Lab 7: pAMP & pKAN ligation

Objective of this lab is to create a ***recombinant plasmid*** constructed from pieces of two different plasmids joined together.

Define: recombinant / cloning / vector / insert

This will be done by:

- Digesting both plasmids with the same restriction enzymes to create compatible ***sticky ends***
- Completeness of enzymatic digestion will be evaluated by running a small aliquot on agarose

  This is crucial because uncut plasmids transform at a MUCH HIGHER efficiency than recombinant ones.

  If uncut plasmid is included in the ligation & transformation, the majority of transformants will contain the *original, uncut plasmids* NOT the recombinant.

  Double transformants (which contain both original, uncut plasmids) will be phenotypically identical to desired clone
- Restriction enzymes will be ***heat inactivated*** to prevent competition with the ligation
- Cut plasmids will be mixed and sticky ends allowed to ***anneal***
- ***DNA ligase*** will be used to covalently link the annealed fragments together

  DNA ligase uses the energy of an ATP molecule (and Mg^{++} cofactor) to restore the phosphodiester bond between the 5' phosphate and 3' OH on the DNA fragments (in other words, ***it seals nicks***).

  Sticky end ligations are performed at <u>room temperature</u>, a compromise between enzymatic efficiency (best at 37C) and annealing of sticky ends (best in the cold)

- Because we are not specifically isolating the two fragments we wish to ligate together, a variety of products of ligation are possible. The desired product (called pAK) must have the following:
  1. An **origin of replication (*ori*)**
  2. The ***amp*** gene for ampicillin resistance
  3. The ***kan*** gene for kanamycin resistance
- Transformants containing this recombinant plasmid will be dual resistant (able to grow on agar containing both ampicillin and kanamycin)

Ampicillin inhibits cell wall synthesis, ***bacteriostatic***
Kanamycin, an aminoglycoside antibiotic, inhibits protein expression (30S subunit of the 70S ribosome), ***bactericidal***

- As a consequence, the *recovery step* following heat shock during transformation is <u>crucial</u>

Blunt end ligations are also possible (though we are not doing any here).

- DNA is cut by restriction enzymes that do *not* leave a single strand overhang (sticky end)
- Or, sticky ends are digested away by ***single-strand specific nuclease*** such as mung bean nuclease, leaving blunt ends
- Blunt end ligations are much less efficient than sticky ones:
  - contact between DNA fragments is transient; no hydrogen bonds hold them together.
  - Nonspecific joining of any fragment to any other fragment

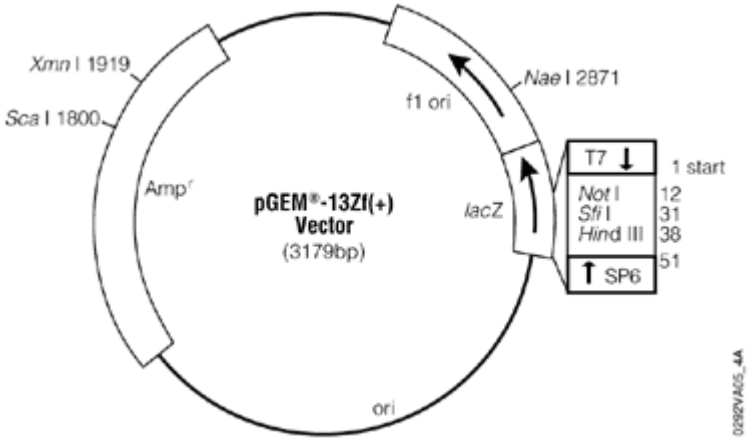
Cloning strategies & issues

Example #1.

You want to clone the human insulin gene into a plasmid vector so you can sequence it.

Human chromosome 11 ····||····[Insulin gene]····||···· Let's say this HindIII fragment is 5000 nt

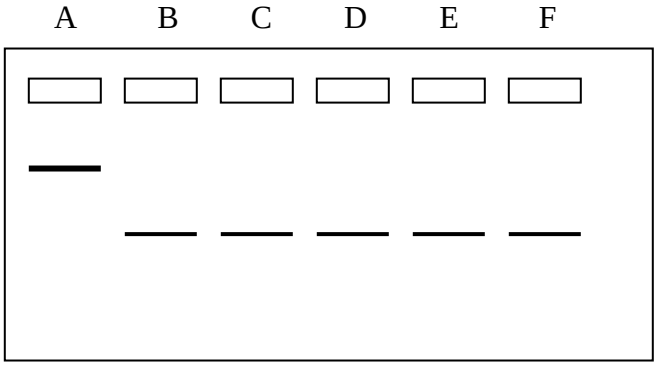
HindIII HindIII



T7 and SP6 are strong viral **promoters** (for initiation of transcription by RNA polymerase)

Cut the insulin gene out of chromosome 11 using HindIII. Purify this fragment.
Open the plasmid pGEM at its multicloning site using HindIII.
Mix and ligate; transform bacteria; select for bacteria that can grow on ampicillin.

You get TONS of colonies/transformants. You miniprep 4 of them, and cut the plasmid DNA with HindIII. Run a gel.



A: Insulin HindIII fragment
B: pGEM cut with HindIII
C: Miniprep #1
D: Miniprep #2
E: Miniprep #3
F: Miniprep #4

What happened? What would a digest of the desired plasmid look like?

Phosphatase enzymes remove exposed phosphate groups from the ends of DNA.

Remember: DNA ligase requires that the DNA pieces it is joining together have a 5' phosphate to form the new phosphodiester bond.

Solution: As before, cut the pGEM vector with HindIII. Next, treat it with a phosphatase (CIP, or calf intestinal phosphatase, is commonly used). This will remove the 5' phosphates and make it impossible for the vector to re-close on itself during the ligation.

Example #2.

Same as above, except insulin gene is now flanked by EcoRI sites, not HindIII.

How can you clone an EcoRI fragment into a HindIII site?

Solution: By **blunt end ligation**.

EcoRI: XXG AATTCXX → XXG CXX
XXCTTAA GXX → XXC GXX

HindIII: XXA AGCTTXX → XXA TXX
XXTTCGA AXX → XXT AXX

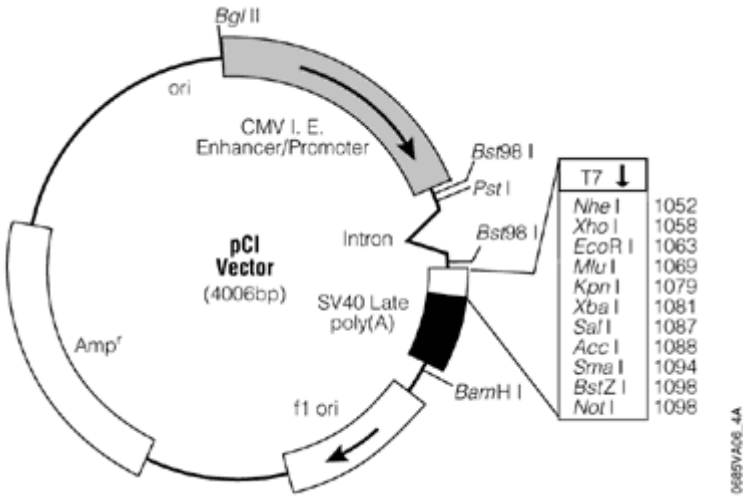
To change a sticky end to a blunt end, you must remove the single strand overhang. This can be done by a **single strand specific nuclease** (a popular choice is mung bean nuclease). It chews up the single stranded part of the DNA end, and stops when it hits the double stranded part. **IMPORTANT:** Creating a blunt end this way **destroys** the enzyme recognition site. After cloning, you cannot pop your insert out again with EcoRI *or* HindIII.

Blunt end ligation is possible. DNA ligase can link blunt ends together just as it can link sticky ends; it is less efficient because the blunt ends are not held together by any hydrogen bonds.

To compensate for this inefficiency, **blunt end ligations** will include a **higher insert:vector ratio** in the ligation mix. By having, say, 10 molecules of your insert DNA for every one molecule of vector, you greatly increase the likelihood that any plasmid being transformed from this ligation will actually include an insert.

Example #3. Directional cloning

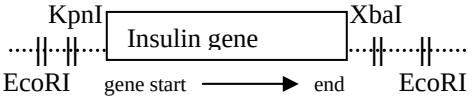
You want to *express* the human insulin gene inside a eukaryotic cell.



This is an example of a plasmid vector that will direct expression of a cloned gene in eukaryotic cells.
KEY POINTS:

- ***Promoter*** drives expression of genes downstream in a specific direction (see arrow)
- ***Orientation*** of inserted gene in multicloning site is crucial (gene is "sense" in only one direction)

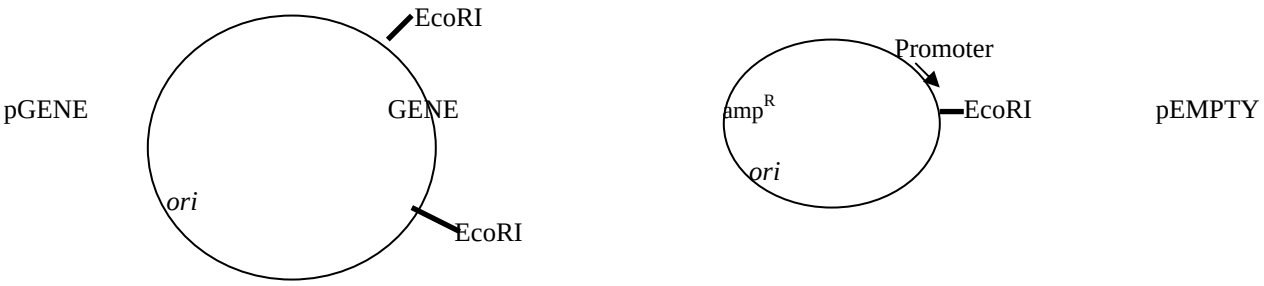
Human chromosome 11



How can you make this clone?

Cloning techniques & strategies

Why use two different restriction enzymes?
Using 2 enzymes to generate fragments with incompatible sticky/cohesive ends greatly improves your chances of obtaining the desired product of ligation (compared to using a single restriction enzyme). Consider the drawing below:



You want to move a cloned gene of interest from pGENE into pEMPTY. You need GENE inserted into the EcoRI site of pEMPTY to place it under the control of a promoter upstream from that site. You cut pGENE and pEMPTY with EcoRI, mix, and ligate.

What products of ligation are generated? Which is the only one you want? How might you enrich for it? Can you think of any way to solve the problem of orientation (i.e., is the gene inserted in the correct direction for expression by the promoter)?

Phosphatase

The main problem with the single enzyme approach is religation of the plasmid backbones. Consider all these different DNA fragments free in solution, with sticky ends searching for each other. At any time, the sticky ends on opposite ends of the same fragment are always close together, and have a high probability of hydrogen bonding back together. {If two different restriction enzymes were used, those tethered sticky ends are incompatible, so this isn't a problem.} As a result, from such a ligation reaction, 99 of 100 plasmids cloned will be empty vectors (with no inserted GENE).

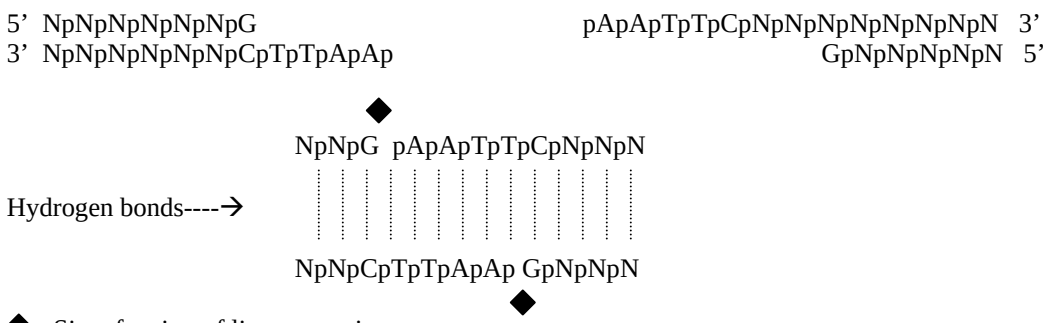
Because it is not always possible or convenient to use 2 different restriction enzymes, a solution has been devised for this problem. One can digest pEMPTY with EcoRI and then treat with a phosphatase. These enzymes remove the 5' terminal phosphate from the exposed sticky ends. This phosphate is required for ligation. Without it, the plasmid cannot reclose on itself. But because your GENE insert still carries 5' phosphates of its own, ligation can occur between GENE and pEMPTY.

Why heat inactivate restriction enzymes before ligation?

Restriction enzymes cleave DNA; ligase links it back together. If both enzymatic activities are active in the same reaction, they compete / have opposite effects. The endonuclease activity is more efficient, so the ligation would fail.

Temperature for ligation reaction

Like most enzymes, DNA ligases are generally most active at 37C. However, a successful plasmid ligation depends on more than enzymatic activity. The ligase must have substrate upon which to work. That substrate is the hydrogen-bonded "sticky ends" of the DNA fragments in your reaction. It is the compatible single-stranded sticky ends generated by restriction enzyme cleavage that brings 2 free floating pieces of DNA together, and hold them in place long enough for DNA ligase to covalently bond them together. The hydrogen bonding of 2 sticky ends to each other is a temperature-dependent process: it occurs best at lower temperatures. At higher temperatures, kinetic energy/molecular motion breaks hydrogen bonds. What is a "low" or "high" temperature? It depends on the number of hydrogen bonds. For example, in an intact plasmid, several thousand base pairs are hydrogen bonded together, and the temperature must raised well above 37C to melt/denature the plasmid. In a ligation reaction, only a few base pairs are involved; in the case of EcoRI, for example, only 5 bases are in the sticky end. A compromise must be made, therefore, between the temperature of greatest enzymatic activity by ligase, and the temperature at which the most sticky ends stick together. In this experiment, room temperature for a long time (overnight) is reasonable.

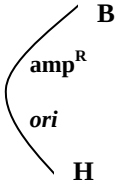


Bio 181: LAB 7 pKAN/pAMP digestion & ligation

Predicting Products of a Ligation

Shown below are the fragments generated by complete digestion of pAMP and pKAN with BamHI and HindIII. Draw the listed ligation products and identify which ones, when transformed into competent bacteria, will "grow" on amp+ plates, kan+ plates, and amp+/kan+ plates.

1

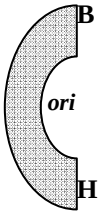


3755 bp
pAMP ori&ampR

2

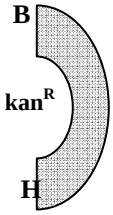
784 bp
pAMP

3



2332 bp
pKAN ori

4



1861 bp
pKAN kanR

Recognize that multimers of more than 2 ligated fragments are rare unless enzymatic digestion was incomplete. This is because it is a rare event for two DNA fragments, floating freely in solution, to find each other at all; it is even more rare for several to bind together before being ligated shut.

Possible products of ligation (indicate which is the desired product):

1+2 1+3 1+4 2+3
2+4 3+4

Growth?
Amp+

Kan+

Amp/Kan+

1+1 2+2 3+3 4+4
1+2+3+4

Growth?
Amp+

Kan+

Amp/Kan+