Wolbachia PCR: Discover the Microbes Within

Insect DNA Extraction

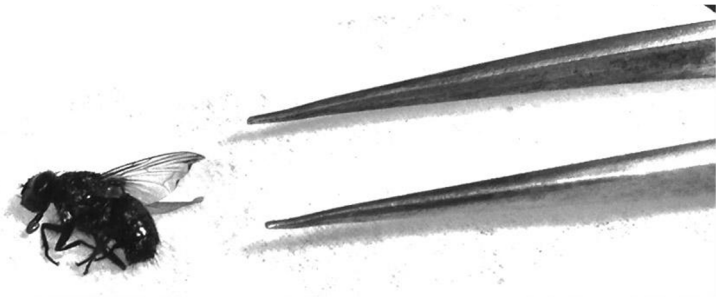
Part 1: Lyse the insect

☐ 1. Label a clean 1.5mL microtube with your ID on the lid and side of the tube. Put 500 µL of isopropanol into the tube. Put your insect directly into the alcohol.

* **Insects can be stored in alcohol for an indefinite amount of time before the lab.** When collecting an insect, the more recently deceased it is, the greater your chances of successfully extracting DNA for PCR.

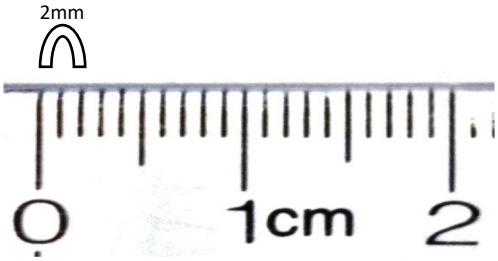
☐ 2. Label a clean 1.5mL microtube with your ID on the lid and side of the tube.

☐ 3. Remove your insect from the alcohol and place on a lab tissue or paper towel to wick away the moisture.



☐ 4. Using a ruler, measure the length of the insect's abdomen. If it is larger than 2mm long/wide, you'll need to **cut off a 2mm portion from the posterior end of the abdomen and use that**. If it is small enough, you can use the whole abdomen or even the whole insect.

1mm is 1/10th of a centimeter, so the 2mm sample size you require is actually very tiny! Note: the ruler pictured may not be to scale.



☐ 5. Place the insect sample into your labeled, clean microtube. The sample should sit about halfway to the 0.1 mL mark – if it is bigger than that, **trim the sample** and try again.

Too large of a sample can create excessive debris and inhibit PCR.

Size of sample sits about halfway to the 0.1 mark

6. Add 200 µL of **Lysis Buffer** and close the lid tightly.

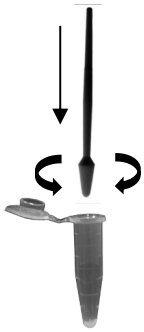
This helps break up the cell to release the DNA into the solution.

Always use a fresh pipette tip when dipping into a new tube of reagent – this prevents contamination

7. Use a small plastic micropestle to macerate your insect sample. Twist down and rotate with force to **crush it as much as possible**.

Crush the sample for at least 1 minute or until only small bits of the chitinous exoskeleton remain and solution has a "soupy" look.

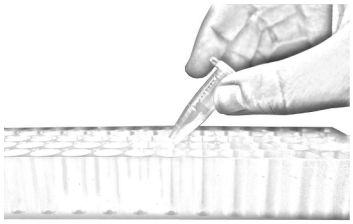
If the insect sample gets stuck at the bottom of the tube, close and flick the tube to resuspend or use a clean pipette tip to dislodge it.



8. Add 800 µL of **Lysis Buffer** and close the lid tightly**.**

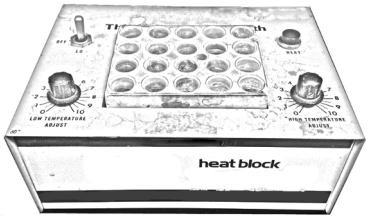
Mix by vortexing or "racking" (keep a finger on the top of the tube to prevent it from opening).

Press down firmly and drag tube across rack to mix contents.



9. Slide a cap lock onto your tube (opposite the tube hinge) to prevent it from popping open when heated. Place it in the 99˚C heat block or water bath for 5 minutes.

slide on



10. After heating, open tube briefly to release pressure, then close.

Close the lid and flick or "rack" the tube to mix.

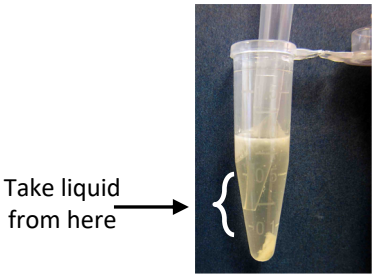
In a centrifuge, spin your tube for **5 minutes** at the highest speed, 12,000 – 14,000 rpm (at least 10,000g).

11. Get another clean 1.5 mL microtube and label it with your **ID** on the lid.

12. Get your tube from the centrifuge and ***carefully*** place it in the rack without shaking it. You should see:
-A pellet at the **bottom** of the tube *(we don't want this)*
-Maybe an oily layer at the **top** of the tube *(we don't want this)*

Hold the tube at eye level and use a p1000 to transfer about 800 µL of liquid **<u>from the middle</u>** of the insect lysate tube to the clean tube you just labeled in step 11.

*Do this **without disturbing the pellet** and without getting a lot of the oily layer. If you do disturb the pellet, re-centrifuge the sample.*

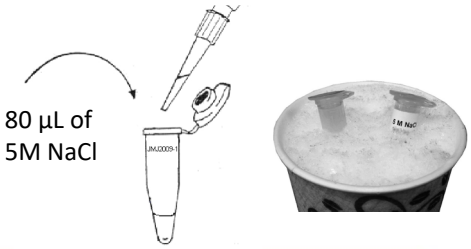


Part 2: Remove impurities from the sample

13. Add 80 µl of **5M NaCl** to the tube.

❑ Shake the tube a few times to mix.

❑ Incubate **on ice** for 5-10 minutes. Solution may become cloudy.



14. Place tube with NaCl into a balanced microfuge and spin again for 5 min at the highest rpm.

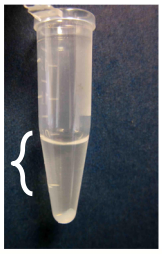
Part 3: Isolate the DNA

☐ 15. Get another clean 1.5 mL microtube. Label the tube "DNA" and your ID number.

☐ 16. Retrieve your tube from the microfuge. **There may or may not be a noticeable pellet** at the bottom of the tube.

Hold the tube at eye level and use a p1000 to transfer 600 µL of liquid **<u>from the top</u>** of the insect lysate/NaCl tube to the clean tube you just labeled "DNA" in step 15.

Take liquid from here →



☐ 17. Add **800 µL ice-cold isopropanol** to your "DNA" tube.

❑ Mix contents by inverting your tube several times

❑ Incubate on ice for 5-10min

Potential Stopping Point: If there's not enough time to complete the DNA isolation, store samples in the freezer for up to 1 week.

☐ 18. Microfuge the tube at **top speed** (**12,000 x g) for 5 minutes.**

VERY IMPORTANT: Orient the hinge of the tube to point outward and away from the middle of the microfuge. Nucleic acids (DNA) will pellet at the bottom-side of the tube under the hinge.

Do NOT place tubes hinge-inward

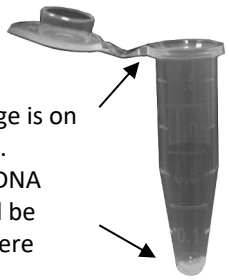
DO place the tubes hinge-outward

☐ 19. **Carefully pour** the liquid out of the tube (we only want the pellet)

Tap the mouth of the tube *hard*, onto a clean paper towel to remove the liquid on the lip of the tube.

The pellet should be stuck to the bottom of the tube as a teardrop-shaped mark or may appear as minute speckles on the hinge-side of the tube. **Do not worry if there is no visible pellet.**

If the hinge is on this side... Invisible DNA pellet will be located here



☐ 20. Open the cap and **air-dry** the pellet for **about 5-10 minutes** to evaporate all remaining isopropanol. **Note:** Residual alcohol will interfere with the PCR reaction.

To speed up the evaporation process, place tubes on a heat block set at about 50-70°C. Keep caps open and monitor for evaporation. If most of the liquid has been removed from the tubes beforehand, this should take less than 5 minutes.

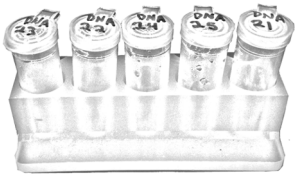
☐ 21. Add **100 µL of TE/RNase buffer** to your tube.

Scrape the side of the tube where the pellet is (or should be) with the micropipette tip to facilitate resuspension. Pipette up and down gently to collect DNA accumulated on the area underneath the hinge of the tube.

☐ 22. Microfuge the tubes for **1 minute** to pellet any particulates that did not dissolve in solution.

Note: This is your isolated insect (and possibly Wolbachia) DNA!

☐ 23. If you are not doing PCR immediately, give the teacher your tube of isolated DNA or place in the class microtube rack.



Be sure that your tube is **labeled clearly!**

☐ 24. Clean micropestles for re-use and soak in ethanol to sterilize. This will reduce cross-contamination when they are used by the next class.

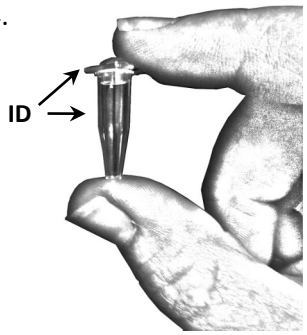
Do not throw these away – clean thoroughly and re-use

Potential Stopping Point: If there's not enough time, store the DNA in the freezer until next class for PCR.

25. Reagents you will need: **Master Mix**, **Primer Mix**, and your **DNA** from Step 24.

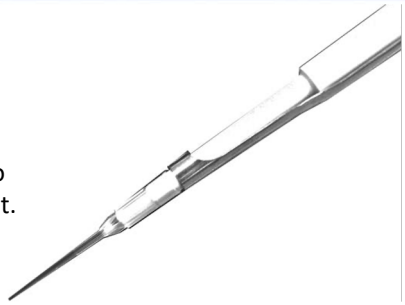
Label a **PCR tube** with your ID on the side and top.

Keep the reagent tubes on ice while setting up the reaction.

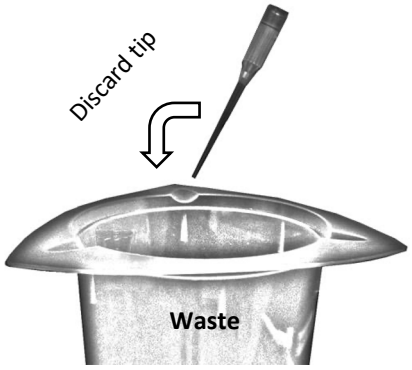


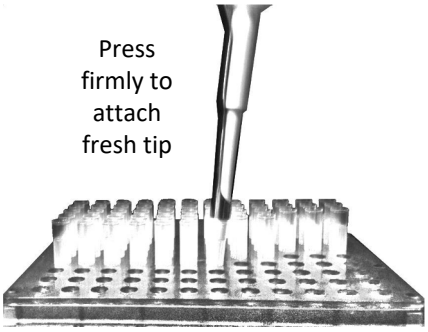
26. Pipette **20 µL of Master Mix** into the PCR tube.

Add all reagents to the **side** of the PCR tube so you can see the droplet.



27. Add **20 µL of Primer Mix** into the PCR tube (use a new tip).





28. **Add 10 µL of your extracted DNA** into your PCR tube (use a new tip).

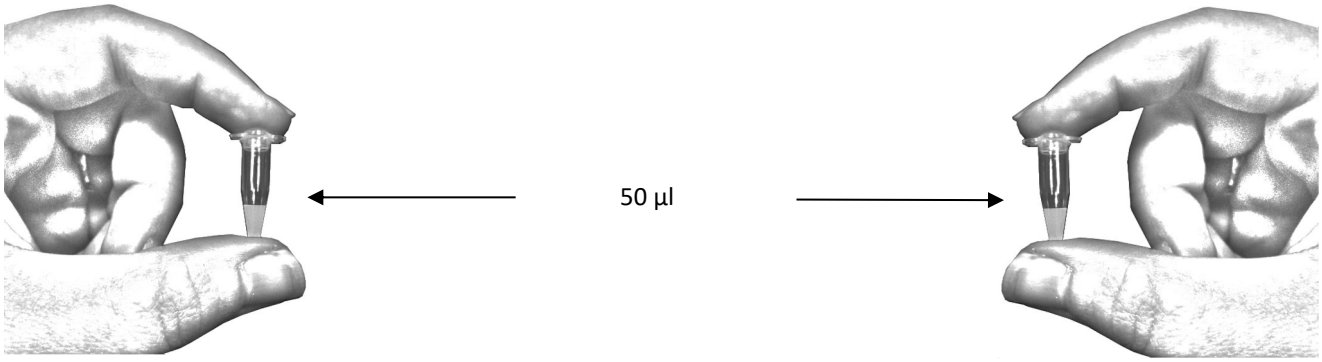
Your final volume should be **50 µL.**

29. Make sure cap is closed tight and **flick** the tube gently to **mix** the contents. Then **fling*** the tube to move the liquid to the bottom.

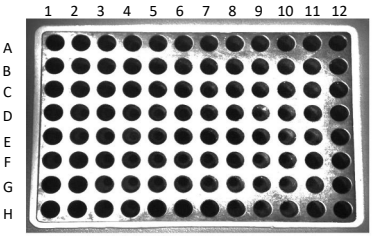
To **fling***: Hold the top of the tube firmly between your fingers
-"Fling" the tube, in a wide downward arc motion with force.

30. Compare the volume of your PCR tube with a reference PCR tube that has 50 µL in it**.**

If the volume of your tube does not match, see your teacher



31. Place your PCR tube into the thermal cycler. Make sure to record the location of your tube on the grid provided by your teacher.



Record your PCR tube location! Example: A11, A12

Wolbachia PCR Thermal Cycler Parameters:

1 – 94°C hold for 2 minutes
2 – 29 cycles of
94°C for 30 seconds
55°C for 45 seconds
72°C for 1 minutes
3 – 72°C hold for 10 minutes
4 – 4°C hold ∞ infinity

☐ 32. Obtain a 2% agarose gel (teachers, see "Preparing the Classroom for the Lab" for options on pouring gels).

Make sure the gel is:
- ❑ placed into the gel box with the **wells oriented towards the negative (black) side.**
- ❑ covered in enough 1X TAE buffer to just cover the gel entirely.

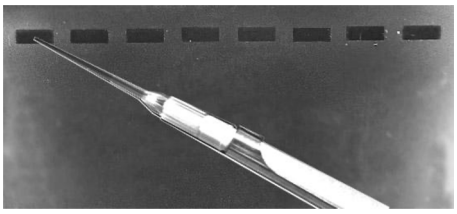
☐ 33. Get your PCR tube and check that all the liquid is at the bottom of the tube. If not, give it a quick **fling** to force the contents to the bottom of the tube.

☐ 34. **Add 5 µL of loading dye to each PCR sample. "Flick**" gently to mix and then "fling" the tube to bring liquid down to the bottom. Be sure to change tips between tubes.

☐ 35. In your lab notebook, or separate paper, draw a picture showing what you are loading into each well of your gel.

1–Ladder **2–**Negative Control **3**– Positive Control **4,5,6,7-**Different PCR samples from your group

36. Your teacher will provide you with **positive** and **negative** controls to load on your gel. Then load **15 µL** of each sample using the picture you made from step 35. **Avoid puncturing the side or bottom of the wells.**

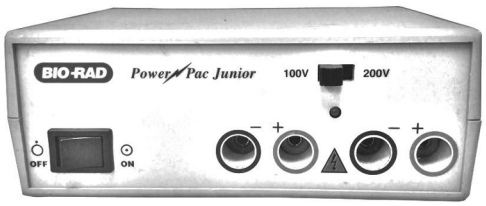


The **loading dye** makes the sample more dense than the buffer. The samples will sink to the bottom of the wells.

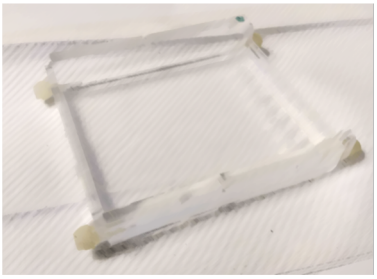
37. When all samples are loaded, attach the electrodes from the gel box to the power supply, **red to red (+) and black to black (-)**. Turn on the power supply and run your samples at 150 volts for 15-20 minutes or until the dye is 1/2 or 2/3 down the gel.

Notes:
Do not leave a running power supply unattended.

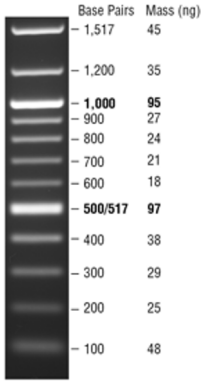
Make sure it is turned off and disconnected before removing gel box lid.



38. When done, turn off the power supply and disconnect the leads. Remove the entire tray from the gel box, and place on a paper towel.



39. Carefully slide the gel off the gel tray directly onto the UV transilluminator for viewing and imaging.



Compare your results to the ladder and the positive control.

You will notice that the 500bp and 1000bp bands are extra bright – this is to help you eyeball the number of base pairs easily.