

Using SNPs to Map Genes that Control Maize Leaf Angle

Section 1: Phenotyping Corn Seedlings

The Phenotype:

In this exercise, maize seedlings of various genetic backgrounds (genotypes) will be phenotyped for their leaf angle. There will be a range of variation in leaf angle across the lines, from very obvious to not visible. The variation in the leaf angle among the genotypes will be recorded across groups and evaluated together. Leaf angles will also be measured using an image-based analysis tool. Students will be given a set of maize genotypes with known leaf angle phenotypes (which span a large diversity so they can see the variation), as well as genotypes that have no existing phenotype data available. Measurements recorded by students will be integrated into our data analysis pipelines for performing GWAS and genomic selection – we will use these data to help make predictions on leaf angle in mature corn plants. The students become an integral part of the project through this activity.

Before Your Class...

Instrument instructions

Timer

Note: Your timer model may differ slightly

The timer is currently set to turn on at 6:00 AM and off at 10:00PM. The plants should stay on a 16 hours of light / 8 hours of dark schedule. However, if you would like to adjust the time when the lights to turn on and off, you can do so.

1. Press “program”.
2. The screen will read 1 ON, and you can program the time that you want the lights to come on. Press “hour” to change to the desired hour. Press “minute” to change to the desired minute.
3. Press “program” again. The screen will read 1 OFF, and you can program the time that you want the lights to turn off. Press “hour” to change to the desired hour. Press “minute” to change to the desired minute.
4. Press “clock” to return to the current time.
5. Connect the power cord of the light bank with the timer, and then plug the timer into a power source.

Thermometer

This thermometer should be ready to use once out of the box, and will constantly display the time, temperature, and humidity. Here, you only need to record the temperature after planting the seeds. Be sure you record the temperature in °C. There is a switch on the back of thermometer to toggle between °C and °F.

List of Materials Needed for Section 1

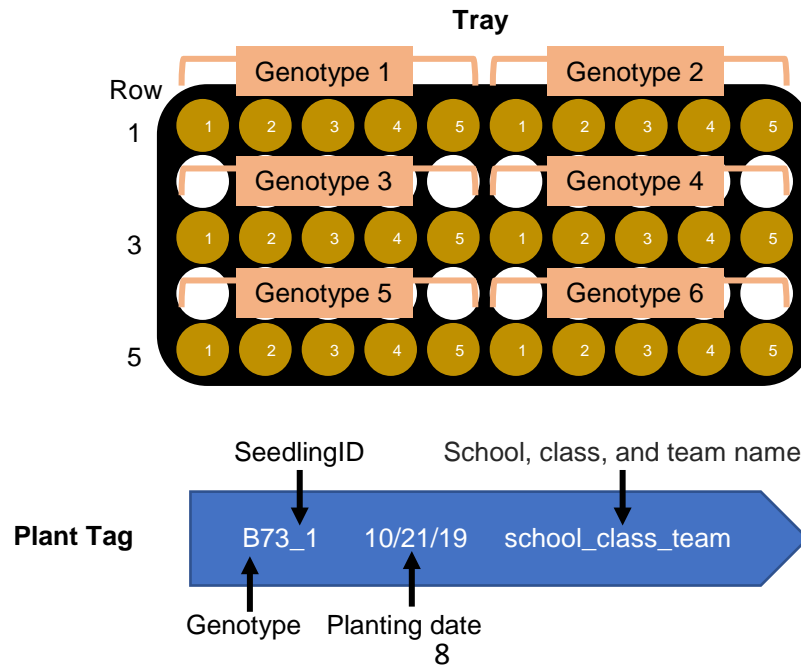
5x10 cone trays
Corn seeds (extra seeds are included for replanting)
Metro Mix 360 with surface blended soil

Light bank / Greenhouse
 Plastic beaker (not included)
 Plastic lids
 Plant tags
 Protractors
 Sharpie markers
 Spray bottle or hose (not included)
 Thermometer
 Timer

Part 1: Planting corn seeds in soil trays

IMPORTANT! Schedule your planting date to avoid the weekends and holidays for phenotyping at 16-18 Days after sowing.

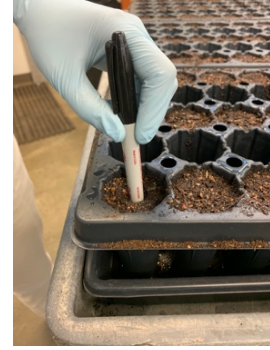
1. Fill rows 1, 3, and 5 of a clean 5x10 cone tray with potting soil (Metro Mix 360 with turface blended soil). This step was pre-prepared at the Donald Danforth Plant Science Center.
2. Moisten the soil by misting with water from a spray bottle or using the “mist” setting on a hose. Hold the spray nozzle about 1 foot above the tray so soil does not fly out of the pots.
3. Each row with potting soil fits 10 seeds from 2 genotypes = 5 seeds per genotype (See diagram below for the arrangement in a tray. The numbers labeled in each cone correspond to the seedling ID on the plant tags). Use the first 5 cones for one genotype and the last 5 cones for another. Label each cone in the tray using the large plant tags provided with: genotype, seedling ID, the date of planting, and your school, class and team name (See diagram below to label your tag for each seedling. Teachers can decide how to group classes and assign genotypes to teams). The seedling ID helps to track individual seedlings throughout the experiment.



4. Use a sharpie marker to punch a 1 cm deep hole in the soil and plant a seed in each cone as labeled. Using a plastic beaker, sprinkle a light layer of soil (about 0.5 cm) over the seeds.

Note: do not plant the seeds too deep.

5. Mist the pots with water again. Be careful to hold the spray nozzle one foot above the tray so that you do not displace soil or the seeds.
6. Repeat steps 1-5 to finish planting another 3 trays and place all 4 trays under the light bank. If a greenhouse is available, just place the trays in the greenhouse. You may have less than 4 trays for your classroom depending on the number of students.



7. Use the thermometer to measure the growth temperature (growth T_m). Record school, class, and team name, genotype, seedling ID, the date of planting, and growth T_m using the *phenotyping table* at the end of this section.
8. Cover the trays with the clear plastic lids until germinating seedlings emerge from the soil. The lid helps keep the humidity high to encourage germination. It takes about 4 to 5 days to see the seedlings geminate. After the plants have emerged, remove the lid to expose the seedlings to your classroom environment.
9. You may replant seeds if some fail to germinate (extra seeds are included in each packet).

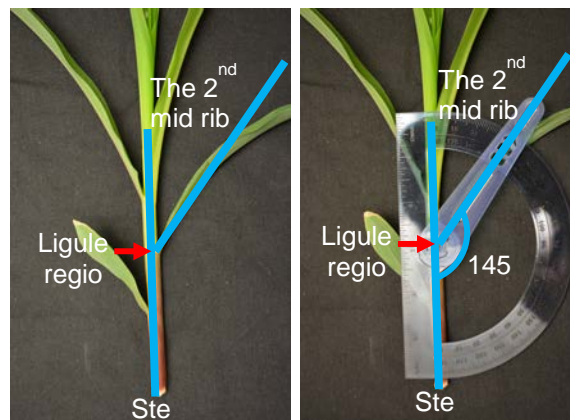
Part 2: Plant care.

IMPORTANT! DO NOT overwater the planting trays.

1. Add 1.5 L of water to the bottom of the tray. Typically, before germination you only need to water the tray when you see the soil obviously dry out. Once the seed germinates you might need to water the tray every other day. Seedlings need more water when they become bigger and you might need to water them every day after 14 days after sowing.
2. After watering the tray, wait about one hour and then pour off any excess water into the sink. Corn does not like to sit in standing water. The soil should feel moist - not dry or too wet / muddy. If the soil in one cone ever feels dry, you can slowly and gently water that cone from the top as well.
3. Coordinate with your teacher to determine a watering schedule, but know that this schedule may need to be adjusted depending on your classroom. Plan out with your classmates when the plants will be watered and who will do this. Make sure to account for weekends and holidays.

Part 3: Data collection.

1. After planting, check on your plants daily and record the date of germination using the [phenotyping table](#). Corn seedlings typically emerge around 4-5 days after sowing. If you observe an obvious delay in germination compared to the other genotypes planted at the same time, adjust the date for phenotyping accordingly.
2. For the first week after germination, try to observe them every other day. After that, observe your plants weekly.
3. Phenotype the leaf angle of corn seedlings at 16-18 days after sowing. Cut a seedling at its root-shoot junction (at the soil line). Lay the seedling on a table with its leaves naturally falling on either side of the stem. **Note: keeping the natural curve of the leaf is very important for the accuracy of data collection.** Place the midpoint of the protractor at the region where the leaf meets the stem. We refer to this as the ligule region. Here, we are choosing the 2nd and 3rd fully-expanded leaves from the bottom. Line up the stem with line zero on the protractor, and the mid rib of the leaf with the mid-line of the swing arm on the protractor. Read the degree at where the swing arm crosses the number scale (See the example below showing the measurement of the 2nd leaf angle of a corn seedling). After taking measurements, the seedlings will be used for imaging in **Section 3: Phenotype leaf angles of corn seedlings using computational methods.**



4. Record the date of phenotyping and the 2nd and 3rd leaf angles using the [phenotyping table](#).
5. Repeat steps 3 and 4 to finish every individual seedling within each genotype.

Section 2: Phenotype leaf angles of corn seedlings using computational methods.

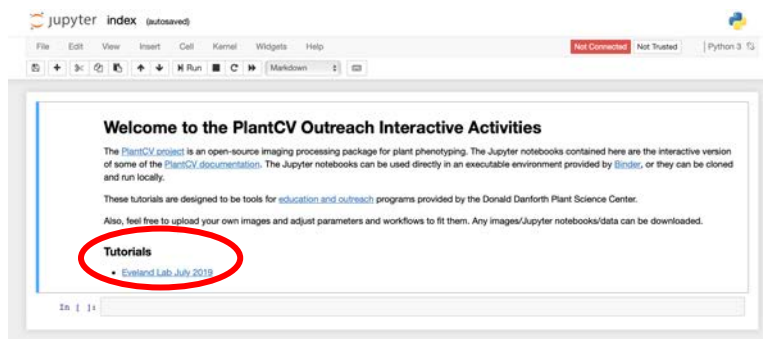
In this exercise, image-based analysis of plant phenotypes will be introduced by using PlantCV. PlantCV is an open-source image analysis software package targeted for plant phenotyping. In the classroom, there is the option to use PlantCV to compare the manual measurements with automated image analysis methods. Hand measurements are typically used to “ground truth” computational based phenotyping to assess accuracy. Here, the students are ground truthing the data from analysis in PlantCV.

List of Materials Needed for Section 2

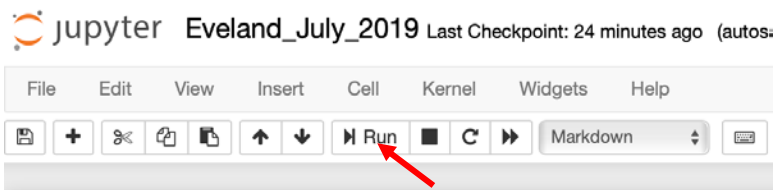
Black background
Computer (not included)
Camera / cell phone with camera (not included)
Corn seedlings
Internet (not included)

Part 4: A tutorial of using *PlantCV* to phenotype corn seedlings

1. Click the link to the website to launch an interactive activity of *PlantCV* via *Binder*. It might take some time to open the interactive activity.
https://mybinder.org/v2/gh/danforthcenter/Eveland_NSF_Outreach/master?filepath=index.ipynb
2. Click the link “Eveland Lab July 2019” in the “Tutorials” section for a tutorial of measuring leaf angle of corn seedlings using *PlantCV*. The tutorial uses an image of a corn seedling (18 days after sowing) from the genotype Hp301 to demonstrate how *PlantCV* extracts the comprehensive phenotypic data from a 2D image.



3. Read the instructions.
4. To launch the analysis, select the first code block by clicking within the block. Once selected, the first line of code will be surrounded by a green box. Then, click the “Run” button (indicated by the red arrow in the screen shot below) on the top, left-hand corner to run the code step-by-step. It might take a few seconds to run through the code within each block and the result of each step will appear beneath each block of code. Do this for each step using the example image.



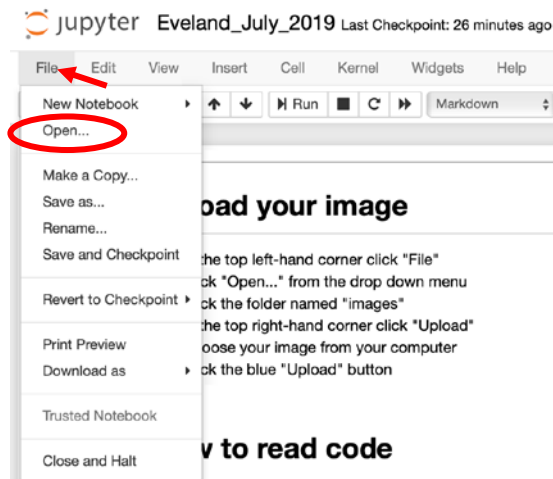
Upload your image

Part 5: Taking your own images of corn seedlings to measure leaf angles via *PlantCV*.

1. Take one corn seedling previously used for manual measurements of leaf angle and lay the seedling on a black background on the table top. Make sure that its leaves naturally fall on either side of the stem on the black background. **Note: keeping the natural curve of the leaf is very important for the accuracy of data.**
2. Place the camera lens parallel to the table top with the photo frame covering the whole seedling.
3. Take an image of the seedling and store the image on your computer.



4. Upload your own image:
 - a. Once your image is somewhere on your computer
 - b. In the top, left-hand corner click "File" (indicated by the red arrow in the screen shot below).
 - c. Click "Open..." from the drop-down menu
 - d. Click the folder named "images"
 - e. In the top, right-hand corner click "Upload"
 - f. Choose your image
 - g. Click the "Upload" button



5. Run each block of code using your own image. Adjust parameters (please read the green, italicized documentation about what each parameter does). Many of the blocks of code

will **NOT** need updating. Parameters that most likely may need adjusting will have little arrows ^ pointing to and describing how to adjust them in the comments.

6. After the last block of code has been run, download your data to your computer:
 - a. To see the text file with data saved, click the “File” tab in the top left corner.
 - b. Click “Open...”
 - c. Download this file to your computer by checking the box directly to the left of the file named “leaf_phenotype_data.txt”
 - d. Click “Download” in the top left corner (indicated by the yellow box in the screen shot below).

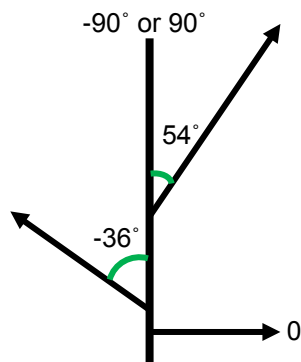
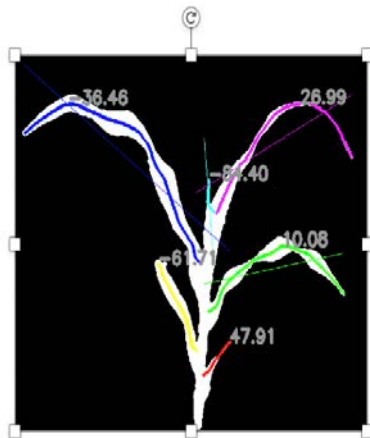


- e. The text file that you downloaded can be imported into Excel for data analysis to compare manual measurements to those collected with *PlantCV*. “segment_insertion_angle” is the variable collected that reflects the manual leaf angle measurements taken. You will see positive and negative leaf angles. Use the graph below to convert the numbers to be similar to those manually recorded in **Section 1: Phenotyping Corn Seedlings**.

```

jupyter leaf_phenotype_data.txt a few seconds ago
File Edit View Language
1 # leaf_id, path_length, eu_length, angle, insertion angle
2 0.00000, 253.95079, 233.62363, -23.32166, 49.30327
3 1.00000, 705.10721, 640.89703, 28.10635, 45.31228
4 2.00000, 1182.18036, 781.72246, -17.20604, 40.01368
5 3.00000, 516.73358, 477.03773, -63.83985, 21.46928
6 4.00000, 1121.62506, 1041.51476, 57.56480, 21.62623
7

```



Part 6: Clean up.

1. When you finish phenotyping, all of your corn seedlings with the soil can be composted. The pots and trays can be rinsed out, dried and boxed for return to the Donald Danforth Plant Science Center.

Section 3: Genotyping SNPs

The Genotype:

In this activity, the individual genotypes of six maize lines will be determined and evaluated. Here, students will use PCR to amplify a piece of DNA from certain maize genotypes that harbor a variable region of the genome. They will use a restriction enzyme designed to cut the amplified sequence at a recognition domain ONLY if a certain allele is present. Performing the restriction digest followed by visualization on an electrophoresis gel will enable dissection of an individual's genotype at the amplified locus.

Polymerase chain reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment.

Derived Cleaved Amplified Polymorphic Sequences (dCAPS) assay is a technique used for detection of Single Nucleotide Polymorphisms (SNPs). In a dCAPS assay, one or more mismatches in a PCR primer are used to create a restriction endonuclease-sensitive polymorphism based on the target SNP. The PCR product modified in this manner is then subjected to restriction enzyme digestion, and the presence or absence of the SNP is determined by the resulting restriction pattern via agarose gel electrophoresis.

dCAPS assays are useful for genotyping known SNPs and genetic mapping of isolated DNAs.

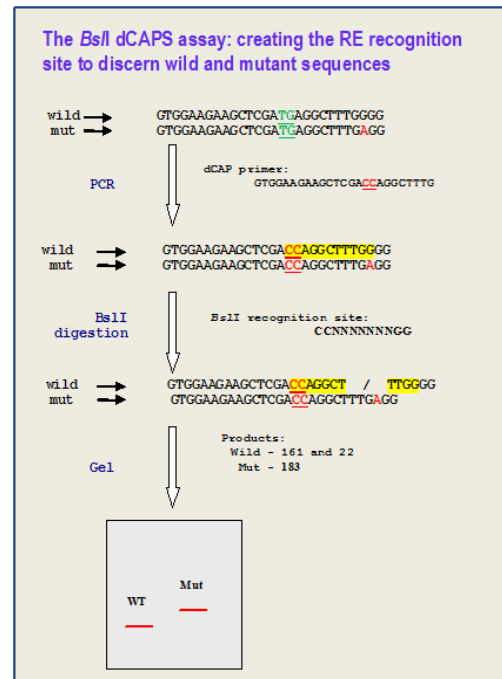
Restriction endonuclease is an enzyme that cleaves DNA into fragments at or near specific recognition sites.

Agarose gel electrophoresis is a method used to separate a mixed population of biomolecules, such as DNA, in a matrix of agarose. By applying an electric field to move the charged molecules through an agarose matrix, the biomolecules are separated by size. In this case, DNA molecules will be separated by length.

Before Your Class...

Set up the program in PCR machine (thermal cycler)

If you use the PCR machine provide by Donald Danforth Plant Science Center, the PCR reaction and Restriction Endonuclease reaction have already been preprogrammed in the machine as “pcr reaction” and “re reaction”, respectively. Connect the power core of PCR machine with power



and turn it on. Select the “*protocol library*” and find “*pcr reaction*” or “*re reaction*” for the corresponding part.

You also can set up the program in your PCR machine.

Program your PCR machine for *PCR reaction* as follows:

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	0.5minute	34 cycles
Annealing	60°C	0.5minute	
Extension	72°C	0.2min	
Final Extension	72°C	5 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

Program your PCR machine for *Restriction Endonuclease reaction* as follows:

Step	Temperature	Time	Number of Cycles
Incubation	37°C	180 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

Make 1XTAE buffer

1. Measure out 14ml of 50xTAE buffer using a 15ml purple cap tube and pour the buffer into a 1L bottle.
2. Fill the bottle with distilled water to 700ml line.
3. Close the cap of the bottle and mix the buffer by shaking the bottle.

dCAP marker information for genotyping corn seedling leaf angles

Marker	Primer pair	RE	Recognition site	SNPs				Allele will be digested
				Chromosome	Position	Names	Alleles	
Marker2	251/252	HindIII	AAGCTT	6	167984060	ss196521458	G/A	G

DNA samples

DNA Sample Name	Genotype
1	CML102
4	B73
7	CML228
10	CML 52
13	P39
22	Oh43

**two of these lines (CML228 and Oh43) were phenotyped in the previous sections

List of Materials Needed for Section 3

- 0.2 ml PCR tubes
- 0.2 ml PCR tubes with DNA samples (kept in freezer)
- 1.5 ml tubes

50xTAE running buffer (kept in room temperature)
 50ml purple cap tube
 15ml purple cap tube
 1L bottle
 8-well comb
 Blue freezer box (kept in freezer)
CutSmart buffer (kept in freezer)
 DMSO (It helps to relieve secondary structures when amplifying high GC templates. **Note: It should be kept in room temperature.**)
 Gel marker (kept in freezer)
 Gel box
 Gel tray
 Gloves (The gel contains SYBR safe DNA Gel Stain, which is a less hazardous alternative to ethidium bromide)
 Ice bucket (not included)
 Lid of gel box
 Marker (dCAPs marker/a pair of primers, kept in freezer)
 Microwave (**Not for Food**)
 Mini Microcentrifuge for 1.5ml tube
 Mini Microcentrifuge for PCR tube strip
 Nuclease-free water
 P-20 micropipette
 P-200 micropipette
 PCR machine (thermal cycler)
 Power supply for gel box
 Pre-prepared 4% agarose gel (kept in room temperature)
Promega GoTaq Master Mix (kept in freezer)
 Restriction enzyme(s) (kept in freezer)
 SYBR safe DNA Gel Stain (kept room temperature)
 Tips for micropipette
 UV box
 Wedges

Part 7: Set up PCRs:

1. Thaw the Promega GoTaq Master mix, Marker, and nuclease-free water (*these will be labeled at each station*). Once they thaw, place them in an ice bucket with ice.
2. Take a 1.5ml tube, label the tube as “Green MasterMix+Marker”, put the tube in the ice bucket.
3. Using P-20 and P-200 micropipettes, add the following to the “Green MasterMix+Marker” tube on ice:

14µl*7	nuclease-free water
4µl *7	Marker
2µl *7	DMSO (kept in room temperature)
25µl*7	GoTaq Green Master Mix

Note: we make “Green MasterMix+ Marker” for 7 reactions but only use 6 reactions in step 6.

4. Gently mix the solution in the “Green MasterMix+ Marker” tube by finger-tapping the bottom of the tube. Quickly spin the solution down for 2-3 seconds in a Mini Microcentrifuge that holds 1.5ml tubes, and place the tube back on ice to keep it cold.
5. Take out the 6, 0.2ml PCR tubes (in a strip) with a DNA sample (5µl) in each tube (*a set of DNA samples that are labeled for each group*).
6. Aliquot 45µl of the “Green MasterMix+ Marker” into each 0.2ml PCR tube for a total volume of 50µl in each tube. Close the cap of 0.2ml PCR tube and your PCR is ready to go.
7. Put the PCR tubes in the PCR machine, or thermal cycler, and start the program. The reaction will proceed as follows (*once programmed, the machine will automate the entire reaction*):

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	0.5minute	34 cycles
Annealing	60°C	0.5minute	
Extension	72°C	0.2min	
Final Extension	72°C	5 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

PCR products can be stored in freezer for months.

Part 8: Set up Restriction Endonuclease reactions:

1. Thaw the 10xCutSmart buffer and nuclease-free water, and once they are thawed place them in the ice bucket with ice.
2. Take out the Restriction endonuclease from the freezer and place it in the blue freezer box (included in the trunk). The Freezer box should be precooled in the freezer the day before. **Note: Restriction endonucleases should always stay cold to keep their enzymatic activity.**
3. Take a new 1.5ml tube, label it as “RE mix”, and put the tube on ice.
4. Using a P-20 micropipette, add the following to the “RE mix” tube on ice:

2µl *7	nuclease-free water
2.5µl*7	10XCutSmart buffer
0.5µl*7	Restriction endonuclease (<i>enzyme assigned to each group</i>)

Note: we make “RE mix” for 7 reactions but only use 6 reactions in step 6.

5. Gently mix the solution in the “RE mix” tube by finger-tapping the bottom of the tube. Quickly spin the solution down for 2-3 seconds in a Mini Microcentrifuge that holds 1.5ml tubes and put the tube back on ice to keep it cold.

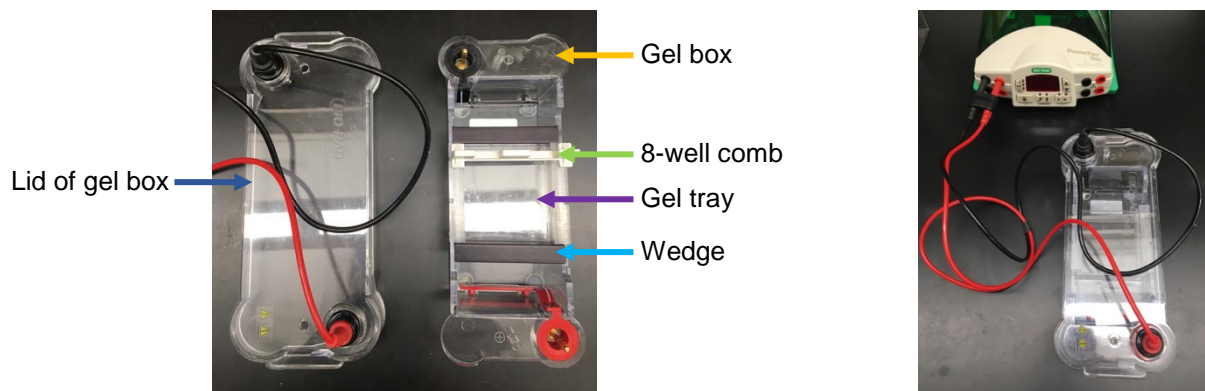
- Take 6 new 0.2ml PCR tubes and label them with “RE+DNA sample name” and aliquot 5µl “RE mix” into each 0.2ml PCR tube. The DNA sample name corresponds to the DNA sample name of each PCR reaction (from **Part 7: Set up PCRs**).
- Pipet 20µl PCR product from the individual PCR reactions (from **Part 7: Set up PCRs**) into the bottom of the corresponding PCR tube with 5µl “RE mix” for a total volume of 25µl. Repeat this step for the other 5 PCR reactions. Close the cap of 0.2ml PCR tube and mix gently by finger-tapping the bottom of the tube. Quickly spin the solution down for 2-3 seconds in a Mini Microcentrifuge that holds PCR tube strips. If a Mini Microcentrifuge that holds PCR tube strips is not available, you can finger-tap the tube gently until the solution settles to the bottom of the PCR tube.
- Put the Restriction Endonuclease reactions in the “RE+DNA sample name” tubes in the PCR machine to incubate at 37°C for 3h.

Restriction Endonuclease reactions can be stored in freezer for months.

Note: DO NOT PUT Restriction Endonuclease reactions at room temperature for a long time.

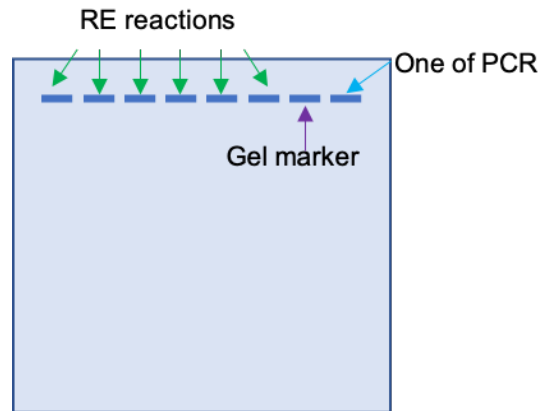
Part 9: Pour a Mini gel for analysis of Restriction Endonuclease reactions.

- Place the gel box on a level surface.
- Place a gel tray in the gel box, and wedges at either end (See image below).
- Set an 8-well comb into the gel tray so that the sample wells are near the cathode (black). DNA fragments will migrate toward the anode (red) during electrophoresis.
- Note: teacher should perform this step.** Loosen the cap of a gel bottle and completely melt the pre-prepared 4% agarose gel in a microwave for 3 minutes. Open the cap of the gel bottle and use P-20 micropipette to add 10µl SYBR safe DNA Gel Stain to the melted gel (**Wear gloves!**). Close the cap of bottle and slowly swirl the gel bottle to mix the Gel stain with melted gel thoroughly. Slowly swirl every other 30 second for about 3 minutes to homogenously cool down the gel. **Note: immediately move to the next step to avoid the 4% agarose gel getting solid.**
- Measure out 30ml of melted 4% agarose gel using a 50ml blue cap tube and pour the gel into the gel tray. Let the gel harden (~10min).

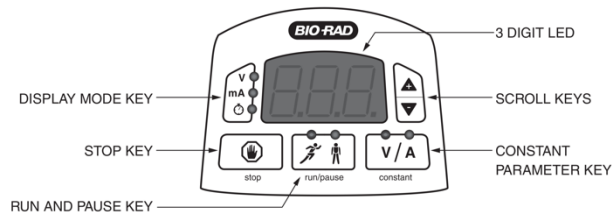


Part 10: Run the Mini gel to analyze Restriction Endonuclease reactions.

- Carefully remove the 8-well comb from the solidified gel and the wedges from gel box.
- Pour 1X TAE running buffer into the gel box to submerge the gel beneath 2 to 6 mm of buffer.
- For the 6 Restriction Endonuclease reactions (RE reactions) (from **Part 8: Set up Restriction Endonuclease reactions**), Pipet 15 μ l of an individual reaction and load it into individual wells (1-6) using a P-20 micropipette. Using a P-20 micropipette, pipet 5 μ l of the gel marker and load it into the 7th well. Also, load 15 μ l of the remaining PCR product from any one of the PCR reactions (from **Part 7: Set up PCRs**) into the 8th well (See diagram below).



- Place the lid on the gel box carefully without disturbing the samples. The lid should attach to the box in only one orientation. To attach the lid correctly, match the red and black banana jacks on the lid with the red and black banana plugs on the box.
- Press the power switch located on the right side of the power supply to turn on power.
- Connect the gel box to the power supply. The power leads are color-coordinated to the output terminals in red and black. **Note: power leads must be inserted perpendicular to the curve of the case.**
- Press the CONSTANT PARAMETER KEY to select voltage, then use the SCROLL KEYS to set voltage to 120 volts.
- Use the DISPLAY MODE KEY to select time, and use the SCROLL KEYS to set to 25 minutes.
- Press the RUN AND PAUSE KEY to start the run. The run LED is lit.



- When a run is completed, i.e., a timed run has ended or an untimed run is stopped, press the STOP KEY and then turn the power switch to the off position.

11. Remove the gel from the gel tray and place it on a UV box in a darkened room.
12. Record the resulting restriction patterns and corresponding DNA sample IDs, marker used for PCR, and restriction endonuclease used for digestion.

Part 11: Clean up.

1. Once finished with reagents, all remaining reagents should be placed back in their storage temperatures.
2. When you finish your genotyping experiment, the remaining PCR reactions and RE reactions in 0.2ml PCR tubes, as well as the gel, can be trashed. Empty the gel box and rinse gel box, gel tray, 8-well comb, and wedges with clean water. Allow them to air dry and repack them in the way they were received.
3. Clean the surface of the UV box with a dampened paper towel.
4. Repack everything in the way they were received.

DNA sample name	Primers	Restriction Endonuclease	Restriction Pattern