agarose 1X TBE
4. Generation of a recombinant clone containing the pGEM vector and the Bgl-Bam restriction fragment:

“Sticky end” ligation reactions: In this part of the cloning exercise you will generate recombinant DNA clones by mixing the vector and insert DNA together with DNA ligase. The two most important parameters in this reaction are
1. the molar ratio of insert to vector DNA
2. the total concentration of DNA.
• What kinds of competing reactions do you have when you mix vector and insert DNA together? Or, in other words, what kinds of DNA molecules will be generated?
• Which ones will give ampicillin resistant transformants?
• If the total DNA concentration is very low, which reaction will be favored?

a. Work out a protocol for a ligation reaction:
Your reaction should include:
- vector DNA
- insert DNA (restriction fragments)
- 10 X ligation buffer **
- T4 DNA ligase* (use 1µl of ligase per reaction)

Reaction conditions:
- The total volume should be 10 µl
- The final DNA concentration should be 10ng/µl
- The molar ratio of insert to vector should be either 2 to 1 or 1 to 1. (We will compare the results with the two different ratios to see which works best.)

b. Mix vector, insert DNA and water. Place at 45°C for 5 minutes.
Place immediately on ice and add ligation buffer and ligase. MIX

c. Let the reaction go overnight at 16°C.
(Why not at 37°C?)

d. Store the ligation reaction in the freezer (-20°C) until you are ready to do the transformations. [This will be taken care of for you.]

*0.01 Weiss unit of DNA ligase is the amount of enzyme required to catalyze the ligation of greater than 95% of the Hind III fragments of 1 µg of λ DNA at 16°C in 20 minutes.

** 10X ligation buffer:
300 mM Tris-HCL pH 7.8
100 mM MgCl2
100 mM DTT 5 mM ATP

Why is ATP in the ligation buffer?
WEEK 7: 5/11 & 5/13 & 5/14
Generation of competent *E. coli* cells for DNA transformation.

Genotype of XL1-Blue strain of *E. coli*:

*Bacterial chromosome:*  
*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*  
*F’ proA* + *B*, *lacI*  
*F*’  
*lacZΔM15 Tn10 (tet*)

The q mutation in the *lacI* gene results in a 10-fold greater synthesis of repressor (as compared to wild-type). The *lacZΔM15* deletion is located in the α–peptide region of the *lac Z* gene.

Examine the genotypes of this strain carefully:

- What is the significance of each mutation on the bacterial chromosome? (See *Molecular Biology LabFax* and/or links posted on 475 website -CT’s version)
- What is the significance of each gene carried on the F’ plasmid
- F’ plasmids are sometimes lost from the cell. What selective medium would you grow each strain on to ensure that it is carrying a copy of the F factor?

**Protocol for generating transformation-competent cells:**

*Sometime during the afternoon or evening before the scheduled lab:*

In a 125ml flask, seed 10-30 ml of L broth with a single colony from a plate of *E. coli* XL1-Blue’s. Incubate overnight at 37°C.

*Late Morning of scheduled lab day:*

Dilute the overnight culture about 1/100 in 50 ml L broth in a 250ml flask. Incubate at 37°C for ~2 hours.

**Before/During lab**

- After about 2 hours of incubation, measure the OD$_{600}$ about every 30 minutes (or less) using the Spec 20 spectrophotometers in the lab. Harvest the cells when the OD$_{600}$ is between at ~0.4 OD. The cells will still be in log phase.
- The cells should be kept on ice at all times. Chill the culture on ice for about 10 min. Transfer 20 ml of the cells to a sterile 30 ml screw-cap centrifuge tube. Centrifuge at 2700 g for 10 minutes in the SA600 rotor (at 4°C).
- Discard the supernatant and allow the residual media to drain from the tube by inverting it for a minute on a pad of paper towels. Resuspend the cells in 6 ml of ice-cold MgCl$_2$ CaCl$_2$. [80 mM MgCl$_2$ 20 mM CaCl$_2$]
- Centrifuge as above. Decant the supernatant and drain the cells as described above.
- **Gently** resuspend the cells in 1.2 ml of ice-cold 0.1 M CaCl$_2$.
- Label the tube and store the cells on ice or at 4°C until you are ready to do the transformations.
Transformation of competent cells with ligated DNA mix and identification of bacterial colonies that contain recombinant plasmids:

Each transformation reaction should contain:
- 200 µl of competent XL1 BLUE cells
- up to 50 ng DNA in a volume of 10 µl or less

Prepare transformation mixes with two different amounts of your ligated DNA mix and be sure to set up two controls: (i) competent cells plus no DNA and (ii) competent cells plus 10 ng supercoiled, uncut pGEM7Z plasmid DNA

1. For each transformation, place DNA and cells in a sterile, chilled 17 X 100 mm polypropylene tube using a chilled micropipette tip. Mix the contents by swirling gently. Store the tubes on ice for 30 minutes.

2. Transfer the tubes to a rack placed in a circulating water bath that has been preheated to 42º C. Leave the tubes in the water bath for exactly 90 seconds. Do not shake the tubes. This heat shock step is a crucial step. It is critical that the cells be raised to exactly the right temperature at the correct rate.

3. Rapidly transfer the tubes to an ice bath. Chill the cells for 1-2 minutes.

4. Add 800 µl of L broth to each tube. Incubate the tubes at 37º C for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

5. For each transformation, spread* 200 µl of cells on an L plate that contains X-gal, IPTG, and ampicillin. (Why are these compounds and why is each added?). Store the remainder of your cells at 4º C.

6. *Use a glass rod which you have flame-sterilized (dip in alcohol and then flame-- don’t catch your lab notebook on fire) to spread your cells around the plate. The spin-girl will help. Get a lesson in spreading cells if you haven’t done this before.

7. Incubate plates at 30º C overnight 18-24 hours

The day after scheduled lab period
- Store plates in refrigerator for a few hours before scoring the clones. (The blue colonies will be more intensely colored after a few hours at 4º C.)
- Count and record the number of blue colonies and white colonies on each plate. Calculate the number of transformant clones (white and blue) per ng of DNA. Determine the % recombinant clones generated by the ligation reaction.
Appendix 1: Working with Restriction Enzymes

1) Keep restriction enzymes cold  Restriction enzymes are proteins and are very sensitive to heat, and freezing and thawing. To keep them cold, they are usually stored at -20 degrees C. To prevent them from freezing, restriction enzymes are usually stored in 50% glycerol. When out of the freezer always keep restriction enzymes on ice. Never hold a tube of enzyme, because you warm hands will immediately warm it up.

2) Never contaminate restriction enzyme stocks.  Always use a fresh sterile tip when going into a restriction enzyme stock. It is simple to ruin an expensive restriction enzyme stock by contaminating it with another restriction enzyme.

3) Use appropriate buffer conditions for digestions.  Restriction enzymes work best in a specific ionic environment. Most enzymes are classified by which type of salt solution they work best in (High, Medium or Low). A high salt buffer is referred to as "H". These buffers are in 10X stocks. They are usually buffered between pH 7.4 and 8.2. Each company that makes a restriction enzyme will send a buffer that it works best with.

4) Use appropriate amount of enzyme.  Restriction enzymes stocks are described in units per volume. One unit of a restriction is usually defined as the amount of enzyme that can digest 1 µg of phage λ DNA in one hour at 37 degrees C. Since enzyme activity may be lower than expected, it is a good rule of thumb to 2-5X the amount of enzyme that is theoretically required.

5) Use appropriate volume for digestion  A good rule of thumb is that the concentration of DNA in the restriction digest should be no more than 100 ng/µl. Sometimes restriction digestions are inhibited at higher DNA concentrations.

6) Final glycerol concentration must not exceed 5%  Glycerol can affect the sequence specificity of a restriction enzyme at concentrations above 5%. Restriction enzymes are stored in 50% glycerol. Therefore the volume of enzyme added must be no more than 10% of the final volume of the digest.

BSA option:  When proteins are diluted they can denature. To keep restriction enzymes more stable, nuclease-free BSA (100 µg/ml final concentration) is often included in restriction digests. BSA (bovine serum albumin) is an inert protein.

DTT option:  Restriction enzymes are sensitive to oxidation. To prevent oxidation, reducing agents such as dithiothreitol (DTT) are often included in restriction digests at final concentrations of 1mM to 5mM.
Appendix 2  pGEM vector
http://www.promega.com/vectors/pgem7zfp.txt

pGEM®-7Zf(+) Vector sequence reference points:

<table>
<thead>
<tr>
<th>Reference Point</th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 RNA polymerase transcription initiation site</td>
<td>1</td>
</tr>
<tr>
<td>SP6 RNA polymerase transcription initiation site</td>
<td>123</td>
</tr>
<tr>
<td>T7 RNA polymerase promoter (-17 to +3)</td>
<td>2981-3</td>
</tr>
<tr>
<td>SP6 RNA polymerase promoter (-17 to +3)</td>
<td>121-140</td>
</tr>
<tr>
<td>Multiple cloning region</td>
<td>10-110</td>
</tr>
<tr>
<td>Phage f1 region</td>
<td>2362-2817</td>
</tr>
<tr>
<td>LacZ start codon</td>
<td>162</td>
</tr>
<tr>
<td>LacZ operon sequences</td>
<td>2818-2978; 148-377</td>
</tr>
<tr>
<td>Lac operator</td>
<td>182-198</td>
</tr>
<tr>
<td>Beta-lactamase coding region</td>
<td>1319-2179</td>
</tr>
<tr>
<td>Binding site of pUC/M13 forward sequencing primer</td>
<td>2938-2954</td>
</tr>
<tr>
<td>Binding site of pUC/M13 reverse sequencing primer</td>
<td>158-174</td>
</tr>
</tbody>
</table>
Appendix 3: Map of pCR 2.1 TOPO vector
pCR 2.1 TOPO vector: sequence and restriction site information:
http://www.invitrogen.com/content/vectors/pcr2_1topo_rest.pdf

Comments for pCR®2.1-TOPO® 3931 nucleotides
LacZa fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809
Appendix 4
Microbiology Issues: ISOLATION OF PURE CULTURES

A pure culture theoretically contains a single species of microorganism. A pure culture may be isolated by the use of enrichment or selective media containing specific chemical and/or physical agents that will allow the growth of a particular organism from among a mixed population of organisms. More simplified methods for obtaining a pure culture include: (i) spread plating on solid agar medium with a glass spreader and (ii) streak plating with a loop. The purpose of spread plating and streak plating is to isolate individual bacterial cells on a nutrient medium so that during subsequent incubation, a cell grows and divides sufficiently to form a single colony. This assumes that each colony arises from a single bacterium.

Both procedures (spread plating and streak plating) require understanding of the aseptic technique. Asepsis can be defined as the absence of infectious microorganisms. However, the term is usually applied to any technique designed to keep unwanted microorganisms from contaminating sterile materials.

The streak plating technique isolates individual bacterial cells on the surface of an agar using a wire loop. The streaking pattern shown in the figure below results in continuous dilution of the inoculum to give well-separated surface colonies. If you have not “streaked for single colonies” in a previous course, be sure to get a lesson from one of your instructors.
Appendix 5

Clones: genetically identical organisms, cells or molecules, all derived from a single ancestor

Recombinant DNA: new combinations of genetic information

If you have a gene or a segment of DNA that you want to study, you must first generate a purified preparation of the gene or segment. That is, you must separate it from all of the other sequences in the genome or the pool of nucleic acids that you are working with. Two processes are available by which relatively large quantities (µg amounts) of a particular DNA sequence or a particular gene can be obtained in a pure form: the polymerase chain reaction (PCR) and molecular cloning using recombinant DNA technology.

Molecular cloning of a segment of DNA requires a cloning vector and an appropriate host cell in which the vector carrying the DNA segment is propagated. In this context, a vector is the molecular vehicle that carries the cloned DNA segment. The choice of vector depends on the size of the DNA segment that you want to clone and the specific experiments that you want to do with the clone. The DNA segment(s) to be cloned can be (i) pieces of genomic DNA, (ii) cDNA generated by reverse transcription of mRNA, (iii) DNA generated by PCR and (iv) fragments of previously cloned DNA. The first two sources of DNA result in heterogeneous mixture of clones (genomic and cDNA libraries, respectively). Your cloning exercise in lab will use fragments of previously cloned DNA.

Types of cloning vectors: plasmids, modified bacteriophage genomes (such as λ), cosmids, YAC's (yeast artificial chromosomes-- for propagation of cloned DNA in yeast), modified animal virus genomes such as retrovirus or adenovirus genomes (for propagation of cloned DNA in mammalian cells)

Features of vectors:
1. Origin of replication recognized by the host cell and required for the propagation of the vector and the foreign DNA inserted into it. Typically a plasmid vector is maintained in the host at a very high copy number (500-700 copies per cell for the pGEM vector that you will use for this experiment). Natural plasmids, such as the F factor, are typically present in one or two copies per cell.
2. “Selectable” genetic marker (such as antibiotic resistance) so that host cells carrying the vector can be easily separated from host cells not carrying the vector by growth on a specific media
3. "Multiple-cloning site" or "polylinker" consisting of an array of restriction enzyme sites. The foreign DNA fragment to be cloned can be inserted into one of these restriction sites.
4. Vectors are often are engineered so that the foreign DNA can be transcribed and translated (expression vectors)
A. Biologically active tetrameric β-galactosidase; non covalent forces (......) maintain quaternary structure.

B. Inactive dimer produced in the deletion, lacZΔM15, which removes amino acids 11-41 (\).

C. Dimer-dimer interaction restored by α-fragment (~15% of amino-terminus of β-galactosidase) supplied by plasmid or M13 vectors.

Figure 6-1. β-galactosidase α-complementation (see Weinstock et al., 1983).