Cloning of genes from genomic DNA:  
Part 3-Restriction Enzyme Digestion and Agarose Gel Electrophoresis

Continuing from our isolation of genomic DNA and PCR amplification of either the even-skipped gene or the twist gene, we will now move on to the third step in the cloning procedure. We will use restriction enzymes to cleave off the ends of the PCR products. The oligonucleotide primers used in the PCR reaction were designed to include either an XbaI or a HindIII restriction site in their sequence. In addition, we will cut our genomic DNA with the enzymes and run a little bit of each (PCR product and genomic) on an agarose gel to see what we have.

Before we can begin the restriction enzyme digests, we need to “clean up” our PCR products. As they are now, the PCR reaction contains the PCR buffer (which is not correct for the enzymes) and lots of unused oligonucleotide primer (which may interfere with our reaction). We will use a DNA purification column that is similar to the one you used for your genomic DNA preps. Once we have “cleaned” PCR product, we can digest the sample with XbaI and HindIII. However, in order to use the digested PCR product in the next step of the cloning procedure (ligation of the PCR product into the pBluescript vector) we have to once again purify our digested DNA sample.

**Why were restriction enzyme sites included in the oligonucleotides for PCR?** Once we have amplified our gene, we wish to ligate it into our plasmid, pBluescript. If the DNA synthesized by PCR were simply a blunt-ended fragment, we could just cut the plasmid with a restriction enzyme that leaves blunt ends as well and ligate the two together (blunt-ended cloning works, but is less efficient than cloning sticky ends). When PCR was first used to clone fragments, many people tried this and had very little success. It was discovered that the PCR enzyme, Taq polymerase, would add a single extra “A” residue onto the 3’ end of each strand it synthesized so that the fragment was no longer blunt-ended (and could not be ligated to a blunt-ended vector). Because we have added restriction enzyme sites into the oligonucleotide primers, we can cut off the ends of the PCR products and create sticky ends (which are easier to clone). We chose enzymes that will allow us to create similar sticky ends in the plasmid as well. In fact, one of the primers has an XbaI site, while the second primer has a HindIII site. By using this technique, both the digested PCR product and digested plasmid will have similar compatible ends and can be ligated together efficiently in the next step.

**Why does each oligonucleotide primer (each end of the PCR product) have a different restriction enzyme site?** To keep it simple, we could have just put an XbaI site on each primer. Then, we would cut both the PCR product and the plasmid with only XbaI. However, by this method, the PCR product can be inserted into the plasmid in either direction. This is known as non-directional cloning. We would not know which way the PCR product went into the plasmid without checking in some way. By using different restriction enzymes, we constrain our PCR product to a particular orientation in the plasmid. This process is known as directional cloning.
**The pBluescript vector.** Below is a map of the plasmid we will use. The expanded area on the right side is a cluster of densely packed restriction enzyme sites, each of which cuts the plasmid only once. This part of the plasmid is called the “multiple cloning site” (MCS, or sometimes called the polylinker) and was designed to give scientists a wide option of restriction enzyme sites to use for cloning any gene.

![Diagram of the pBluescript vector.](image)

Figure 1. Diagram of the pBluescript vector. The PCR products for either sim or sna will eventually be cloned into the pBluescript vector using the restriction enzymes XbaI and HindIII. The XbaI and HindIII sites cut the plasmid only once in the MCS region (blue) which is at the beginning of the LacZ gene.

**So in summary for today:** You will first save a small amount of your PCR reaction for an agarose gel. Then you will clean up the PCR product(s) resulting from the PCR amplification using a DNA purification column. Following this, you will digest the ends of the PCR product(s) with XbaI and HindIII. (Additionally, you will digest some of your genomic DNA with XbaI and HindIII as a control.) Once digested, your PCR product(s) will be cleaned up from the restriction enzyme digest using another column (notice a pattern here?). Samples of the genomic DNA (undigested and digested with XbaI/HindIII) and the PCR reactions (undigested and cleaned, digested with XbaI/HindIII) will then be electrophoresed through an agarose gel in order to determine the number of bands, their sizes and amounts within the samples. The remainder of the PCR reaction sample that has been digested and cleaned must be saved for a ligation reaction next week.
**Qia Quick PCR Clean Up Kit (Qiagen, cat # 28104):**

This kit will remove the buffer, salts, dNTP’s, and primers from the PCR reaction and increase the efficiency of future enzymatic reactions, like the restriction enzyme digestions following this step. It uses silica gel technology just like you used when purifying your genomic DNA samples last week.

1. *(Sample for gel later)* Place 3 µl of your PCR reaction into a separate 1.5 mL microfuge tube and add 7 µl of dH₂O to the sample. Label this tube with your initials sim PCR und or sna PCR und depending on what your group picked (und = undigested). Add 1 µl of the blue loading dye to your tube and save this sample for the gel later.

2. Add the remainder of your PCR reaction to 5 volumes of buffer PB. So, place 235 µl of buffer PB into a clean 1.5 mL microfuge tube and add the remaining 47 µl of your PCR reaction.

   *This buffer corrects the pH and salt conditions in your sample in order to allow the DNA, your PCR product(s) in this case, to bind to the silica gel in the column.*

3. Transfer your sample to the spin column and centrifuge at max speed for 1 minute to allow the DNA in your sample to bind to the column.

4. Discard the flow-through appropriately and place the column back in the same 2 ml collection tube.

5. Wash step: add 750 µl of buffer PE and centrifuge at max speed for 1 minute.

6. Discard the flow-through appropriately and place the column back in the same 2 ml collection tube. Centrifuge again at max speed for 1 minute; this removes any residual ethanol from the column. *This second centrifugation is critical!*

7. To elute the PCR product(s) from the column, place the column in a clean microfuge tube and add 36 µl of dH₂O. Let this sit for 1 minute and then centrifuge at max speed for 1 minute. Label this tube either sim dig or sna dig (dig = digest) accordingly. You will actually set up your digest on the PCR product(s) in this tube in the next step.

**Restriction Enzyme Digestion:**

You will digest both your PCR product and your genomic DNA with XbaI and HindIII. Thus each group will have two digestes, be sure these are labeled clearly to identify everyone’s samples. Remember, the goal of digesting your PCR sample is to prepare the ends of your PCR product(s) for ligation into the digested pBluescript vector. (To save time and resources pBluescript will be digested with XbaI and HindIII for you).

1. You will set up two digestions: A) a 40 µl digestion of your PCR product in your tube labeled sim dig or sna dig and B) a 20 µl genomic DNA digestion.

   40 µul PCR sample digestion:
   ~36 µl cleaned PCR product (all of the cleaned sample from step 7 above, use same tube)
   4 µl of 10x Buffer 2
Add 1 µl of XbaI and 1 µl of HindIII directly to your solution. Flick the tube to mix and then pulse-spin the sample to get any drops back down to the bottom of your tube.

*Save your undigested genomic DNA on ice again for gel electrophoresis later.*

*This is called a double digest. Both enzymes will cut the DNA well in the same conditions (buffer, temperature) and so they can be performed together at the same time.*

2. Incubate your samples at 37°C for 1 to 2 hours.

*If the time is too short you will get inefficient cutting and end up with something called a partial digest where not all of the DNA will get digested. If the time is too long you can get ‘star’ activity where the enzyme(s) begin to cut the DNA nonspecifically, at places other than their specific restriction enzyme site.*

34. When the digest is finished, save your Gen dig sample on ice to use for the gel. For the sim dig or sna dig sample, you will continue on to the next step and purify the digested PCR product(s) using a column purification kit again.

**Qia Quick PCR Clean Up Kit (Qiagen, cat # 28104):**

1. Add 5 volumes of buffer PB to your sim (or sna) dig sample. So, for a 40 µl double digest sample you will want to add 200 µl of buffer PB.

2. Transfer your sample to the spin column and centrifuge at max speed for 1 minute to allow the DNA in your sample to bind to the column.

3. Discard the flow-through appropriately and place the column back in the same 2 ml collection tube.

4. Wash step: add 750 µl of buffer PE and centrifuge at max speed for 1 minute.

5. Discard the flow-through appropriately and place the column back in the same 2 ml collection tube. Centrifuge again at max speed for 1 minute; this removes any residual ethanol from the column. *This second centrifugation is critical!*

6. To elute the DNA from the column, place the column in a clean microfuge tube and add 30 µl of dH2O. Let this sit for 1 minute and then centrifuge at max speed for 1 minute. Label this tube sim dig clean or sna dig clean. Store on ice until you gel loading sample has been prepared.
7. For the gel, 3 ul of this sample will be used (see agarose gel handout) while the remainder is saved at -20°C for the ligation into the pKS vector.