

Chapter 2

Inheritance of Aldehyde Oxidase in *Drosophila melanogaster*

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Introduction

This exercise differs from traditional *Drosophila* exercises in that it utilizes biochemical assays to investigate the inheritance of the gene for the enzyme aldehyde oxidase. As a result of this exercise students should be able to discuss why *Drosophila* is one of the most important organisms used in eucaryotic genetics, they should increase their understanding of Mendelian patterns of inheritance, and they should be able to determine the phenotypes and genotypes of parents by observing offspring phenotypes. One experiment introduces students to the concept that genes are expressed sequentially in the development of an organism. Another experiment requires that students collect data from a cross involving linked genes and use this data to map the location of those genes. Students are required to analyze the data they collect using a chi-square test.

This exercise was written to be used in an introductory biology course for science majors and lab takes approximately 3 hours to complete. If the fly crosses are prepared for the students, preparation extends for approximately 9 weeks. Preparation the day of the laboratory is minimal. Alternatively, for smaller classes, students can do the crosses, and the instructor's preparation time is reduced considerably.

Persons handling the flies and making crosses should be experienced in these techniques. The chemicals for the assay mixture should be fresh and the mixture be made fresh daily (see Notes for the Instructor). In our laboratory we use ethyl ether to anesthetize the flies, but carbon dioxide may be used. If ethyl ether is used, be sure there is adequate ventilation and that there are no sparks or flames in the room. The assay mixture is a carcinogen, so students should be warned not to touch the liquid and to wash their hands after the experiments.

Notes for the Instructor

Materials (per two students)

Fly Vials 1–5, paper towels and Kimwipes, 10–15 ml of assay mixture in an amber dropper bottle, one dropper bottle filled with ethyl ether, two toothpicks and a small brush to manipulate the flies, one “re-etherizer” (we use a petri dish lid with an absorbent pad for the ether taped inside—this is placed over the flies if they begin to wake up), one large and one small index card, two clean spot plates, and a fly masher (a short glass rod rounded on one end).

If the students' vials for Experiment 1 do not have larvae, pupae, and adults to use in Experiment 6, you should have several additional wild-type fly cultures available.

Notes on Preparation

Assay Mixture

You should prepare enough for 10–15 ml per pair of students. It is best to prepare the mixture fresh daily; however, left-over mixture which is the original yellowish color is still okay to use. If it has turned bluish, it should be discarded. The pH of the solution is critical to the life of the solution. If the pH is not exactly 8, it quickly becomes unusable.

Formula for the Assay Mixture

<i>per 100 ml</i>	<i>per 500 ml</i>
25 mg NBT (nitroblue tetrazoleum)	125 mg NBT
2 mg PMS (phenazine methosulfate)	10 mg PMS
150 μ l anisaldehyde (1 drop)	0.75 ml anisaldehyde (7–10 drops)
100 ml 0.1 TRIS-HCl, pH 8*	50 ml 1M TRIS-HCl, pH 8
	deionized (or distilled) water to 500 ml

Store in amber bottles. Make fresh daily. Just before the laboratory dispense into small amber dropper bottles, about 10–15 ml per bottle.

* To make 1M TRIS-HCl (Tris[hydroxymethyl]-aminomethane): 121.14 g in 700 ml deionized water, add drops of HCl until pH is 8, and add deionized water to 1 liter.

Fly Vials (see Appendix A)

Vial 1: Flies with the *aldox*⁺ (wild-type) phenotype, about 10–20 flies, both males and females.

Vial 2: Flies with the *aldox* genotype (negative), about 15 flies, (minimum of 6, 3 of each sex).

Vial 3: 20–30 flies (minimum of 10 males and 10 females) from a mating between a female from a stock homozygous for the *aldox* allele and a male from a stock homozygous for the *aldox*⁺ allele. (Since I already know that all of these flies will be *aldox*⁺ phenotype, I sometimes just use wild-type flies for this vial rather than making the cross.)

Vial 4: At least 25 flies from a mating between two heterozygotes. The phenotypic ratio will be 3 *aldox*⁺ to 1 *aldox*.

Vial 5: Vial with the offspring from the following cross (i.e., a test cross):

$$\frac{sepia^+ aldox^+}{sepia aldox} \times \frac{sepia aldox}{sepia aldox}$$

Student Outline

Introduction

The experiments in this exercise have been chosen because they exemplify some of the classic areas of genetic research. The study of genetics focuses on an understanding of the principles which govern the inheritance of specific traits. Initial experiments were concerned with the transmission of hereditary factors from generation to generation and led to the discovery of specific laws which define the pattern of inheritance of individual genes. Later experiments identified chromosomes as the physical structures where the units of heredity reside and provided firm cytological evidence for the theorems of transmission genetics. More recent investigations are concerned with the biochemical and molecular basis of gene expression.

Drosophila melanogaster

One of the classic tools of genetic research is the fruit fly, *Drosophila melanogaster*. This organism has been employed in genetic studies for nearly 80 years and has played an important role in the development of our knowledge of heredity. *Drosophila melanogaster* has been a particularly important eukaryotic genetic system for such studies because it has a very low chromosome number. The haploid (N) number of chromosomes is 4 and the chromosomes are designated X(1), 2, 3, and 4. The 2, 3, and 4 chromosomes are the same in both sexes and are referred to as autosomes to distinguish them from the X and Y sex chromosomes. Female *Drosophila* are characterized by having two X chromosomes while male *Drosophila* have an X and a Y chromosome. The Y chromosome only has genes necessary for male fertility, and does not carry any other genes. The 4 chromosome is so small, constituting only about 5% of the total genome, that, for all practical purposes, it can be ignored. Thus, almost the entire genetic content of the *Drosophila* genome resides on only three chromosomes: X, 2, and 3.

Another characteristic of *Drosophila* that makes it an excellent genetic research tool is its short generation time. At 25°C, a *Drosophila* culture will produce a new generation in 10 days: 1 day in the egg (embryo) stage, 5 days in the larval stage, and 4 days in the pupal stage. Each experiment in this exercise will make use of the fruit fly, *Drosophila melanogaster*.

Laboratory Objectives

1. To become familiar with *Drosophila melanogaster* as a genetic research tool.
2. To become familiar with certain biochemical aspects of genetic research.
3. To use biochemical tests to demonstrate some basic genetic principles.

Enzyme Spot Test

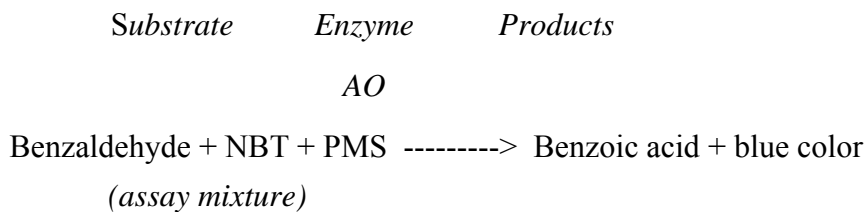
The trait that will be studied in each experiment is the presence or absence of an enzyme, aldehyde oxidase (AO), which catalyzes the oxidation of a number of aldehydes, including acetaldehyde and benzaldehyde. AO activity is controlled by one gene. Although *Drosophila* possess AO activity, its physiological importance to the organism is not well understood. Mutant strains which exhibit no AO activity are available and their viability and fertility are normal. This

latter observation indicates that AO activity is not a vital enzyme activity for the fly when reared in a laboratory setting.

The technique used to test for AO activity is called a colorimetric **spot assay**. This test operates on the following principle:

In the presence of AO, the substrate—benzaldehyde, mixed with a color indicator, nitroblue-tetrazolium (NBT), and phenazine methylsulfate (PMS)—will oxidize to form benzoic acid and a blue color. Without AO, the reaction will not proceed and no blue color will be produced.

This reaction can be diagrammed as follows:



Experiments 1 and 2: Enzyme Reaction Controls

The first experiments will demonstrate the positive and negative enzyme reactions as seen in the spot assay. Vial 1 contains flies which have the enzyme and can be expected to give a positive blue reaction. Vial 2 contains flies which do not have the enzyme and can be expected to give no reaction.

Procedure

1. Anesthetize the flies in Vials 1 and 2 as follows:
 - (a) From your ether drop bottle, place 2 or 3 drops of ether on the cotton plug of your vial. Be sure to re-cap the ether bottle tightly. Keep in mind that ether fumes are explosive.
 - (b) Invert your vial so that the adult flies will fall asleep on the cotton plug rather than on the culture medium.
 - (c) When flies have become immobilized on the cotton plug, they can be removed for examination.
 - (d) Flies can be examined on a white card and moved with a toothpick using the stereoscopic microscope.
 - (e) A fly “re-etherizer” is provided in case the adults begin to awaken before phenotype classification is concluded.
2. From each vial, identify 2 or 3 females and 2 or 3 males and return the remainder to their appropriate vial. (Vial 1 will be used again in later experiments.) The following criteria may be used to distinguish adult males and females (Figure 2.1).
 - (a) *Size*: The female is generally larger than the male.

(b) *Shape of abdomen:* The female abdomen is larger and the tip is more pointed than the male abdomen.

(c) *Abdominal pigmentation:* Alternating dark and light dorsal bands can be seen on the entire rear portion of the female abdomen; the last few segments of the male are uniformly pigmented.

(d) *External genitalia:* On the ventral portion of the abdomen, the female has anal plates and *lightly pigmented* ovipositor plates. The male has anal plates and a *darkly pigmented* genital arch and penis.

(e) *Sex comb:* On males, there is a tiny brush-like tuft of hairs on the basal tarsal segment of the first pair of forelegs. This is the most accurate method for sexing males.

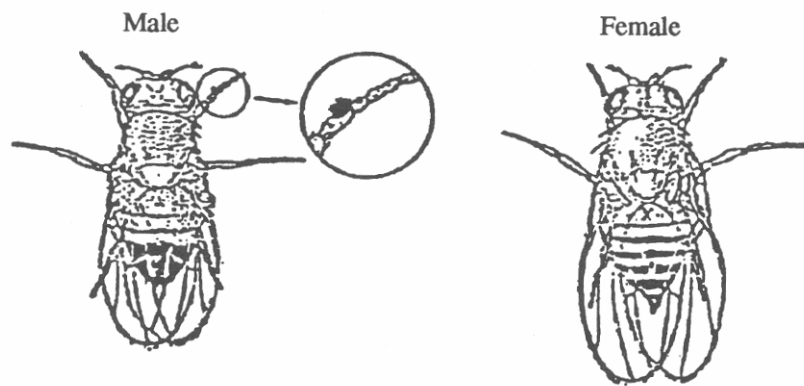


Figure 2.1. Characteristics of adult male and female *Drosophila melanogaster*; male sex comb (inset).

3. Keeping track of the sexes, place the flies from Vial 1 in one row of a spot assay plate, one fly per well.
4. Place the flies from Vial 2 in different row.

Note: The next two steps need to be done quickly since the assay mixture is light sensitive. Cover the spot plate with an index card if there is any delay between steps.

5. Add 1 drop of assay mixture to each well.
6. Homogenize the flies (why?) with a “fly masher.” Wipe off the masher after each fly.
7. Place the spot plate in a desk drawer.
8. After 5 minutes, check the reactions and record your results in Table 2.1.
9. Rinse and shake off the excess water from your spot plate before proceeding to Experiment 3.

Table 2.1. AO activity in male and female flies from Vials 1 and 2.

Vial	Fly #	Sex (F, M)	AO activity (+, -)
1	1		
	2		
	3		
	4		
	5		
	6		
2	1		
	2		
	3		
	4		
	5		
	6		

Experiment 3: Pattern of Inheritance I

The third experiment initiates our study of the pattern of inheritance of the *aldox* gene. Vial 3 contains the F₁ progeny from a mating between a female from a stock homozygous for the *aldox* allele and a male from a stock homozygous for the *aldox*⁺ allele.

What is the genotype of the F₁ progeny if the gene is sex-linked?

If the gene is not sex-linked?

Hypothesize (“guess”) the genotype of the parent flies, and proceed with the experiment.

Procedure

1. Anesthetize the flies in Vial 3.
2. Count out 10 males and 10 females.
3. Perform the spot assay as described in Experiment 1 with the following addition: from Vial 1 select a single fly and assay it with this and all following experiments. This will provide you with a “positive control” (PC) to compare with your unknown assays. Record your results when you see the “positive control” has turned blue—indicating the assay is working.
4. Record the results in Table 2.2.

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5. Rinse out the spot plate.

Questions

1. From this experiment, can you tell which allele is dominant? If so, which?
2. Can you tell if the *aldox* gene is on the X chromosome or on an autosome?

Table 2.2. Data sheet for Experiment 3.

Fly #	Sex (F, M)	AO activity (+, -)	Fly #	Sex (F, M)	AO activity (+, -)
1			11		
2			12		
3			13		
4			14		
5			15		
6			16		
7			17		
8			18		
9			19		
10			20		
Positive control:					

Experiment 4: Pattern of Inheritance II

Vial 4 contains F₁ progeny from a mating between unknown parents.

Procedure

1. Anesthetize the flies in Vial 4.
2. Count out 25 flies and place them in individual spot plate wells.
3. Perform the spot assay including a “positive control” from Vial 1.
4. Record the results on *two* data sheets: Table 2.3 and the class data sheet.

Table 2.3. Data sheet for Experiment 4.

Fly #	Sex (F, M)	AO activity (+, -)	Fly #	Sex (F, M)	AO activity (+, -)
1			14		
2			15		
3			16		
4			17		
5			18		
6			19		
7			20		
8			21		
9			22		
10			23		
11			24		
12			25		
13			–		
Positive control:					

Questions

Using your data, hypothesize what the genotypes of the parent flies must be by reviewing the following questions, some answered by previous experiments:

1. Is the trait sex-linked?
2. Is one allele dominant (from Experiment 3)?
3. What is the ratio of the two traits? (Round-off the ratio.)
4. What parental cross can account for the above ratio, taking into consideration the answers to questions 1 and 2?
5. What are the expected values for each trait, given the rounded ratio between the traits and the total number of observations?

Enter your hypothesis below:

Total the class data and, using this data, test your hypothesis concerning the genotypes of the parents using a chi-square test as outlined in Table 2.4. (For a review of chi-square refer to an appropriate textbook.)

Table 2.4. Chi-square calculations using result from Experiment 4.

Value	AO activity present	AO activity absent
Observed value (o)		
Expected value (e)		
Deviation ($o - e$)		
Deviation ² (d^2)		
d^2/e		
Chi-square (X^2) = $\Sigma d^2/e$		
Degrees of freedom (df)		
Probability (P) (see a X^2 table)		

1. Do the data support your hypothesis?
2. Does this experiment support or contradict your conclusions concerning the pattern of inheritance derived from Experiment 3?

Experiment 5: Cross Involving Two Genes

Vial 5 contains the F₁ progeny from a mating between parents having the following genotypes:

$$\frac{sepia^+ aldox^+}{sepia aldox} \times \frac{sepia aldox}{sepia aldox}$$

Where *sepia*⁺ represents the dominant, wild-type eye color allele that produces red eye color and *sepia* represents the mutant, recessive allele that produces a dark brown eye color.

Procedure

1. Anesthetize the flies in Vial 5.
2. Count out 50 flies and classify them on the basis of eye color (red or brown).
3. Keeping the eye colors separate, perform the spot assay including a “positive control” from Vial 1.
4. Record the results in Table 2.5.
5. Rinse out the spot plate.

Questions

1. From the mating outlined, what are the *expected* phenotypic classes and respective ratios?
2. What phenotypic classes were observed, and in what approximate ratio?

The AO gene and the *sepia* gene are located on the same chromosome (#3). This means that, when meiosis takes place, the two genes will NOT assort independently, but will be linked together, moving into the same gamete *unless crossing over has taken place*. Only if crossing over takes place will *recombinant classes* of phenotypes be observed. A *recombinant chromosome* is one emerging from meiosis with a combination of alleles not present on the chromosomes entering meiosis.

3. What are the recombinant classes of phenotypes?

The map distance between two genes is related to the frequency of recombinants produced during meiosis. The specific relationship between map units and recombinants is the following:

$$\text{Map units} = 100 \times \frac{\text{Number of recombinants}}{\text{Total}}$$

4. How frequent are the recombinant classes?
5. How far is the *aldox* locus from the *sepia* locus?
6. What is the exact map position of the *aldox* locus if the *sepia* locus is 26.0? Are you sure?

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Table 2.5. Data sheet for Experiment 5: gene location.

Fly #	Eye Color	AO (+, -)	Fly #	Eye Color	AO (+, -)
1			26		
2			27		
3			28		
4			29		
5			30		
6			31		
7			32		
8			33		
9			34		
10			35		
11			36		
12			37		
13			38		
14			39		
15			40		
16			41		
17			42		
18			43		
19			44		
20			45		
21			46		
22			47		
23			48		
24			49		
25			50		
Positive control:					

Experiment 6: Developmental Profile

The following experiment is designed to demonstrate the expression of AO activity during the development cycle of the fly. There are four distinct stages in the life cycle of the fruit fly as shown in Figure 2.2

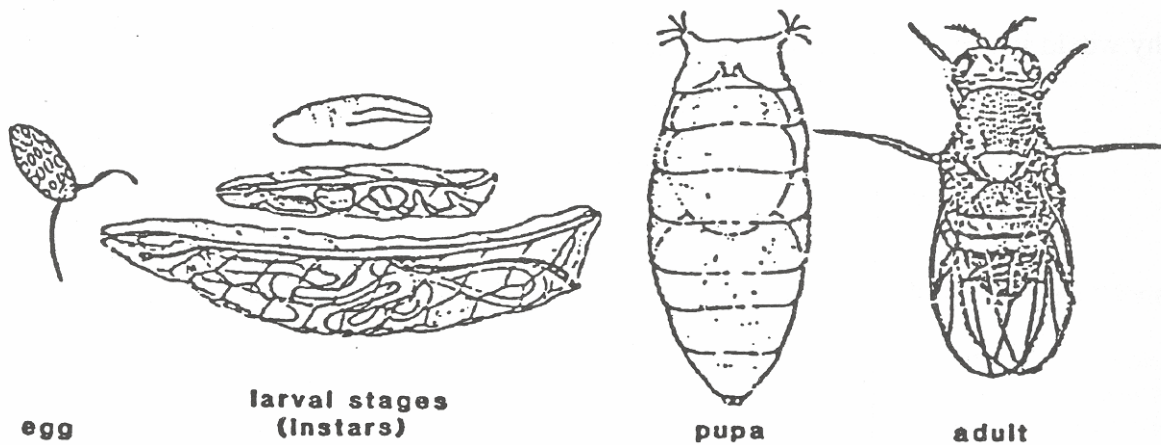


Figure 2.2. The developmental stages of *Drosophila melanogaster*.

Procedure

Use Vial 1 which contains various development stages from a true-breeding *aldox*⁺ strain.

1. Anesthetize the flies in Vial 1.
2. Select five large larvae, five pupae (*not* the clear, empty pupae cases), and five adult flies.
3. Put each developmental stage in a separate row on the spot plate.
4. Perform the spot assay *simultaneously* on all stages.
5. After 5 minutes, compare the degree of coloration of the various assays. Select the darkest stage and assign it an arbitrary value of 10. Then assign values for the other two stages relative to the 10.
6. Plot the above number on the developmental profile in Figure 2.3.

Questions

1. Is AO activity present at each developmental stage?
2. Are some stages more active than others?
3. If flies which are AO-negative are normally viable and fertile, why does the enzyme exist at all?
4. Why would it vary in developmental stages?

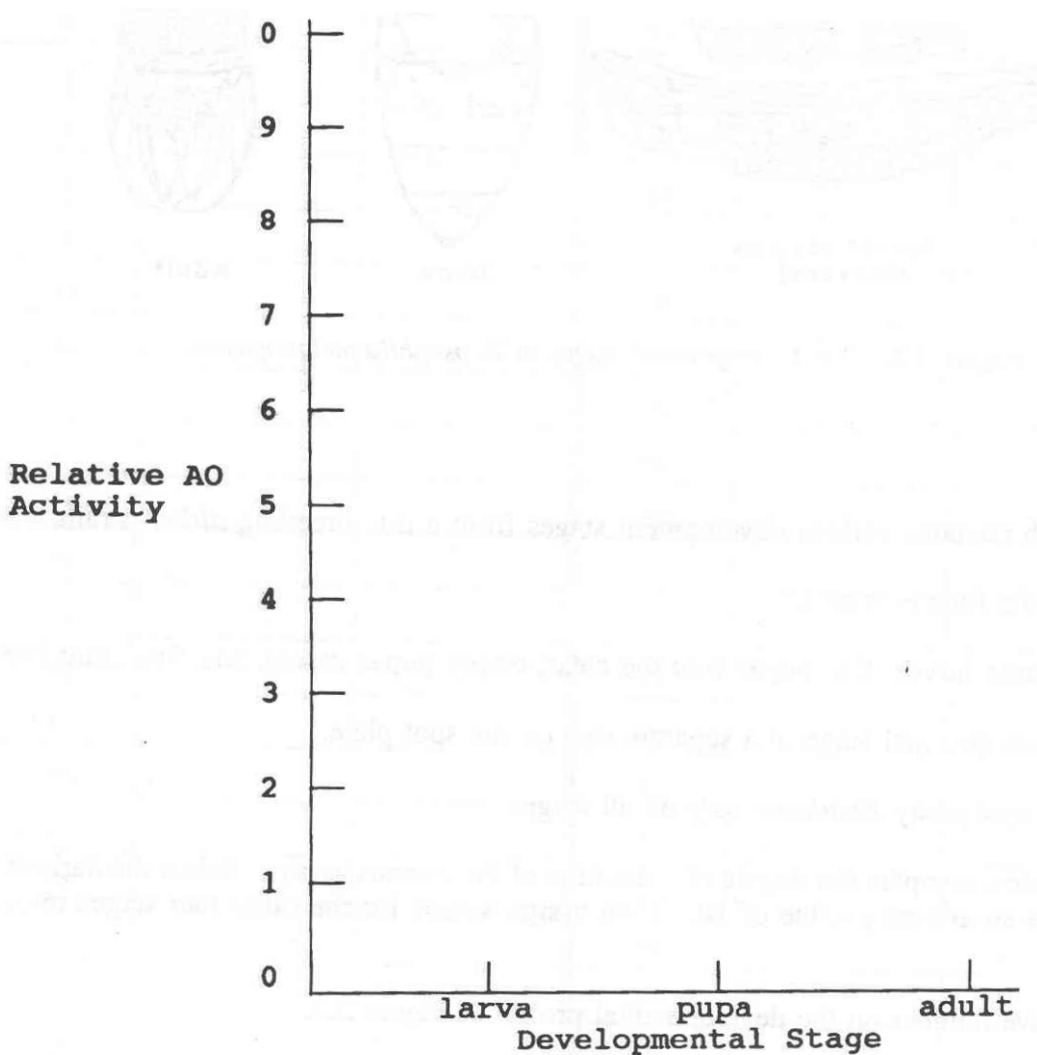


Figure 2.3. Plot of AO developmental profile from Experiment 6.

APPENDIX A

Obtaining Cultures and Schedule for Crosses

Stock to start your cultures of wild-type and *sepia-aldox* mutant flies may be requested from the first author of this chapter.

9–6 weeks before laboratory

Begin to expand stocks of *sepia*⁺ *aldox*⁺/*sepia*⁺ *aldox*⁺ and *sepia aldox/sepia aldox* flies. Every 3–4 days transfer flies to new medium to allow parents to give the best yield of F₁.

Note: At 25°C, the life cycle is 10 days; at 20°C, 15 days.

6 weeks before laboratory

Collect virgins of each phenotype (this will take several days). Save males separately to use for reciprocal crosses.

There are two methods for virgin collection. The traditional method is to keep flies at room temperature and collect every 8–10 hours before emerging flies mate. An alternate method, however, is based on the principal that females emerging in jars lacking old adult males will not mate with their young brothers for 24 hours if kept at 16–18°C. Therefore, when collecting, an easy schedule is as follows: on *day 1*, as soon as pupae darken, clear jars of all adults and place at 18°C. Collect virgin females the next morning, leave at room temperature or 25°C for 8 hours, collect again, and leave overnight at 18°C. If you want to collect only once per 24 hours, first clear and then keep the cultures at 16–18°C.

5 weeks before laboratory

Make the cross: *sepia*⁺ *aldox*⁺/*sepia*⁺ *aldox*⁺ × *sepia aldox/sepia aldox*. This produces the heterozygotes for later crosses. Be sure to keep the *sepia aldox/sepia aldox* cultures at a reasonable level as they will be used to produce the cross for Vial 5.

3 weeks before laboratory

1. Discard the parents and begin to collect virgin flies (heterozygotes) for the Vial 5 cross.
2. After a sufficient number of virgin females have been collected, transfer the heterozygotes to fresh media and allow them to mate to produce the 3:1 ratio for Vial 4.
3. Collect virgins of the original cultures and cross to produce Vial 3. (Since I know this vial will show all positive offspring, in an emergency I sometimes use flies from the wild-type cultures for this vial).

2 1/2 weeks before laboratory

Make the cross for Vial 5.