Linking Genotype to Phenotype in *Drosophila melanogaster*: PCR Genotyping the White-one Eye Mutation

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This lab exercise adds a molecular genetics component to a traditional undergraduate lab that investigates eye color inheritance in *Drosophila melanogaster*. This two-week activity is based on polymerase chain reaction (PCR) and is used to DNA fingerprint (genotype) wild-type (red-eyed) and mutant (white-eyed) flies. The use of this genotyping exercise as a companion activity to a traditional *Drosophila* investigation provides students “hands-on” experience establishing the link between genotype and phenotype. In addition, this lab-based activity introduces students to common techniques used in molecular genetics, including the extraction of DNA, PCR, and agarose gel electrophoresis.

**Keywords**: *Drosophila*, PCR, white-one mutation, genotyping, phenotype, wild-type, eye color, DNA gel electrophoresis

**Introduction**

This lab exercise was developed to add a molecular genetics component to a traditional undergraduate lab that investigates eye color inheritance in *Drosophila melanogaster*. At Hood College, our sophomore-level cell biology and genetics course includes a multi-week lab exercise that uses *Drosophila* to investigate the classic sex-linked inheritance of “white eyes” discovered by Thomas Hunt Morgan in the early twentieth century. Students culture their own fly stocks, set up a genetic cross examining eye color inheritance and follow the cross through the F₂ generation. Statistical analysis of data is also included. To broaden the learning experience of students, we have developed a genotyping exercise that instructors can use as a companion activity to this classic lab investigation. This two-week activity is based on polymerase chain reaction (PCR) and is used to DNA fingerprint (genotype) wild type (red-eyed) and mutant (white-eyed) flies. The use of this genotyping exercise as a component of a classical *Drosophila* laboratory experience provides students “hands-on” experience establishing the link between genotype and phenotype, reinforcing the connection between classic and molecular genetics. In addition, this lab-based learning activity introduces students to common techniques used in molecular biology, including the extraction of DNA, PCR, and agarose gel electrophoresis.

To help students with the understanding of the biological concepts associated with this lab, we present a Powerpoint presentation during the first week of the two-week activity. This presentation summarizes the fly crosses students have been conducting and provides background information on the cell and molecular biology associated with the red and white-eyed phenotypes. In addition, the presentation introduces students to the principles and applications of PCR.
Student Outline

Introduction

This semester we have investigated the inheritance of eye color in *Drosophila melanogaster* first discovered by Thomas Hunt Morgan in the early twentieth century (Morgan 1910). Wild-type *Drosophila* have red eyes and Morgan's observation of a male with white eyes in his laboratory culture led to the discovery of sex-linked inheritance and the establishment of the chromosomal basis for heredity. Since the pioneering work of Morgan, the genetics and molecular biology of *Drosophila melanogaster* have been extensively studied, including the creation of FlyBase, a database that lists and describes *Drosophila* gene sequences and functions (Tweedie et al. 2009).

This model organism has four pairs of chromosomes designated I, II, III, and IV; females have one pair of X chromosomes, males have one X and one Y chromosome (Fig. 1).

Figure 1. Comparison of female (left) and male (right) *Drosophila melanogaster*.

The sex-linked inheritance of eye color discovered by Morgan (1910) is controlled by the white allele found on the X chromosome. This wild-type allele (X\textsuperscript{w+}) is composed of a gene characterized by 6 exons and 5 introns, encoding a protein (designated “white”) of 687 amino acids that is located in the membrane of eye cells (and cells of other tissues). White protein partners with the protein “brown” encoded by the brown gene to produce a heteromeric transporter protein. In wild-type flies, the transporter moves extracellular precursors for eye pigments into the cytoplasm of eye cells. Once inside the cell, the precursors are biochemically converted to the final pigment products that give wild-type *Drosophila* eyes that are red in color (Tweedie et al. 2009). In the white-one mutation (X\textsuperscript{w1}), transcription from the white gene is disrupted, the white protein is not synthesized, and the functional transporter protein is not produced. Therefore, this mutation blocks the ability of eye cells to uptake pigment precursors and the eyes appear white (Fig. 2).
Work on the molecular genetics of the white-one ($w1$) mutation has determined that this mutation results from insertion of a 4,700-bp Doc retroposon in the promoter region of the white gene resulting in inactivation of the promoter and, thus, disruption of transcription (Driver et al. 1989). In addition, DNA sequence analysis of the 4,700-bp Doc element has been completed and it has been shown to be closely related to other retroposons reported for Drosophila (O’Hare et al. 1990).

**Objectives**

The goal of this two-week lab activity is to analyze individual female and male flies from the $F_1$ and $F_2$ generations you have worked with this semester. Each student will choose a fly (male or female; red-eyed or white-eyed), extract fly DNA, and conduct the polymerase chain reaction (PCR) procedure (Bloom et al. 1996; Dieffenbach and Dveksler, 2003) followed by agarose gel electrophoresis to identify the genotype of the fly.

The promoter region for the wild-type ($X^w+$) and mutated ($X^w1$) alleles, including the location of the three primers which will be used to genotype individual flies using PCR are shown in Figure 3. Primers P1 and P3 are specific for the wild-type allele, whereas, primer P2 is specific for the mutated allele. Amplification of the wild-type allele by PCR is expected to produce a product of 467 bp while amplification of the white-one allele is expected to produce two products: 5,211 bp and 704 bp. The upper limit of size for PCR products using *Thermus aquaticus* (Taq) DNA polymerase approximates 5,000 bp (Barnes, 1994; Dieffenbach and Dveksler, 2003), therefore, the 5,211-bp product generated by primers P1 and P3 is not made in amounts that are detectable by agarose gel electrophoresis.
The nucleotide sequences for primers P1, P2, and P3 are presented in Table 1. In addition, the sequences for the wild type and white-one alleles are available from your instructor, including the location of the binding (annealing) site for each primer.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GTGCAAGGTGGTGAATT</td>
<td>20</td>
</tr>
<tr>
<td>P2</td>
<td>TCTGGGAGTTCATCTGACA</td>
<td>20</td>
</tr>
<tr>
<td>P3</td>
<td>GAGAGGAGTTTTGGCACAGC</td>
<td>20</td>
</tr>
</tbody>
</table>

This is a schematic diagram of an agarose gel used to analyze the DNA fragments generated by analyzing individual female flies with primers P1, P2, and P3 in a single PCR reaction. Lane M represents DNA fragments that are size markers (standards). In lanes 1, 2, and 3, sketch in the DNA fragments expected to be produced by PCR for female Drosophila homozygous for wild-type, heterozygous, and homozygous for the white-one mutation, respectively:
Analysis of male *Drosophila* using the same primers would produce two genotypes. In lanes 1 and 2, sketch in the results expected for wild-type males and those with the *white-one* mutation, respectively:

```
<table>
<thead>
<tr>
<th>bp</th>
<th>Lane 1</th>
<th>Lane 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
<td></td>
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<tr>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

**Flowchart for Two-Week Lab Activity**

1. **Extract Fly DNA**
2. **PCR using Primers**
   - P1, P2, and P3 in a single reaction
3. **Agarose Gel Electrophoresis**
4. **Analyze Gel Results to Determine Fly Genotype**

*Week 1*

*Week 2*
Extraction of DNA from Individual Flies - Week 1

Place a single fly in a 0.5-mL tube and then report to the instructor’s station where the instructor will transfer 50 µL of fly squishing buffer to this tube. After returning to your bench, use a 200-µL pipet tip to squish (mash) the fly for 2 minutes at room temperature. After the fly is squished (yes, you will still see fly parts when you are done!), add 25 µL of mineral oil to the tube and quick-spin (5 seconds) in the microcentrifuge. This ensures the mineral oil forms the top layer, thus forming a barrier against evaporation when the tube is incubated. (Note: the mineral oil is omitted if a thermal cycler with a heated lid is used). Following the instructor’s directions, transfer your tube to the thermal cycler which is programmed to incubate the sample at 55ºC for 60 minutes followed by 94ºC for 2 minutes and then cooling to 4ºC. At the completion of this process, you will use 4 µL of the fly DNA lysate as a template for PCR. The residual lysate will be stored at -20ºC.

How does this process extract fly DNA? The squishing buffer contains proteinase K (Gloor et al. 1993). This enzyme is a broad-spectrum protease that is very active at 55ºC, resulting in the digestion (hydrolysis) of cell membrane proteins and the lysis of cells which releases (extracts) fly DNA from the nucleus. The subsequent heating of the tube for 2 minutes at 94ºC inactivates the proteinase K.

Polymerase Chain Reaction (PCR) – Week 1

A. As summarized below, you will conduct a 40-µL PCR reaction that uses 4 µL of the fly DNA lysate you prepared. Components 1-8 will be provided to you as a 36-µL aliquot (termed a “master mix”) by your instructor:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Distilled Water</td>
<td>27.4 µL</td>
<td></td>
</tr>
<tr>
<td>2. 10X PCR Buffer</td>
<td>4.0 µL</td>
<td>10 mM Tris (pH 8.3), 50 mM KCl</td>
</tr>
<tr>
<td>3. MgCl₂, 50 mM</td>
<td>1.2 µL</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>4. dNTPs, 10 mM</td>
<td>0.8 µL</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>5. Primer P1, 2.5 µM</td>
<td>0.8 µL</td>
<td>0.050 µM</td>
</tr>
<tr>
<td>6. Primer P2, 10 µM</td>
<td>0.8 µL</td>
<td>0.200 µM</td>
</tr>
<tr>
<td>7. Primer P3, 10 µM</td>
<td>0.8 µL</td>
<td>0.200 µM</td>
</tr>
<tr>
<td>8. Taq DNA Polymerase (5 Units/µL)</td>
<td>0.8 µL</td>
<td>25 Units/mL</td>
</tr>
<tr>
<td>9. Fly DNA Lysate</td>
<td>36.0 µL Components 1-8, from Instructor</td>
<td></td>
</tr>
</tbody>
</table>

4.0 µL 40.0 µL Total Volume

B. Preparation of one PCR reaction by each student:

1. Label a PCR (0.5-mL) tube and aliquot 25 µL of mineral oil into the tube. As described for the fly DNA extraction, the mineral oil is used as a barrier to minimize evaporation during the PCR process. (Note: the mineral oil is omitted if a thermal cycler with a heated lid is used.)

2. Report to the instructor’s station where the instructor will transfer 36 µL of the “master mix” to your tube.

3. Return to your bench and transfer 4 µL of your fly DNA lysate to the tube. Your tube now contains mineral oil (25 µL), master mix (36 µL), and your fly sample (4 µL). Close the tube completely and quick-spin (5 seconds) in the microcentrifuge to ensure the mineral forms the top layer. Place the tube on ice.

C. Use of the Thermal Cycler:

1. Following the instructor’s directions, place your tube in the DNA Thermal Cycle 480. After all students have transferred tubes to the machine, press “Start” for File #50. The total PCR run time will approximate 3 hours.

2. After the completion of the PCR method, store the reaction tubes at -20ºC for analysis by agarose gel electrophoresis. This analysis will reveal the genotype of your fly (next week).
D. Description of PCR method

<table>
<thead>
<tr>
<th>File #</th>
<th>Steps</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1. 94°C, 1 minute</td>
<td>• Preamplification denaturation of fly DNA</td>
</tr>
<tr>
<td></td>
<td>2. 1 cycle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. linked to File 49</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>1. 94°C, 1 minute</td>
<td>• Denaturation of DNA templates</td>
</tr>
<tr>
<td></td>
<td>2. 55°C, 1 minute</td>
<td>• Annealing (binding) of primers</td>
</tr>
<tr>
<td></td>
<td>3. 72°C, 2 minute</td>
<td>• Extension (synthesis) of new DNA</td>
</tr>
<tr>
<td></td>
<td>4. 35 cycles</td>
<td>• Amplification (steps 1 to 3, repeated 35X)</td>
</tr>
<tr>
<td></td>
<td>5. linked to File 48</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>1. 72°C, 5 minutes</td>
<td>• Final extension (synthesis) of DNA to ensure products (amplicons) are completely synthesized</td>
</tr>
<tr>
<td></td>
<td>2. linked to File 47</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>1. 4°C, hold</td>
<td>• Method completed</td>
</tr>
</tbody>
</table>

Gel Electrophoresis – Week 2

A. Sample Preparation

Label a 1.5-mL tube and transfer 8 µL of your PCR reaction to this tube followed by 2 µL of 5X DNA loading buffer (25% glycerol, 0.05% bromophenol blue) to produce a total volume of 10 µL. The addition of loading buffer adjusts the sample to 5% glycerol and 0.01% bromophenol blue. The glycerol increases the density of the sample for loading into the well of the agarose gel and the bromophenol blue provides a “dye front” to monitor the length of time for electrophoresis. (Bromophenol blue migrates at a rate equivalent to a 300-bp fragment of DNA.)

B. Gel Electrophoresis

Load your 10-µL sample into the appropriate well of 1.0% agarose gel. Be sure to reserve one well (lane) for DNA size markers. Electrophorese for approximately 60-70 minutes at 80 volts (constant voltage) until the bromophenol marker dye migrates 3/4 the length of the gel.

---

Lane Description
1  
2  
3  
4  
5  
6  
7  
8  

• Electric potential applied

• DNA fragments migrate at a rate inversely related to size (molecular weight)

• Electrophoresis completed

• Gel is incubated with ethidium bromide or SYBR® Safe stain to visualize DNA fragments via exposure to UV light
C. Gel Staining and Documentation

The lab instructor will demonstrate how to stain the gel with ethidium bromide or Sybr® Safe stain and to produce a gel photograph. The final photograph will be posted on Blackboard.

Questions

1. Based on the results of agarose gel electrophoresis, what is the genotype of the fly you processed by PCR. Explain your reasoning.

2. The DNA extraction protocol includes the heat inactivation of proteinase K. Why is this step necessary?

3. Melting temperature ($T_m$) is the temperature at which a primer dissociates from its complementary target during the PCR process. This parameter is an important consideration for PCR because annealing of primers to target sequences occurs 3 to 5 degrees below the calculated $T_m$ for each primer. In addition, for primers less than 21 bases in length, the following formula can be used to calculate $T_m$:

$$T_m \text{ (in °C)} = 4(G + C) + 2(A + T)$$

Using this formula, complete the following table by calculating the $T_m$ values for the primers employed to genotype the flies by PCR (show all work):

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Length</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GTGCAAGGTTGGTCAATTT</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>TCTGGGAGTTTCATCTGGACA</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>GAGAGGATTTTGGCAAGC</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

4. In the formula provided in Question 3, the (G + C) component makes a greater contribution to the value of $T_m$ as compared to (A + T). Based on your knowledge of DNA structure, explain why this makes sense.

5. Predict the expected outcome in terms of the size of PCR product if a mutation occurs in the promoter by a retroposon that is 300 bp in size. The two primers ($P_a$ and $P_b$) used in this new PCR method produce a product 250 bp in size for the normal allele. Diagram the promoter with and without the retroposon, similar to Figure 3.

6. Your instructor discovers a new “white-eyed” mutant of D. melanogaster. In addition, cell biology students confirm that white and brown proteins are present in the membrane of eye cells of this mutant fly. Based on this information (and Figure 2), suggest one hypothesis for this novel mutation.

7. As for many genes, the link between genotype and phenotype is rarely simple. In fact, the protein encoded by the wild-type allele (white gene) is expressed in tissues other than eyes, thus mutations in this gene can alter more than eye color producing a pleiotropic effect. Based on information available at the website (http://www.sdbonline.org/fly/aimain/1aahome.htm) for “The Interactive Fly,” describe three characteristics (other than eye color) of Drosophila that are associated with the white-one gene.
Materials

The section describes reagents and supplies for twenty students.

Drosophila and Supplies

For a Pair of Students

- Culture of Wild-type Drosophila – Cat. No. 172100 (Carolina Biological Supply)
- Culture of White-one Drosophila – Cat. No. 172220 (Carolina Biological Supply)
- FlyNap® Anesthetic Kit – Cat. No. 173010 (Carolina Biological Supply)
- Metal pan, labeled with names of students
- Soft paint brush
- 3 x 5 index cards
- Timer

Shared Material

- Stations for setting up vials of medium: well-sealed bins of Formula 4-24 Instant Medium, Blue – Cat. No. 173210 (Carolina Biological Supply)
- Yeast (if used)
- Vials
- Sterilized stoppers
- Disposal bags for used medium
- Spatulas for scooping medium from vials
- 10-mL pipettes
- Sterile tap water
- Labels and markers for vials
- Alcohol “morgues” for disposing of flies
- Stereoscopes

DNA Extraction

- Fly squishing buffer (10 mM Tris, pH 8.0, 25 mM NaCl, 1 mM EDTA, and 200 µg/mL proteinase K) - 1.5 mL, prepared fresh for each lab, placed on ice.
- DNA Thermal Cycler 480* (Perkin Elmer, Inc.)
- 0.5-mL PCR tubes
- P-20, P-200, & P-1000 micropipettors
- Aerosol-resistant pipet tips
- Ice buckets
- Markers to label tubes
- Mineral oil*

Polymerase Chain Reaction (PCR) - Equipment and Plasticware

- DNA Thermal Cycler 480* (Perkin Elmer, Inc.)
- 1.5-mL microcentrifuge tubes
- 0.5-mL PCR reaction tubes
- P-20, P-200, & P-1000 micropipettors
- Microcentrifuge
- Aerosol-resistant pipet tips
- Ice buckets
- Markers to label tubes
- Mineral oil*

Polymerase Chain Reaction (PCR) - DNA and Reagents (stored at -20°C)

- Student-prepared Fly DNA lysate samples (50 µL per sample)
- Primer P1 (2.5 µM) - 100 µL
- Primer P2 (10 µM) - 100 µL
- Primer P3 (10 µM) - 100 µL
- 10X PCR buffer (100 mM Tris, pH 8.3, 500 mM KCl) - 100 µL
- 10 mM dNTPs -100 µL
- 50 mM MgCl2 - 100 µL
- PCR-grade distilled water
- Platinum Taq DNA Polymerase (5 Units/µL) – Cat. No. 10966-026 (Invitrogen)

Gel Electrophoresis

- Mini-sized horizontal gel unit – Model No. Horizon 58 (Whatman)
- Agarose
- 50X TAE buffer (2.0 M Tris-acetate, pH 8, 0.05 M EDTA)
- 5X DNA loading buffer (50 mM EDTA, pH 8.0, 25% glycerol, 0.05% SDS, and 0.05% bromophenol blue)
- Ethidium bromide (10 mg/mL) or SYBR® Safe stain (10,000X concentrate) – Cat. No. S33102 (Invitrogen)
- 100-bp DNA ladder – Cat. No. 15628-019 (Invitrogen)
- UV-Transilluminator
- Power supply
- Camera system

Notes for the Instructor

Drosophila Crosses

We have found students to be more invested in this semester-long project if they maintain Drosophila cultures and are responsible for doing their own crosses. It is very helpful to have teaching assistants maintain fly stocks and to conduct crosses simultaneously with students in the class to provide “spare” cultures or crosses when needed. Individual students conduct one cross through the F1 generation, either Cross A or Cross B. These are reciprocal crosses and produce the
complete range of fly genotypes. We recommend periodic fly checks where students must fill out forms documenting their progress with the crosses. Students must also show their *Drosophila* cultures when turning in the forms to complete the fly check. Students store flies (F1 and F2 generations) at -20°C in 1.5-mL microcentrifuge tubes which are later processed for DNA extraction and PCR analysis. During the time students are making their crosses through the F2 generation, students should maintain counts of phenotypic classes. These counts can be used for Chi Square calculations.

The genotypes and phenotypes for the two *Drosophila* crosses through the F2 generation are summarized in the figure at the bottom of the page.

**DNA Extraction – Preparation of Fly Squishing Buffer**

Label a 1.5-mL microcentrifuge tube “FSB” (for fly squishing buffer) and place on ice. In the order indicated, add the following components to the tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Distilled Water</td>
<td>1,320 µL</td>
</tr>
<tr>
<td>2. 10X TNE</td>
<td>150 µL</td>
</tr>
<tr>
<td>3. Proteinase K (10 mg/mL)</td>
<td>30 µL</td>
</tr>
<tr>
<td></td>
<td>1,500 µL Total</td>
</tr>
<tr>
<td></td>
<td>Volume (1.5 ml)</td>
</tr>
</tbody>
</table>

**Polymerase Chain Reaction (PCR)**

As outlined in the student handout, each student conducts a single PCR reaction using 4 µL of fly DNA lysate, and a 36-µL volume of all the other components that allow amplification of the DNA via PCR. This reaction uses more than two primers, therefore, is termed a “multiplex” PCR protocol (Dieffenbach and Dveksler, 2003).

For a class of 20 students, we provide the 36-µL amounts from a 25-reaction (900-µL) master mix. To prepare the master mix, we label a 1.5-mL microcentrifuge tube “MM” (for master mix), place on ice, and add the components in the order described in Table 2.

This is followed by directing the students to the instructor station where you add the 36 µL aliquot to each student’s tube. Students then return to their bench and add 4 µL of their fly DNA lysate to the tube followed by a brief microcentrifugation step (5 seconds). When all students have completed this step, the tubes are placed in the DNA Thermal Cycler 480 and File #50 is started.

The genotypes and phenotypes for the two *Drosophila* crosses through the F2 generation are summarized in the figure at the bottom of the page.
Table 2. Components for PCR master mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>1 Reaction Vol.</th>
<th>25-Reaction Master Mix Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Distilled Water</td>
<td>27.4 µL</td>
<td>x 25 = 685 µL</td>
</tr>
<tr>
<td>2. 10X PCR Buffer</td>
<td>4.0 µL</td>
<td>x 25 = 100 µL</td>
</tr>
<tr>
<td>3. MgCl₂, 50 mM</td>
<td>1.2 µL</td>
<td>x 25 = 30 µL</td>
</tr>
<tr>
<td>4. dNTPs, 10 mM</td>
<td>0.8 µL</td>
<td>x 25 = 20 µL</td>
</tr>
<tr>
<td>5. Primer P1, 2.5 µM</td>
<td>0.8 µL</td>
<td>x 25 = 20 µL</td>
</tr>
<tr>
<td>6. Primer P2, 10 µM</td>
<td>0.8 µL</td>
<td>x 25 = 20 µL</td>
</tr>
<tr>
<td>7. Primer P3, 10 µM</td>
<td>0.8 µL</td>
<td>x 25 = 20 µL</td>
</tr>
<tr>
<td>8. Tag DNA Polymerase (5 Units/µL)</td>
<td>0.2 µL</td>
<td>x 25 = 5 µL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>900 µL Total Volume</td>
</tr>
</tbody>
</table>

Table 3. Primer sequence information & \( T_m \) values.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Length</th>
<th>GC%</th>
<th>( T_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GTGCAAAGGTGGTGCAGAATT</td>
<td>20</td>
<td>45</td>
<td>58</td>
</tr>
<tr>
<td>P2</td>
<td>TCGGAGGATTCATGTCGACA</td>
<td>20</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>P3</td>
<td>GAGAGGAGTTTGCAGACAGC</td>
<td>20</td>
<td>55</td>
<td>62</td>
</tr>
</tbody>
</table>

We have presented the nucleotide sequences for the promoter regions of the wild-type and white-one alleles in Appendix A and B, respectively. We recommend using this information and the primer sequences to conduct computer-assisted PCR via the Primer3 (http://frodo.wi.mit.edu/primer3/) website (or a similar website) to verify the primer annealing sites and the size of the expected amplicons. Table 4 summarizes the primers and sequences to use to verify PCR product sizes of 467 and 704 bp for the wild-type and white-one alleles, respectively. For example, to confirm the PCR product associated with the wild type allele, go to the Primer3 website (do not change the default options) and import the sequence from Appendix A, followed by the sequences for the left (P1) and right (P3) primers from Table 2. To run Primer3 and to produce the output file showing the annealing sites and PCR product size, click on “Pick Primers.” Repeat these steps for the white-one allele using the information in Table 4.

Table 4. List of primers and sequences to use with Primer3 to confirm primer annealing sites and PCR product sizes.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Left Primer</th>
<th>Sequence</th>
<th>Right Primer</th>
<th>PCR Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>P1</td>
<td>Appendix A</td>
<td>P3</td>
<td>467 bp</td>
</tr>
<tr>
<td>white-one</td>
<td>P2</td>
<td>Appendix B</td>
<td>P3</td>
<td>704 bp</td>
</tr>
</tbody>
</table>

We also encourage you to conduct this activity with students because it introduces them to a useful bioinformatics tool that reinforces PCR concepts. Please note, however, that Primer3 will report \( T_m \) values slightly different from those reported in Table 3 because we employed the formula \( T_m \) (in °C) = 4(G + C) + 2(A + T) and Primer3 uses an alternative calculation for \( T_m \).

Preparation of Primer Stock Solutions (100 µM)

Primers were synthesized commercially (Invitrogen) and reconstituted to a final concentration of 100 µM in TE buffer (10 mM Tris, pH 7.2, 1 mM EDTA) followed by storage at -20°C. For the lab activity, primers P1, P2, and P3, were diluted to concentrations of 2.5, 10, and 10 µM, respectively, using distilled water and stored at -20°C.

Table 5 summarizes the volume of TE buffer added to each lyophilized primer to produce stock solutions with concentrations of 100 µM.

Table 5. Preparation of primers to yield stock solutions of 100 µM.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Quantity in tube (from Invitrogen)</th>
<th>Vol. TE buffer added to tube</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>27.62 nmoles</td>
<td>276 µL</td>
<td>100 µM</td>
</tr>
<tr>
<td>P2</td>
<td>26.26 nmoles</td>
<td>263 µL</td>
<td>100 µM</td>
</tr>
<tr>
<td>P3</td>
<td>28.66 nmoles</td>
<td>287 µL</td>
<td>100 µM</td>
</tr>
</tbody>
</table>

Gel Electrophoresis

1. Electrophoresis Buffer: Prepare 250 mL of 1X TAE buffer (pH 8.0) by mixing 5 mL of 50X TAE buffer with 245 mL of distilled water.
2. Gel Preparation: A 1.0% agarose gel is prepared by adding 0.25 g of agarose to 25 mL of 1X TAE buffer in a 125-mL Erlenmeyer flask. Bring to a boil by microwaving (1 minute). Swirl and pour into the gel unit that has a comb inserted. Allow to solidify (20 minutes).
3. Sample Preparation: Transfer 8 µL of each PCR reaction to a 1.5-mL tube followed by the addition of 2 µL of 5X DNA loading buffer. Mix briefly.

4. Gel Electrophoresis: Pour the remaining amount of 1X TAE buffer (225 mL) into the gel unit and remove the comb. Load each 10-µL sample into the appropriate well. Conduct electrophoresis for approximately 60-70 minutes at 80 volts (constant voltage). The bromophenol blue dye front should migrate about 3/4 the length of the gel.

5. Gel Staining: At the completion of electrophoresis, transfer the gel to a staining tray and add 100 mL of 1X TAE buffer followed by the addition of 5 µL ethidium bromide (10 mg/mL) [caution: ethidium bromide is a mutagen] or 10 µL SYBR® Safe stain (10,000X concentrate). Mix and incubate for 15 minutes. At the completion of staining step, photograph the gel using a UV-transilluminator.

6. Important Note: Handle ethidium bromide and SYBR® Safe stain, including waste disposal, in accordance with the safety regulations of your institution.

**Expected Results for PCR Genotyping Activity**

For a class of 20 students, we suggest dividing the class into three groups and running three DNA gels. It is important, of course, that students in each group have a variety of fly samples that will demonstrate the full range of fly genotypes.

The expected results for gel electrophoresis are summarized in the figure at the bottom of the page.

**Sample Student Results**

During the spring semester of 2010, Biology 203 students sampled and stored flies at -20ºC followed by genotyping via PCR the week of May 3rd as shown on the next page.

**Lessons Learned on the “Fly”**

- Fresh flies are preferable to using frozen flies, but often the timing of crosses does not allow students to use fresh flies, particularly from the F₁ generation. Therefore, be very sure that students properly label fly samples that are stored at -20ºC.
- If students are inexperienced using micropipettors, have them practice the proper technique for using micropipettors before they aliquot the small volumes used in this exercise.

---

**DNA fragment (size) standards**
**Major Workshop: PCR Genotyping *Drosophila White-one* Eye Mutation**

- Squishing the fly for DNA extraction is an important step that students often do not do properly. We have found that toothpicks DO NOT work well during the squishing step. There seems to be a chemical in the wood that interferes with the DNA extraction or the PCR process. When macerating the fly, do this vigorously and hold the 0.5 mL tube on a solid surface so more force can be used.

- To facilitate gel loading by students, we have found it is helpful to have students practice loading a “mock gel” before they load their sample. We use 1% agarose (or agar) in a Petri dish that holds an electrophoresis gel comb the same size as the one used for the gel to make the mock gel. After the mock gel is overlaid with water, students practice pipeting 1X DNA loading buffer into the wells.

### Results for Wednesday Lab Section

- **Lane** | **Description**
  | M 100-bp ladder (size marker) |
  1 | red-eye ♀ (heterozygous) |
  2 | white-eye ♀ |
  3 | red-eye ♂ |
  4 | red-eye ♀ (heterozygous) |
  5 | red-eye ♀ (heterozygous) |
  6 | white-eye ♂ |
  7 | white-eye ♂ |

### Results for Thursday Lab Section

- **Lane** | **Description**
  | M 100-bp ladder (size marker) |
  1 | red-eye ♂ |
  2 | red-eye ♀ (heterozygous) |
  3 | red-eye ♂ |
  4 | red-eye ♀ (homozygous) |
  5 | red-eye ♀ (heterozygous) |
  6 | white-eye ♂ |
  7 | white-eye ♂ |
Internet Resources

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlyBase</td>
<td>Database of Drosophila genes</td>
<td><a href="http://flybase.org/">http://flybase.org/</a></td>
</tr>
<tr>
<td>Primer3</td>
<td>Design of primers for PCR</td>
<td><a href="http://frodo.wi.mit.edu/primer3/">http://frodo.wi.mit.edu/primer3/</a></td>
</tr>
</tbody>
</table>

Acknowledgements

The development of this lab activity was supported by a Roberta Williams Laboratory Teaching Initiative Grant from the Association of Biology Laboratory Educators (ABLE). We thank our Biology 203 students and undergraduate teaching assistant, Dianna Watson, for helping us test this activity in the lab. We also express grateful appreciation to the college and university educators who attended our workshop at the 32nd ABLE Conference (Halifax, Nova Scotia), providing us with peer evaluations and constructive feedback for this activity. We also thank Life Technologies (Invitrogen) Corporation (Frederick, Maryland) for the generous donation of PCR-related supplies for our workshop presentation.

Literature Cited


About the Authors

Oney P. Smith is an Associate Professor of Biology at Hood College. His education includes a Ph.D. in Entomology from Texas A&M University, a M.S. in Entomology from the University of Maine, and a B.S. in Biology from the University of Vermont. In addition to teaching an introductory laboratory course for the Core Curriculum, “Newsstand Biology,” Oney teaches two laboratory courses for biology majors, “Introduction to Cell Biology and Genetics” and “Principles and Methods of Molecular Genetics.” His commitment to biology education also involves the active mentoring of undergraduate students on research projects, including the use of molecular genetic approaches to study the biology and gene expression strategies of aphid-transmitted luteoviruses.

Kathy F. Falkenstein is an Associate Professor of Biology and Department Chair at Hood College. She received her B.A. degree in Biology from Gettysburg College, a M.S. in Biology from West Virginia University and her Ph.D. in Botany from The Pennsylvania State University. For two years, Kathy held the position of Lecturer and Post Doctoral Research Associate at Princeton University. At Hood College, Kathy teaches a variety of laboratory biology courses including, “Secret Lives of Plants,” “Physiology of Plants and Animals,” and “Introduction to Cell Biology and Genetics.” She also developed a unique upper-level course for the Core Curriculum entitled, “Reaping the Harvest: Advances in Biotechnology and Global Agriculture.”
Appendix A

DNA Sequence for the Promoter Region of the Wild-type Allele

DNA sequence of the promoter region (abridged) for the wild-type allele (Xw+). This is a partial sequence (1,040 bp) obtained from Genbank accession X02974 and was used to select primers P1 and P3 for PCR by Primer3 analysis:

<table>
<thead>
<tr>
<th>P1 primer binding site - bases 171 to 189</th>
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</thead>
<tbody>
<tr>
<td>1  aaatcatgca tgcaaaagtac agtgcaaaccc cccgaaacgg gacgacaaca</td>
</tr>
<tr>
<td>51 ggcggattaa caagaacctt cttattcaccg ataagaagac gcctcccact</td>
</tr>
<tr>
<td>101 caacctaactc agtatctcaaa gacagccact cagctttatg atactgccca</td>
</tr>
<tr>
<td>151 ggtgggggtac tatcatatgg gtgcaaaagtg ggtcgaattt ttaattatct</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P3 primer binding site - bases 618 to 637</th>
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</thead>
<tbody>
<tr>
<td>201 tgaacggaac acatagctica aacataaaca tgggtctact agtatgtatg</td>
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</tr>
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<tr>
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</tr>
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<tr>
<td>801 agtagtacat gtcgatattg tttaaatttt cttcattatt atctttttagc</td>
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<td>851 attgcaacact ctaatttgtc gcggcgtgag aactgcgacc cacaattatc</td>
</tr>
<tr>
<td>901 cacaaccgca atgcacacaa ctaattatga cacaagacag aatttttccg</td>
</tr>
<tr>
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</tr>
<tr>
<td>1001 acatctaaag gcattcattt tcgactacat ttttttttac</td>
</tr>
</tbody>
</table>
Appendix B

DNA Sequence for the Promoter Region of the White-one Allele

DNA sequence for the promoter region (abridged) of the white-one allele (X<sup>wo</sup>). This is a partial sequence (5,784 bp) derived from Genbank accession X02974 and X17551 and was used to select primers P2 and P3 for PCR by Primer3 analysis:

<table>
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<tr>
<th>P1 primer binding site - bases 171 to 189</th>
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</thead>
<tbody>
<tr>
<td>P2 primer binding site - bases 4678 to 4697</td>
</tr>
<tr>
<td>P3 primer binding site - bases 5362 to 5381</td>
</tr>
<tr>
<td>Doc retroposon - bases 556 to 5280 (upper-case letters)</td>
</tr>
</tbody>
</table>

1  aaatcatgca tgcaaggtac a tgtgcaacc cccgaaacgg gacgacaaca
51  ggccgattaa caagaacctct cttattcagc ataagaagac gctttccact
101 caacctaatc agtatccaaaa gacagccact cagcttatga gtactgccca
151 ggtggggtctc tatactatgg gtgcaaggtt ggtcgaattt ttaattatgt
201 tgaacggaac acattagcta aacaataaca tgtgttcact agtatgtatg
251 taagtttaata aacccccccc tttggagaatg tagatttaaa aaacacatat
301 tttttttttat tttttactgc actggacatc attgaactta tctgatcagt
351 ttaatattta cttcgatceta agggatattt aagttccagg tcttttctgt
401 tacctctcac tcaaaatgac attccactca aagtcacgc ttttttcctc
451 cttctctgct gacaggaata tcgctgcttc tttgccgcgt gcgtccgcta
501 tctcttttgc caccgtttttt agcgttacct agcgtcagtc tcaatgtcgc
551 ccttcGACAT TCGGCAATCC ACAGTCTTTCG GGTGGAGACG TGTTTCTTTTC
601  AAGCTACGAA TAGCAAGTTC TAAAAACTAC AACAGTATAG TGAAAGTTAA
651  ACACAAAGTG TAAAGGCGAT CTTGCACTAC TAAACATAG TGAATATTGTT
701  AATTATTTAC TAAAATAAAT AAATATTCCA TATTTTGCTG GTAATTGTTA
751  TATGTGACCT TAGACAAARG AATCAAACAG AATAAATTTG CTCGTATGT
801  TGTGAAACTG AGGAGCAGCG GCTTCTCCTTA CAACGCAACA ATGCAACTT
851  TCTTTTGCCT TCACCGCAAA TCGGTGACCT AGCCACCTCA CTTTCAACTA
901  ACTCGAAACT TTTGCCCTCA GCGAAGCACA GACCGCGTTC TTGCTCTCCC
951  TCTCTGACCT TAACCGGCTCA AAGTCTGTTG AGCAGGAGAG CCACCTCTCC
1001 TACCCCGCTC CTCTCGGGAC GCAAAACGAC GTGCGGGGS AACTGGAACA
1051 CTGCAATAAAC GAGTGCAGTG ACCTCAGCT CTGGCCGACA AATGATGAC
1101 TCCTCGCAGG CCAACTAAT TATCGCTGTTT AGCACTGAA ACAATTAGAC
1151 AGACTGCAAC GTGTCGCAAG ACAATAATGC ACGTAAAGAA GAATCAAAA
1201 AAAAGCAGAA GTCGATTTTC ACTTGCAGAG TCTGCAAGT TCTGCAACTC

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1251 CAAATCAAGA GAAAGCTCAG CCCCCAAAAC AATAAGGCAG GTAATCAACC
1301 CAAAATCAAT CGAACCACA ACAGGCAATGA AAACCTCTGCA GTAATAATT
1351 CAAACCGATA TGCTATCTTG GCTGATTCTG CGACCGAACA ACCCAACGAA
1401 AAAACGCTAG GGAACCAAAA AAAGACCAGG CCTCCACCAA TTTTCATACG
1451 AGAACAATAGT ACAATGCAAC TTTGAAAAAA ACTCAGTGCT TGTATTGAGT
1501 ACAGCAAGTT CCCACATTAC CCACCTAAAAA AAGGAAATAT TCATGAAATA
1551 AAACCTACAG ACAAACACGA AGGACAGCAG CGTATAGTGA CTAAATACCT
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1701 ATAAATGAGG CTCTGAAAGGC CAAAAACTTT TCTGCAAAAAG CAGCTATTTA
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1851 TTACAGTACT TGCTACATCG GAGGATCACC GTGGAGGAGG CGCAAAAACG
1901 TATCAATCCA GTCGATGATA CTATGTGCCA AAGATACGGG CACACAAAGG
1951 CATCACTGCAC CTTAAATGCC GTATGTTTGG TCTGAGCGA ACCCTCATCT
2001 ACCGCAAACCT GCCCCAAAAA CAAGGAGGT AAGTCTCTTG AAGATACGGG
2051 TAATGTGGGG GAAAAACACT CTGCACATAA CAGAGGCTGT GTGGTGATAC
2101 AAGAATTTGA GAGCAGGCTTA AACAAATCAG TTTGCCAGAC GCATACATAC
2151 AACAAAGGGA ATTTTCTACTC TCCGCAACGG ATTTTTCACC CACCCCCAAC
2201 TGTCGCAAGC ACTACTCCAA CAATTTCTTT CGCTGAGCGC CTAAAAACCCG
2251 GACTAGAAGT GCCCGCCTCC CCGACAGAGA CTGTGCTCTC CGAACATACA
2301 CGCACAACACA TCCAAACAAA ACAAGAACGT GGCATCGAAG CTATGAGGCT
2351 ATCCCTACAG CAAAGCATGA AAGATCTCAT GACGTTCTAG CAAAATACCT
2401 TGCAAGAGCT CATGAAAACC CAAAATATCC TGAATCCTAC TCAATGTATCT
2451 TCAAAATCCC CTATGGCTG TCCCTACGGA TATCTCTTGG QAACGCAAAT
2501 GGCAGTACGC GGCATACAAG AAGATCACA CAGATTTTT CAGGGAAAAA
2551 CATCGAGCTA ATGTCACTAT CATGAAAGGC CTCACAAAAA AAAAACAAATT
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2651 GCTCATGGAAG GCATCTGGAAT ACTCATCAAG ATATCGAATA AACACCACCA
2701 CTTAAACAAAT TTTGCCAAGA ACTACTTACA ATCTAGGTCC ATAGCCTTAC
2751 AACCTAACAA TGTCTCCAGG ACTCTAGCGG CAGTCTACTG CCCACCGCGC
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```
4351  TTGCGCAATC GAAGCTGGAG TGCCGCAAGG CAGTGTACTG GGTCCAATCT
4401  TATACACCCCT GTATACGCGG GATTTCCTCA TAGACTACAA TCTAACAACC
4451  TCCACGTTCG CTTAGGTGAT CCGGATACTC AGTGCCTCGA AATGACCAAT
4501  AAAAAACCACGC CACTCCTCAT CCCGACACTT AACATCTGTA GAACGATGGC
4551  TTGCGCAGCTG GAGAATTTCA ATAATGTTC AAAAATGCAA GCAGGTACC
4601  TTTACCTTTAA ACAAAACAAAC ATGCCCACAAC CTGCTTTCTGA ATAACATATG
4651  CATTCCACCAA GCGGAGCGAG GAACTATATCT GGGAGTTTCAT CTGGACAGGC
4701  GGCTCACTTG GCGCAAAACT ATAGAAGCCAA AATCGGAACA TCTTTAACCT
4751  AAAGCAAGGA ACCTCCACTG GCCTCAAATT GCTCGCTCTC CACTTAGCTC
4801  GGAGTTCAAAC GCTTTTCTAT ACAACTCCGT CTTTAAACCT ATCTGGACTT
4851  ATGGCTCTCGA GTCTGTGGGGG AAGCAGATCAA GAAGTAACAT AGACATTATT
4901  CAGCGAGCACC GCTCAAGAATT CTGAGAAATT ATCAGCTCGAG CGCGGTGGTA
4951  CTTGCGAACA GAAAACATAC ACAGAGACCT AAAATCAAAC TTAGTAACCG
5001  AAGTAATAGCA TGAGAAAAAAA ACGAGATATA ACGAAAAGCT GACCACCACAT
5051  ACAAAATCCCA TGCGAGAGAAA ACTAATCACA GTATGCAGTC AAAGCCGGCT
5101  GCACCGCAAC GACCTCCAG CCCAGCAAATA AACTTATTAG GGCATTAATG
5151  AAAAAAAAAAA ACTATCATAA AGTAGAAGTTT AATTAGTGTA GATCAGATT
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5251  TAAAAATTTAA AAAAAAAA AAAAAAAA AAAAAAAA atatgctccct ttcagttgca
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5351  aacacaaag aatgagttgca aatcctctct cgctttcttat ttttttttgt
5401  ttttctctgtg tattggttt tgggtggtgtga agcaggagga
5451  agtctgtgaa aatcccgctta aagggcagga ggctcaggag ctataatttc
5501  gcggagggcag cacaacaccn tctgcgctcg acctgaacaa tttgtgagtt
5551  acatgctctct atcttttaag ttcactttga ctataggaac ttcgattggca
5601  acatcataatt ggtctgcggcg cggacacagca acacaccaac aatcccgacac
5651  cgcaatcgcg cacaacacatt gttgaacgga acagattatt cggctgtctg
5701  tggctgctatt ataagacact ttttaagatc atatcatgat caagacactt
5751  aaagccatc attttcgact atatcttttt ttac
```
Appendix C

Supplier Addresses

Carolina Biological Supply Co.
2700 York Road
Burlington, North Carolina 27215
Phone: 800-334-5551
Website: http://www.carolina.com

Invitrogen Corp.
5791 Van Allen Way
P.O. Box 6482
Carlsbad, California 92008
Phone: 800-955-8288
Website: http://www.invitrogen.com

Whatman, Inc.
Building 1
800 Centennial Avenue
Piscataway, New Jersey 08854
Phone: 800-942-8626
Website: http://www.whatman.com

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