

Protocol for MT DNA PCR and Sequencing

Cheek cell extraction

1. Scrape inside of cheek with sterile loop.
2. Twirl loop in 200µl of 10% Chelex buffer.
3. Add 10ul of 2mg/ml PK to tube at front bench
4. Incubate for 15-30 minutes @ 56°C
5. Vortex for 10 seconds
6. Spin at max speed for 20 seconds
7. Boil for 8 minutes in a 110°C heat block.
8. Vortex for 10 seconds
9. Spin at max speed for 2-3 minutes.
10. Use the top, aqueous solution, called the supernatant, STAYING AWAY FROM the pellet on the bottom, for your PCR reaction.

Behind the scenes:

PCR of MT DNA

Kit components are thawed, vortexed, and spun down and a master mix is made as follows: for each sample to be amplified, we add to a single tube:

8.6 µl of ddH₂O
1µl Enhancer
12.5µl of Taq 2X Master Mix
0.2 µl of 5' primer (15pmol/ul) (5'-TTAACTCCACCATTAGCACC-3')
0.2 µl of 3' primer (15pmol/ul) (5'-GAGGATGGTGGTCAAGGGAC-3')
Mix by inverting with quick spin

22.5µl was aliquoted into tubes

For Class:

PCR of MT DNA

Students should come up to the front bench with gloves on

- select one of the tubes prepared above with their number on it
- add 2.5 µl of your cheek cell DNA (from the TOP of the aqueous layer) to your labeled tube
- tap tube with finger to mix (DO NOT VORTEX-you will kill the enzyme)
- place tube back in rack on ice.

Tubes will be spun quickly in a centrifuge to pool all reagents in the bottom and then put in a PCR machine which will take your tube through the following temperature changes:

94°C 10 minutes

52°C 5 minutes

35 cycles: 72°C for 30 seconds

94°C for 30 seconds

52°C for 30 seconds

72°C for 10 minutes

4°C til we take out of machine

This program will take about 3 hours to run.

Check PCR Reactions on a gel:

PCR products and many other DNA manipulations can be visualized by gel electrophoresis. DNA is negatively charged and will therefore run toward a positive electrode (anode) when placed in an electric field.

Agarose comes from seaweed and provides a matrix through which DNA can migrate based on size (smaller fragments move through faster than larger fragments).

So we make a gel mold out of agarose with little wells in it in which we load our DNA. Then the gel mold is placed in a buffer chamber with electrodes on either end. Your DNA will be combined with a dye that is heavier than the buffer so that it will sink down into the wells. The electrodes are plugged in (with the one at the bottom of your gel being plugged into the positive end) and the DNA will migrate through the gel.

To actually see your DNA fragments, prior to loading the gel, we stain it with SYBR Safe Green, which binds in the crevices of the double helix. Then when it is exposed to excitation wavelengths in our blue light box, it glows, allowing you to see where your DNA is. By comparing to size markers one can determine the size or sizes of DNA in a given sample.

Loading the gel:

Students will come up to the front bench and practice loading blue juice and water into gel wells.

When each feels ready, they can go to the back bench and add 15 μ l of their PCR reaction (**Note: this should not be all of your reaction**) into a tube containing 3 μ l of 5X blue juice, and load this entire mix into the gel well assigned to you on the board.

This 2% agarose gel, stained with a SYBR DNA stain called Gel Green, will be run at 275V for 20 minutes, then viewed in a PrepOne Sapphire and photographed. The photo will be transferred to a computer and labeled.

At this point everyone should have a band in their gel lane. All bands should be the same size because the differences that we are looking for are too small to be detected by an agarose gel. They can only be detected by sequencing the DNA and looking for polymorphisms in the sequence.

Sequencing your MT DNA

Behind the Scenes:

A seq. master mix was made for 40 rxns as follows: 160µl Big Dye Terminator Mix
40µl of 5' primer (15pmol/ul)
80µl ddH₂O

7µl was aliquoted into PCR tubes.

For Class:

Students should come up to the front bench with gloves on

- select one of the tubes prepared above with their number on it
- add 3 ul of your MT DNA PCR product to your labeled tube
- tap tube with finger to mix (DO NOT VORTEX-you will kill the enzyme)
- place tube back in rack on ice

Tubes will be spun quickly in a centrifuge to pool all reagents in the bottom and then put in a PCR machine which will take your tube through 25 cycles of the following temperature changes:

96°C 30 seconds
50°C 15 seconds
60°C 4 minutes

This program will take about 3 hours to run.

Purify your MT DNA Sequencing Reaction:

Sequencing reactions must be purified to get rid of all unincorporated NTPs. This will make your sequence much neater and easier to read.

Students should come up to the front bench and choose a spin column from the rack.

- Label the column **on the top** with your initials and place in the microcentrifuge.
- The tubes will be spun at 850 x g for 3 minutes. While it is spinning, label an eppie tube on the top with the number assigned to you on the board (you can put your name on the side if you like).
- When the microcentrifuge is done, retrieve your column, dispose of the bottom waste tube and put your column in your fresh labeled eppie tube (be careful not to jostle column, it is just resin beads lightly packed down).
- Bring the column/eppie assembly back to the front bench and load your entire sequencing reaction (10ul) onto the top of the column without touching the column.
- Place column back in centrifuge for another spin at 850 x g for 3 minutes.
- Remove the column from the tube and dispose. Close the eppie, make sure it is labeled properly, and bring it back to the front bench to place in a rack on ice.
- Your sequences will be loaded onto the sequencing machine and your sequences will be ready to read in about 4 hours (we will give you a tour of the machine).

On the 3130xl Genetic Analyzer

10 μ l of your samples will be added to 15 μ l of water in a plate that will be loaded on the genetic analyzer. The analyzer robotically sticks positively charged metal needles into the samples, calling all DNA in the sample to the tip of the needle. The charge is then transferred to the end of the capillary filled with polymer that is attached to the needle. The charge pulls the DNA through the capillary while the polymer separates the DNA by size smallest to largest just like an agarose gel. However, in this case your DNA fragments only differ in size by one base pair. You would not be able to see a 1bp difference in agarose but with a very thin polyacrylamide, you can. So the fragments are put in order smallest to largest with a florescent tag at the end of each one indicating which nucleotide is at the end. The machine and computer put all of this information together into a chromatogram. I will email a pdf copy of your chromatogram to you.