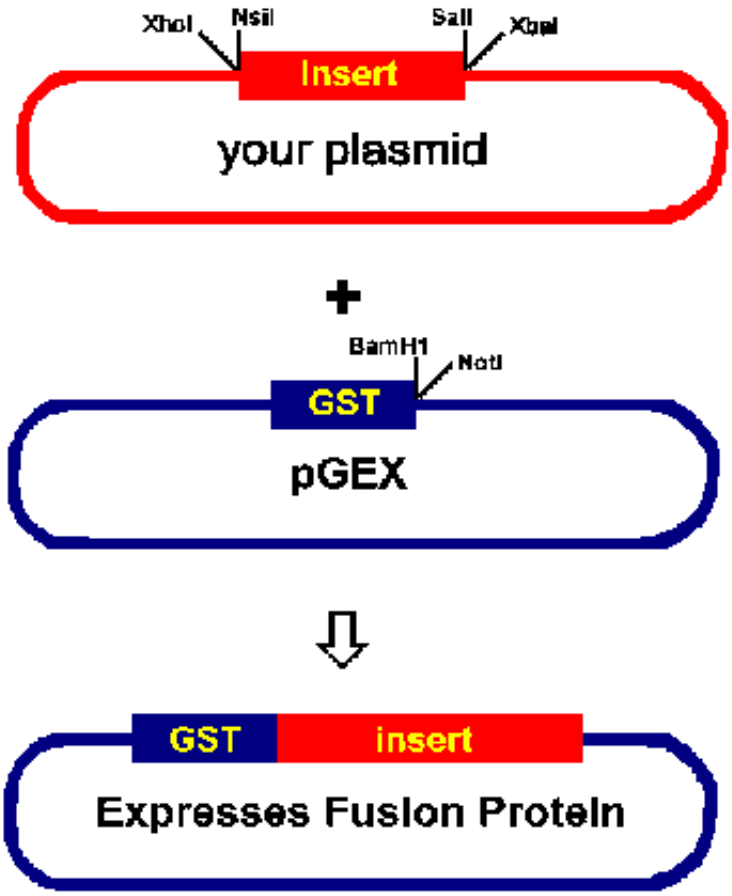
A Solution to Homework #10

The goal is to identify restriction enzymes that will allow you to subclone (i.e., move) the 'insert' fragment from its current vector (pBluescript) in to the expression vector pGEX so that the insert is in the correct orientation and in the correct reading frame. This is illustrated schematically in the following figure:

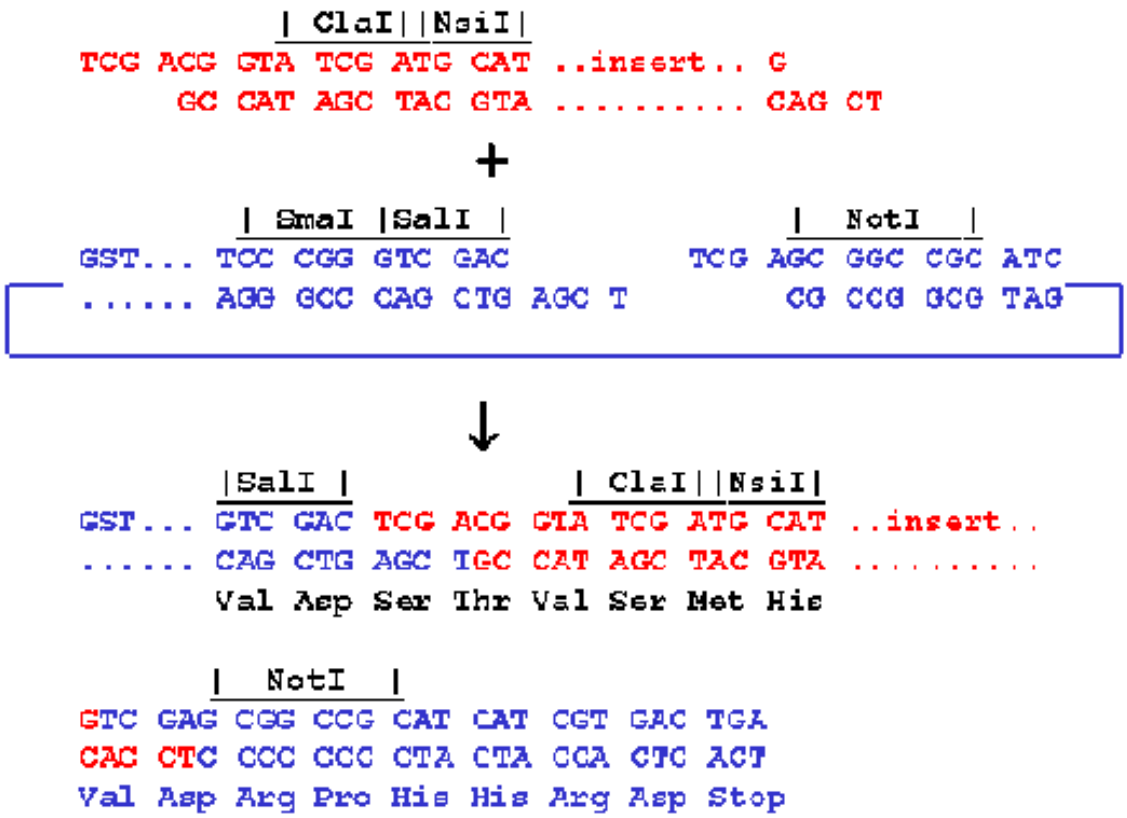


The first step in performing this subcloning is to identify restriction sites on both sides of the insert fragment which are compatible with the restriction sites on the C-terminal side of GST. (By convention, the N-terminus is depicted on the left.) A quick inspection reveals that almost all of the sites that are in common between pBluescript and pGEX would result in the insert being in the wrong orientation (i.e., backwards).

The only exception to this is the *Sal*I site since there are two *Sal*I sites that flank the insert. Using the *Sal*I site does present a minor problem in that both orientations are possible. However, the orientation can be easily determined after the subcloning (see below). Therefore, the plasmid should be digested with *Sal*I and the fragment corresponding to the insert isolated. (Approaches based on adaptors or PCR strategies could also be utilized.)

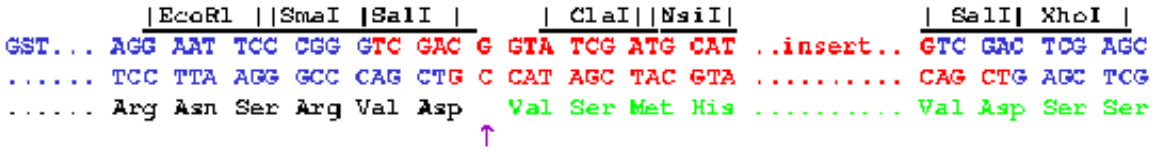
The vector could also be digested with *Sal*I. However, this results in the insert being out of frame (see example) which would need to be corrected (see appendix in handout on recombinant protein expression). A simpler solution is to digest pGEX with *Xho*I which is compatible with *Sal*I. In other words they have the same overhangs. Following digestion with *Xho*I, the vector should also be treated with phosphatase to minimize self-ligation of the vector.

The next step is to mix the *Sal*I fragment (in red) with the *Xho*I-treated vector (in blue) in the presence of ligase. Approximately half of the *Sal*I fragments will anneal with the vector so that the insert is in the correct orientation and reading frame as depicted in the following:



Following transformation, a few colonies (3-4) are selected, plasmids isolated and the orientation checked. The orientation is easily checked by digesting with restriction enzymes that should flank the insert in a particular orientation. For example, digestion with *Nsi*I and *Not*I should produce two fragments--the vector and the insert--if the insert is in the correct orientation. If the insert is in the opposite orientation, a single fragment corresponding to the combined sizes of vector and insert will be observed. Orientation can also be check by testing clones for the expression of the fusion protein of interest. PCR can also be used by designing primers that span the splice sites or using one primer from the vector and one primer from the insert.

Result of ligation into the *Sal*I site of pGEX:



After confirming the proper orientation (eg., *Eco*RI + *Xho*I digest producing insert fragment), the -1 frameshift could be carried out by digesting with *Eco*RI, filling in with Klenow, and religating. Alternatively, the frame shift could have been introduced before excising the *Sal*I fragment by digesting with *Cla*I and filling in.

Another possible solution to the problem would be to convert a restriction site on either plasmid to a more appropriate restriction site. This is accomplished by cutting with a restriction enzyme and annealing with a synthetic oligonucleotide containing the new restriction site. For example, converting the *Bam*HI site of the pGEX vector to a *Cla*I site would allow the use of *Cla*I and *Eco*RI. However, this approach does involve more work.

Similarly, PCR primers could be designed so the appropriate restriction sites are engineered into the insert fragment.