

Bulleted/Summarized Teacher Instructions

For the complete and thorough fly-handling protocol, activity, background, explanations, and instructions, please see “Detailed Teacher Instructions.”

Teacher In-Advance Prep:

1. Prepare 3 culture vials with fly food by reconstituting Formula 4-24 Medium according to package instructions.
2. Place adults from the white-eyed *D. melanogaster* heat shock strains 60739 and 60740 and wild-type 60741 strain into new vials of food once you receive them. Leave them at room temp.
3. Ensure that you see eggs laying on top of the food media within 1-3 days of the date you set up the vial. Once you see many larvae and the food appears soupy, you can place the adults into a new vial of fresh food to continue to perpetuate the strains.
4. When you see larvae crawling out of the food and becoming pupae, begin heat shock treatment on white-eyed 60739 vials only! Continue to allow 60740 and 60741 strains to develop at room temp. See below for heat shock instructions.
5. Determine the number of flies you will need to collect for your students. You need 4 females and 4 males per cross. We recommend setting up 5 crosses per 6-10 pairs of students.
6. Prepare culture vials with fresh food for virgin female and male collecting.
7. Isolate virgin 60739 females from heat shock vials and place them into vials with fresh food. Collect more than you need to account for random mortality.
8. Isolate 60740 males from vials left at room temp and place them into vials of fresh food. (We recommend putting only 4-5 males per vial). Collect more than you need to account for random mortality.
9. Continue to maintain population of wild-type 60741 flies. You do not need to manipulate them at this point!
10. Keep virgin 60739 females and 60740 males in isolation for 4-5 days before beginning Teacher Cross 1.

Heat Shock instructions:

1. When pupae start to appear in the vial, place the vial in an incubator set at 37°C for one hour a day.
2. Repeat step 1 every day or every other day until adults begin to emerge.
3. When adults emerge, females can be collected until there are no more adults in the vial and heat shock treatment continued until all females needed for the activity are collected.
4. Be sure to double check that all adults in heat shock vials are females during collection.

Teacher Cross 1:

1. Determine the number of crosses you will need to set up (Recommended: 5 crosses per 6-10 pairs/groups of students).
2. Prepare culture vials with fresh food.
3. Set up the number of crosses you need. Each cross consists of placing four virgin 60739 females and four 60740 males together in a vial containing fresh food.

4. Leave adults in the vial until there are many larvae and the food media appears soupy (usually 2-4 days later), then discard the adults in a fly morgue.
5. Approximately 11 days after the crosses are set up, offspring from your crosses should emerge.
6. Any unused flies should NOT be released into the wild. Discard of them in a fly morgue (a bottle filled with ethanol or oil).

Teacher Cross 2:

1. Prepare culture vials with fresh food.
2. Place the offspring of Teacher Cross 1 into fresh vials of food and allow brother-sister matings to occur. No virgin females are necessary here. We recommend setting up double the number of Cross 2 vials as Cross 1 vials (i.e. if you set up 5 Cross 1 vials, set up 10 Cross 2 vials). Leave adults in the vial until you see larvae and the food media is soupy, then discard adults.
3. On the day you set up the brother-sister matings, place your population of wild-type 60741 flies into a fresh vial of food. You will need one 60741 male per pair/group in the next cross. We recommend starting at least 1-2 vials of 60741 flies per 6-10 pairs/groups of students.
4. When pupae start to emerge in your brother-sister mating vials, begin heat shock treatment on half of the vials (i.e. 5 of your 10 brother-sister mating vials will start heat shock treatment).
5. Prepare vials with fresh food for virgin female and male collecting.
6. Collect virgin white-eyed females from your heat shock vials and place them in vials with fresh food. You will need four females per student pair/group. Collect more than needed to account for random mortality and if you want to set up control vials.
7. Collect white-eyed males emerging from your non-heat shock brother-sister mating vials and place them in vials with fresh food. You will need four males per student pair/group. Collect more than needed to account for random mortality and if you want to set up control vials.
8. Collect wild-type red-eyed males emerging from 60741 vials and place them in vials with fresh food. You will need one male per student group. Collect more than needed to account for random mortality.
9. Keep white-eyed females, white-eyed males, and red-eyed males isolated for 4-5 days.
10. Prepare new food media (2 vials per student pair/group with extra if you choose to do control crosses) and set up the following vials the day before you plan to start the activity with students:
 - Per Student Pair/Group for Experimental Crosses
 - 1 vial with four white-eyed females
 - 1 vial with four white-eyed males and one red-eyed male
 - Control Crosses (your choice)
 - 1 vial with four white-eyed females
 - 1 vial with four white-eyed males

Activity Day 1 with Students: Crossing the Flies and Observing:

1. Give a brief presentation about inheritance and natural selection and any other background material deemed necessary about the activity. Sample PowerPoints for the entire activity can be found at <https://sites.google.com/site/noorlabduke/fly-evolution-advanced>. More basic intro PowerPoints can be found at <https://sites.google.com/site/noorlabduke/flyevolutionk12>. You will need to modify them accordingly.

2. Divide students into pairs or groups and give each pair/group 1 vial with four white-eyed females and 1 vial with four white-eyed males and one red-eyed male.
3. Give students the opportunity to become familiar with telling apart the differences between males and females and eye color (white vs. red).
4. Demonstrate how to properly combine the males and females in separate vials into one vial.
5. Recommended: Stand behind student groups as they transfer their flies to ensure that as little flies as possible escape.
6. Have students label their cross vials with lab tape (i.e. names and period) so the correct offspring are returned to them.
7. Allow students to watch the behavior of the flies, specifically looking at differences between the red and white-eyed males. Students should see courtship (male extending one wing and vibrating it behind the female) and potentially copulation, with the red-eyed male most likely to mate with a female first.
8. Reiterate the fact that the better vision and health associated with the male having red eyes gives him a fitness advantage over white-eyed males in terms of finding mates and passing his genes on to future generations. Tie this back in to natural selection and evolution and ask students to predict what they might expect to see in future generations of offspring.

Teacher Fly Care between Activity Day 1 and 2:

1. Remove and discard adults from student vials when larvae appear and media looks soupy.
2. When the larvae of the crosses set up by students in Day 1 emerge as adults (Generation 1), prepare culture vials with food (1 per student group) and place the adults in new food. Transfer the student label.
3. Allow them to mate.
4. When there are larvae present, remove the adults and place them into fresh food to hand back to students on Activity Day 2 for manipulation. You can rubber band the vial of larvae to the vial of adults to keep the label with both of the vials.
5. Return the vial of larvae and vial of adults to students on Activity Day 2, emphasizing that they will only be manipulating the vial of adults that day. This is done since we do not want the larvae for the next generation to be exposed to FlyNap.

Activity Day 2: Generation 1:

1. Recap the main concepts discussed on Activity Day 1: fitness differences between red and white-eyed males, what they observed, what natural selection is, etc.
2. Give students back their offspring from the cross on Activity Day 1 (Generation 1 adult vial and larvae vial). Again, tell them not to manipulate the vial of larvae.
3. Demonstrate how to use FlyNap anesthesia to put the adult flies to sleep, then have students do this themselves.
4. Have the students draw a four-quadrant square on a piece of paper and label each quadrant with white male, white female, red male, red female.
5. Have them sort their flies by sex and eye color on the piece of paper using a paintbrush or pencil to move the flies around.
6. Double check that they sorted their flies correctly. There should not be any red-eyed males.

7. Have students calculate the percentage of each type of fly they have and put it into a spreadsheet to be projected to the class.
8. Discuss why there are no red-eyed flies. Eye color in *Drosophila* is inherited on the X-chromosome. You may wish to do a Punnett square to demonstrate the cross set up in Activity Day 1.
9. Collect larvae vials with labels from the students. The adult flies can be discarded in a fly morgue.

Teacher Fly Care between Activity Days 2 and 3:

1. Prepare vials with fresh food as needed when transferring adults during this portion of the activity.
2. The larvae you collect from students on Activity Day 2 will turn into Generation 2 adults. Once adults emerge, place them in new food and allow them to mate. Make sure to transfer the vial label.
3. When larvae appear in the vial and media appears soupy, the adults can be discarded. These larvae will turn into Generation 3 adults. Once adults emerge, place them in new food and allow them to mate.
4. When larvae appear in the vial, the adults can be discarded. These larvae will turn into Generation 4 adults. These adults will be returned to students for Activity Day 3.

Note: You can use Generation 3 adults in Activity Day 3 if you want as well.

Teacher Prep between Activity Days 2 and 3:

1. Prepare squishing buffer and Proteinase-K solution. See page 8 of the detailed teacher instructions for protocol.

Activity Day 3: Evolution by Natural Selection and DNA extraction:

Part 1:

1. Return the Generation 4 (or 3) adults back to students.
2. Have the students anesthetize their flies with FlyNap and then sort them on a piece of paper just as they did on Activity Day 2.
3. Once students count their flies, they should see that about half of the flies will have red eyes. Compile percentages of each type of fly in an excel sheet.
4. Discuss how students have witnessed evolution by natural selection. The abundance of the red eye allele changed and most, if not all, vials should have over 11% red-eyed adults. See detailed teacher instructions for hints on how to discuss vials where the red-eyed phenotype may not have spread as anticipated (pg. 9) and class discussion questions (pg. 10).

Part 2:

1. Give a brief presentation about how scientists use genetic markers that are variable in an organism's genome to look for signs of evolution and introduce the NEAR and FAR markers the students will be looking at in their flies.
2. Have students select 7 **male** flies to prepare for DNA Extraction. Students should select a representative sample of their flies (but make sure to include both white and red-eyed males).

3. With tweezers or fingers, students should place one **male** fly into each well of the 8-well strip tube except one for a control, noting the eye color on the provided handout and on the top and sides of the tube with a fine-tipped Sharpie®.
4. With a 100 or 200 µl pipettor, students should pick up 30 µl of the DNA Extraction Buffer solution and, without ejecting, stab the fly repeatedly with the pipettor tip. They can eject a small amount of liquid to help squish the fly. Remind them not to press too hard – the tube or tip can break! After the fly is squished (~20 seconds), instruct the students to eject the remaining liquid over the fly.
5. Repeat for remaining flies, but be sure to change tips between flies- NEVER put a used tip back into the DNA extraction master mix solution. This will contaminate the DNA preps! Red-eyed flies will produce a pink colored liquid, white-eyed flies will remain colorless. The body of the fly should be visibly disassembled.
6. Keep on ice until all preps are done. When all done, place in thermal cycler for 37°C for 30 minutes and 95°C for 2 minutes.

Teacher PCR Prep for Activity Day 4:

1. Order five primers to amplify the “NEAR” and “FAR” markers from IDT. See pages 11-12 of the detailed teacher instructions for primer sequences and step-by-step instructions.
2. When you receive the primers, they will come in a powdered form. You will need to reconstitute the powder into a liquid by finding the number of nm on the tube, multiplying it by two, and adding that many microliters of cold 1X TE buffer to the tube. Vortex after adding the TE buffer to mix. This will give you a 0.5 mM primer concentration, and is your primer STOCK solution.
3. Make dilutions of your primer stock to use for the PCRs in a 1:50 ratio. For every 1 µl of stock primer you use, add 49 µl of water. (i.e. 1 ml diluted primer = 20 µl stock primer and 980 µl dH₂O).
4. Put stock and diluted primers in the -20°C freezer until you are ready to use them.
5. Prepare PCR master mixes for both the “NEAR” and “FAR” markers. See page 13 of the detailed teacher instructions.
6. Before class on Activity Day 4, aliquot 200 µl of both the “NEAR” and “FAR” master mixes into 1-2 ml microcentrifuge tubes for each pair/group. Keep them on ice.

Activity Day 4: PCR:

1. Return DNA extractions to students.
2. Have students label each set of strip tubes. One set of strip tubes will be for the “NEAR” marker and one set will be for the “FAR” marker.
3. Vortex both tubes of master mixes.
4. Using a 20 µl pipettor, students will pipette 19 µl of PCR master mix for the NEAR marker into each strip tube well for the NEAR marker. They can use the same tip for this step.
5. Using a different tip, students will pipette 19 µl of PCR master mix for the FAR marker into each strip tube well for the FAR marker. If students run out of master mix, they may not have pipetted correctly.
6. Using the 2 µl or 10 µl pipettor, students will pipette 1 µl of DNA from their DNA prep strip tubes to the master mix NEAR marker strip tubes. They should use a different tip for each fly prep! Have students close the lid after adding a fly prep to remind them which they have already done.

7. Make sure they have labeled everything and know which fly is going into which strip tube well. Cap the tubes tightly afterwards.
8. Repeat by putting the same fly DNA into the master mix for FAR marker strip tubes.
9. Now the strip tubes will be put in the thermal cycler. Program: Step 1: 1 x (95°C, 2 min), Step 2: 30 x (95°C, 30s; 60°C, 30s; 72°C, 1 min), Step 3: 4°C ∞
10. Refrigerate samples after thermal cycler is complete until ready to run on gels.

Teacher Prep for Activity Day 5:

1. Prepare a 1.8% LB agarose gel for each pair/group.
 1. Add 1.8 grams of agarose and 100 ml 1X LB buffer to 50 ml flask.
 2. Heat in microwave until large bubbles appear. Swirl and continue to heat until the liquid is clear.
 3. Add 10 µl SYBR Safe DNA gel stain, swirl, and let cool for a couple minutes.
 4. Pour into a casting tray with a 20-well comb, move any bubbles in the gel to the side with a pipette tip, and let cool for 45 minutes. Cover gel with foil while setting since the SYBR Safe is light sensitive.
2. Prepare ladder loading dye solution. Mix 1 µl 100-bp ladder, 3 µl 6X loading dye, and 14 µl 1X LB buffer in microcentrifuge tube for each pair/group. Store the ladder loading dye in the -20°C freezer until ready to use.
3. Optional: Prepare practice loading gels in petri dishes for each group.

Activity Day 5: Gel Electrophoresis

1. Return PCR products to students.
2. Have students put on gloves.
3. Optional: Students practice loading gels with the practice gel in the petri dish and loading dye before loading their actual PCR samples.
4. Students will prepare samples by adding 4 µl of 6X loading dye into each well of the strip tubes. Close the caps and tap gently to mix. They should now have 24 µl of sample in each well. Make sure they use a different pipette tip for each well.
5. Students will then add 18 µl of the 100-bp ladder-loading dye into one well, then carefully add 20 µl of each sample from strip tubes into the remaining individual wells of their gel. They should label the placement of each sample on their worksheet (it is very important to keep track of which sample went into which well).
6. PCR samples for both markers should be run for 45 minutes at 300 V.
7. Gels can be visualized using a Gel Doc station with UV light. Alternatively, one can use a stain per manufacturer instructions.

Activity Day 6: Data Analysis:

1. Students will use their gel printouts to answer the discussion questions on their student handouts.
2. Discuss answers as a class, and reiterate the big picture of the activity. See pages 16-19 of the detailed teacher instructions for answers and sample data.