Cloning of genes from genomic DNA
Part 1 and 2: DNA Isolation and PCR

The objective of this lab is to demonstrate how the DNA of a particular gene in an organism’s genome can be copied, isolated and cloned into a plasmid vector. Once in this vector, the cloned gene can be used for many things and can also be sequenced and studied. This experiment will illustrate some of the basic techniques often found today in laboratories that use recombinant DNA technology. The basic outline of the experiment can be seen below for which half the lab will clone a portion of the *single-minded* gene (*sim*) and the other half a portion of the *snail* gene (*sna*) into the plasmid pBluescript (pKS) over the next 5 weeks of lab.

1. Isolation of genomic DNA from your organism (*w^{118} Drosophila*, of course).

2. Amplification of the gene of interest (*sim* or *sna*) by the polymerase chain reaction (PCR).

3. Restriction enzyme digests of both amplified DNA (*sim* or *sna* insert) and plasmid DNA (pBluescript, the cloning vector). We will check our products at this point by agarose gel electrophoresis.

4. Ligation of amplified DNA and plasmid DNA to create a recombinant DNA molecule (a cloning vector containing an insert).

5. Transformation of the ligation products to produce bacteria which will carry and copy our reproduced molecules for us.

6. Plasmid purification from bacteria and restriction enzyme digestion to check the insert cloned into the vector.

7. Agarose gel analysis of the final plasmid hopefully containing either *sim* or *sna* cloned into pBluescript.

From this cloning project, *sim* and *sna* antisense RNA (RNA that is the reverse complement to the mRNA sequences) will be made for you from the clones produced in the lab. This antisense RNA provides you with a way to examine RNA expression in *Drosophila* embryos, using a technique called In-situ Hybridization. This experiment will be performed in the 6th and 7th week of lab and is the culmination to the cloning project.

How does one isolate genomic DNA? There are several methods but almost all of them require separation of tissue into individual cells (or at least small clumps of cells) followed by lysis of the cells, often through addition of detergent and proteases. After lysis, protocols tend to vary but the idea of each one is to isolate the DNA from contaminating molecules including proteins. We will use commercially prepared kits that contain proprietary resins that can specifically bind DNA under certain conditions and will release the DNA under others. Many of these resins are based upon silica compounds (glass) which will bind to DNA under high salt conditions but release under low salt
conditions. From our genomic DNA isolation method, we expect fragments of ~50,000 basepairs (~ 50 kbp) in length to be produced.

**What is the polymerase chain reaction?** Our gene of interest is a minute part of the genome. If your gene is 2 kbp long, it would represent approximately 0.00001% of the human genome or 0.0002% of the fly genome. Even though your gene is present in isolated genomic DNA, it is difficult to study it or do anything with it while all of the other DNA is present. However, if we have some knowledge of the sequence around the gene, we can copy it again and again by the polymerase chain reaction (PCR). PCR involves copying your gene using DNA polymerase and two specific short stretches of DNA (oligonucleotide primers) that flank your gene. If we heat the genomic DNA, it will denature (a.k.a. “melt” - the base pairs will break leaving us two long single stranded pieces of DNA). If we lower the temperature, the oligonucleotide primers will base pair with the complementary flanking sequence on one of the strands. The binding of the primers is called “annealing”. If we raise the temperature to the optimal working temperature for Taq DNA polymerase (68-72° C), this enzyme will synthesize a complementary strand of DNA. This cycle of denaturation, annealing, synthesis is then repeated numerous times and the number of copies of our DNA increases exponentially. If you started with one copy of your gene, 30 cycles should produce $2^{30}$ copies, in theory. Below, I’ve given a number of links that discuss or illustrate different aspects of the technique. Feel free to visit them and browse (one of them contains a rather lame game).

http://www.dnalc.org/ddnalc/resources/pcr.html
http://sunsite.berkeley.edu/PCR/whatisPCR.html
http://nobelprize.org/chemistry/educational/pcr/

![Diagram illustrating the basic PCR technique.](image)
I. Isolation of Genomic DNA

1. Place the flies at -20°C for 30-60 minutes. (This will already be done for you prior to lab.)

2. Weigh out 0.03g of tissue (~28 flies) and place in a 1.5 ml microcentrifuge tube. Keep the flies as cold as possible at all times to avoid DNA degradation.

3. Using a pestle in a drill, grind the flies to a powder while holding the tube in liquid nitrogen. You are done when you do not see any fly “chunks”.

4. Quickly add 180 µl of ATL buffer and then 20 µl of Proteinase K. Resuspend the sample by carefully pipetting up and down a few times or until all of the powder is clearly in suspension. Remember to clearly label your samples. If your solution is frozen, you can warm it up in your hand – you no longer need to keep your sample cold.

5. Incubate your sample at 55°C for 1 hour.

6. Vortex your sample on the highest setting for 10-15 seconds (use the same conditions each time you vortex for this procedure).

7. Add 200 µl of AL buffer and immediately vortex.

8. Incubate at 70°C (use the heat block) for 10 minutes.

9. Add 200 µl of 100% ethanol (EtOH) and vortex.

10. Add the solution and any precipitate that may have formed to the column in a 2 ml collection tube. Again, remember to clearly label your tube.

11. Centrifuge your sample at ≥ 6000g for 1 minute.

12. Discard the liquid in the specially designated waste container and transfer the column to a clean 2 ml collection tube.

13. Add 500 µl of AW1 buffer to the column and centrifuge as described in Step 11.

14. Discard the liquid in the specially designated waste container and transfer the column to a clean 2 ml collection tube.

15. Add 500 µl of AW2 buffer to the column. Centrifuge your sample at 20,000g or maximum speed for 3 minutes.

16. Place the column in a clean microcentrifuge tube (the ones in the orange boxes on your bench). Add 100 µl of AE buffer directly onto the center of the membrane in the column. Incubate for 1 minute at room temperature and then centrifuge as described in Step 11. Remember to clearly label this tube.
II. Polymerase Chain Reaction

1. Keep your reagents and samples on ice. Set up a 50 µl reaction in a labeled 0.2 ml tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Description</th>
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<tbody>
<tr>
<td>32 µl dH₂O</td>
<td></td>
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</tr>
<tr>
<td>10 µl genomic DNA</td>
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</tr>
<tr>
<td>2 µl forward and reverse primer pair</td>
<td></td>
<td>(Use either sim or sna)</td>
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<tr>
<td>5 µl PCR Buffer</td>
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<td></td>
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<tr>
<td>1 µl 10 mM dNTP</td>
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<td>(this is a mixture of dATP, dGTP, dTTP, and dCTP)</td>
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2. Then add 1 µl of Taq DNA polymerase directly to your solution, cap your tube and flick to mix. Pulse-spin your samples using the adapters in the mini tabletop centrifuge and then store them on ice until ready to load into the thermocycler.

3. The amplification program that you will use for either sim or sna is set for the following:

   1x  95°C     2 min

   Repeat 30x
   95°C     20 sec
   55°C     20 min
   72°C     1 min

   1x  72°C     10 min
   1x  4°C     ∞

This whole process will take several hours and once completed the samples will be stored at -20°C until next week’s lab (this will be done for you).