

HCS604.03  
Exercise 1  
Dr. Jones  
Spring 2005

**Recombinant DNA (Molecular Cloning) exercise:**

The purpose of this exercise is to learn techniques used to create recombinant DNA or clone genes. You will clone a cDNA into the plasmid vector pGem7zf+ (Promega), transform the construct into *E.coli* (JM109; Promega), and then confirm the construct using restriction digests and agarose gel electrophoresis. The clone will be further confirmed by sequencing. You will create a restriction map for your construct. Using the sequence provided to you, you will determine the putative identity of the cDNA clone using the BLAST search at GenBank (<http://www.ncbi.nih.gov/Genbank/>). You will then confirm the restriction enzyme recognition sites within the sequence.

**Schedule:**

**March 31, 2005 (Day 1)**

Ligate cDNA clone into pGem7zf+ plasmid

**April 5, 2005 (Day 2)**

Transform *E. coli* with DNA constructs

**April 6, 2005**

Start 3 ml O/N liquid cultures

**April 7, 2005 (Day 3)**

Plasmid DNA miniprep

**April 12, 2005 (Day 4)**

Restriction digest analysis  
Prepare samples for sequencing at MCIC

**Notebooks are due- April 14, 2005**

## **Introduction to the exercise:**

*Molecular cloning* includes techniques for manipulating DNA for a wide variety of purposes. Molecular cloning has 5 requirements.

### **1. Host organism:**

The DNA of interest can be amplified

- Usually *E.coli*
- *S. cerevisiae* (Yeast- eukaryotic host).

### **2. A vector:**

Contains the DNA of interest and facilitates its replication within the host cell.

- *Plasmids*

### **3. A technique for getting the DNA of interest into the vector:**

- *Restriction endonucleases*
- *Ligation*

### **4. A method for getting the recombinant DNA construct into the host cells:**

- *Transformation-*
- *Transfection-* involves *in vitro* packaging of DNA into phage particles
- *Electroporation*

### **5. A method of selection and/ or screening that allows for the identification of host cells that contain the desired DNA construct.**

- *Selection-* usually antibiotic resistance
- *Screening-* Means of differentiating cells that contain the construct of interest, example: blue-white selection ( $\beta$ -galactosidase activity)

### ***Plasmid vectors:***

The plasmids used as cloning vectors are usually small, only several thousand base pairs. They are circular, extrachromosomal DNA. They contain an origin of replication, for replication of the plasmid in bacteria. They also contain a polylinker or a multiple cloning site that has several different restriction enzyme recognition sites that are unique to that site (only found once in the plasmid). They also contain a gene for antibiotic resistance that allows for selection of cells that contain the plasmid. They may also contain a marker used to screen for plasmids that contain an insert.

**In our experiments we will use the plasmid pGEM<sup>®</sup>-7Zf(+)** (Promega): See website below for a map of the pGEM<sup>®</sup>-7Zf(+) plasmid. Sequence information is also available on GenBank (Accession #X65310).

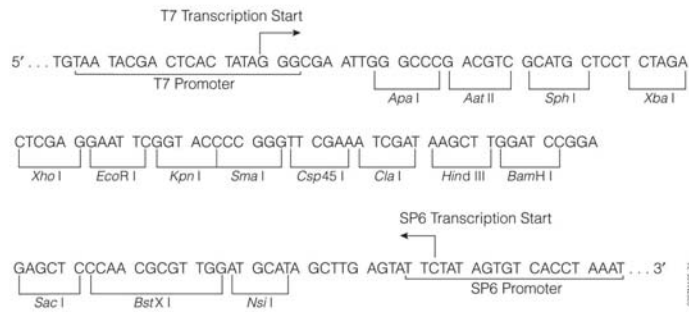
<http://www.promega.com/tbs/tb048/tb048.pdf>

The pGEM<sup>®</sup>-7Zf(+) Vector is a derivative of the pGEM<sup>®</sup>-3Zf(+) Vector and contains the origin of replication of the filamentous phage f1. The plasmid serves as a standard cloning vector, as a template for in vitro transcription, and can be used for the production of circular ssDNA. The plasmid contains SP6 and T7 RNA polymerase promoters flanking the multiple cloning sites. The multiple cloning site (or polylinker) includes unique restriction sites for *Apa* I, *Aat* II, *Sph* I, *Xba* I, *Xho* I, *Eco*R I, *Kpn* I, *Sma* I, *Csp*45 I, *Cla* I, *Hind* III, *Bam*H I, *Sac* I, *Bst*X I and *Nsi* I. This arrangement is designed specifically for generation of unidirectional deletions with Promega's Erase-a-Base<sup>®</sup> System. The polylinker contains restriction enzyme sites that produce 5' overhangs or blunt ends (sensitive to Exonuclease III) flanked on both sides by blocks of restriction sites that generate 3' overhangs (resistant to Exonuclease III).

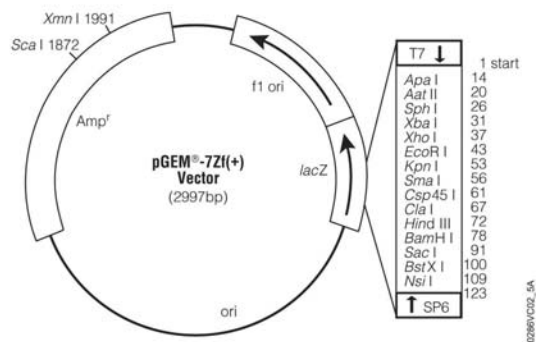
**Selection:** pGEM<sup>®</sup>-7Zf(+) confers ampicillin resistance to host cells. It contains the gene that encodes  $\beta$ -lactamase, a protein that can destroy ampicillin.

**Blue-white colony screening:** is a technique used to identify positive recombinant clones. Plasmid cloning vectors (like pGem7) that contain the *lacZ* fragment of the  $\beta$ -galactosidase coding sequence will produce  $\beta$ -galactosidase by  $\alpha$ -complementation when in the proper host, including *E. coli* strain JM109. JM109 is deficient in  $\beta$ -galactosidase activity due to deletions in both genomic and episomal copies of the *lacZ* gene. The deletion in the episomal (F factor) copy of the *lacZ* gene (*lacZ* $\Delta$ M15) is located in the  $\alpha$ -peptide region, and as such,  $\beta$ -galactosidase activity can be complemented by addition of a functional  $\alpha$ -peptide. This is referred to as  $\alpha$ -complementation. When plated on indicator media containing X-Gal (substrate) and IPTG, this host and plasmid combination will result in bacterial colonies that are blue. The multiple cloning site in the plasmid is located within the  $\alpha$ -peptide coding region of  $\beta$ -galactosidase. When this is disrupted by insertion of foreign DNA, complementation does not occur and  $\beta$ -galactosidase activity is produced. Bacterial colonies harboring these recombinant plasmids remain white.

### III. pGEM<sup>®</sup>-7Zf(+) Vector Multiple Cloning Region and Circle Map



**Figure 1. pGEM<sup>®</sup>-7Zf(+) Vector promoter and multiple cloning site sequence.** The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is complementary to the ssDNA strand produced by this vector.



**Figure 2. pGEM<sup>®</sup>-7Zf(+) Vector circle map and sequence reference points.**

### ***Transformation of E. coli cells:***

In our experiment we will be using Promega's *E. coli* Competent Cells, prepared according to a modified procedure of Hanahan, 1985 (DNA Cloning, vol 1, Glover D ed., IRL Press, Ltd., 109-135). The competent cells can be used for many standard molecular biology applications, and are available for convenient transformation in two efficiencies, High Efficiency at greater than  $10^8$  cfu/ $\mu$ g and Subcloning Efficiency at greater than  $10^7$  cfu/ $\mu$ g. We will be using subcloning efficiency JM109 competent cells. JM109 cells are an ideal host for many molecular biology applications. They are capable of alpha-complementation and can therefore be used for blue/white screening as noted above. *E. coli* cells are competent if they can take up DNA and are made competent by treating with  $\text{CaCl}_2$ . See the class website for a protocol for making competent cells. *E. coli* cells will take up DNA following a heat shock or an electric shock (electroporation).

A laboratory exercise describing *E. coli* transformation using electroporation can be found at the following website:

<http://userpages.umbc.edu/~jwolf/m7.htm>

### ***Ligation:***

The technique of molecular cloning requires the recombining of two DNA fragments usually from different sources. This joining of two linear fragments of DNA is called ligation. The enzyme *T4 DNA Ligase*, which originates from the T4 bacteriophage, catalyzes the joining of two strands of DNA between the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides in either a compatible cohesive-ended ("sticky" or blunt-ended configuration. The enzyme has also been shown to catalyze the joining of RNA to either a DNA or RNA strand in a duplex molecule, but will not join single-stranded nucleic acids. The optimum incubation temperature for T4 DNA ligase is 16° C and when very high efficiency is desired, like in the construction of libraries, this temperature is recommended. For routine subcloning, ligation reactions are often performed at 4° C overnight or at room temperature for a couple of hours. If a single restriction site is used to digest a plasmid for DNA cloning, the plasmid should be treated with alkaline phosphatase (SAP, Shrimp alkaline phosphatase; or CIAP, calf intestinal alkaline phosphatase) prior to ligating to the insert DNA. Alkaline Phosphatase catalyzes the removal or dephosphorylation of 5' phosphates from both termini and prevents recircularization and religation of linearized cloning vectors. SAP is active on 5' overhangs, 5' recessed and blunt ends.

### ***Restriction enzymes or endonucleases:***

Restriction endonucleases cleave the sugar-phosphate backbone of DNA. Several thousand restriction endonucleases have been isolated and they are named after their hosts of origin (mostly bacteria). A nice overview of the discovery of Restriction enzymes can be found at:

<http://www.bioteach.ubc.ca/MolecularBiology/RestrictionEndonucleases/index.htm>

The substrates for restriction endonucleases are specific sequences of double stranded DNA called recognition sequences or restriction sites. Catalogs from companies that sell restriction enzymes are a good source of information about the recognition sequences for specific enzymes. The length of the recognition sequence is usually 6 bp but can be 8 or 4 bp. The length of the restriction site determines the frequency that an enzyme will cut a random sequence of genomic DNA. Different restriction enzymes may have the same recognition site. These enzymes are called isoschizomers. Most recognition sequences are palindromes, which means they read the same forward and backward. Restriction enzymes hydrolyze DNA between the deoxyribose and phosphate groups. This leaves a DNA fragment with a 5' phosphate and a 3' hydroxyl on both strands. Cuts from restriction enzymes can leave 3 types of ends:

1. Blunt ends (ex. Sma I)- no overhang
2. Sticky end (or cohesive end) – 5' overhang (ex. BamHI)
3. Sticky end (or cohesive end) – 3' overhang (ex. KpnI)

For further information of restriction enzymes and using restriction enzymes to map DNA see the website below or other references given on the course website.

<http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/index.html>