Some miscellaneous notes for labs 7-10

Understand and be able to fill out a table of possible ligation products like we did for lab 7. This includes predicted which products of ligation would be propagated (requires an origin of replication) and which would confer antibiotic resistance.

Cloning strategies handout ex. #3

Be able to determine appropriate restriction sites that give a desired orientation after ligation of the insert DNA into a vector. {In this case, EcoRI + XbaI, or KpnI + XbaI, but NOT EcoRI + KpnI} Consider additional factors:

1. Compatibility of chosen enzymes for simultaneous double digestion. While it is possible to do one digest first, purify the DNA, and then perform the second digestion, this is not desirable. It is best if the two enzymes can ***work in the same buffer*** and ***at the same temperature.*** If one enzyme is slightly less efficient, this can be compensated for by ***increasing the # of units of enzyme used, or increasing the time of digestion.***
2. Proximity of the two cut sites in the multicloning site: If they are very close, digestion with one enzyme may leave the second enzyme's recognition site at the end of the DNA molecule. Some restriction enzymes will not bind and cut near the ends of DNA; some will. You would need to check this out.
3. Always minimize the amount of "junk" DNA in your construct. Choose restriction enzymes that cut as closely as possible to the exact region you wish to clone. This is particularly important if you want to express a gene, when extra DNA can cause problems with transcription or translation.

Transformation results: 9 plates

	A	K	L	
Expected growth patterns:	+	-	+	amp plates
	-	+	+	kan plates
	-	-	+	amp/kan

- Satellite colonies on amp not kan plates: ampicillin resistance is mediated by an enzyme (β lactamase) that degrades the antibiotic. This creates a locally reduced concentration of antibiotic around each resistant colony. Kanamycin resistance is NOT mediated by the destruction of the antibiotic, so no zone of lower concentration exists, even around resistant colonies. Furthermore, kanamycin is bactericidal, not bacteriostatic.
- For transformations of the ligation: Most colonies on amp, fewer on kan, fewest on amp+kan plates. Why? 1) All products of the ligation that we discussed in lab 7 are equally likely to form in the ligation reaction. However, once transformed into bacteria, more of these products can confer ampicillin resistance than kanamycin resistance; and only one can confer resistance to both antibiotics. Also, with kanamycin, a long recovery period is crucial for survival of transformed cells (due to immediate lethal effect). If the recovery period is somewhat abbreviated, one expects to see more colonies on the more forgiving ampicillin.

Calculate transformation efficiency for pAMP & pKAN only

Lab 9: Replica plating (sample data below):

From: Ligation, plated on amp (#1-10)
Ligation, plated on kan (#11-20) Satellite colonies from Amp plates shouldn't grow anywhere

AMPICILLIN

*	*	*	*	*
*	*	*	*	*
0	0	0	*	0
*	0	0	0	0

Kanamycin

0	0	0	0	*
*	0	0	*	0
*	*	*	*	*
*	*	*	*	*

* = growth
0 = no growth

Lab 10: Interpretation of gels (the products of pAMP/pKAN ligation)

- First, determine which of the four fragments are present (there must be at least 2)
- Second, look at bands of UNCUT miniprep DNA and compare sizes with UNCUT pAMP or pKAN. This will help you determine whether the plasmid has 2 or 4 fragments.
- Use these data to determine whether 1 or 2 plasmid types are present; and which DNA fragments they are made of. **{Importance of double transformants:** bacteria can be transformed by more than one plasmid**}**

Cloning in general

Understand and be able to describe the steps in cloning a gene/piece of DNA into a plasmid vector. We did all of these in labs 7-10.

1. **Plan** a cloning strategy. {This protocol will assume two different sticky ends are used.}
2. **Digest** both *vector* and the source of the *insert DNA* with the same two restriction endonucleases. If possible, do these as double digests (both enzymes at the same time).
3. **Confirm** that *complete digestion* has occurred. Run an aliquot of each sample on a gel to look for evidence of partial digestion. *If partial digestion is found*, add more enzyme and/or continue digesting for more time. {This is to prevent having intact plasmid in the transformations as intact plasmid transforms at a much higher efficiency than ligated plasmid, confounding your ability to get the recombinant plasmid you want.}
4. **Heat inactivate** the restriction enzymes so they do not compete with DNA ligase.
5. **Ligate.** Mix your cut vector and cut insert DNA together. Add DNA ligase and buffer containing ATP & Mg++. Incubate at room temperature to encourage annealing of sticky ends, which is diminished at higher temperatures.
6. **Transform.** Add your ligated DNA to competent cells prepared from mid-log phase broth cultures. Depending on the antibiotic(s) to be used for selection of transformed cells, allow an adequate recovery period for expression of the antibiotic resistance gene(s) on the plasmid vector.
7. **Screen.** Observe and interpret the growth patterns on your plates. Select colonies from your desired selection plate (in our experiment, Ligated DNA onto Amp+Kan). Grow some of them in broth cultures. Isolate plasmid DNA by miniprep method. Perform appropriate restriction digests on miniprepped plasmids to characterize which clone (colony) carries the recombinant plasmid you want.