EDVOTEK®

THE **BIOTECHNOLOGY EDUCATION** COMPANY®

REVISED & UPDATED

Edvo-Kit # 337

Edvo-Kit #337

Drosophila Genotyping Using PCR

Experiment Objective:

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

See page 3 for storage instructions.

LyphoPrimer™

LyphoTemplate™

NOTE:
PCR Cycling Conditions have changed. Please review your PCR program before performing the experiment.

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Safety Data Sheets:

Now available for your convenient download on **www.edvotek.com/Safety-Data-Sheets**

Experiment Components

	Component	Storage	Check (✓)
•	PCR EdvoBeads™ *(Each PCR EdvoBead™ contains: dNTP Mixture, Taq DNA Polymerase Buffer, Taq DNA Polymerase, and $MgCl_2$)*	Room Temp.	❑
A	Universal DNA Buffer	-20° C Freezer	❑
B	TE Buffer	-20° C Freezer	❑
C	EdvoQuick™ DNA Ladder	-20° C Freezer	❑
D	*Drosophila* LyphoPrimer™ Mix	-20° C Freezer, desiccated	❑
E	*Drosophila* WT LyphoTemplate™	-20° C Freezer, desiccated	❑
F	Potassium Acetate	-20° C Freezer	❑
G	DNA Extraction Buffer	-20° C Freezer	❑
•	Proteinase K	-20° C Freezer	❑

NOTE: Components D and E are now supplied in our LyphoPrimer™ and LyphoTemplate™ form and require reconstitution prior to setting up PCR reactions.

Experiment #337 is designed for 10 groups.

All experiment 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.

Reagents & Supplies *(included with this experiment)*

Store all components below at room temperature.

Component	Check ✓
• Wild-type and White *Drosophila* ***(request 2 weeks in advance)***	❑
• UltraSpec-Agarose™	❑
• Electrophoresis Buffer (50x)	❑
• SYBR® Safe Stain	❑
• FlashBlue™ Liquid Stain	❑
• Microcentrifuge Tubes	❑
• PCR tubes (0.2 mL - for thermal cyclers with 0.2 mL template)	❑

NOTE: Drosophila must be requested two weeks in advance.

Experiment Requirements *(NOT included with this experiment)*

- Thermal cycler (EDVOTEK Cat. # 541 highly recommended) or three water baths*
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- Water bath (70º C) (EDVOTEK Cat. #539 highly recommended)
- UV Transilluminator or Blue light visualization (EDVOTEK® Cat. #557 or #558 highly recommended)
- White light visualization system (OPTIONAL - use if staining with FlashBlue™)
- UV safety goggles
- Automatic micropipettes (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 water baths. However, a thermal cycler assures a significantly higher rate of success.

Background Information

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.

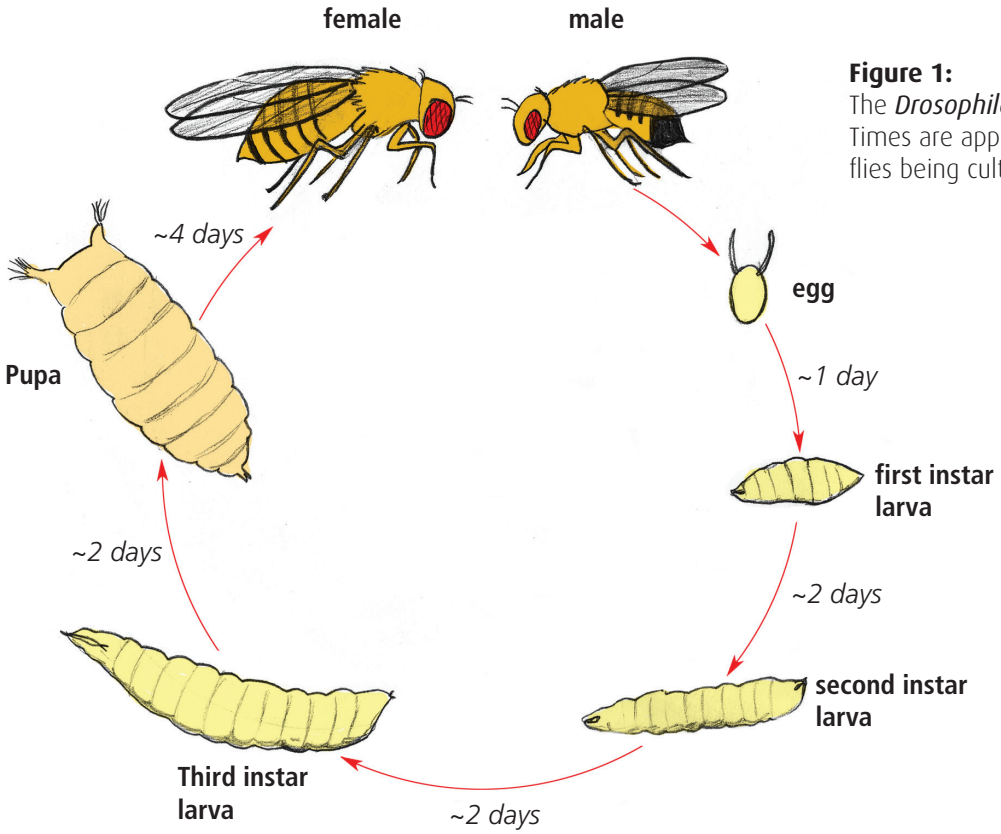


Figure 1: The *Drosophila* life cycle. Times are approximate for flies being cultured at 25° C.

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.

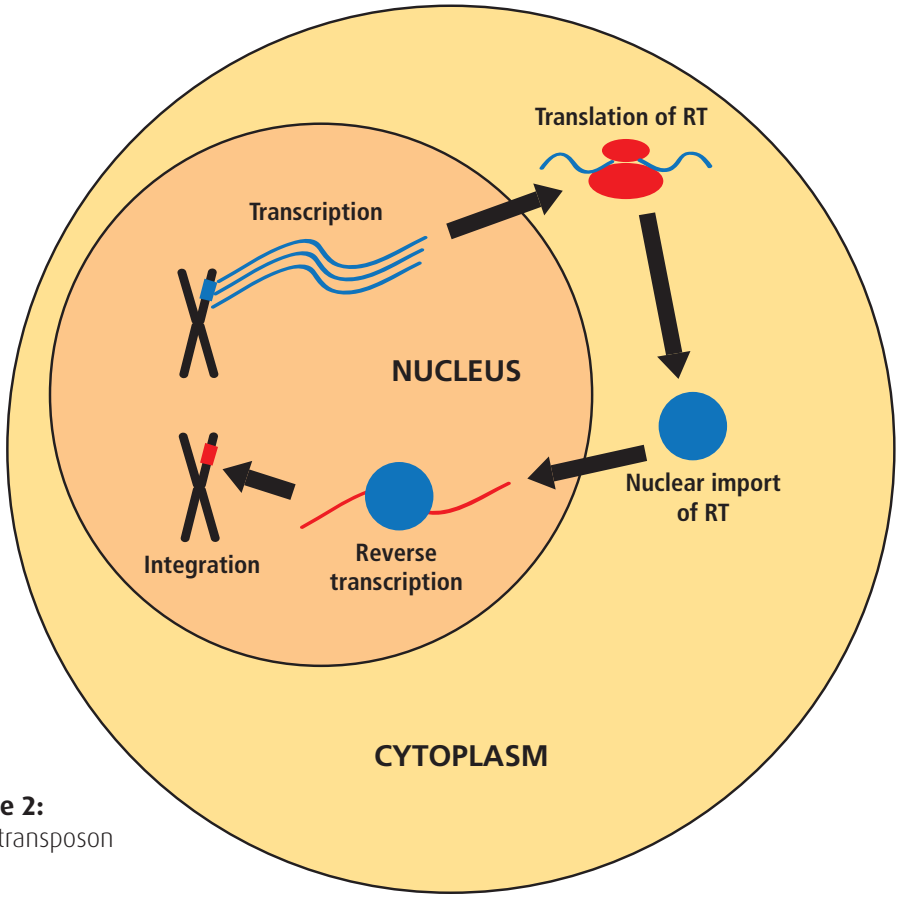


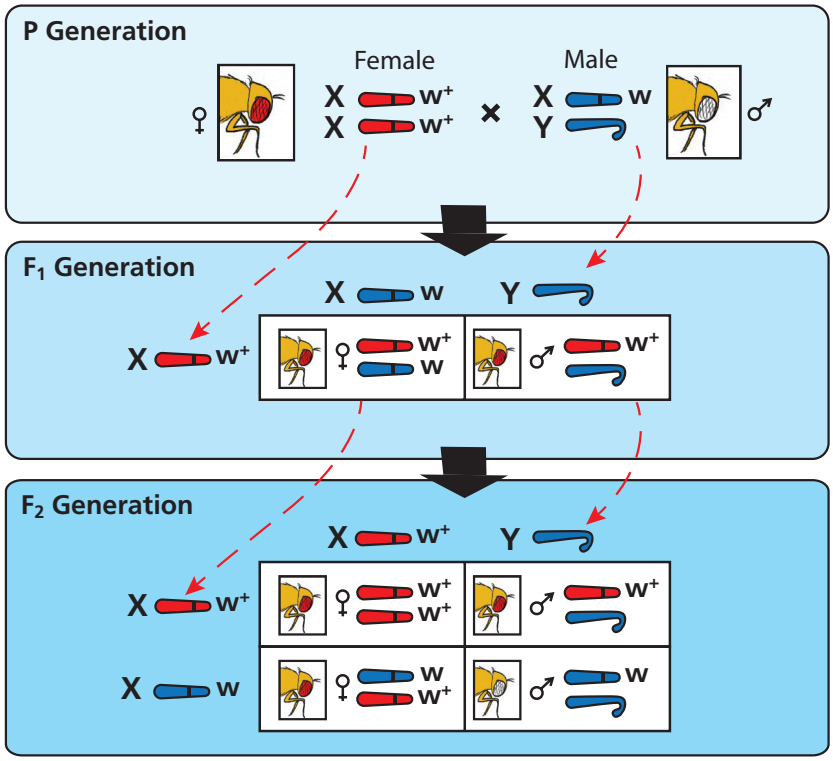
Figure 2:
Retrotransposon

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*.

Figure 3:
White-eyed and red-eyed flies.

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.



KEY:
w+: wild type white gene
w: mutant white gene

Figure 4:
Linking genes to chromosomes.

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.

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.

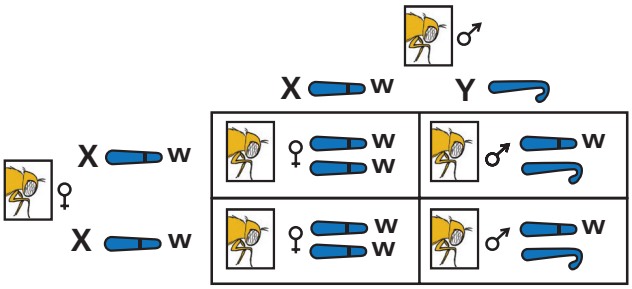


Figure 5:
Cross between white-eyed female and male flies.

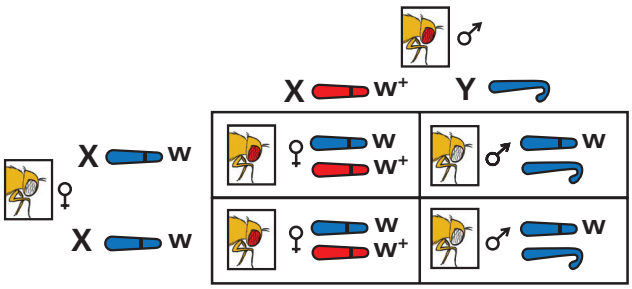


Figure 6:
Cross between white-eyed female and red-eyed male flies.

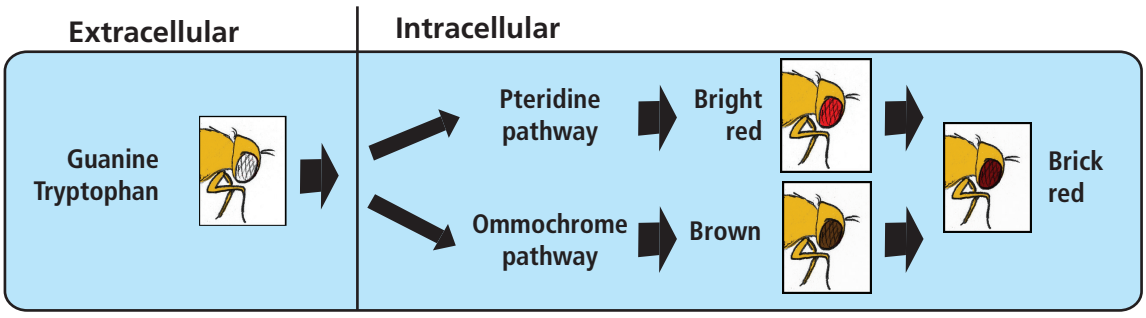


Figure 7:
Current Model of Eye Color Determination in *Drosophila.*

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.

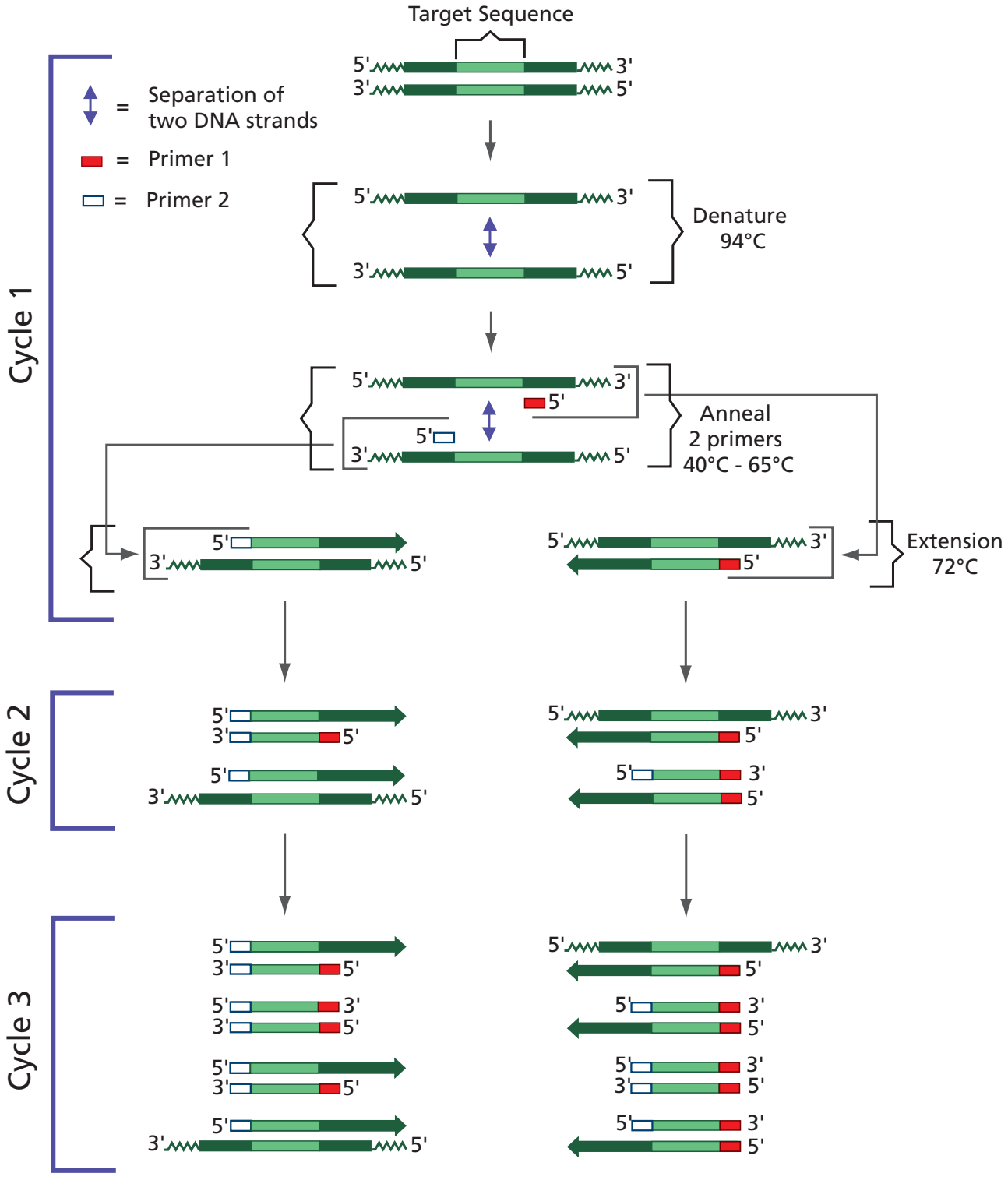
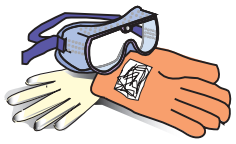


Figure 8: Polymerase Chain Reaction

Experiment Overview

EXPERIMENT OBJECTIVE:

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



Wear gloves and safety goggles

LABORATORY SAFETY:

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 7 cm (short tray)
Number of sample wells required	6
Placement of well-former template	First set of notches
Gel concentration required	1.0%

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I – 50 min.
Isolation of *Drosophila* DNA

↓

Module II – 120 min.
Amplification of the Extracted DNA

↓

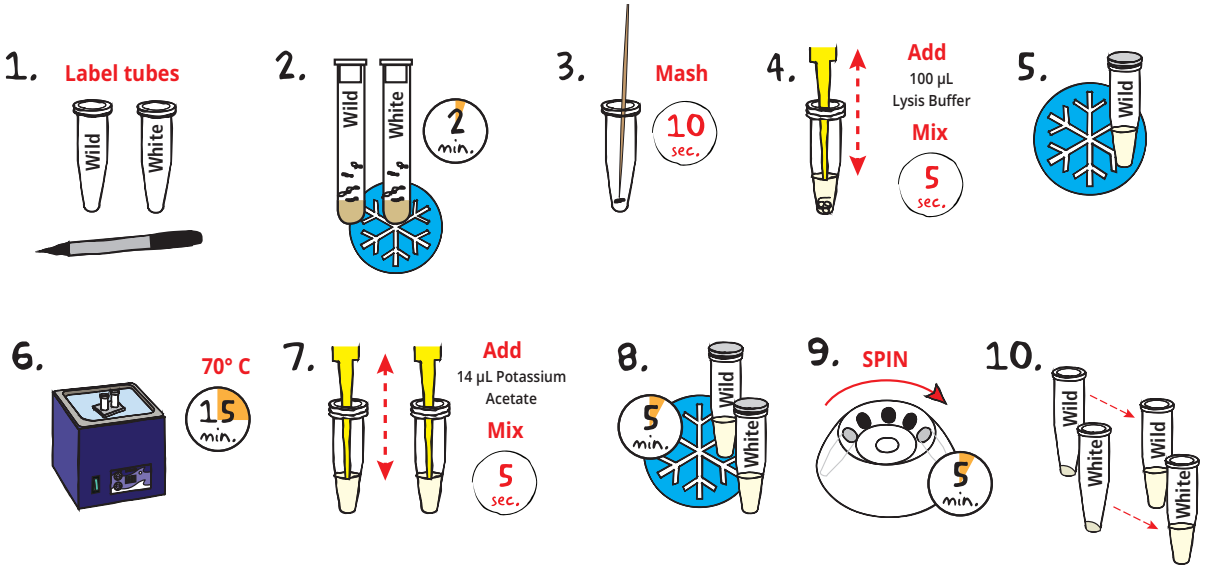
Module III – 30-45 min.
Separation of PCR Products by Electrophoresis

↓

Module IV – 5 min.
(OPTIONAL) Staining with FlashBlue™

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" plus your Group #.

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 pipette tip or toothpick, **MASH** the fly in the tube (about 10 seconds).

4. Using a fresh pipette tip, **ADD** 100 µL of the prepared Lysis buffer to the fly. **MIX** the sample by pipetting up and down.

WARNING!
Use only screw-cap tubes when incubating in the water bath for DNA isolation. Do not use snap-top tubes.

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

6. **INCUBATE** both samples in a 70° C water bath for 15 minutes.

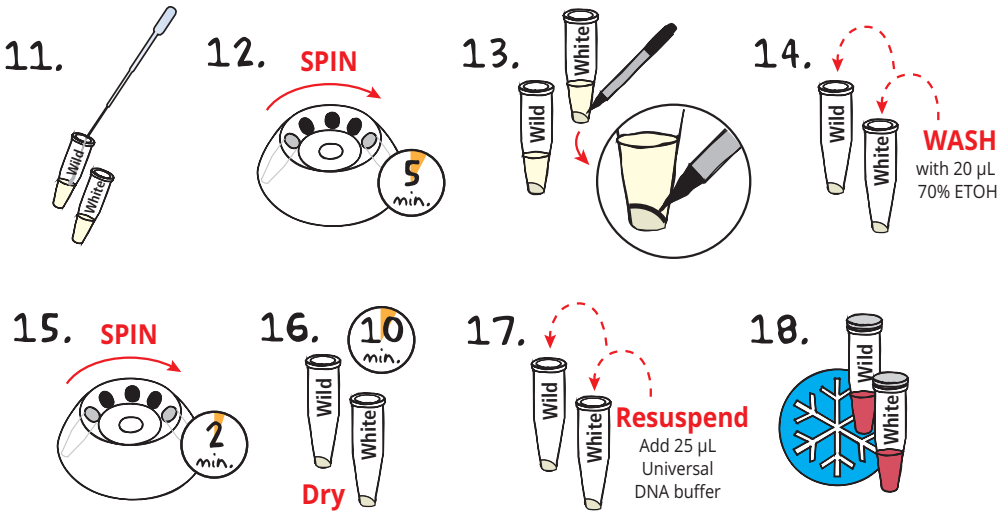
7. **ADD** 14 µL of potassium acetate to each sample and **MIX** for 5 seconds.

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.

Module I: Isolation of *Drosophila* DNA, continued



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 both tubes. With a marker, **CIRCLE** the location of the DNA pellets.

14. Carefully **REMOVE** and **DISCARD** the supernatant. Take care to avoid the DNA pellets while removing the supernatant. Carefully **WASH** the pellets 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)

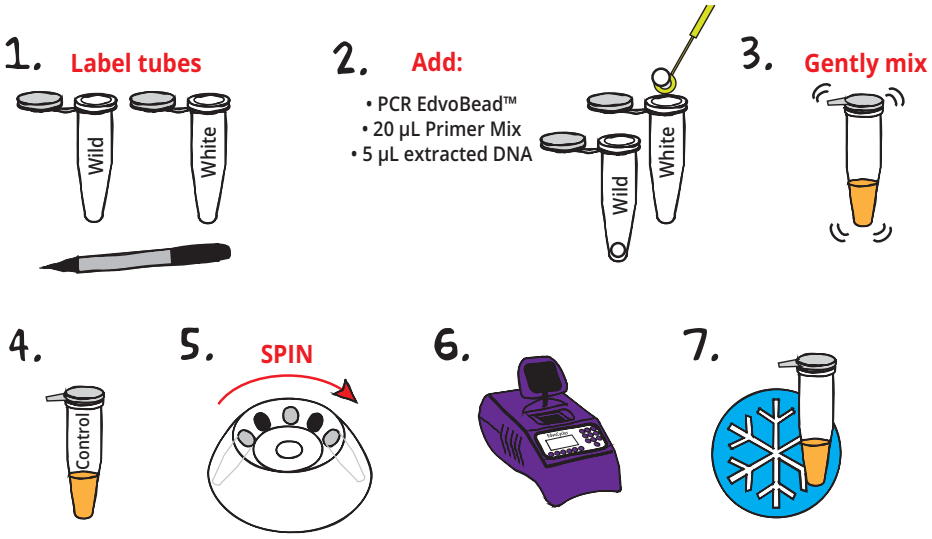
17. **RESUSPEND** the DNA pellet in 25 µL of Universal DNA buffer.

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



NOTES AND REMINDERS:

This kit includes enough DNA to set up one control reaction for every 2 groups to share on a gel. All 5 control reactions should be run.

PCR REACTION:

1. **LABEL** two PCR tubes "Wild" and "White" plus your Group #.
2. To each tube, **ADD** one PCR EdvoBead™, 20 µL Primer Mix (yellow), and 5 µL extracted DNA (red).
3. **MIX** the PCR samples gently. Make sure the PCR EdvoBeads™ are completely dissolved in each tube. ***NOTE: Double-check that both the primer and DNA have been added by looking at the color of the mixture in the PCR tube. The mixture should be orange with the primer and DNA mixed together.***
4. **OBTAIN** one tube of positive control per 2 groups.
5. **CENTRIFUGE** to collect the samples at the bottom of the tubes.
6. **AMPLIFY** DNA using PCR.

PCR Cycling Conditions:
Initial denaturation 94°C for 2 minutes
94°C for 45 seconds
55°C for 45 seconds } 35 cycles
72°C for 90 seconds
Final Extension 72°C for 5 minutes

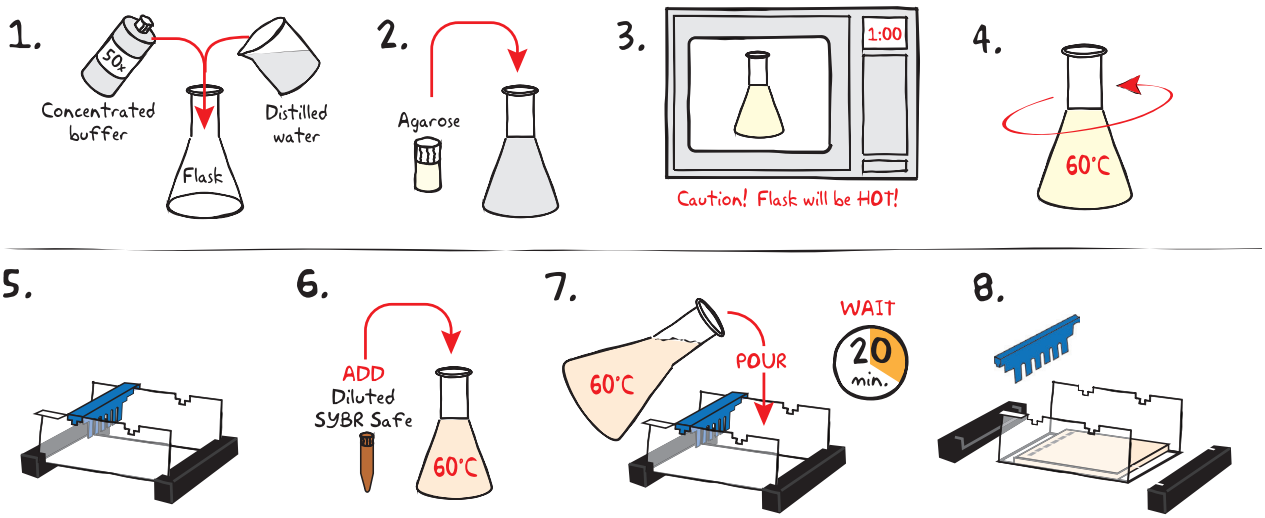
NOTE:
PCR Cycling Conditions have changed. Please review your PCR program before performing the experiment.

7. **STORE** on ice until ready for electrophoresis. **PROCEED** to Module III: Separation of PCR products by Electrophoresis.

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



PREPARING THE AGAROSE GEL WITH SYBR® SAFE STAIN

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** the agarose to 60° C by carefully swirling the flask to promote even dissipation of heat.
5. While the agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the comb in the appropriate notch.
6. Before casting the gel, **ADD** diluted SYBR® Safe stain to the cooled molten agarose and swirl to mix (see Table A).
7. **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.
8. **REMOVE** the end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

IMPORTANT:

For this experiment, each agaorse gel should be shared by two student groups. 7 x 7 cm gels are recommended. Place the 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**

OPTIONAL STOPPING POINT

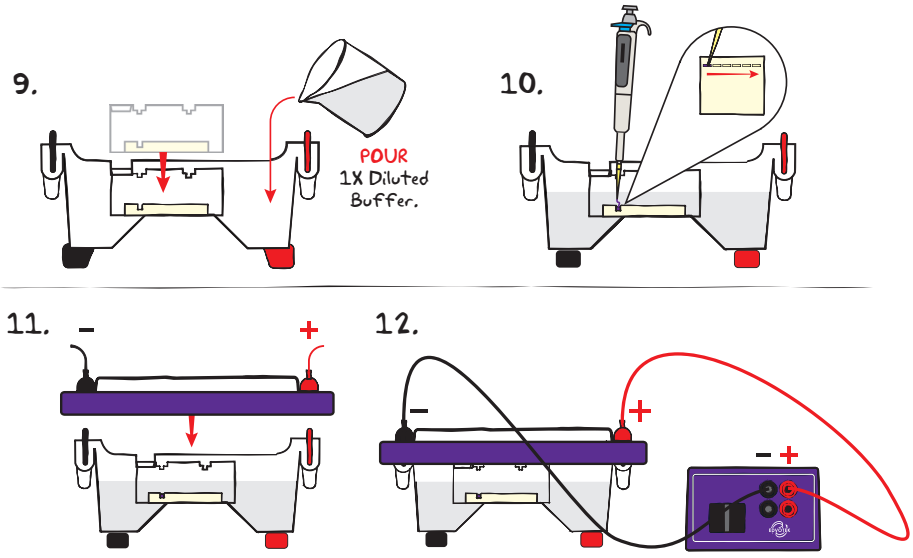
Gels can be stored overnight submerged in electrophoresis buffer, in the fridge, and protected from light.

Table A

Individual 1.0% UltraSpec-Agarose™ Gel with SYBR® Safe Stain

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.5 mL	24.5 mL	0.25g	25 mL	25 µL
7 x 14 cm	1.0 mL	49.0 mL	0.50 g	50 mL	50 µL

Module III: Separation of PCR Products by Electrophoresis, continued



Reminder:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

Wear gloves and safety goggles

RUNNING THE GEL

9. **PLACE** the gel (on the tray) into the electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. Using Table 2 as a guide, **LOAD** the entire sample (25 µL) into the wells in consecutive order.
11. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

OPTIONAL STOPPING POINT

Gels can be stored for several days. Place gel in a watertight plastic bag with 2 mL of electrophoresis buffer and store in the refrigerator.

Table 1

Lane	Recommended	Sample Name
1	EdvoQuick™ DNA ladder	
2	Control PCR Product	
3	Group 1 White PCR Product	
4	Group 1 Wild-type PCR Product	
5	Group 2 White PCR Product	
6	Group 2 Wild-type PCR Product	

Table B — 1x Electrophoresis Buffer (Chamber Buffer)

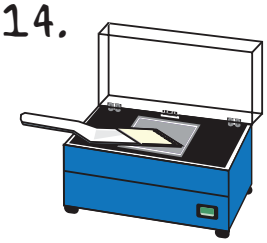
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
M6+ & M12 (new)	300 mL	6 mL	294 mL
M12 (classic)	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C — Time and Voltage Guidelines (0.8% – 7 x 7 cm Agarose Gel)

Volts	Recommended Time: Minimum	Maximum
150	10 min.	20 min.
125	20 min.	35 min.
70	35 min.	1 hour

Module III: Separation of PCR Products by Electrophoresis, continued

14.



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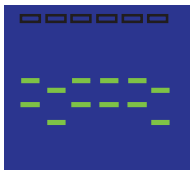
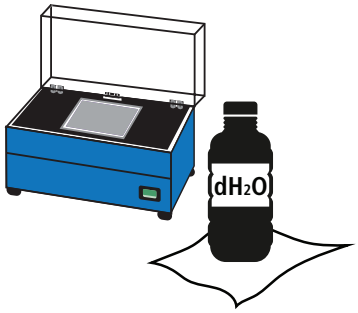


16.



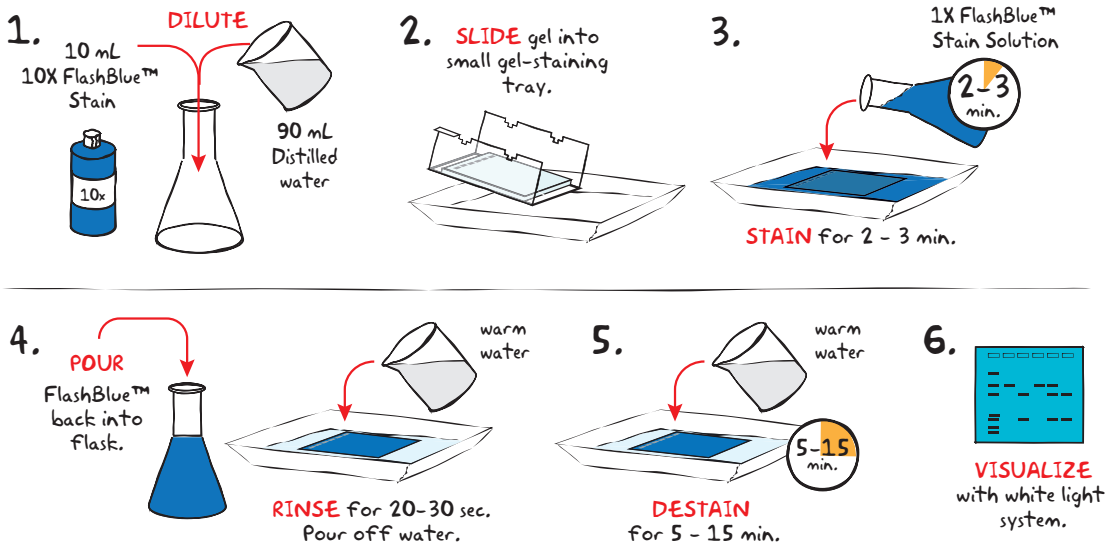
VISUALIZING THE SYBR® GEL

14. **SLIDE** the gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. **ADJUST** the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.
15. **PHOTOGRAPH** the results.
16. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water.

Be sure to wear UV goggles if using a UV transilluminator.

Module IV: OPTIONAL Staining with FlashBlue™

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. ***IF staining with both SYBR® Safe and Flash Blue, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.***



1. **DILUTE** 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.
2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.
3. **COVER** the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. **POUR** the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45° C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.
5. **COVER** the gel with clean, warm water (40-45° C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
6. Carefully **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.

Wear gloves and safety goggles

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

1. **DILUTE** 1 mL of 10X FlashBlue™ stain with 499 mL distilled water.
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. Carefully **REMOVE** the gel from the staining 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

Depending on the size of your class, you may choose to have students work in groups for Modules 1 and 2. For Modules 3 & 4, each agarose gel can be shared by two student groups. The EdvoQuick™ DNA Ladder and a positve control reaction should be run on each agarose gel. Enough reagents have been provided for up to five 7 x 7 cm agarose gels.

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation For:	What to do:	When:	Time Required:
Module I: Isolation of *Drosophila* DNA	Prepare and aliquot various reagents.	Up to one day before performing the experiment.	30 min.
	Prepare and aliquot Lysis Buffer.	Prepare on the day the students will be performing the experiment **OR** freeze for up to one week.	15 min.
	Equilibrate water bath at 70° C.	Anytime before performing the experiment.	5 min.
Module II: Amplification of the Extracted DNA	Prepare and aliquot various reagents (Primer, control, ladder, etc.).	One day to 30 min. before performing the experiment.	30 min.
	Program Thermal Cycler.	Anytime before performing the experiment.	15 min.
Module III: Separation of PCR Products by Electrophoresis	Prepare electrophoresis buffer and dilute SYBR® Safe Stain.	Up to one week before performing the experiment.	45 min.
	Prepare molten agarose and pour gel.		
Module IV: Staining Agarose Gels with FlashBlue™ (OPTIONAL)	Prepare staining components.	Up to 10 min. before the class period.	10 min.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

NOTE:
The PCR cycling conditions may have changed. Before running the experiment, confirm that the program matches the settings below:

- Initial denaturation 94° C for 2 minutes
- 94° C for 45 seconds
- 55° C for 45 seconds } 35 cycles
- 72° C for 90 seconds
- Final Extension 72° C for 5 minutes.

Pre-Lab Preparations: Module I - Isolation of *Drosophila* DNA

Preparation of Lysis Buffer

Note: The Lysis Buffer must be mixed with Proteinase K before performing the experiment. Once prepared, the lysis should be used the same day or frozen.

1. Add 100 µL of DNA Extraction buffer (G) to the tube of Proteinase K and allow the sample to hydrate for several minutes. After the sample is hydrated, pipet up and down several times to thoroughly mix the material.

2. Transfer the entire amount of the rehydrated Proteinase K solution Back to the bottle of DNA Extraction buffer (G).

3. Cap and invert the bottle several times to mix. Label this as "Lysis Buffer".

NOTE: The Lysis Buffer should be clear and free of any undissolved clumps.

4. Aliquot 250 µL of Lysis Buffer into 10 labeled microcentrifuge tubes.

NOTE: At this point, the Lysis Buffer should be stored on ice for use within the same day (up to 6 hours) or frozen.

5. Distribute one tube of "Lysis Buffer" to each group. If frozen, the Lysis Buffer can be quickly thawed in a 37° C water bath or by students warming the tube in their hands.

FOR MODULE I
Each group receives:
- Two 0.5 mL screw cap tubes
- 250 µL Lysis Buffer
- 40 µL Potassium Acetate
- 70 µL Universal DNA Buffer
- 100 µL Isopropanol
- 50 µL 70% Ethanol

Reagents to be shared by the class:
- Vial of white flies
- Vial of wild-type flies

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.

Additional Preparations

1. Aliquot 40 µL of Potassium Acetate (F) per group into labeled tubes.

2. Aliquot 70 µL of Universal DNA Buffer (A) per group into labeled tubes.

3. Aliquot 100 µL Isoproanol per group into labeled tubes.

4. Aliquot 50 µL 70% Ethanol per group into labeled tubes.

Flies

- The class will recieve 1 vial of wild-type flies and 1 vial of white flies to be shared.
- Upon arrival, most flies should be alive with enough for the class.
- If you would like to maintain a colony of *Drosophila* in your classroom, refer to Appendix D for additional information.

Pre-Lab Preparations: Module II - Amplification of the Extracted DNA

The PCR primers are provided as a lyophilized mixture that must be rehydrated by the instructor before performing the experiment. The PCR EdvoBeads™ can be distributed prior to setting up the PCR – students or instructors can gently transfer the PCR EdvoBeads™ using gloved hands. Alternatively, beads can be gently "poured" from the vial into individual PCR tubes. After distributing the beads, it is important to close the PCR tubes securely to prevent the beads from absorbing moisture and becoming hard to resuspend prior to the experiment. ***NOTE: The PCR EdvoBeads™ are fragile, use care to not crush the beads while transferring to a PCR tube.***

FOR MODULE II
Each group receives:
- 50 µL *Drosophila* Primer Mix
- 2 PCR tubes
- 2 PCR EdvoBeads™

Two groups should share:
- 1 tube positive control

This kit features the NEW EDVOTEK® LyphoPrimer™ and LyphoTemplate™. The reagents are also color coded so that a correctly assembled PCR reaction should appear orange in color. These innovations will help ensure experimental success.

Preparation of the *Drosophila* Primer Mix Concentrate:

1. Thaw the TE buffer (B). Mix well before using.
2. Before preparing the primer mix, make sure the solid material is at the bottom of tube of *Drosophila* LyphoPrimer™ Mix (D). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
3. Dilute the *Drosophila* LyphoPrimer™ by adding 1 mL of TE Buffer to the tube. Cap and mix well and place on ice. The solution should be clear and yellow in color, and no solid pieces should remain.
4. Pipette 50 µL of the diluted *Drosophila* Primer Mix into microcentrifuge tubes. Label these tubes “Primer Mix". Distribute one tube per student group.

Preparation of the Positive Control Reaction:

1. Before preparing the control reaction, make sure the solid material is at the bottom of tube of *Drosophila* WT LyphoTempate™ (E). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
2. Dilute the LyphoTemplate™ by adding 30 µL of TE buffer (B) to the tube. Pipette the solution up and down to mix.
3. Obtain five 0.2 mL PCR tubes and label them "control". To each tube, add 20 µL diluted LyphoPrimer™, 5 µL diluted LyphoTemplate™, and one PCR EdvoBead™. Distribute one tube per two student groups sharing a gel.

NOTE: Enough reagents are provided to create 5 positive control reactions. We STRONGLY recommend running the control reaction on each gel. If you do not plan to run the reaction immediately, it may be stored at -20° C until ready to run.

Additional Materials:

- Each student group will need two 0.2 mL PCR tubes and two PCR EdvoBeads™.

PCR Amplification:

The thermal cycler should be programmed as outlined in Module II in the Student’s Experimental Procedure.

- Accurate temperatures and cycle times are critical. A pre-run for one cycle (takes approximately 3 to 5 min.) is recommended to check that the thermal cycler is properly programmed.
- For thermal cyclers that do not have a heated lid, it is necessary to place a layer of wax or mineral oil above the PCR reactions in the PCR tubes to prevent evaporation. Visit **www.edvotek.com** for instructions.

Pre-Lab Preparations: Module III - Separation of PCR Products by Electrophoresis

Preparation of 1X Electrophoresis Buffer:

For this experiment, we recommend preparing the 1X Electrophoresis Buffer in bulk for sharing by the class. Unused diluted buffer can be used at a later time. See Appendix C for instructions.

Preparation of Agarose Gels:

For this experiment, one 1.0% agarose gel can be shared by two student groups. For best results, we recommend using 7 x 7 cm gels. You can choose whether to prepare the gels in advance or have the student groups prepare their own. Allow approximately 30-40 minutes for this procedure.

SYBR® Safe Stain Preparation

1. Following the instructions in Appendix C, prepare 1x Electrophoresis Buffer by combining 10 µL of 50X Concentrated Buffer with 490 µL of distilled water.

2. Add 250 µL of the 1X buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times. The SYBR® Safe Stain is now diluted and ready to be used during agarose gel preparation.

Individual Gel Preparation
Two student groups can be responsible for casting their own agarose gel to share prior to conducting the experiment (see Module III in the Student's Experimental Procedure). Each 7 x 7 cm gel will require 50X electrophoresis buffer, distilled water, agarose powder, and 25 µL diluted SYBR® Safe Stain.

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

Preparing Gels in Advance
Agarose gels may be prepared in advance and stored for later use. Solidified gels can be stored for up to 1 week in the refrigerator in water-tight bags with a small amount of buffer to prevent drying. We recommend adding 2 mL of buffer to the bag; excess buffer can lead to diffusion of SYBR® Safe out of the gels.

Do not store gels at -20° C as freezing will destroy them.

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.

NOTE:
Accurate pipetting is critical for maximizing successful experiment results. This experiment is designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students are unfamiliar with using micropipettes, we recommended performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

FOR MODULE III

For casting each gel, you will need:
- 50X concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe

For loading each gel, you will need:
- EdvoQuick™ DNA ladder
- 1 tube positive control reaction (from Module II)
- Samples from 1 or 2 student groups (from Module II)

NOTE:
QuickGuide instructions and guidelines for casting various agarose gels can be found our website. **www.edvotek.com/quick-guides**

Additional Materials:

Each 1.0% gel should be loaded with the EdvoQuick™ DNA ladder, a positive control reaction, and samples from 1 or 2 students groups.

- Pipette 30 µL of the EdvoQuick™ DNA ladder (C) into labeled microcentrifuge tubes and distribute one tube per gel.

Pre-Lab Preparations: Module IV - Staining Agarose Gels

OPTIONAL Staining with FlashBlue™

Wear gloves and safety goggles

FlashBlue™ can be used as an alternative or in addition to SYBR® Safe in this experiment. If only staining with FlashBlue™, you can omit SYBR® Safe from the gel preparation. However, FlashBlue™ is less sensitive than SYBR® Safe and will take a longer time to obtain results. Alternatively, gels can be visualized first with SYBR® Safe and then with FlashBlue™.

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 develop in the destaining solution, resulting in dark blue DNA bands that contrast with 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 if they are refrigerated, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels should be discarded in the garbage and destaining solutions should be disposed of down the drain.

FOR MODULE IV

Each gel will need:

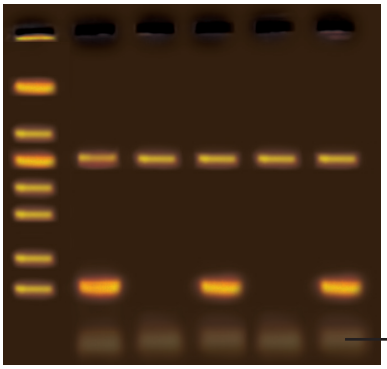
- 10 mL 10X concentrated FlashBlue OR 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

Photodocumentation of DNA (Optional)

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

Expected Results

The gel results photo 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: EdvoQuick™ DNA ladder.

Lane 2: PCR products from *Drosophila* WT LyphoTemplate™:
A 1,000 bp control band and a 200 bp band from the *white* gene can be observed.

Lanes 3 & 5: 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* gene is absent.

Lanes 4 & 6: 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 Prep of Electrophoresis Buffer and Agarose Gels with SYBR® Safe
D Culturing *Drosophila*

Safety Data Sheets:
Now available for your convenient download on **www.edvotek.com/Safety-Data-Sheets**

Appendix A
EDVOTEK® Troubleshooting Guides

DNA EXTRACTION

PROBLEM:	CAUSE:	ANSWER:
My fruit flies have died.	*Drosophila* are sensitive to very hot or very cold temperatures.	Positive results can be obtained using DNA extracted from dead flies.
	Drosophila have a short life cycle.	Positive results can be obtained using DNA extracted from dead flies.
Poor DNA Extraction	Samples not mixed well enough during extraction	In addition to flicking the tube, vortex or pipet up and down to mix the sample.
	Proteinase K inactive because it was prepared too far in advance.	Prepare Proteinase K within one hour of use.
	Water bath not at proper temperature	Use a thermometer to confirm water bath set point.
	Not enough DNA	Repeat extraction. Be careful that you do not aspirate pellet. Try using two flies.
There is no pellet in the tube after the isopropyl alcohol precipitation.	Fly not adequately homogenized.	Make sure that the fly is completely mashed before proceeding.
	Sample not centrifuged at an appropriate speed.	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.
	Pellet was aspirated.	Repeat extraction. Be careful that you do not aspirate pellet.
The extracted DNA is very cloudy.	Cellular debris from pellet transferred to tube	Centrifuge sample again and move supernatant to a fresh tube. Take care to avoid pellet.
	Cellular debris not separated from supernatant	Centrifuge sample again. If possible, centrifuge at a higher speed. Move cleared supernatant to a fresh tube.

Appendix A
EDVOTEK® Troubleshooting Guides

PCR AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
There is very little liquid left in tube after PCR	Sample has evaporated	Make sure the heated lid reaches the appropriate temperature.
		If your thermal cycler does not have a heated lid, overlay the PCR reaction with wax (see Appendix B for details)
		Make sure students close the lid of the PCR tube properly.
	Pipetting error	Make sure students pipet 20 µL primer mix and 5 µL extracted DNA into the 0.2 mL tube.
The ladder, control DNA, and *Drosophila* PCR products are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		Gels of higher concentration (> 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.
		The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.
	The gel was not stained properly.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained for a sufficient period of time.	Repeat staining protocol.
After staining, the ladder and control PCR products are visible on the gel but some *Drosophila* samples are not present.	*Drosophila* DNA sample was not concentrated enough.	Poor DNA extraction. Repeat Module I (Isolation of *Drosophila* DNA)
	Drosophila DNA sample was degraded.	If DNA is not used right after extraction, store sample at -20°C.
	Wrong volumes of DNA and primer added to PCR reaction.	Practice using pipettes
Some *Drosophila* samples have more/less amplification than others.	Concentration of DNA varies by sample.	There is an inherent variability in the extraction process.
Low molecular weight band in PCR samples	Primer dimer	Low concentration of extracted DNA in PCR reaction.
DNA bands were not resolved.	Tracking dye should migrate at least 6 cm from the wells to ensure adequate separation.	Be sure to run the gel at least 6 cm before staining and visualizing the DNA (approximately one hour at 125 V).
DNA bands fade when gels are kept at 4°C.	DNA stained with FlashBlue™ may fade with time	Re-stain the gel with FlashBlue™

Appendix B

Performing the PCR Experiment Using Three Water Baths

This experiment can be modified to use three water baths in place of a thermal cycler. In this method, the PCR samples are cycled between three water baths, each maintained at a different temperature, for a specified period. The sequential placement of the reaction sample between the three water baths constitutes one PCR cycle. Please note that results obtained when using three water baths are often variable. ***A thermal cycler assures a significantly higher rate of success.***

We recommend EDVOTEK's Digital PCR Water Bath (Cat. #544) if you do not have access to a thermal cycler.

Set up the samples as outlined in Module II steps 1-4. Before cycling the samples, be sure to:

- Allow at least 15 minutes for the water baths to reach the temperatures specified in Module I (94° C, 55° C, and 72° C).
- Cover the water baths when not in use to maintain the proper temperature and to prevent water evaporation.
- The volume of the PCR sample is small and can evaporate easily. To prevent this, transfer one wax bead to each PCR sample. The melted wax bead forms a barrier over the PCR sample to prevent its evaporation during heating.
- Make sure that the sample remains undisturbed at the bottom of the tube. If necessary, centrifuge or shake the tube to get the sample to the bottom of the tube.
- Place the PCR samples in a water bath float before placing them in the water bath.

Continue with step 5 (thermal cycling), following the protocol below:

- Initial denaturation at 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

Handle the samples carefully when shifting between water baths. Use forceps to carefully raise/lower the float into the water baths.

Remove samples at specified time points. Take care to avoid the liquid wax layer when removing the sample. We recommend placing the tube on ice for a few seconds to solidify the wax. Use a clean pipette tip to gently break through the wax layer, making enough room to fit a clean pipette tip. Using a fresh, clean pipette tip, remove the PCR product and transfer to the appropriate tube.

PLACE tubes on ice. **PROCEED** to Module III: Separation of PCR Products by Electrophoresis.

Appendix C

Bulk Prep of Electrophoresis Buffer and Agarose Gels with SYBR® Safe

To save time, the 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 solution can be remelted.

BULK ELECTROPHORESIS BUFFER

Quantity (bulk) preparation for 3 liters of 1X electrophoresis buffer is outlined in Table D.

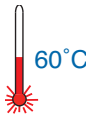
Table D: Bulk Preparation of 1X Electrophoresis Buffer

50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 mL		2,940 mL	3000 mL (3 L)

BATCH AGAROSE GELS WITH SYBR® SAFE

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. Add the entire volume of diluted SYBR® Safe (from page 23) to the cooled agarose and mix thoroughly.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 30 mL for a 7 x 7 cm tray, 50 mL for a 7 x 10 cm tray, and 60 mL for a 7 x 14 cm tray. ***For this experiment, 7 x 7 cm gels are recommended.***
8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Gels can be used immediately or stored in a small amount of buffer in the refrigerator for several days.

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.

NOTE:
QuickGuide instructions and guidelines for casting various agarose gels can be found our website.
www.edvotek.com/quick-guides

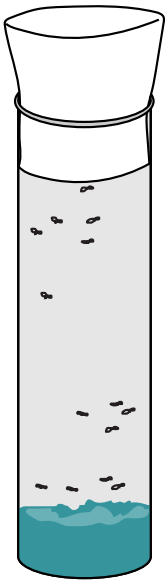
Table E: Batch Prep of 1.0% UltraSpec-Agarose™

Amt of Agarose	+ Concentrated Buffer (50X)	+ Distilled Water	= TOTAL Volume	Cool to 60° C, then ADD:
3.0 g	6.0 mL	294 mL	300 mL	Entire Tube of Diluted SYBR Safe

Appendix D
Culturing *Drosophila*

Drosophila melanogaster is a small fruit-fly commonly used for research. If desired, one can culture and breed *Drosophila* right in the classroom. Their care is quite simple, and can be typed male or female, white or WT eyed, and mated accordingly.

TEMPERATURES AND CONDITIONS



Drosophila can be grown at room temperature, simply placed on a lab bench. The optimal conditions for growth at 25° C (77° F), however within 10-15 degrees of that is suitable. Try to keep *Drosophila* away from direct sunlight and heat sources, as to avoid drying out the media.

PREPARING VIALS WITH MEDIA

Drosophila can be grown in a number of different types of bottles and vials, anything with air access and space will work. We recommend well-cleaned test tubes with a cotton or styrofoam stopper at the top to prevent flies from escaping.

Media, or fly food, can be purchased from many biological supply companies. We recommend Ward's instant *Drosophila* media (ready-to-use), which can be easily re-hydrated with water. Simply add one teaspoon of drosophila media into the test tube followed by one teaspoon of water. Repeat until there is ~ 0.5" of fly media at the bottom of the test tube or vial.

Once added, flies will survive 2-4 weeks before needing to be transferred to fresh media.

TRANSFERRING FLIES

Flies can be anesthetized by placing their vial on its side in a -20° C freezer for 3 minutes. You can then remove the cotton or styrofoam topping, and tap the flies into their new container. Be sure to cover the container quickly after transfer, as the flies will begin to wake up as their temperature increases.

Always wear gloves when working with and transferring *Drosophila*.