Part A: Generating a map of *let-418* with mutations and PCR primers

We will generally use "A plasmid Editor" (ApE) as our DNA analysis platform. If you're familiar with another program you'd like to use, feel free, but in-class guidance will be focused towards use of ApE.

This is open-source software and can be downloaded here: https://jorgensen.biology.utah.edu/wayned/ape/

ATPase deletion genotyping - predicting the results:

1. Use ApE to open the *let-418* nucleotide sequence, and add features for the following:
    a. The ATPase deletion found in Ter6 worms
    b. Each of the three primers used for genotyping the Ter6 mutation and the WT allele
        i. Save this file for reference later
2. Determine the fragments which will be generated by each set of primers for each allele under our PCR conditions for this strain, using the following primers:

Name	Sequence
ATPase fwd	5' CTTGGGTCTCCGAAATGCTG 3'
ATPase rev-1	5' CACGGTACGACTCGTTATCAT 3'
ATPase rev-2	5' CCTCCGGTTTCCGTAACGTA 3'

    a. It may be helpful to make a separate ApE file for the *let-418* deletion mutant, with primers at appropriate locations in this file.
        i. If you make a second file here, save it as well
    b. Refer to our PCR conditions to help understand the limits on PCR product length. This is the Taq polymerase we use for reference: https://www.neb.com/products/m0270-taq-2x-master-mix#Protocols,%20Manuals%20&%20Usage

Answers:

- The WT fragment (ATPase fwd – ATPase rev-2) is 517 bp
- The mutant fragment (ATPase fwd – ATPase rev-1) is 918 bp
- The ATPase fwd – ATPase rev-1 primers do not create a product in WT allele; they're separated by ~2500 bp and Taq Polymerase only replicates ~1kb/minute; our extension time was 90 seconds, so the longest product would be approx 1.5kbp

3. In the space below, sketch a gel showing the predicted results from your PCR reactions. Assume lane 1 contains a DNA ladder, lane 2 contains lysate from a WT (N2) animal, lane 3 contains lysate from a heterozygote, and lane 4 contains lysate from a homozygous (null/null) worm.

Answers:

- Lane 2 (WT) should have band at ~500 bp only
- Lane 3 (GFP+ worm, heterozygote) should have 500 and 900bp bands
- Lane 4 (GFP- worm, homozygous for null allele) should only have the 900bp band

EC1 genotyping & understanding RFLP:

1. Open a fresh *let-418* nucleotide sequence file and create a version with the substitution mutations specified below:
    a. 3235-6237: GAT → GAC
    b. 3247-3249: CAC → CGT
2. Figure out how to translate the sequence in ApE - how do these nucleotide substitutions impact the protein sequence? What is the correct nomenclature for the EC1 mutation at the amino acid level?
        i. The GAT → GAC is silent
        ii. The CAC → CGT is the histidine to arginine mutation at position 1027
        iii. Nomenclature is H1027R
    a. Save this version of *let-418* as well.
3. Determine the fragments which will be generated by each set of primers for each allele under our PCR conditions for this strain, using the following primers:

Name	Sequence
EC1 fwd	5' TATCATATATGATTGTGA 3'
EC1 rev	5' TTTCCTCCCAATCCTGCACG 3'

    a. Do you notice anything odd about either of these primers? What?
        i. There is a substitution in the forward primer at the 3rd position away from the 3' end (**G**TGA 3'). This does not perfectly match the sequence of *let-418*.
    b. Will the thing you should notice in (a) impact the PCR reaction? Why or why not?
        i. Maybe subtly by changing primer binding characteristics, but it's a single base substitution and there should still be product, even if it isn't the full amount which would be made with a primer w/ 100% identity to the target; there are some papers where this is actively investigated, if you're ahead on time or want to integrate discussion they could look for one of these to support their position
4. After running the PCR of a worm heterozygous for the EC1 allele and a worm that is homozygous for the EC1 allele, will you be able to distinguish the PCR products on a gel as we did when genotyping the ATPase deletion mutants? Why or why not?
        i. No – the products will all be the same length, regardless of WT vs substitution mutation

EC1 genotyping, part 2 - the RFLP analysis:

1. What does RFLP stand for? How might we be able to use this technique to analyze our EC1 PCR products?
    a. **Restriction fragment length polymorphism** (abbreviated RFLP) refers to differences (or variations) among people in their DNA sequences at sites recognized by restriction enzymes. Such variation results in different sized (or length) DNA fragments produced by digesting the DNA with a restriction enzyme.
    b. (don't tell them this, let them think) Maybe one or more of the substitution mutations introduced a restriction site….. hmmm….
2. Find the datasheet for MaeIII on the Sigma-Millipore website.
3. Can you find the restriction site for MaeIII in either PCR product for EC1? If so, how will treatment of your PCR product with MaeIII allow you to discriminate between the EC1 and WT alleles?

a. MaeIII cut site is:
    i. GTNAC, where N is any nucleotide
    ii. The WT PCR product has a GTGAT sequence. The "G" mutation in the first position is introduced by the single point mutation in the primer, but the WT allele has a "T" in the fifth position, so the enzyme does not recognize a site or cut the DNA. A single band at 195 nucleotides will be produced.
    iii. The mutant PCR product has GTGAC as a result of the point mutation in the primer as mentioned above, but *also* has a point mutation to introduce the C into the fifth position. This provides the full cut site for MaeIII, and the allele with the H1027R mutation will be cut by the enzyme, generating a small (19 nucleotide) and large (176) nucleotide fragment.

4. How will we need to alter the DNA gel conditions to visualize the result in (3) above? Why?
    a. Need to use a high percentage gel (usually 3% or more) rather than the usual 0.7-1% gel so we can discriminate very small changes in fragment size.
        i. The H1027R product (after digestion) will be approximately 19bp shorter than the WT allele product

EC2 genotyping:

We will genotype this strain by sending the purified PCR product for sequencing, but it is important that you know what size fragment to expect so we can confirm the PCR amplified the proper region of the genome prior to sending for sequencing.

1. Open a fresh *let-418* nucleotide sequence file and create a version with the substitution mutations specified below:
    a. 3281 G to A
    b. 3282 T to G
    c. 3285 C to G
2. Figure out how to translate the sequence in ApE - how do these nucleotide substitutions impact the protein sequence? What is the correct nomenclature for the EC2 mutation at the amino acid level?
        i. The overall mutation is CGTCTC in WT to CAGCTG
        ii. This is spread across two codons. The first is CGT→CAG which results in R1038Q (Arginine to glutamine); the second is CTC→CTG which is silent
        iii. Nomenclature is R1038Q
    b. Save this version of *let-418* as well.
3. Determine the fragments which will be generated by each set of primers for each allele under our PCR conditions for this strain, using the following primers:

Name	Sequence
EC2 fwd	5' TGGGACAAATGAGACAAGATGC 3'
EC2 rev	5' TAACTTTATGCTTCTGGCC 3'

Answer: all PCR products should be 207 nucleotides.