LAB 1
MOLECULAR GENOTYPING OF *Arabidopsis*

STUDENT GUIDE

Information from two NSF sponsored workshops was used in writing this lab: *Greenomes: Plant Molecular Genetics and Genomics,* taught at the University of Texas Austin, July 2004 by the Dolan DNA Learning Center staff from Cold Spring Harbor Laboratory; and *Genomics: from Mendel to Microchips,* taught by the Partnership for Plant Genomics Education at the University of California Davis, July 2005.

GOAL
The goal of this laboratory is to introduce students to the action of transposons, to simple plant DNA extraction and to the use of polymerase chain reaction (PCR) to compare diploid genotypes.

OBJECTIVES
After completion**,** the student should be able to:
1. Isolate plant DNA and perform PCR using that DNA.
2. Explain the molecular process of PCR and the necessary reagents.
3. Interpret the results of the PCR reaction, given information about the *Arabidopsis* clf-2 mutation.
4. Explain how a transposon functions.
5. Answer questions regarding diploid genotype when given PCR results.
6. Describe several applications of PCR.
7. Cast an agarose gel for chromosomal DNA verification.
8. Explain how agarose gel electrophoresis separates DNA fragments.
9. Construct a standard curve from gel electrophoresis results and use it to determine the size of sample DNA fragments.

TENTATIVE TIMELINE
Day 1: Plant *Arabidopsis* seeds and prepare solutions
Day 2: DNA extraction and PCR
Day 3: Gel electrophoresis and analysis

BACKGROUND
Additional background reading can be found at: http://www.greenomes.org/ "Detecting a Transposon Tag in *Arabidopsis*".

Transposable Elements
Movement of transposons causes the multicolored kernel phenotypes in Indian corn and many other phenotypes in other organisms. Transposons are fragments of DNA that do not exist independently because they cannot be replicated, having no origin of replication site (ORI) where DNA polymerase binds. They range in size from 750 bp to 1,500 bp and can be found in bacteria, plants and animals. Transposons are spliced out of DNA sites and transferred to others by an enzyme encoded by the transposon. This enzyme, called transposase, recognizes inverted repeats on the ends of the transposon and cuts both strands of the DNA forming blunt ends. The transposase enzyme also recognizes a 5-9 bp

target sequence within the genome, and cuts it asymmetrically leaving sticky ends. The transposase holds the transposon in place at the new site while DNA polymerase adds complementary nucleotides to the single stranded ends. Then ligase enzyme seals the nicks to form direct repeats in the sequence on either side of the newly inserted transposon (**Figure 1**).

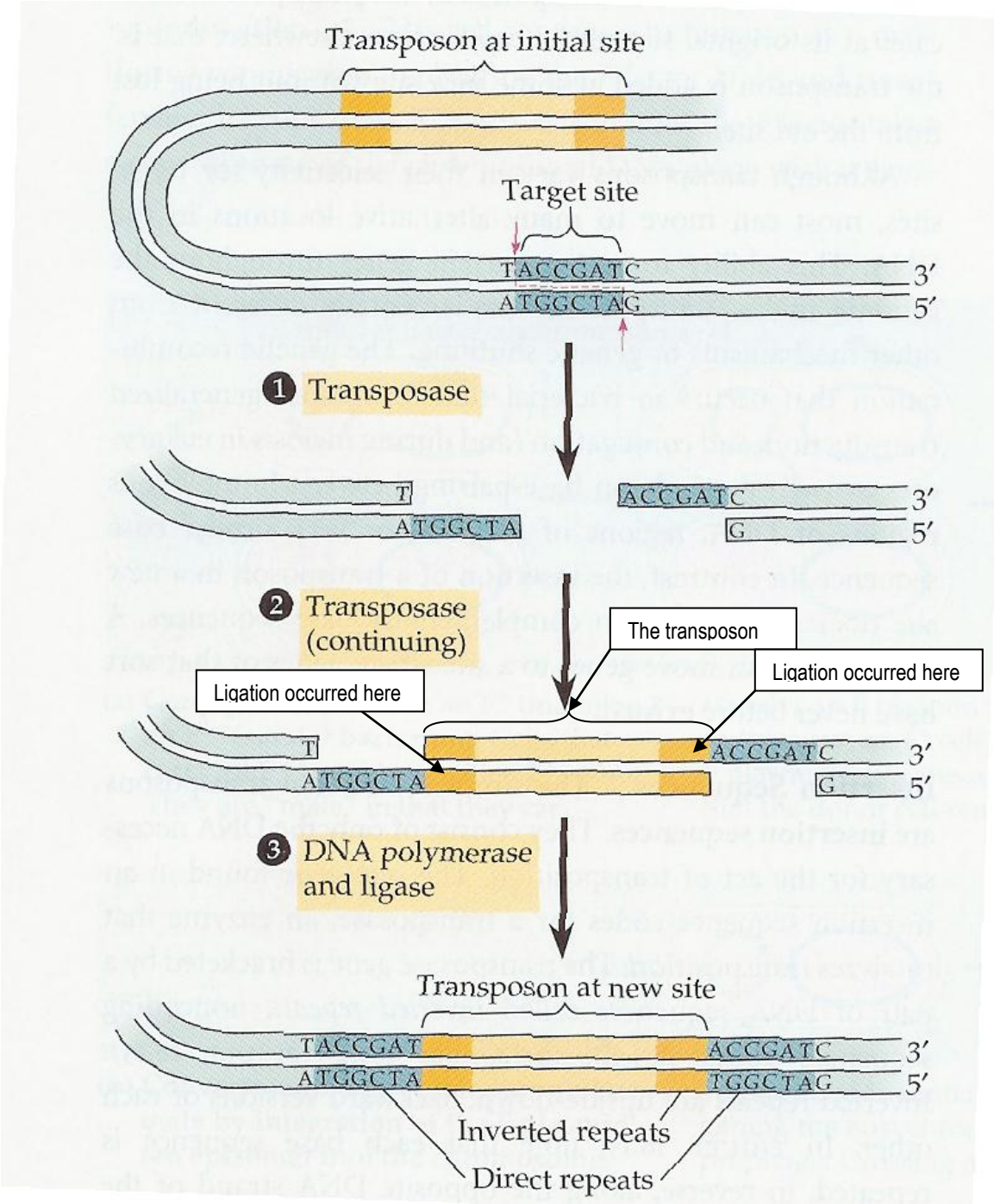


Figure 1. Transposon insertion (from *Biology* Fifth Edition by Campbell, Reece and Mitchell, page 336, ©1999, Benjamin Cummings, publishers)

These "jumping genes" were first discovered by Barbara McClintock who was doing research on the variegation of Indian corn while working at Cold Spring Harbor Laboratory, New York, in the 1940's. McClintock named the transposable DNA element she discovered "Dissociator" (Ds) after the way it disassociates from the chromosome on which it is carried. When it then inserts into a gene that codes for a pigment, such as anthocyanin, the pigment is not produced and the corn kernel is white instead of purple. If the Ds element is transposed from the anthocyanin gene after the kernel starts developing, some cells within the kernel produce pigment while others do not, resulting in a mottled effect where purple spots appear within the white background **(Figure 2)**.

Figure 2. Indian Corn (http://community.webshots.com/photo/93492638/93495658hDbgjG)

The Ds element is not the entire story, however. McClintock found that another gene she called "Activator" (Ac) is necessary for the transposition of Ds. Ds is believed to be a mutant transposon, lacking the gene for transposase. Ac carries the transposase gene, and no matter where in the genome it is located, it supplies the enzyme for the transposition of Ds. It is possible to cross breed Indian corn so that the Ac gene is removed from the genome. If Ac is missing, Ds will not move, giving a stable phenotype. Barbara McClintock's publication in 1953 documenting this non-classical genetics answer to phenotypic traits rattled the scientific world that thought of genes as static structures. It was not until the advent of modern biotechnology and DNA manipulation techniques that McClintock was proven right. She was awarded the Nobel Prize in 1983 for her work.

The *Arabidopsis* CURLY LEAF Gene

Researchers are now able to use the Ac/Ds system to knock out genes in order to characterize them not only in corn, but in tobacco, tomato and the fast growing *Arabidopsis thaliana*. *Arabidopsis* is a model plant for genetic research because it is diploid, fast growing, small, self pollinating, and easy to grow in the laboratory. It is to plant genetic research as the lab mouse is to animal genetic research. Once an interesting mutant phenotype is observed, the plant is backcrossed to get rid of the Ac element, making the mutation permanent. Since most genes are discovered and characterized when a mutation changes the observable phenotype, genes are named for the mutation they cause when the gene is defective. For example, *Arabidopsis thaliana* has a gene called CURLY LEAF, or *CLF*. When an *Arabidopsis* plant is homozygous recessive for

mutated curly leaf (genotype *clf/clf*), the leaves are curled instead of straight, the plant flowers early, has fused sepals, small petals, and grows slowly compared to wild type. The mutation causes a decrease in both the extent of cell elongation and in the number of cells, making the leaf appear curly (**Figure 3)**.

A. **B.** **C.**

Figure 3. *Arabidopsis thaliana;* A. Wild type rosette; B. wild type adult; C. *clf-2* curly leaf mutant (http://www.bio.davidson.edu/courses/genomics/arab/arab.html and http://www.biology.ed.ac.uk/plant/pages/staff_pages/J_Goodrich_staffpage.htm)

CLF is a homeotic gene that encodes a histone methyltransferase enzyme (Narita et. al, 2004). Homeotic genes are active in early embryonic development of an organism, controlling body plans and the development of groups of cells (Campbell, Reese and Mitchell; 1999). The defects observed in *CLF* mutants arise because histone methyltransferase fails to repress *AGAMOUS,* a gene controlling cell division and elongation during flower development. When *AGAMOUS* is expressed prematurely, it affects vegetative development and leaf morphogenesis, causing leaves to curl (Kim, Tsukaya, and Uchimiya; 1998).

Polymerase Chain Reaction (PCR)

PCR is DNA replication in a test tube. In a cell, several enzymes are required to replicate DNA prior to cell division. One enzyme specializes in unwinding the double helix, while another unzips the two polymers of DNA by breaking the hydrogen bonds between the base pairs. Single strand binding proteins stabilize the two strands while another enzyme adds a short complementary RNA primer to the site where replication will begin on one of the strands (**Figure 4)**.

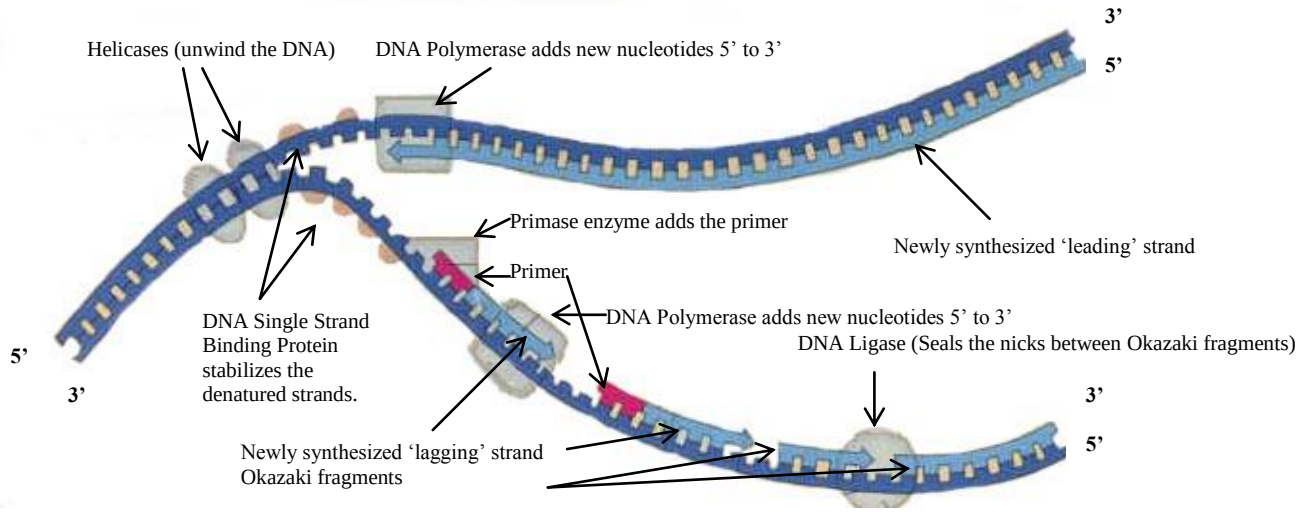


Figure 4. The enzymes of DNA Replication. Synthesis always occurs in the 5' to 3' direction. Therefore, the lagging strand must be synthesized in short pieces called Okazaki Fragments, which are later ligated together. The Leading strand is synthesized in a continuous fashion, 5' to 3'. (Modified from *Biology* Fifth Edition by Campbell, Reece and Mitchell, page 336, ©1999, Benjamin Cummings, publishers)

The primer is required because the replication enzyme, DNA polymerase, must have a free 3' hydroxyl group (-OH) in order to fit onto the DNA strand and then to add the next nucleotide. See **Figure 5**. Single nucleotides are then added opposite their complementary nucleotides to build two complete DNA molecules, each with one old strand and one new strand.

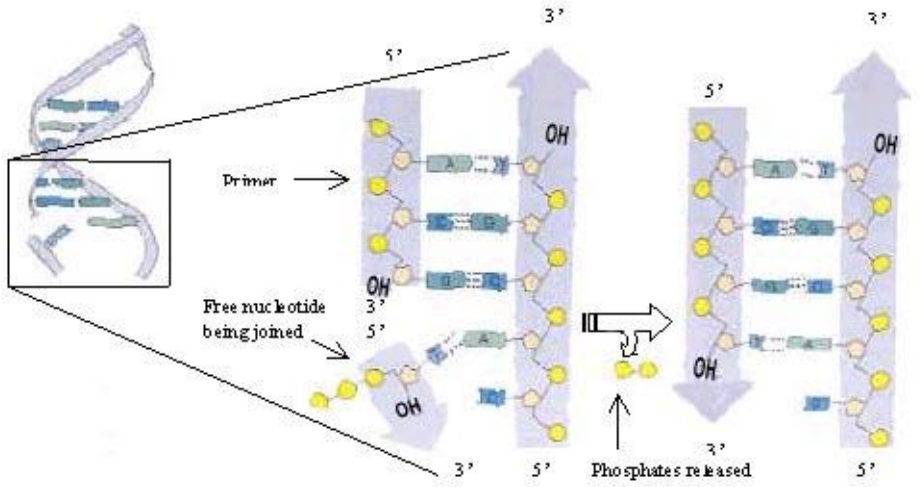


Figure 5. DNA Replication. Shows how new nucleotides are joined to a primer.
(Modified from *Biology* Fifth Edition by Campbell, Reece and Mitchell, page 336, ©1999, Benjamin Cummings, publishers)

The primer concentration is very important in PCR; if it is too high, amplification of nonspecific products may result. If too low, not enough of the target product will be

amplified. The ideal concentration is between 0.1 and 0.5 µM. Primers are sold as solid DNA by some companies and diluted in water by other companies. A certificate of analysis packed with the primers give the mass, number of moles and if diluted, the concentration in either µM or µg/µl or both. It can be difficult to determine how to dilute and mix the primers for use in PCR so that the concentration is between the optimum 0.1 and 0.5 µM. **Table 1** and the sample calculation, below, may help.

Table 1. Molar conversion for Primer Concentration*

Primer length	**pmol/µg**	**20 pmol****
18-mer	168	119 ng
20-mer	152	132 ng
25-mer	121	165 ng
30-mer	101	198 ng

* From Qiagen News, Issue 5 1997
** 20 pmol of primer in a 100 µl PCR reaction gives a primer concentration of 0.2 µM

SAMPLE CALCULATION for DILUTION OF LIQUID PRIMER DNA

Suppose a primer concentration is given as 40 µM and 0.32 µg/µl. You need to make up 300 µl of primer/loading dye mix. The protocol states that 22.5 µl of the primer/loading dye is used in the 30 µl reaction. The final concentration of each of the primers in the 30 µl reaction must be 0.1 – 0.5 µM.

1. The primer's final concentration in the reaction must be 0.1 – 0.5 µM, so dilute the primer to 0.5 µM and use this to set up the reaction.
2. Use $C_1 \times V_1 = C_2 \times V_2$ to calculate the volume of working solution to use.
   C_1 = 40 µM
   V_1 = X
   C_2 = 0.5 µM
   V_2 = 300 µl

   (40 µM) X = (0.5 µM) (300 µl)
   X = 3.75 µl

Add 3.75 µl of the primer as supplied by the manufacturer to enough loading dye and the other reagents to give a total of 300 µl. When 22.5 ul of this mix is used in the reaction, the final concentration of this primer will be 0.375 µM, which is within the 0.1 – 0.5 µM final concentration limits.

(0.5 µM) (22.5 µl) = X (30 µl)
X = 0.375 µM

For PCR, the only enzyme required is DNA polymerase. Magnesium chloride is added to the buffer because magnesium acts as a co-enzyme, fitting into the three dimensional structure of the protein to activate it. Too much magnesium can reduce the ability of the polymerase to stay attached to the template (i.e., fidelity is decreased), so the right concentration (1.0 – 3.0 mM) is crucial. Heating to 94 - 95°C denatures the DNA by increasing the kinetic energy of the atoms, breaking the hydrogen bonds between the base pairs. Primers (oligo-deoxyribonucleotides) are synthesized commercially to match the known DNA sequences that surround the fragment to be amplified. These primers anneal (H-bond) to their complementary sequences as the temperature is lowered to 50 - 65°C (the temperature depends on the sequence of the primers). The temperature is then increased to 72°C, which serves to keep the single strands of template DNA apart. The polymerase adds complementary nucleotides starting from the 3' -OH end of the primers. Enough time is allowed, (30 seconds – 1 minute) for the enzyme to work. The concentration of the deoxynucleotides in the reaction is also critical. For example, if the concentration of dNTPs is too high, fidelity of the polymerase (ability to stay attached to the template DNA) will decrease. When the cycle is complete, two double-stranded DNA molecules will result, each containing primer sequences on either side of the target DNA. The reaction is heated again to 94 - 95°C to denature the strands and begin another cycle of replication. Since all newly synthesized DNA molecules have both primer sequences, these steps can be repeated to yield an exponential increase in the target DNA. After about 30 cycles, the target is replicated to give over one million copies. See **Figure 6**.

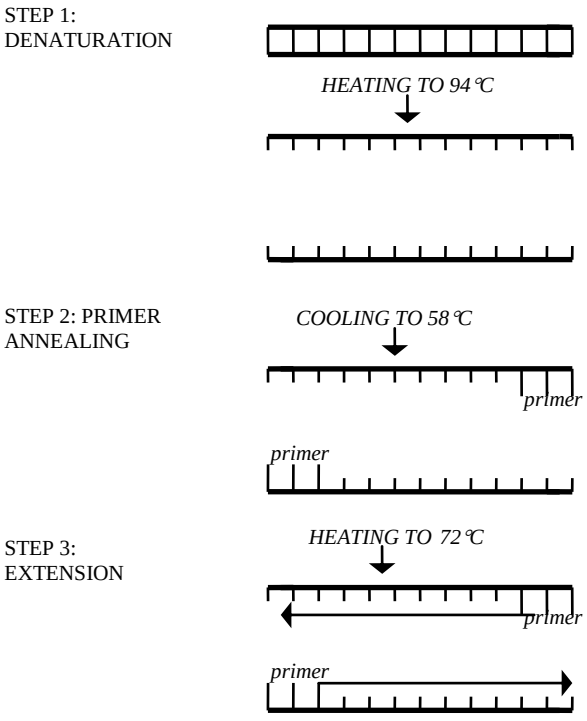


FIGURE 6. Steps of PCR

In review, PCR requires inorganic $MgCl_2$, and four types of organic molecules: template DNA, DNA polymerase, deoxynucleotides, and DNA primers. PCR consists of four basic steps:

1. Denaturing the template DNA
2. Annealing the primers
3. Extension (replication) of complementary strands by DNA polymerase
4. Repeating the cycle 30 - 35 times

PCR Genotyping of Arabidopsis

The *Arabidopsis thaliana* plants used for PCR in this lab will be grown from seed collected from heterozygous plants. Since *A. thaliana* self pollinates, this F2 generation will exhibit a 1:2:1 genotypic ratio (1 homozygous dominant: 2 heterozygous: 1 homozygous recessive) and a 3:1 phenotypic ratio (3 wild type: 1 curly leaf). The seedlings used in this experiment may be too small to determine the phenotype, but even if wild type and curly leaf were distinguishable, only the genotype of the mutant would be known because wild type phenotype could be either homozygous or heterozygous genotype. PCR primers for this lab are of three kinds: forward and reverse primers for a 250 bp fragment of the *CLF* gene, and a reverse primer homologous to sequence within the transposon **(Figure 7)**. When a forward *CLF* primer is used with a reverse *Ds* primer, the product will be 750 bp long if the transposon is present. Theoretically, the forward and reverse primers could be used to amplify either fragment, but in reality, the distance between these two sites in the mutant is too long for PCR to give reliable results. Therefore, two reactions will be run on the same plant DNA, one with the two *CLF* primers and the other with the forward *CLF* primer and the reverse *Ds* primer.

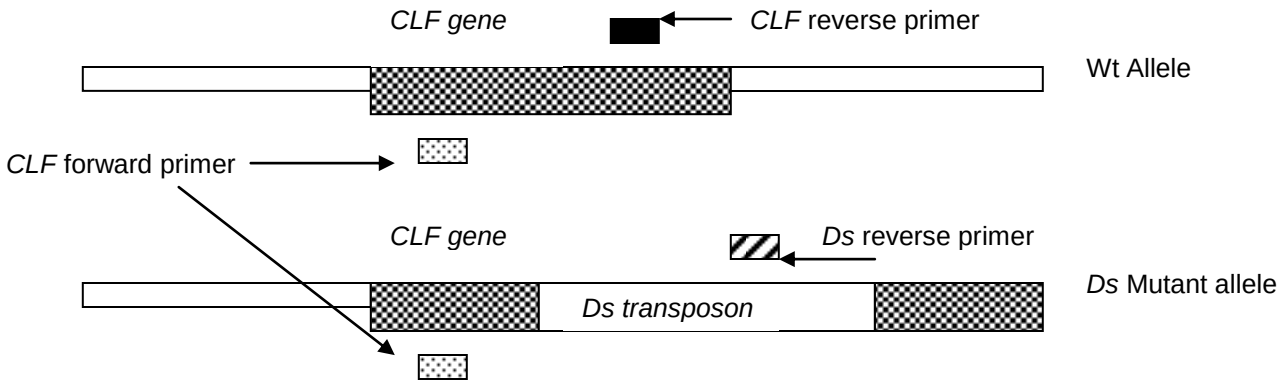


Figure 7. Maps of the *CLF* gene and primers

Gel Electrophoresis

The two PCR reactions from one plant will be combined and run on a 2% agarose gel to separate different sized alleles, giving a definitive genotype. Agarose is a derivative of agar, a polysaccharide from algae. An agarose gel of relatively high concentration (vis-à-vis 0.7 - 0.8% for standard gels) is used because fragments are small (in this case, 250 and 750 bp) and concentrated agarose works better to resolve small DNA fragments. The

DNA fragments, including some molecular weight markers, are often heated to 65°C prior to electrophoresis to straighten any loops formed along the length of the molecules by weak forces of attraction such as hydrogen bonding. The pH of the PCR reaction ionizes the phosphate groups so that the DNA has a negative charge. Once loaded into the gel, an electric current is applied and the negatively charged molecules of DNA move through the gel toward the positive electrode. The gel has a sieve effect, allowing the smaller fragments to move more quickly than the larger fragments so separation is based on the size of the molecules. The gel is exposed to ethidium bromide, a flat or planar molecule that intercalates (slides between the stacked base pairs) the DNA. Ethidium bromide fluoresces orange in UV light, making it possible to visualize the DNA (**Figure 8)**. It can be added to the DNA in various ways: placed in the buffer while the gel runs; added to the agarose during gel casting; or the gel can be incubated with ethidium bromide after electrophoresis. By running marker DNA of known size and concentration on the gel as in lane 1, the DNA concentration can be calculated.

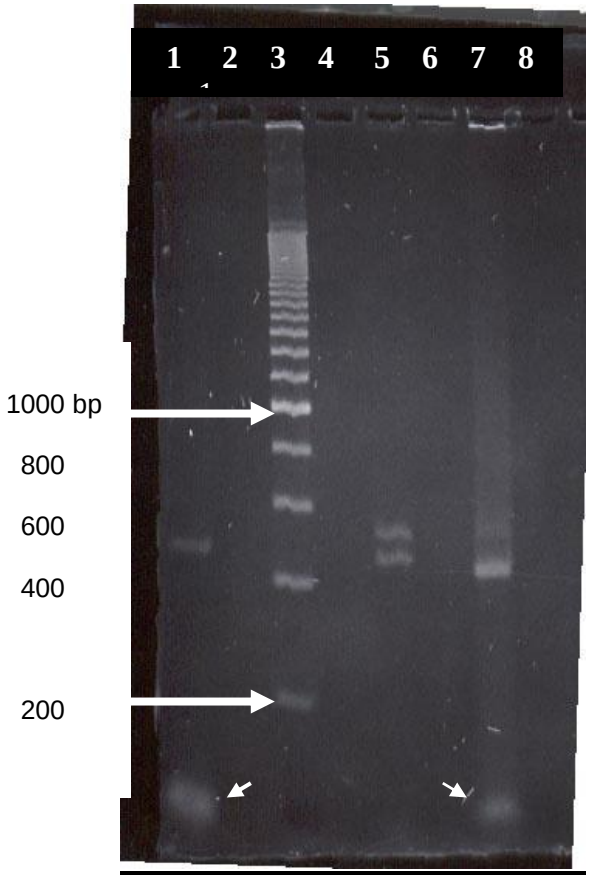
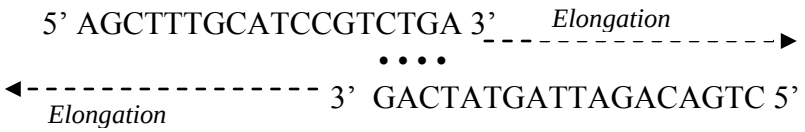


Figure 8. Polaroid photo of DNA fluorescing in UV light. Bands of DNA previously incubated with ethidium bromide fluoresce in UV light at 302 nm. Lane 3 contains DNA standard markers. Lanes 1 & 7 show homozygous alleles; Lanes 5 shows heterozygous alleles. Lanes 1 and 7 also show evidence of primer dimmer formation during amplification (small arrows).

Primer Dimers

Primer dimers are frequent artifacts of PCR, appearing as bright smears of DNA of approximately 50 bp or less. They are due to unintentional annealing between primers and their subsequent elongation, which competes with elongation of the desired PCR product. The design of primers is of critical importance, for if there is homology between

the forward and reverse primers, especially at their 3' ends, annealing can occur. For example, a forward and reverse primer could anneal in the following manner:



Primers with inverted sequence can also anneal to themselves, forming hairpin structures. Primer design programs are available so that such errors are avoided, but poor technique can also lead to primer dimmer formation. The most popular PCR polymerase is called Taq, short for *Thermus aquaticus*, a thermophilic bacterium that is found in hot springs and geysers. Taq is used in programmed PCR reactions because it is not denatured in the 95ºC heating step in each cycle. The optimum temperature at which *Taq* polymerase works is 70ºC, but it also has activity at room temperature. When reagents are mixed at room temperature, nonspecific annealing is enhanced and once amplification begins in the thermal cycler, these extended primers can produce many copies. To decrease primer dimers, always keep PCR reagents and the final reaction mix on ice until transfer to the thermal cycler; then start the cycler immediately upon loading.

Even with good primer design and good technique, primer dimers can form. Hot-start products and kits are commercially available PCR reagents designed to increase target DNA amplification by decreasing primer dimers. Hot-start products work by coating reagents such as the magnesium or the Taq with wax, preventing the polymerase from working. The first step in the PCR program is a 1-2 minute heating step that melts the wax, freeing the reagents. Another hot-start method is to complex the Taq with monoclonal antibodies that prevent enzyme activity until the initial heating step denatures the antibodies, freeing the enzyme. Brands of enzymes differ. Be sure to follow each manufacturer's directions.

Estimating DNA Size by Standard Curve

Once the PCR reactions are amplified and the DNA products separated by gel electrophoresis, the size of the DNA fragments can be determined by setting up a standard curve using the size of the molecular weight markers (the STANDARDS) and the distance each marker band migrated from the well. First, measure the distance (in millimeters) that each band of the molecular weight DNA standards migrated from the bottom of the well to the middle of each band and record in a data table like **Table 2,** below. Since the speed of movement increases exponentially as the size of the DNA decreases, semi-log graph paper is used to graph the standard curve. The molecular weight of each marker is placed on the Y (ordinate) axis of the graph and the distance of migration is placed on the X (abscissa) axis. Draw a best fit straight line between the XY coordinates to create the standard curve. Next, measure the distances that each PCR fragment migrated, from the bottom of the well to the middle of each band and record them in the data table. Use the standard line on the graph to determine the size of the PCR fragments by marking where the migration distance for a fragment on the X axis intersects the standard line. This point will correspond with a size on the y axis, giving an estimate of the size of the PCR fragment +/- 10%. See a sample graph in **Figure 9**.

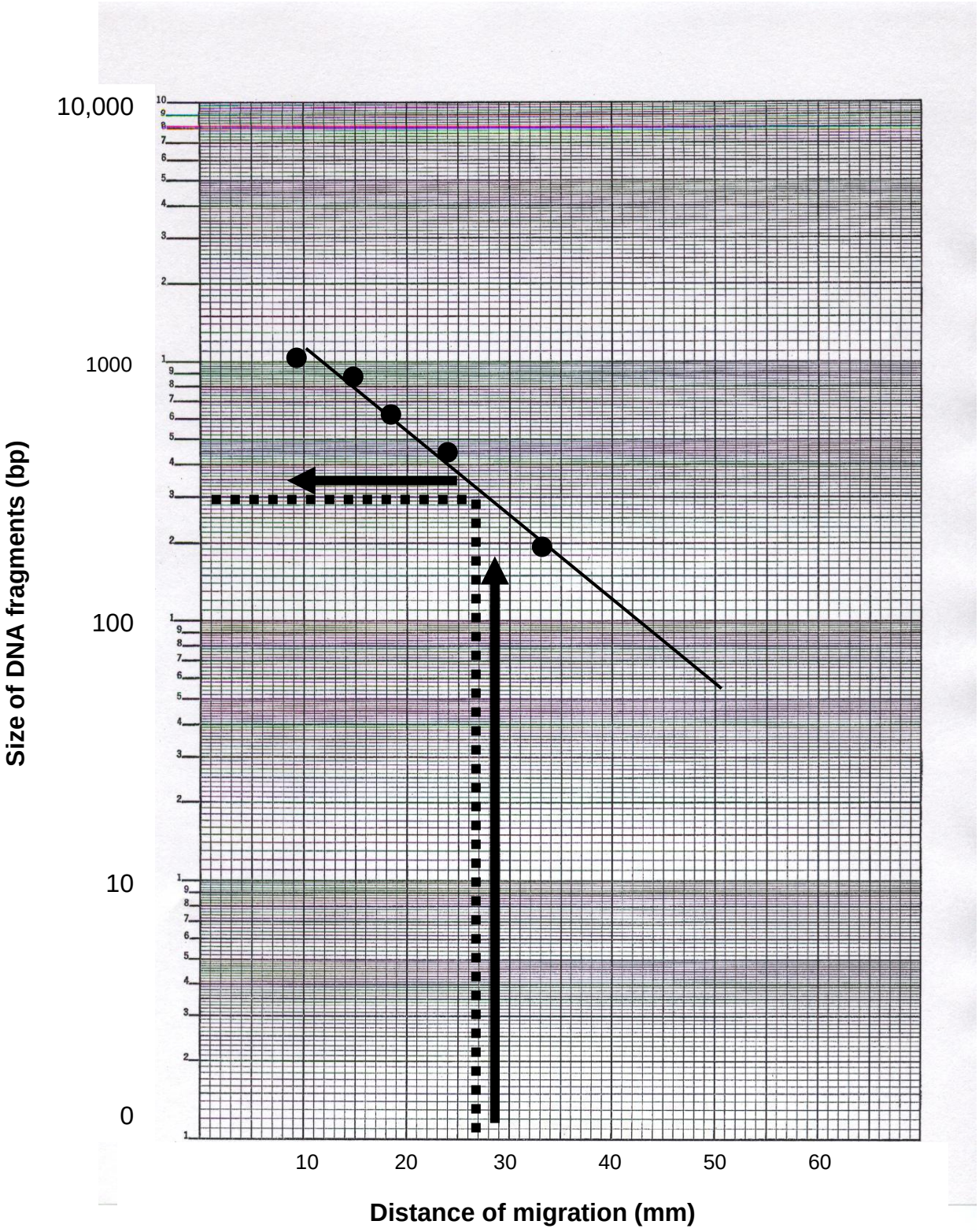


Figure 9. **Standard curve of DNA electrophoresis results.** If one of the PCR fragments migrated 27 mm, find where this intersects the best fit line drawn through the coordinates of the standard marker DNA data points. The corresponding Y value is the size of the amplified DNA band (350 bp ± 10%).

Table. 2 Sample Data Table for Standard Curve Graph

Sample ID	Molecular Weight (Y axis)	Distance of Migration (X axis)
Mwt Marker	1000 bp	10 mm
"	800 bp	15 mm
"	600 bp	18 mm
"	400 bp	24 mm
"	200 bp	33 mm
PCR fragment 1	*	27 mm
PCR fragment 2	*	20 mm
PCR fragment 3	*	25 mm

* Use the standard curve graph to determine these sizes

LABORATORY OVERVIEW

In this lab, you will use *Arabidopsis* seedlings as a source of DNA. For PCR analysis, the DNA Stock Center for *Arabidopsis* at Ohio State University routinely grows plants on agar and harvests them when the first true leaves appear. After adding sterilized seeds to the agar, the plates are given a 3 day cold treatment in darkness, then harvested 7 to 10 days after transferring out of the cold into the light and warmth. The cells of the tiny 7-10 day old seedlings will be lysed and the DNA will serve as the template for PCR. After amplification, the PCR products will be run on a gel to determine if the plant has two different alleles (heterozygous *CLF/clf*) or two identical alleles (homozygous *CLF/CLF* or *clf/clf*). The base pair size of each allele will be estimated using a standard curve.

MATERIALS for growing *Arabidopsis*

Arabidopsis seeds (Carolina Biological Supply or abrc@arabidopsis.org for seed ordering)
Each student needs one tube of 25-50 *clf*-2 seeds (from a heterozygous plant)
Modified MS agar plates, one per student
Growth chamber set on continuous light
Sterile 1000 ml pipette tips
30% bleach solution (students will prepare)
Sterile dH_2O – one tube or bottle per group
Sterile 0.1% agarose solution (students will prepare)
Aluminum foil

MATERIALS for DNA Isolation and PCR

2 per group Ready-To-Go PCR Beads (**PuReTaq Ready-To-Go™ PCR Beads** from http://www1.amershambiosciences.com 96 reactions #27-9559-01, NC9711585 from Fisher, will last ≥ 1.5 years; cost $227.50)
Each pellet reconstituted to 25 µL contains 1.5 units Taq polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 µM of each dNTP.
Primers can be ordered from http://invitrogen.com
0.4 uM minimum *CLF* (wildtype) primers/loading dye mix – 25 µL per Team
FORWARD 5'-TTAACCCGGACCCGCATTTGTTTCGG-3'
REVERSE 5'-AGAGAAGCTCAAACAAGCCATCGA-3'
0.4 uM minimum *clf*-2 (mutant) primers/loading dye mix – 25 µL per Team
CLF FORWARD 5'-TTAACCCGGACCCGCATTTGTTTCGG-3'
Ds REVERSE 5'-GTCGGCGTGCGGCTGGCGGCG-3'
Edward's Buffer, 2 ml per team (Students will prepare)
Cresol Red Loading Dye
Isopropanol, aliquoted – 2 ml per team
Pellet pestles – one per team
Dissecting microscopes – 1 per team
Thermal cycler
Hair dryer
TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA)

MATERIALS for Electrophoresis

100 - 1000 bp ladder or pBR322 DNA-BstN I digest (inexpensive marker from NE Biolabs)
Zip Lock bags for storing gels
Heating block or water bath set at 50°C
50X TAE electrophoresis buffer (students will dilute to 1X working solution)
D.C. power supplies – per 2 groups
Practice loading dye
10 mg/ml ethidium bromide stock solution

MATERIALS THAT SHOULD BE AVAILABLE OR KEPT IN THE LAB

10 mg/mL ethidium bromide stock solution (stored at 4°C)
Microwave oven and hot gloves
Electric pipette pumps
Thumb-operated pipette pumps
Serological pipettes
Kim wipes
Lab diapers
Box of plastic wrap
Sharpie markers
Sterile 1.5 ml microcentrifuge tubes
Microcentrifuge tube trays
Microcentrifuges
Personal microcentrifuges
Micropipetters and tips (2.5 µl and P-1000)
Vortex mixers – 4 per lab
Ice buckets and crushed ice
Horizontal gel rigs
Labeling tape
Electrophoresis grade agarose
Electric balance, weigh paper/boats, spatulas, clean-up brush
250 ml Erlenmeyer flasks
Beakers and bottles of various volumes
25, 100, 500 & 1000 ml graduated cylinders
Molecular grade water
UV transilluminator, camera, film, face shields, spatulas, plastic dishes
Carboy of dH_2O
disposal for Ethidium Bromide contaminated items
Dishpan for dirty glassware

RECIPES:

Modified MS agar:

4.3 g MS salts (half the amount used for bacteria culture)
900 ml distilled water
Use 1.0 M NaOH or KOH to adjust to pH 5.7
BTV of 1 Liter

Then add 8 g agar and autoclave for 20 minutes at 120°C. One liter makes 40-50 10 cm diameter plates.

Cresol Red loading dye

1% Cresol Red Dye Stock – yield 50 ml
Add 500 mg to 50 ml of ddH_2O in a 50 ml tube or bottle
Shake to dissolve
Store at room temperature

Cresol Red Loading Dye – yield 50 ml of working solution
Add 17 g of sucrose to 49 ml of ddH_2O in a 50 ml tube or bottle
Shake to dissolve
Add 1 ml of 1% Cresol Red Dye
Shake tube to mix. Store at 4°C.

Edward's Buffer – yield 50 ml (solid NaCl and concentrated stocks will be used)
Mix the ingredients in a 50 ml bottle. Can be stored at room temperature, indefinitely.

32.5 ml ddH_2O
10 ml of 1 M Tris pH 8
2.5 ml of 5 M NaCl
2.5 ml of 0.5 M EDTA
2.5 ml of 10% SDS

Recipes (cont.)

50x Concentrated TAE Electrophoresis Buffer (40 mM Tris-acetate, 2 mM EDTA)

Add the following to dH_2O to give a final volume of 1 liter
242 g Tris base (Tris [hydroxymethyl] aminomethane)
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8)

Students will dilute to a 1X working solution

Primer/Loading Dye Mix

Final concentration of components:
0.4 uM of each primer, 13.9% sucrose, and 0.0082% cresol red in Tris-low EDTA (TLE) buffer (10 mM Tric-HCl, pH 8.0; 0.1 mM EDTA).

SAFETY GUIDELINES

GLP requires wearing gloves and eye protection.

Ethidium bromide is a strong mutagen. Gloves must always be worn when handling gels or buffers containing this chemical.

Boiling agarose can cause burns. Wear hot gloves when removing agarose from hot plate or microwave oven.

The **electric current** in a gel electrophoresis chamber is extremely dangerous. Never remove a lid or touch the buffer once the power is turned on. Make sure the counter where the gel is being run is dry.

UV light, used to illuminate the DNA stained with ethidium bromide, is dangerous. Eye protection must be used.

PROCEDURE
PART I. Prep

Teams should verify all calculations with the instructor before proceeding.

Prepare 100 ml of 0.1% agarose for use by the whole class. Verify calculations with the instructor before proceeding. Weigh out the correct amount of agarose and place into a 250 mL Erlenmeyer flask. (Clean up the balance when finished!) Add dH_2O and swirl gently. Microwave on high for 1 minute intervals until no particles of agarose can be seen floating in the liquid when held up to the light. Use the P-1000 pipetter, sterile tips and sterile technique to aliquot 1 ml into a sterile 1.5 mL microcentrifuge tube for each student + 1 extra. Cap tubes and distribute one to each student.

Prepare the Edward's Buffer according to the recipe given in the materials section of this lab. Aliquot 0.5 mL to each team. (Note: This solution does not have to be sterile, but MAKE SURE THE NaCl is IN SOLUTION.)

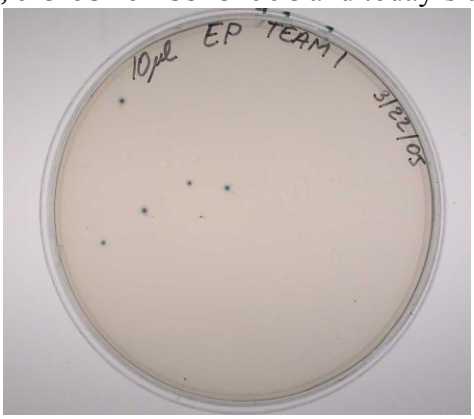
Prepare 30 mL of 30% bleach solution using dH_2O. Verify your calculation with the instructor before proceeding. Aliquot 1 mL in a 1.5 mL microcentrifuge tube for each Team.

Dilute 50X TAE buffer to make 1 liter of 1X TAE buffer.

PART II. Planting *Arabidopsis* seeds

Each student should have a 1.5 ml microcentrifuge tube with 25-50 *clf*-2 seeds that must be sterilized, rinsed, and mixed with 0.1% agarose before inoculating the MS agar plate. (NOTE: The *Arabidopsis* seeds are packaged with about 50 in each 1.5 ml tubes, so each student gets one tube of seeds. These seeds are very tiny, so DON'T SNEEZE!)

1. Gather all your materials and supplies before you begin. Label your MS agar plate around the edges on the bottom of the plate (see example, below) with your Team number, your initials, the lab number or title and today's date.



2. Each student should get one 1.5 ml microcentrifuge tube containing *Arabidopsis* seeds. Add 0.5 ml of 30% bleach solution to the tube. Let soak for 3 minutes, shaking once each minute.

3. Let the seeds settle to the bottom and then remove the bleach solution with a micropipetter. You can place the used bleach in the sink at your bench or in a beaker for waste.

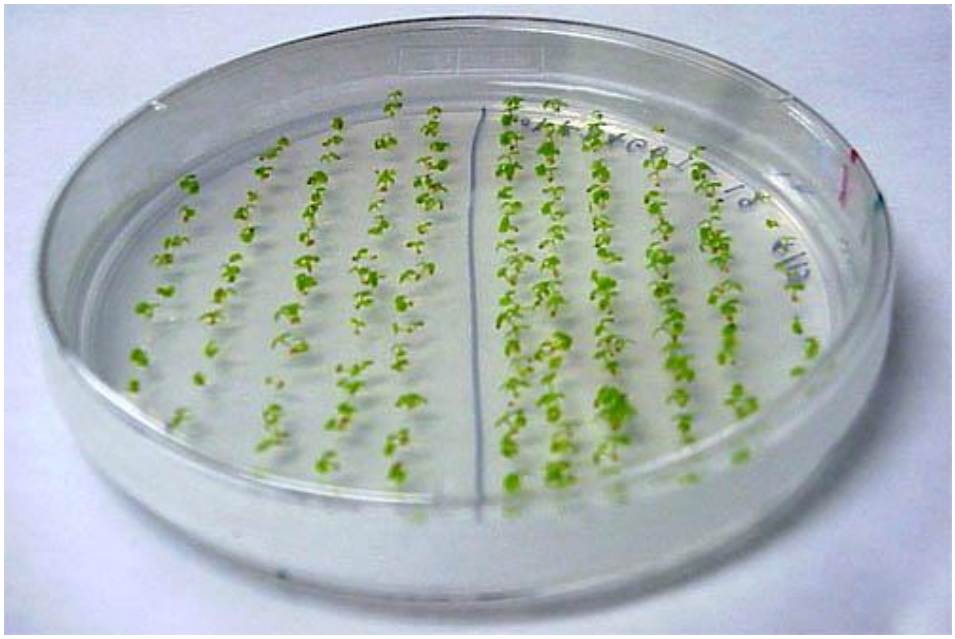
4. Add 200 µL <u>sterile</u> dH_2O to rinse the seeds. Let the seeds settle again and remove the water.

5. Repeat the above step two more times. The seeds are now sterilized.

6. Use a P-1000 pipetter and a sterile tip to add 100 µL of cooled 0.1% agarose to the sterilized seeds.

7. Use a P-1000 digital pipetter to carefully pipette the seeds + agarose up and down to mix. Set the pipetter for 1 ml and draw all into the tip. (NOTE: seeds get caught in smaller tips, so use the P-1000.)

8. Use sterile technique to carefully dot the agarose-seed solution onto an MS agar plate so as to get growth as seen in the photo, below.



9. Wrap the plate in aluminum foil to keep out all light and place at 4°C for three days.

10. After the incubation at 4°C, remove the aluminum foil and transfer the plates to a 37°C incubator for approximately 5 hours. Then transfer plates to the plant growth chamber and grow in constant light for 7-10 days.

Part III. Isolating *Arabidopsis thaliana* DNA

1. Make sure you have all materials and supplies. Place the isopropanol on ice.

2. Obtain your team MS agar plate with seedlings from the plant growth chamber. Observe your plants using a dissecting microscope. Record any observations regarding the seedling you choose to use. A digital camera can be used to take a photo of the seedling, if desired. Your instructor can demonstrate this.

3. Pick one of the larger seedlings from your MS agar plate and place into a 1.5 mL microcentrifuge tube. Brush off any excess agar clinging to the roots. (Note: it is no longer necessary to keep the plant sterile.)

4. Use a disposable micro pestle to grind the plant for approximately 1 minute. Notice that the tip of the pestle does not go flush into the bottom of the tube, so you may need to use a pipette tip to pull the tissue up along the side of the tube and grind it more. The tissue should have a liquid consistency.

5. Add 400 µL of Edward's Buffer. Grind briefly to get all tissue off the pestle.

6. Vortex the capped tube for 5 seconds and then let sit at room temperature for 5 minutes.

7. Spin for 3 minutes at maximum speed in a microcentrifuge. There should be a tight pellet of debris in the bottom of the tube.

8. Do not disturb the pellet as you transfer 350 µL of the supernatant, which contains the DNA, to a new labeled 1.5 mL tube. If you disturb the pellet, centrifuge again. Label with your team number and your initials.

9. Add 400 µL of cold isopropanol to the 350 µL of supernatant. Rock the tube to mix and place on ice for 3 minutes. This step precipitates the DNA.

10. Place the tube into the microcentrifuge so that the tube's hinge is on the outer circumference of the rotor. Spin for 5 minutes at maximum speed. The DNA pellet will form at the bottom of the tube on the hinge side. This small pellet of DNA may not be visible, so it is important to know where it is in the tube.

11. Remove the isopropanol by decanting and then keep the tube inverted on a paper towel. The slight amount of alcohol in the top of the tube can be removed with a Kim wipe or sterile swab.

12. Resuspend the DNA in 50 µL of TE Buffer. Tap the tube with your finger to mix. It is not always a good idea to pipette DNA up and down or to vortex because either can shear chromosomal DNA.

13. If there are flecks of plant debris still visible, centrifuge the DNA-TE to pellet the debris. You will only need a small amount for the PCR reaction, so you will have plenty, and you can centrifuge again just before you use it.

Part IV. Amplifying DNA using PCR

Before beginning this part of the lab, the instructor will show one group how to program the thermal cycler. Then the groups will tell each other in a round-robin style until all have heard it and explained it to another group or to the instructor.

1. Gather all supplies and materials required.

2. Label two Ready-To-Go PCR pellet-containing tubes with your Team number and date. Mark one tube as Wt (for wild type, which will contain the forward and reverse wild type primers) and one tube as Ds (which will contain the transposon Ds Primer + forward Wt primer).

3. Add 22.5 µL of the correct primer/loading buffer mixture into the appropriately labeled tube. Tap each tube to dissolve the beads.

4. Add 2.5 µL of your Team's DNA-TE mixture into the tube with the dissolved bead and primer/loading buffer. Close the lid and tap the PCR tubes with your finger to mix and then sharply rap the tubes on the bench top to move all the liquid to the bottom. Place the tubes on ice.

5. Place your tubes into the thermal cycler when all teams are ready to amplify their DNA.

6. Place the heat bonnet on the top of the tubes and start. The program for amplification of these products is:

95°C 5 minutes (to ensure complete denaturation of the template DNA)
94°C 30 seconds
65°C 30 seconds } **repeat these three steps for 30 cycles**
72°C 30 seconds

4°C hold

Part V. Casting a 2.0% agarose gel

Agarose gels can be poured a few days to a week in advance of their use and kept at 4°C. However, microorganisms capable of producing enzymes that degrade DNA can grow in gels. Electrophoresis buffer can be poured over a gel in the rig and refrigerated, but if it will be longer than three days until use, do not add buffer. Instead, remove the gel from the rig and wrap it in plastic or place in a zipper-type plastic bag and store at 4°C. A gel should be removed from the cold and brought to room temperature just prior to the time it is needed.

Note: Since up to 8 teams can load on one gel, the instructor will demonstrate how to cast the gel. In the future, gels will be cast by the students.

1. Gel rigs are manufactured by different companies, so set up the electrophoresis chamber according to your instructor's directions. If the rig needs to be cleaned before using, DO NOT dry it with paper towels, which leave paper fragments that float in your molten agarose after pouring. (Note: For gel rigs with adjustable combs, set the depth of the comb to about 2-3 mm from the bottom of the gel chamber. The correct depth is approximately the height of two stacked microscope slides. Adjust the teeth of the comb so they sit on the slides. Remove the slides and comb before pouring the gel.)

2. To cast a 2% agarose gel, weigh out 1.0 gram of agarose and place in a 250 ml flask. (GLP: C*lean up the balance when finished!*) Add 50 ml 1X TAE Electrophoresis Buffer and microwave in 30 second intervals, removing the flask and gently swirling to mix after each heating. (Make sure you use hot mitts!) Continue until the agarose is completely melted, 1-3 minutes. Hold the flask up to the light and make sure that all the agarose is melted. The agarose will appear thick and may contain bubbles. Check

the volume after heating by pouring the molten gel into a graduated cylinder. Add more dH_2O to bring the volume to 55 ml. (Even thought the concentration is slightly less than 2%, this volume is necessary for the wells to accommodate the samples to be loaded.)

3. Let the agarose cool until you can hold your hand on the bottom of the flask for 30 seconds. Pour the slightly cooled melted agarose into the casting tray. Hold an automatic pipette tip and use it to pop any bubbles that remain in the agar – they can interfere with the progress of the DNA's movement through the gel. Insert the comb about 1 centimeter from the end that will have the negative electrode attached. (To make this determination, note how the lid fits on the gel rig.)

4. Allow the gel to cool and solidify for about 20 minutes. Do not disturb the gel during this time. It should look opaque, not translucent, when solid.

5. Gently remove the comb from the solidified agarose by pulling it straight upwards in one motion. (Note: if the gel is not completely solidified, the wells will be malformed or will collapse.) If the gel is to be used later, carefully place it into a small Zip Lock bag and store it at 4°C. Clean the rig and air dry, upside down.

Part VI. Gel Electrophoresis of PCR products

YOU MUST BE WEARING YOUR GLOVES. **Set a heating block to 50°C.**

Two groups can use one gel to run their reactions. Each team will need the following supplies:

- Horizontal electrophoresis unit
- Power supply, to be shared with another gel rig
- Molecular Weight DNA Ladder (DNA standards)
- A heating block or water bath set to 50°C.
- 2.0% agarose gel, brought to room temperature
- 1x TAE Electrophoresis Buffer
- Your team's PCR reactions
- The instructor will have the 10 mg/mL stock of Ethidium bromide

1. If your gel was stored, remove it from the zipper bag and place into the gel rig. Add enough 1x TAE electrophoresis buffer to fill both end reservoirs and cover the gel by about 5 mm. (Note: too much buffer over the gel can slow the movement of DNA; too little buffer can cause the gel to melt from the heat generated by the electric current.)

2. If you have not loaded an agarose gel before, and need practice, use some of the sample gel loading dye. Your instructor can demonstrate the correct way to load a gel. There is no need to put the pipette tip into the well when delivering the sample. The gel loading dye added to each sample is denser than the electrophoresis buffer and will carry the DNA down into the well when delivered directly above it.

3. Thaw your PCR reactions if they were just removed from the -20°C freezer.

4. Pulse spin your PCR reactions to move the fluid to the bottom of the tubes.

5. Combine both of the reactions that each team member set up and pulse spin the mixture.

6. Heat your reactions along with the molecular weight DNA ladder in a heating block set at 50°C for **two minutes**.

7. Immediately pulse spin the reactions & DNA ladder to move all the fluid to the bottom of the tubes. Place on ice.

8. Slowly load each sample in the order directed by your instructor. Include a molecular weight marker DNA sample in at least one lane of each gel.

9. Add 10 µL of 10 mg/mL ethidium bromide stock to the buffer. (Note: since the total buffer is about 100 mL, the final concentration of ethidium bromide is 1.0 µg/mL.)

10. Place the cover on the electrophoresis unit, making sure to have the negative electrode at the well end of the gel.

11. Insert the electrode cords into the proper input of the power supply. Set the power supply on 'low' and turn it on. Set the voltage at 90-95 volts. Check to make sure bubbles are forming in the buffer on the platinum wire electrodes along the ends of the gel box. Watch the gel closely for 2 – 5 minutes to make sure that the dye is migrating in the correct direction.

12. Allow the tracking dye to migrate 4-5 centimeters from the wells so that the molecular weight marker fragments separate sufficiently.

13. Turn off the power supply, unplug it, and disconnect the electrodes from it. Then remove the lid from the gel box. Remove the gel tray to a plastic container and transfer it to the transilluminator.

14. All those observing the gel must have UV face shields on, to protect their eyes and skin. If your transilluminator has more than one setting, make sure it is on the 302 nm wavelength. Cover the gel with the camera hood and turn on the transilluminator. Press the trigger on the camera and hold for 3-4 seconds, pull the film tape and remove the print. Let the print sit for about 1 minute before peeling apart. If the wells and DNA bands are not both visible, a longer exposure time may be required. The photo can be copied or scanned so that each team member has a photo to place into his or her lab notebook.

15. After photo documentation of your results, dispose of the gel and buffer and anything contaminated with ethidium bromide according to your instructor's directions.

(NOTE: more information on decontamination of ethidium bromide can be found on the Blackboard website for the class.)

16. Clean the gel rig and its parts and air dry.

DATA ANALYSIS

Determine the sizes of the Molecular Weight DNA Ladder fragments. This information is supplied by the manufacturer. Use these known DNA fragment sizes to estimate the size(s) of the PCR products on your gel. Note whether the plant your team analyzed was heterozygous or homozygous. Tape the photo of your gel into your laboratory notebook.

QUESTIONS

1. Explain how Ethidium Bromide interacts with the DNA so that it can be visualized.
2. Why would larger fragments of DNA be more intensely stained with ethidium bromide than small fragments of DNA?
3. What would happen to your DNA samples if the (+) and (–) leads on the electrophoresis chamber were accidentally reversed?
4. Why should paper towels never be used to dry electrophoresis rigs?
5. Explain, in a concise manner, how PCR works to amplify DNA. Discuss the different reagents and the steps of thermal cycling in your answer.
6. Give three methods for preventing primer dimer formation.

For the following questions, see "How to dilute solid primers" at the back of this lab.

7. Custom PCR primers arrive in the lab. The forward primer contains 25 nmoles and the reverse 40 nmoles.
    a. Explain how you would dilute each primer to a stock solution. To a working solution.
    b. Calculate the volume of working solution of each primer that you would use to prepare 100 µl of primer/dye solution so that the working concentration of each primer is 0.25 pmoles/µl.
    c. If you set up 30 µl reactions using 22.5 µl of the primer/loading dye mix, what is the final concentration of each primer in the reaction?
8. Complete the PCR PRIMER CALCULATION Practice sheet on the next page.

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PCR PRIMER CALCULATION Practice

Remember that the concentration of a primer working stock should be about 10x higher than its concentration in the Master Mix.

1. You have to set up 10 PCR reactions, each with a total volume of 25 uL (20 uL primer master mix + 5 uL of the target DNA + PCR reaction pellet). The forward primer is 40 uM and the reverse primer is 30 uM. Fill in the blanks below that show how you will make up the primer master mix using each of the primers and ddH_2O. Then answer the two questions regarding primer concentrations.

   a. What concentration will you make your working stock for each primer? _____ uM
   b. How much of each are you going to make? _____ uL

   FORWARD PRIMER WORKING STOCK

   ddH_2O _____ uL
   Forward Primer_____ uL
   _____uL T.V.

   REVERSE PRIMER WORKING STOCK

   ddH_2O _____ uL
   Reverse Primer _____uL
   _____uLT.V.

   MASTER MIX

   ddH_2O _____ uL
   Forward Primer _____ uL
   Reverse Primer_____uL
   _____ uL Total volume

   c. What will be the final concentration of each primer in your master mix? _____
   d. What will be the final concentration of each primer in your PCR reactions? _____

2. You have to set up 5 PCR reactions, each with a total volume of 50 uL (45 uL primer master mix + 5 uL of the target DNA + PCR reaction pellet). The forward primer is 30 uM and the reverse primer is 30 uM. Fill in the blanks below that show how you will make up the primer master mix using each of the primers and ddH_2O. Then answer the two questions regarding primer concentrations.

   a. What concentration will you make your working stock for each primer? _____ uM
   b. How much of each are you going to make? _____ uL

   FORWARD PRIMER WORKING STOCK

   ddH_2O _____ uL
   Forward Primer_____ uL
   _____ uL T.V.

   REVERSE PRIMER WORKING STOCK

   ddH_2O _____ uL
   Reverse Primer _____ uL
   _____uL T.V.

   MASTER MIX

   ddH_2O _____ uL
   Forward Primer _____ uL
   Reverse Primer_____ uL
   _____ uL Total volume

   c. What will be the final concentration of each primer in your master mix? _____
   d. What will be the final concentration of each primer in your PCR reactions? _____

HOW TO DILUTE SOLID PRIMERS for PCR
Work out all calculations BEFORE doing any dilutions!
[NOTE: pmoles/uL = uM]

1. Solid primers are sold in moles of primer, and once diluted, the concentration can be determined in picomoles/microliter, which is the same as uMolar.
2. For PCR, you need two primers, so make **concentrated master stocks** for both that you then keep at -20 degrees C, or -80 degrees C for long term storage. When you first dilute a primer, you should make sure it is more concentrated, about 100 uM would be appropriate. You can always dilute something, but once diluted, it is pretty difficult to make it more concentrated.
3. Use the concentrated stock to make a **working stock of each primer** that is ten times more concentrated than what you need for your reaction mix.
4. Use the working stock for each primer for the reaction mix OR for combining with Cresol Red loading dye that will be used in the reaction mix.
5. When you add all components to the reaction mix, the final concentration of each primer in the reaction should be between **0.1 and 0.5 uM.**

EXAMPLE PROBLEM:

1. The forward primer contains 25 nmoles and the reverse primer contains 40 nmoles.
   Forward primer = 25,000 picomoles
   Reverse primer = 40,000 picomoles

2. **CONCENTRATED MASTER STOCKS**
   Forward Primer concentrated master stock:
   Dissolve solid in 250 uL of ddH_2O to give a concentration of 100 uM.
   25,000 pmoles/250 uL = 100 pmoles/uL = 100 uM

   Reverse Primer concentrated master stock:
   Dissolve solid in 400 uL of ddH_2O to give a concentration of 100 uM.
   40,000 pmoles/400 uL = 100 pmoles/uL = 100 uM

3. **WORKING STOCKS –** The final concentration of the combined primers + Cresole Red loading dye should be about 10X more concentrated than the final reaction mix, and 2.5 uM is midrange (0.25 uM is midrange of 0.1 to 0.5 and 0.25 x 10 = 2.5 uM)

   Forward Primer working stock:
   Dilute the master stock 1/40 to give a final concentration of 2.5 uM.
   $C_1V_1 = C_2V_2$
   $(100\ uM)\ V_1 = (2.5\ uM)\ (100\ uL)$
   $V_1 = 2.5\ uL$
   Mix 2.5 uL of master stock with 97.5 uL of ddH_2O

To be more accurate, it would be better to prepare a serial dilution of the master stock by diluting it 10/100 (→ 10 uM) and then 25/100 (→ 2.5 uM). This yields 100 uL of [2.5 uM] primer.

Reverse Primer working stock:
Dilute the master stock 1/40 to give a final concentration of 2.5 uM, as above.

4. **PRIMER-DYE SOLUTION PRIMER MIX**
   Mix the reverse primer, forward primer and Cresole Red loading dye so that the concentration of each primer is 0.25 uM in a final volume of 100 uL.

   $$C1V1 = C2V2$$
   $$(2.5\text{ uM})\ V1 = (0.25\text{ uM})\ (100\text{ uL})$$
   $$V1 = 10\text{ uL}$$

   Mix 10 uL of each primer working stock and 80 uL of Cresole Red loading dye

5. Calculate the final concentration of each of the primers in the final reaction mix to make sure that it is within the acceptable range of concentration (0.1 – 0.5 uM). If the final volume of the reaction is 25 uL, which includes 2.5 uL of the DNA template solution, then the volume of the primer-dye mix added is 22.5 uL.

   $$C1V1 = C2V2$$
   $$(0.25\text{ uM})\ (22.5\text{ uL}) = C2\ (25\text{ uL})$$
   $$C2 = 0.225\text{ uM}$$

   This final concentration applies to each of the primers in the final reaction to be PCR amplified.