EXPERIMENT OBJECTIVE:

The objective of this experiment is to introduce students to Drosophila genotyping using the Polymerase Chain Reaction.

This experiment is designed for DNA staining with InstaStain® Ethidium Bromide.
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Material Safety Data Sheets can be found on our website:
www.edvotek.com
Experiment Components

Experiment # 337 contains material for up to 10 lab groups.

Sample volumes are very small. It is important to quick spin the tube contents in a microcentrifuge to obtain sufficient volume for pipetting. Spin samples for 10-20 seconds at maximum speed.

**NOTE:**

*Drosophila must be requested two weeks in advance.*

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**Component**

<table>
<thead>
<tr>
<th>A</th>
<th>Tubes with PCR EdvoBeads™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Each PCR EdvoBead™ contains</td>
<td></td>
</tr>
<tr>
<td>• dNTP Mixture</td>
<td></td>
</tr>
<tr>
<td>• Taq DNA Polymerase Buffer</td>
<td></td>
</tr>
<tr>
<td>• Taq DNA Polymerase</td>
<td></td>
</tr>
<tr>
<td>• MgCl₂</td>
<td></td>
</tr>
<tr>
<td>• Reaction Buffer</td>
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<table>
<thead>
<tr>
<th>B</th>
<th>Primer mix concentrate</th>
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<table>
<thead>
<tr>
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<table>
<thead>
<tr>
<th>D</th>
<th>Control DNA concentrate</th>
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<table>
<thead>
<tr>
<th>E</th>
<th>TE buffer</th>
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<table>
<thead>
<tr>
<th>F</th>
<th>Proteinase K</th>
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<table>
<thead>
<tr>
<th>G</th>
<th>Potassium Acetate</th>
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<tbody>
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</table>

<table>
<thead>
<tr>
<th>H</th>
<th>DNA extraction buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Store at Room temperature</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Components B and D are now supplied in concentrated form.

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**Reagents & Supplies**

Store all components below at room temperature.

**Component**

- Wild and White *Drosophila* *(request 2 weeks in advance)*
- UltraSpec-Agarose™
- Electrophoresis Buffer (50x)
- 10x Gel Loading Solution
- InstaStain® Ethidium Bromide
- FlashBlue™ Liquid Stain
- Conical tube (15 ml)
- Microcentrifuge Tubes
- PCR tubes (0.2 ml - for thermal cyclers with 0.2 ml template)
- Calibrated transfer pipets
- Wax beads (for waterbath option or thermal cyclers without heated lid)

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All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.
**Experiment Requirements** *(NOT included in this experiment)*

*If you do not have a thermal cycler, PCR experiments can be conducted, with proper care, using three waterbaths. However, a thermal cycler assures a significantly higher rate of success.*

- Thermal cycler (EDVOTEK Cat. # 541 highly recommended) or three waterbaths*
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- Waterbath (70°C) (EDVOTEK Cat. # 539 highly recommended)
- UV Transilluminator or UV Photodocumentation system *(use if staining with InstaStain® Ethidium Bromide)*
- UV safety goggles
- White light visualization system *(optional - use if staining with FlashBlue™)*
- Automatic micropipets (5-50 µl) with tips
- Microwave or hot plate
- Pipet pump
- 250 ml flasks or beakers
- Hot gloves
- Disposable laboratory gloves
- Distilled or deionized water
- Ice buckets and ice
- Isopropanol

*If you do not have a thermal cycler, PCR experiments can be conducted, with proper care, using three waterbaths. However, a thermal cycler assures a significantly higher rate of success.*
WHY STUDY DROSOPHILA?

A model organism is any plant, animal or microorganism that allows us to study fundamental questions in biology that may be hard to study directly in complex organisms like humans. For almost a century, the fruit fly *Drosophila melanogaster* has been a valuable model organism for research in genetics, developmental biology and evolutionary biology. In the early 1900’s, Thomas Hunt Morgan used the fruit fly to illustrate one of the core principles of modern genetics – the linkage of a gene with a particular chromosomal location. For this groundbreaking work in genetics, Morgan was awarded the Nobel Prize in Physiology or Medicine.

Fruit flies were initially chosen as a model organism because they have a simple genome, a fast generation time, and they breed in large numbers. A new generation of fruit flies can be produced every two weeks (Figure 1). True-breeding fruit flies are put together in a culture vial and allowed to mate. *Drosophila* larvae will hatch twenty-four hours after the female fly deposits fertilized eggs on special growth media. The larvae will then molt one, two, and four days after hatching, increasing in size each time. After the third larval molt, the fly pupates for four days. After metamorphosis, *Drosophila* emerges from the pupa as a winged adult with brick red eyes and yellow-brown bodies with black rings on the abdomen. Adults are fertile after two days, which means that these flies can quickly be used for further genetic studies.

![Figure 1: The *Drosophila* life cycle. Times are approximate for flies being cultured at 25°C.](image)
GENETIC MUTANTS IN FLIES

Today, the entire Drosophila genome has been completely sequenced and several thousand genetic mutants are available for study. This allows scientists to correlate changes at the DNA level with changes in phenotype. Many genes found to be crucial in human health and development are conserved in Drosophila. In fact, about 75% of the genes that cause disease in humans have homologs in the fruit fly. This makes the humble fruit fly an important model system in today’s research laboratory.

The wide array of Drosophila mutants available to geneticists has been generated through many different means, including X-rays, mutagenic chemicals and mobile genetic elements known as transposons. There are many different types of transposons found in the Drosophila genome, including P-elements, Doc, copia and gypsy. When transposons “jump” into relatively random sites throughout the Drosophila genome, they can disrupt genes – in fact, transposons account for over 50% of spontaneous mutations identified in Drosophila!

The Doc element belongs to a group of retrotransposons known as long interspersed elements, or LINEs, which are found in abundance in eukaryotic genomes. Like other retrotransposons, the Doc element is believed to “hop” through the Drosophila genome using an RNA intermediate (Figure 2). In brief, the Doc element is transcribed into mRNA using the fly’s native DNA polymerase. The Doc mRNA codes for an enzyme called Reverse Transcriptase (RT), which can synthesize a strand of DNA using an RNA template. After the RT mRNA is translated in the cytoplasm, the protein is transported back into the cell’s nucleus, where it converts the Doc mRNA into DNA. The newly synthesized DNA integrates at a new location in the genome, while the original copy of Doc transposon remains at the same genomic location. For this reason, eukaryotic genomes often contain thousands of copies of LINE retrotransposons.
LINKING GENES TO CHROMOSOMES

Thomas Hunt Morgan used eye color mutants to discover the relationship between genes and chromosomes. While sorting through his flies, he identified a single white-eyed male among the red-eyed flies (Figure 3). This mutation, called white-1, was the first genetic mutant identified in *Drosophila*.

Morgan crossed the white-eyed fly with true-breeding red-eyed female flies. All of the flies in the first filial generation (or F1) had red eyes, suggesting that white allele was recessive to the red allele (summarized in Figure 4). To confirm this, Morgan crossed male and female flies from the F1 generation. The second generation (second filial, or F2) observed the classical Mendelian ratio of three red-eyed flies for every one white-eyed fly. However, all of the female flies in the second generation were red-eyed. Half of the males were red-eyed, while the rest had white eyes like the original mutant. From this data, Morgan reasoned that the gene responsible for the white color of the eye was located on the X-chromosome, providing the first evidence of a gene being linked to a specific chromosome.

**DEFINITIONS:**

*Allele* – alternative forms of the same gene

*Dominant allele* – produces the same phenotype whether heterozygous or homozygous.

*Recessive allele* – produces a phenotype only when homozygous.
Since white is a sex-linked gene, males will exhibit the white-eyed phenotype with one copy of the white-1 mutant, as they have one X chromosome and one Y chromosome. Females must have two copies of the recessive allele to see the white-eyed phenotype. As such, crosses between white-eyed females and males will only produce white eyed offspring (Figure 5). In contrast, crossing white-eyed females with wild-type males will result in female offspring with red eyes and males with white eyes (Figure 6).

**EYE COLOR DETERMINATION IN DROSOPHILA**

Today, scientists have shown that eye color in Drosophila results from a combination of pigments known as the pteridines and the ommochromes. Two separate enzymatic pathways work in concert to synthesize the pigments from amino acid precursors. The pteridine pathway converts the tryptophan into a bright red pigment and the ommochrome pathway converts the guanine into a brownish pigment (Figure 7). The white gene codes for a special transporter protein that moves the precursor amino acids into the developing eyes, allowing the pigments to be synthesized.

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**Figure 5:**
Cross between white-eyed female and male flies.

**Figure 6:**
Cross between white-eyed female and red-eyed male flies.

**Figure 7:**
Current Model of Eye Color Determination in Drosophila.
The *white-1* mutation initially described by Morgan was caused by the insertion of the 4.4 kilobase pair (kb) *Doc* transposon into the promoter region of the white gene. The large transposon blocks transcription of the messenger RNA, meaning that the transporter protein cannot be produced. Without this protein, neither of the pigments can be synthesized, so *white-1* flies have white colored eyes. Because of this, *white* is considered to be the master eye color gene.

**USING PCR TO IDENTIFY MUTATIONS**

In 1984, Dr. Kary Mullis revolutionized the field of molecular biology when he devised a simple and elegant method to copy specific pieces of DNA. Recognizing that an initial step in DNA replication in a cell’s nucleus is the binding of RNA primers, Mullis discovered that he could replicate DNA in vitro using short, synthetic DNA primers and DNA Polymerase I. Furthermore, because researchers can specify a primer’s sequence to target a specific gene, this method allowed for the rapid amplification of a selected DNA sequence *in vitro*. For the development of this technique, known today as the Polymerase Chain Reaction (or PCR), Mullis was awarded the Nobel Prize in Chemistry in 1993.

Because of its ease of use and its ability to rapidly amplify DNA, PCR has become indispensable in life sciences labs, replacing the time-intensive Southern blot as the method of choice to characterize genetic differences at the molecular level. To perform PCR, purified double-stranded DNA is mixed with primers (short synthetic DNA molecules that target DNA for amplification), a thermostable DNA polymerase (*Taq*) and nucleotides. The mixture is heated to 94°C to denature the DNA duplex (i.e., unzip it into single strands). Next, the sample is cooled to 45°C-60°C, allowing the primers to base pair with the target DNA sequence (called “annealing”). Lastly, the temperature is raised to 72°C, the optimal temperature at which *Taq* polymerase will extend the primer to synthesize a new strand of DNA. Each “PCR cycle” (denaturation, annealing, extension) doubles the amount of the target DNA in less than five minutes (summarized in figure 8). In order to produce enough DNA for analysis, twenty to forty cycles may be required. To simplify this process, a specialized machine, called a “thermal cycler” or a “PCR machine”, was created to rapidly heat and cool the samples to the designated temperature for each of the three steps.

PCR is a simple, fast and reliable method to identify specific genetic mutations. For example, wild type and *white-1* flies can be distinguished using primers that flank the *Doc* element present in the white promoter. After PCR amplification, DNA extracted from flies without the *Doc* transposon will produce a 220 base pair (bp) amplicon, whereas DNA extracted from *white-1* flies will produce a 4.6 kb amplicon. However, since *Taq* polymerase is not efficient at amplifying DNA fragments greater than 2 kb, this large fragment is not usually produced. An unlinked gene coding for a wing protein is simultaneously amplified as a positive control; this PCR product is 1000 bp.
Figure 8: Polymerase Chain Reaction
**Experiment Overview and General Instructions**

**EXPERIMENT OBJECTIVE:**

The objective of this experiment is to introduce students to *Drosophila* genotyping using the Polymerase Chain Reaction.

**IMPORTANT**

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.

**GEL SPECIFICATIONS**

This experiment requires a gel with the following specifications:

- **Recommended gel size**: 7 x 14 cm (long tray)
- **Number of sample wells required**: 4
- **Placement of well-former template**: first set of notches
- **Gel concentration required**: 1.0%

**NOTE:** Experimental times are approximate.
Module I: Isolation of *Drosophila* DNA

1. **LABEL** one 0.5 ml screw-cap tube with “wild” and a second 0.5 ml screw-cap tube with “white”.

2. **ANESTHETIZE** the fruit flies by placing the vials in the freezer for one to two minutes.

3. **TRANSFER** one wild type fly from the vial to the tube labeled “Wild”. Using a clean pipet tip or toothpick, **MASH** the fly in the tube (about 10 seconds).

4. Using a fresh pipet tip, **ADD** 100 µl of the DNA extraction buffer to the fly. **MIX** the sample by pipetting up and down.

5. **PLACE** the “Wild” tube on ice. **REPEAT** steps 3-4 with the White-eyed flies using fresh tubes and tips.

6. **INCUBATE** the samples in a 70° C waterbath for 15 minutes.

7. **ADD** 14 µl of potassium acetate to each sample and **MIX** for 5 seconds.

8. **INCUBATE** on ice for 5 minutes.

9. **CENTRIFUGE** the samples at maximum speed for 5 minutes.

10. Carefully **TRANSFER** the supernatant to clean, labeled 0.5 ml snap-top microcentrifuge tubes. Avoid the pellet at the bottom of the tubes. After removing the supernatant, **DISCARD** the tubes with the pellet.

**WARNING!**
Use only screw-cap tubes when incubating in the waterbath for DNA isolation. Do not use snap-top tubes.
Module I: Isolation of *Drosophila* DNA

11. **ADD** 45 µl of room temperature isopropanol to each sample to precipitate the DNA.

12. **CENTRIFUGE** the samples at maximum speed for 5 minutes.

13. After centrifugation, a very small DNA pellet should be visible at the bottom of the tube. With a marker, **CIRCLE** the location of the DNA pellet.

14. Carefully **REMOVE** and **DISCARD** the supernatant. Take care to avoid the DNA pellet while removing the supernatant. Carefully **WASH** the pellet with 20 µl of 70% ETOH.

15. **CENTRIFUGE** the tubes at maximum speed for 2 minutes.

16. Carefully **REMOVE** and **DISCARD** the supernatant. **ALLOW** the pellet to completely dry (5-10 minutes)

17. **RESUSPEND** the DNA pellet in 25 µl of TE buffer.

18. **PLACE** tubes in ice. **PROCEED** to Module II: Amplification of the Extracted DNA.

**OPTIONAL STOPPING POINT:**
The extracted DNA may be stored at -20°C for amplification at a later time.
Module II: Amplification of the Extracted DNA

PCR REACTION:

1. **LABEL** two PCR tubes "wild" and "white". If assigned control DNA, **LABEL** a third PCR tube "control".
2. To each tube, **ADD** one PCR EdvoBead™, 20 µL Primer Mix, and 5 µl extracted DNA (either Wild, White, or Control DNA).
3. **MIX** the PCR samples gently. Make sure the PCR EdvoBeads™ are completely dissolved in each tube.
4. **CENTRIFUGE** to collect the samples at the bottom of the tubes.
5. **AMPLIFY** DNA using PCR:
   - **PCR Cycling Conditions**:
     - Initial denaturation 94°C for 2 minutes
     - 94°C for 45 seconds
     - 55°C for 45 seconds
     - 72°C for 90 seconds
     - 35 cycles
     - Final Extension 72°C for 5 minutes
6. **ADD** 5 µl of 10x Gel Loading Solution to the samples.
7. **STORE** on ice until ready for electrophoresis. **PROCEED** to Module III: Separation of PCR products by Electrophoresis.

**NOTES AND REMINDERS:**

This kit includes enough DNA to set up 3-4 control reactions. At least one control reaction should be performed per class to confirm that PCR was successful.

If your thermal cycler does not have a heated lid, it is necessary to overlay the PCR reaction with wax to prevent evaporation. See Appendix B for guidelines.

**OPTIONAL STOPPING POINT**

The PCR samples may be stored at -20°C for electrophoresis at a later time.
Module III: Separation of PCR Products by Electrophoresis

1. DILUTE concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
2. MIX agarose powder with 1X buffer in a 250 ml flask (see Table A).
3. DISSOLVE agarose powder by boiling the solution. MICROWAVE the solution on high for 1 minute. Carefully REMOVE the flask from the microwave and MIX by swirling the flask. Continue to HEAT the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. COOL agarose to 60° C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, SEAL the ends of the gel-casting tray with the rubber end caps. PLACE the well template (comb) in the appropriate notch.
6. POUR the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. REMOVE end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

NOTES:
7 x 14 cm gels are recommended. Each gel can be shared by 4 students. Place well-former template (comb) in the first set of notches.
If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at www.edvotek.com

<table>
<thead>
<tr>
<th>Size of Gel Casting tray</th>
<th>Concentrated Buffer (50x)</th>
<th>Distilled Water</th>
<th>Amount of Agarose</th>
<th>TOTAL Volume</th>
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<td>7 x 14 cm</td>
<td>1.0 ml</td>
<td>49.0 ml</td>
<td>0.50 g</td>
<td>50 ml</td>
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</table>

Module III: Separation of PCR Products by Electrophoresis

8. PLACE gel (on the tray) into electrophoresis chamber. COVER the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.

9. LOAD the entire sample (30 µL) into the well. RECORD the position of the samples in Table 1, below.

10. PLACE safety cover. CHECK that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.

11. CONNECT leads to the power source and PERFORM electrophoresis (See Table C for time and voltage guidelines). After electrophoresis is complete, REMOVE the gel and casting tray from the electrophoresis chamber and proceed to STAINING the agarose gel.

Table 1

<table>
<thead>
<tr>
<th>Lane</th>
<th>Recommended</th>
<th>Sample Name</th>
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<tbody>
<tr>
<td>1</td>
<td>200 bp ladder</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control PCR Product</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>White PCR Product</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Wild-type PCR Product</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
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Table B

<table>
<thead>
<tr>
<th>EDVOTEK Model #</th>
<th>Total Volume Required</th>
<th>50x Conc. Buffer + Distilled Water</th>
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<tr>
<td>M6+</td>
<td>300 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>M12</td>
<td>400 ml</td>
<td>8 ml</td>
</tr>
<tr>
<td>M36</td>
<td>1000 ml</td>
<td>20 ml</td>
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Table C

<table>
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<tr>
<th>Volts</th>
<th>Minimum</th>
<th>Recommended Time</th>
<th>Maximum</th>
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<tbody>
<tr>
<td>125</td>
<td>55 min.</td>
<td>1 hour 15 min.</td>
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</tr>
<tr>
<td>70</td>
<td>2 hours 15 min.</td>
<td>3 hours</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3 hours 25 min.</td>
<td>5 hours</td>
<td></td>
</tr>
</tbody>
</table>
Module IV-A: Staining Agarose Gels using InstaStain® Ethidium Bromide

1. Carefully REMOVE the agarose gel and casting tray from the electrophoresis chamber. SLIDE the gel off of the casting tray on to a piece of plastic wrap on a flat surface.

DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.

2. MOISTEN the gel with a few drops of electrophoresis buffer.

3. Wearing gloves, REMOVE and DISCARD the clear plastic protective sheet from the unprinted side of the InstaStain® card(s). PLACE the unprinted side of the InstaStain® Ethidium Bromide card(s) on the gel. You will need 2 cards to stain a 7 x 14 cm gel.

4. With a gloved hand, REMOVE air bubbles between the card and the gel by firmly running your fingers over the entire surface. Otherwise, those regions will not stain.

5. PLACE the casting tray on top of the gel/card stack. PLACE a small weight (i.e. an empty glass beaker) on top of the casting tray. This ensures that the InstaStain® Ethidium Bromide card is in direct contact with the gel surface. STAIN the gel for 3-5 min. for an 0.8% gel or 8-10 min. for a gel 1.0% or greater.

6. REMOVE the InstaStain® Ethidium Bromide card(s). VISUALIZE the gel using a long wavelength ultraviolet transilluminator (300 nm). DNA should appear as bright orange bands on a dark background.

BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!
**Module IV-B: Staining Agarose Gels using FlashBlue™**

1. **DILUTE** 10 ml of 10x concentrated FlashBlue™ with 90 ml of water in a flask and **MIX** well.

2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray into a small, clean gel-staining tray.

3. **COVER** the gel with the 1x FlashBlue™ stain solution. **STAIN** the gel for 5 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 5 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**

4. **TRANSFER** the gel to a second small tray. **COVER** the gel with water. **DESTAIN** for at least 20 minutes with gentle shaking (longer periods will yield better results). Frequent changes of the water will accelerate destaining.

5. **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

**Alternate Protocol:**

1. **DILUTE** one ml of concentrated FlashBlue™ stain with 149 ml dH₂O.
2. **COVER** the gel with diluted FlashBlue™ stain.
3. **SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
4. **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.
Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. What are some advantages of using *Drosophila* to study genetics?

2. What is a sex-linked gene? How do we know that the *white* gene is sex-linked?

3. What is a retrotransposon? How does the *Doc* retrotransposon affect eye color in *Drosophila*?

4. Determine the genotype and the phenotype of the offspring generated by a cross between a white-eyed male fly and a true-breeding red-eyed female fly.
Instructor’s Guide

OVERVIEW OF INSTRUCTOR’S PRELAB PREPARATION:
This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

<table>
<thead>
<tr>
<th>Preparation For:</th>
<th>What to do:</th>
<th>When:</th>
<th>Time Required:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Module I: Isolation of Drosophila DNA</td>
<td>Prepare and aliquot various reagents (potassium acetate, Lysis buffer)</td>
<td>Up to one day before performing the experiment. IMPORTANT: Prepare the Lysis buffer no more than one hour before performing the experiment.</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>Equilibrate waterbath at 70 ° C.</td>
<td>One hour before performing the experiment.</td>
<td>15 min.</td>
</tr>
<tr>
<td>Module II: Amplification of Extracted DNA</td>
<td>Prepare and aliquot various reagents (Primer, DNA template, ladder, etc.)</td>
<td>One day to 30 min. before performing the experiment.</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>Program Thermal Cycler</td>
<td>One hour before performing the experiment.</td>
<td>15 min.</td>
</tr>
<tr>
<td>Module III: Separation of PCR Product by Electrophoresis</td>
<td>Prepare diluted TAE buffer</td>
<td>Up to one day before performing the experiment.</td>
<td>45 min.</td>
</tr>
<tr>
<td></td>
<td>Prepare molten agarose and pour gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Module IV: Staining</td>
<td>Prepare staining components</td>
<td>The class period or overnight after the class period.</td>
<td>10 min.</td>
</tr>
</tbody>
</table>

NOTE:
PCR Cycling Conditions have changed. Please review your PCR program before performing Module II.
Pre-Lab Preparations: Module I - Isolation of Drosophila DNA

**FOR MODULE I**
Each Group should receive:
- Two 0.5 ml screw cap microtest tubes
- Calibrated transfer pipet
- 0.25 ml DNA Extraction Buffer
- 40 µl potassium acetate
- 70 µl TE buffer

Reagents to be Shared by the Class:
- Vial of white flies
- Vial of wild type flies
- Isopropanol

**PREPARATION OF LYSIS BUFFER**
(Prepared no more than one hour before starting the experiment)

1. Add 200 µl of the DNA Extraction Buffer (H) to the tube of lyophilized Proteinase K (F); cap the tube.
2. Allow the sample to hydrate for a minute and vortex and mix completely to dissolve the enzyme.
3. Add all of the rehydrated Proteinase K solution to the remaining DNA Extraction buffer and mix well.
4. Dispense 0.25 ml per group and store on ice or frozen until ready to use.

**Additional Preparations:**

1. Dispense 40 µl of potassium acetate (G) per group into labeled tubes.
2. Dispense 70 µl of 1x TE buffer (E) per group into labeled tubes.
3. Obtain isopropanol.
4. Designate at least one of the groups to use the control DNA to perform a control PCR reaction.

**Warning !!**
Remind students to only use screw-cap tubes when heating their DNA samples. The snap-top tubes can potentially pop open and cause injury.
Pre-Lab Preparations: Module II - Amplification of the Extracted DNA

Preparation of the *Drosophila* Primer Solution

1. Thaw the *Drosophila* Primer Solution on ice.
2. Add 1 ml of TE Buffer (E) to the tube of Primer Mix Concentrate (B). Cap tube and mix.
3. Aliquot 50 µl of the diluted Primer Mix into 13 labeled microcentrifuge tubes.
4. Distribute one tube of diluted *Drosophila* Primer Solution to each student pair.

Preparation of the Control DNA

1. This kit includes enough DNA to set up 4 control reactions. At least one control reaction should be performed per class to confirm that PCR was successful.
2. Thaw the tube of Control DNA Concentrate (D) on ice.
3. Add 20 µl of TE buffer (E) to the tube containing Control DNA Concentrate. Pipet up and down to mix.
4. Dispense 7 µl of the diluted control DNA for each control reaction.

PCR AMPLIFICATION

The Thermal cycler should be programmed for the following cycles.

<table>
<thead>
<tr>
<th>Initial Denaturation</th>
<th>35 cycles @</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C for 2 min.</td>
<td>94°C for 45 seconds</td>
<td>72°C for 5 min.</td>
</tr>
<tr>
<td>55°C for 45 seconds</td>
<td>55°C for 45 seconds</td>
<td></td>
</tr>
<tr>
<td>72°C for 90 seconds</td>
<td>72°C for 90 seconds</td>
<td></td>
</tr>
</tbody>
</table>

At the completion of the cycling, the thermal cycler can be programmed to hold the samples at 4°C overnight.

**NOTE:**

PCR Cycling Conditions have changed. Please review your PCR program before performing Module II.
Pre-Lab Preparations - Module III: Separation of PCR Product by Electrophoresis

PREPARATION OF AGAROSE GELS

This experiment requires one 1.0% agarose gel per student group. For best results, we recommend using a 7 x 14 cm gel. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Individual Gel Preparation:
Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Module III in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water and agarose powder.

Batch Gel Preparation:
To save time, a larger quantity of agarose solution can be prepared for sharing by the class. See Appendix C.

Preparing Gels in Advance:
Gels may be prepared ahead and stored for later use. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks.

Do not freeze gels at -20º C as freezing will destroy the gels.

Gels that have been removed from their trays for storage should be “anchored” back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

Additional Materials:
Each 1.0% gel should be loaded with the 200 base pair ladder and samples from 1 or 2 students. The control PCR reaction can also be loaded in one of the wells.

• Aliquot 30 µl of the 200 base-pair ladder (C) into labeled microcentrifuge tubes and distribute one tube of ladder per gel.
Pre-Lab Preparations - Module IV: Staining

STAINING WITH INSTASTAIN® ETHIDIUM BROMIDE

InstaStain® Ethidium Bromide provides the sensitivity of ethidium bromide while minimizing the volume of liquid waste generated by staining and destaining a gel. An agarose gel stained with InstaStain® Ethidium Bromide is ready for visualization in as little as 3 minutes! Each InstaStain® card will stain 49 cm² of gel (7 x 7 cm). You will need 2 cards to stain a 7 x 14 cm gel.

Use a mid-range ultraviolet transilluminator (Cat. #558) to visualize gels stained with InstaStain® Ethidium Bromide. BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!

- Standard DNA markers should be visible after staining even if other DNA samples are faint or absent. If bands appear faint, repeat staining with a fresh InstaStain card for an additional 3-5 min. If markers are not visible, troubleshoot for problems with electrophoretic separation.
- Ethidium bromide is a listed mutagen. Wear gloves and protective eyewear when using this product. UV protective eyewear is required for visualization with a UV transilluminator.
- InstaStain® Ethidium Bromide cards and stained gels should be discarded using institutional guidelines for solid chemical waste.

STAINING WITH FLASHBLUE™

FlashBlue™ can be used as an alternative to Ethidium Bromide in this experiment. However, FlashBlue™ is less sensitive than InstaStain® Ethidium Bromide and will take a longer time to obtain results.

FlashBlue™ stain, however, is optimized to shorten the time required for both staining and destaining steps. Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid overnight. This will allow the stained gel to “equilibrate” in the destaining solution, resulting in dark blue DNA bands contrasting against a uniformly light blue background. A white light box (Cat. #552) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks with refrigeration, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels can be discarded in solid waste disposal. Destaining solutions can be disposed of down the drain.

PHOTODOCUMENTATION OF DNA (OPTIONAL)

Once gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.
Experiment Results and Analysis

The idealized gel result shows the PCR products from Wild and White genotypes.

Note: Depending on the PCR conditions used, a diffuse, small-molecular weight band, known as a "primer dimer", may be present below the 200 bp marker. This is a PCR artifact and can be ignored. Other minor bands may also appear due to nonspecific primer binding and the subsequent amplification of these sequences.

Lane 1: Standard 200 bp ladder.

Lane 2: PCR products from control DNA: A 1,000 bp control band and a 200 bp band from the white gene can be observed.

Lane 3: PCR products from white eyed flies: A 1,000 bp control band can be observed. The 220 bp PCR product from the wild-type white (italics) gene is absent.

Lane 4: PCR products from wild-type flies: The 1,000 bp control and the 220 bp PCR product from the wild-type allele are present.
Please refer to the kit insert for the Answers to Study Questions
Appendices

A  EDVOTEK® Troubleshooting Guide
B  Preparation and Handling of PCR Samples With Wax
C  Bulk Preparation of Agarose Gels

Material Safety Data Sheets:
## EDVOTEK® Troubleshooting Guides

### DNA EXTRACTION

<table>
<thead>
<tr>
<th>PROBLEM:</th>
<th>CAUSE:</th>
<th>ANSWER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>My fruit flies have died.</td>
<td><em>Drosophila</em> are sensitive to very hot or very cold temperatures.</td>
<td>Positive results can be obtained using DNA extracted from dead flies.</td>
</tr>
<tr>
<td>Sample not centrifuged at an appropriate speed.</td>
<td><strong>Drosophila</strong> have a short life cycle.</td>
<td>Positive results can be obtained using DNA extracted from dead flies.</td>
</tr>
<tr>
<td>Poor DNA Extraction</td>
<td>Samples not mixed well enough during extraction</td>
<td>In addition to flicking the tube, vortex or pipet up and down to mix the sample.</td>
</tr>
<tr>
<td>Proteinase K inactive because it was prepared too far in advance.</td>
<td></td>
<td>Prepare Proteinase K within one hour of use.</td>
</tr>
<tr>
<td>Water bath not at proper temperature</td>
<td></td>
<td>Use a thermometer to confirm water bath set point.</td>
</tr>
<tr>
<td>Not enough DNA</td>
<td></td>
<td>Repeat extraction. Be careful that you do not aspirate pellet. Try using two flies.</td>
</tr>
<tr>
<td>There is no pellet in the tube after the isopropyl alcohol precipitation.</td>
<td>Fly not adequately homogenized.</td>
<td>Make sure that the fly is completely mashed before proceeding.</td>
</tr>
<tr>
<td>Sample not centrifuged at an appropriate speed.</td>
<td></td>
<td>Spin cells at maximum speed (17,000 x g) for two minutes. If your centrifuge does not reach this speed, spin at highest available speed for four minutes.</td>
</tr>
<tr>
<td>Pellet was aspirated.</td>
<td></td>
<td>Repeat extraction. Be careful that you do not aspirate pellet.</td>
</tr>
<tr>
<td>The extracted DNA is very cloudy.</td>
<td>Cellular debris from pellet transferred to tube</td>
<td>Centrifuge sample again and move supernatant to a fresh tube. Take care to avoid pellet.</td>
</tr>
<tr>
<td>Cellular debris not separated from supernatant</td>
<td></td>
<td>Centrifuge sample again. If possible, centrifuge at a higher speed. Move cleared supernatant to a fresh tube.</td>
</tr>
</tbody>
</table>
## PCR AND ELECTROPHORESIS

<table>
<thead>
<tr>
<th>PROBLEM:</th>
<th>CAUSE:</th>
<th>ANSWER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>There is very little liquid left in tube after PCR</td>
<td>Sample has evaporated</td>
<td>Make sure the heated lid reaches the appropriate temperature.</td>
</tr>
<tr>
<td></td>
<td>Pipetting error</td>
<td>Make sure students close the lid of the PCR tube properly.</td>
</tr>
<tr>
<td></td>
<td>Sample has evaporated</td>
<td>If your thermal cycler does not have a heated lid, overlay the PCR reaction with wax (see Appendix B for details)</td>
</tr>
<tr>
<td>The ladder, control DNA, and <em>Drosophila</em> PCR products are not visible on the gel.</td>
<td>The gel was not prepared properly.</td>
<td>Ensure that the electrophoresis buffer was correctly diluted.</td>
</tr>
<tr>
<td></td>
<td>The gel was not stained properly.</td>
<td>Gels of higher concentration (&gt; 0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of “clumps” and glassy granules before pouring gels.</td>
</tr>
<tr>
<td></td>
<td>The gel was not stained properly.</td>
<td>The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.</td>
</tr>
<tr>
<td></td>
<td>Malfunctioning electrophoresis unit or power source.</td>
<td>Repeat staining.</td>
</tr>
<tr>
<td></td>
<td>Malfunctioning electrophoresis unit or power source.</td>
<td>Contact the manufacturer of the electrophoresis unit or power source.</td>
</tr>
<tr>
<td>After staining the gel, the DNA bands are faint.</td>
<td>The gel was not stained for a sufficient period of time.</td>
<td>Repeat staining protocol.</td>
</tr>
<tr>
<td>After staining, the ladder and control PCR products are visible on the gel but some <em>Drosophila</em> samples are not present.</td>
<td><em>Drosophila</em> DNA sample was not concentrated enough.</td>
<td>Poor DNA extraction. Repeat Module I (Isolation of <em>Drosophila</em> DNA)</td>
</tr>
<tr>
<td></td>
<td><em>Drosophila</em> DNA sample was degraded.</td>
<td>If DNA is not used right after extraction, store sample at -20°C.</td>
</tr>
<tr>
<td></td>
<td>Wrong volumes of DNA and primer added to PCR reaction.</td>
<td>Practice using pipettes</td>
</tr>
<tr>
<td>Some <em>Drosophila</em> samples have more/less amplification than others.</td>
<td>Concentration of DNA varies by sample.</td>
<td>There is an inherent variability in the extraction process.</td>
</tr>
<tr>
<td>Low molecular weight band in PCR samples</td>
<td>Primer dimer</td>
<td>Low concentration of extracted DNA in PCR reaction.</td>
</tr>
<tr>
<td>DNA bands were not resolved.</td>
<td>Tracking dye should migrate at least 6 cm from the wells to ensure adequate separation.</td>
<td>Be sure to run the gel at least 6 cm before staining and visualizing the DNA (approximately one hour at 125 V).</td>
</tr>
<tr>
<td>DNA bands fade when gels are kept at 4°C.</td>
<td>DNA stained with FlashBlue™ may fade with time</td>
<td>Re-stain the gel with FlashBlue™</td>
</tr>
</tbody>
</table>
Preparation and Handling of PCR Samples With Wax

ONLY For Thermal Cyclers WITHOUT Heated Lids, or Manual PCR Using Three Waterbaths

Using a wax overlay on reaction components prevents evaporation during the PCR process.

HOW TO PREPARE A WAX OVERLAY

1. Add PCR components to the 0.2 ml PCR Tube as outlined in Module II.
2. Centrifuge at full speed for five seconds to collect sample at bottom of the tube.
3. Using clean forceps, add one wax bead to the PCR tube.
4. Place samples in PCR machine and proceed with Module II.

PREPARING PCR SAMPLES FOR ELECTROPHORESIS

1. After PCR is completed, melt the wax overlay by heating the sample at 94° C for three minutes or until the wax melts.
2. Using a clean pipette, remove as much overlay wax as possible.
3. Allow the remaining wax to solidify.
4. Use a pipette tip to puncture the thin layer of remaining wax. Using a fresh pipette tip, remove the PCR product and transfer to a new tube.
5. Add 5 µL of 10x Gel Loading Buffer to the sample. Proceed to Module III to perform electrophoresis.
Bulk Preparation of Agarose Gels

To save time, electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel can be remelted.

**BULK ELECTROPHORESIS BUFFER**

Bulk preparation of 1X electrophoresis buffer is outlined in Table D.

**BATCH AGAROSE GELS (1.0%)**

Bulk preparation of 1.0% agarose gel is outlined in Table E.

1. Use a 500 ml flask to prepare the diluted gel buffer
2. Pour the appropriate amount of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60°C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.
6. Dispense the required volume of cooled agarose solution for casting each gel. The volume required is dependent upon the size of the gel bed.
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Proceed with electrophoresis (Module II) or store the gels at 4°C under buffer.

**Table D**

<table>
<thead>
<tr>
<th>50x Conc. Buffer</th>
<th>Distilled Water</th>
<th>Total Volume Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 ml</td>
<td>2,940 ml</td>
<td>3000 ml (3 L)</td>
</tr>
</tbody>
</table>

**Note:**
The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle’s plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

**Table E**

<table>
<thead>
<tr>
<th>Amt of Agarose</th>
<th>50x Conc. Buffer</th>
<th>Distilled Water</th>
<th>Diluted Buffer (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 g</td>
<td>8.0 ml</td>
<td>392 ml</td>
<td>400 ml</td>
</tr>
</tbody>
</table>