Fall 2013 Biology 322 Arabidopsis thaliana: *Phenotypic and genotypic analysis of F2 segregants from aha-3/+ heterozygotes*. **Part 2**: ***Direct detection of Genotype using PCR***

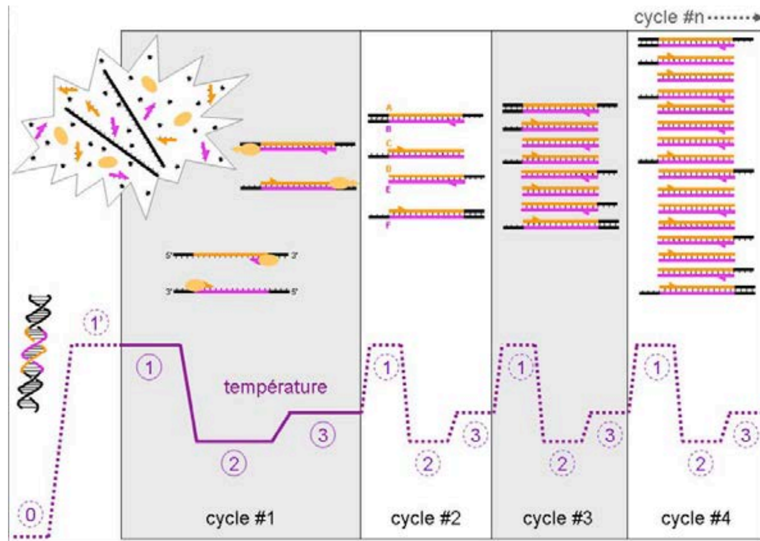


Table of Contents: *Schedule:* this page

- *Tips for Success:* pgs 2-3
- *Writing up Molecular Protocols in your Lab Notebook:* pg 4
- *Selecting and Processing Seedlings pg 5*
- *DNA preparation* using the ZR Plant/Seed DNA Microprep pgs. 6-7
- *Info on PCR including primer sequences* pgs 8-11
- *Agarose Gel Electrophoresis* Info on Tracking Dyes & Hi Size Standards: pg 12

Mon Oct 14 Lecture

- Introduction to **Forward and Reverse Genetics**, *Arabidopsis*, the *aha* gene family and T-DNA induced mutations
- Review PCR in your genetics text or at the links below
  Background information on PCR:
  http://fire.biol.wwu.edu/trent/trent/13.05.20lecture.pdf
  PCR Animation
  http://www.dnalc.org/resources/animations/pcr.html

Tues Oct 15

- *Extract genomic DNA (one seedling per extraction).* ***This protocol MUST be written out in your notebook BEFORE you come to lab—see additional comments pg 5***
- Subject each prep of DNA to two PCR reactions each: one to detect wild-type template and one to detect the presence of the T-DNA insertion. ***These protocols MUST also be written out in your notebook BEFORE you come to lab. Work up a diagram of your two strips of 8 tubes and indicate what master mixes and templates will go in each.***

Thurs Oct 17

- Agarose Gel electrophoresis of PCR reaction and analysis of results
- While gels are running, continue discussion of this experiment
- Collect Class Data and discuss Lab report which will be due on or around ??

TIPS for SUCCESS using Molecular Techniques

- Use your lab notebook for Planning, Recording and Troubleshooting
- Work up the protocol for each exercise BEFORE you come to lab
- At the start of each lab session, check the chalk-board for late-breaking information on the experiment.
- For those labs where you have divvied up the responsibilities with your lab partner, maintain continuous communication with him/her about the progress of your experiment; you are responsible for knowing about and recording (in your lab notebook) all steps of each experiment
- ASK QUESTIONS

Sex shock as fetal error gives cloners bull fright

São Paulo Note to cloning researchers: always check the label on samples before beginning an experiment.

The dangers of getting samples mixed up were exposed by scientists at the University of São Paulo in Brazil. The birth of a cloned bull calf stunned researchers there, as they were expecting a female calf. They thought they had created a cloned embryo using cells from a cow's ear. But genetic profiles last week showed that male fetal cells that were stored in the same freezer had been used instead.

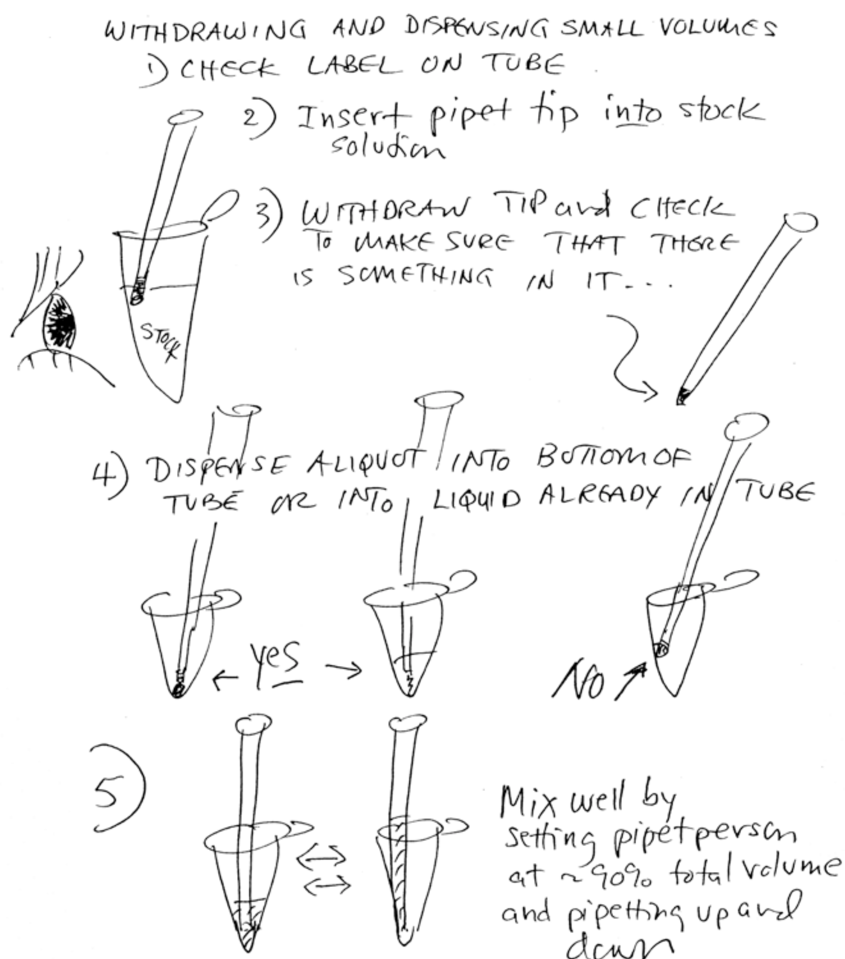
"It was a surprise," admits Jose Visintin, head of the cloning project. The team intended to clone both the adult and fetal cell lines to explore differences in chromosome ageing between the resulting clones. Two other expectant cows, due in August and January, are now expected to bear bulls too.

Nature 417:111 Mary 9, 2002

- *Do not use a stock reagent without reading the label. Make sure it is what you want and that you have recorded the concentration correctly.*
- Be sure to label your own tubes carefully. This is especially important for preps that will need to be retrieved later from the refrigerator (4ºC) or from the freezer (-20ºC). Be sure to put your initials***, the date and enough additional info so that you know the identity of the contents.*** Codes are OK, but be sure to record a key in your notebook.

- Be sure you use clean, sterile pipette tips to take aliquots from stock solutions. Cross contamination of reagents is a royal pain, is sometimes very hard to sort out, and may ruin an experiment for the entire class. ***If you aren't sure that your pipet tip is clean (ie. unused), discard it and get a clean one from your box.***
- When measuring aliquots repeatedly from a personal or group STOCK (of an inexpensive reagent such as sterile water or broth), pipet an aliquot into a 1.5 ml, 15ml or 50ml tube. Use this aliquot as a working stock and discard after use.
- When using a reagent that has been frozen (such as 10X enzyme buffer), be sure to thaw it completely and mix thoroughly (by pipeting or vortexing) before you pipette an aliquot. NOTE: this instruction this does NOT apply to enzymes, which are stored in glycerol (which acts as an anti-freeze).

- As a matter of course, when setting up any reaction or prepping (or diluting) a reagent, be sure to MIX, MIX, MIX. For small volumes (say less than 200 µl) pipet up and down. For larger volumes, you can use the vortex mixer.
- *If you make a mistake in carrying out an experiment, fess-up and talk to your lab partner, lab instructor or TA about it.* You may be able to easily correct the mistake.
- ASK QUESTIONS
- Pay attention to the components of buffers and other reagents and try to figure out what the significance of each component of a reaction.
- ***Be a good lab citizen. Clean up after yourself in the lab.***
- Return common supplies to "the commons"
- Be sure to use the appropriate water source: tap, tap-distilled, nanopure and nanopure-sterile…
- Be sure that you know how to do all manipulations associated with a particular exercise.
- Be sure to trade-off "jobs" (such as pouring or photographing your agarose gels) with your lab partner.



IMPORTANT INFO ABOUT LAB NOTEBOOK/RECORDS:

Information concerning the DNA preps follows on the next two pages.

→ ***For Tues Oct 15, keep the Google form simple – don't include the DNA preparation protocol or the details of PCR when you fill out the form.***

→ ***BUT, BEFORE LAB,*** *transfer DNA prep and PCR protocol information directly to your lab notebook in recipe (not paragraph) form. Be sure to note, where your DNA is at any given step (ie on the column or in the flowthrough?)*

- *one protocol step per line*

- *with plenty of space between steps for notes that might include:*
  - changes in protocol

  - mistakes made or other problems encountered in executing the protocol – for example, *OOPS. I dropped my seedling (or tube) on the floor*

  - any other information or observations (expected or unexpected) that might prove to be important later on – for example, *I accidentally skipped this step OR there were huge pieced of plant material after the bashing bead step OR…*

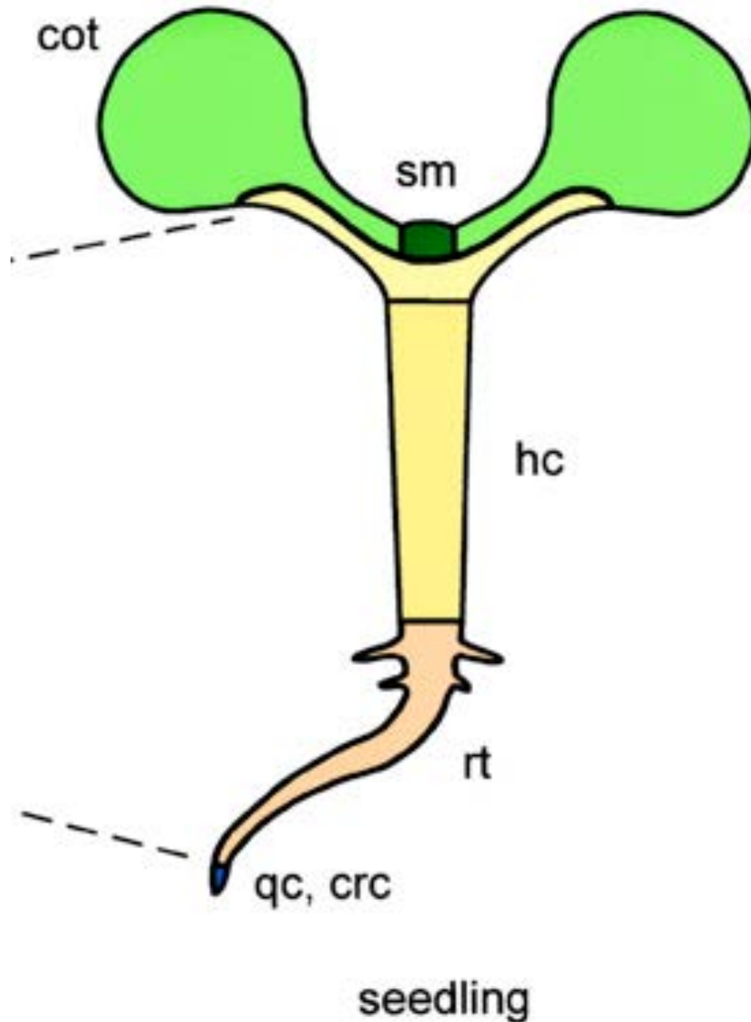
  - if you are not sure that a piece of info is important, write it down just in case

- *One strategy you might use is to write out the protocol in black ink and use a different color pen to record observations or other info while you are actually doing the preps*

- *Be sure to include a* ***Big Reminder*** *at the beginning of your Protocol NOT to throw away any tubes or columns until the prep is completed.*

Selecting and Processing Seedlings for DNA preps:

Review Part 1 for instructions on assessment seedling phenotypes



Check the chalkboard for instructions concerning the selection of seedlings for DNA preps

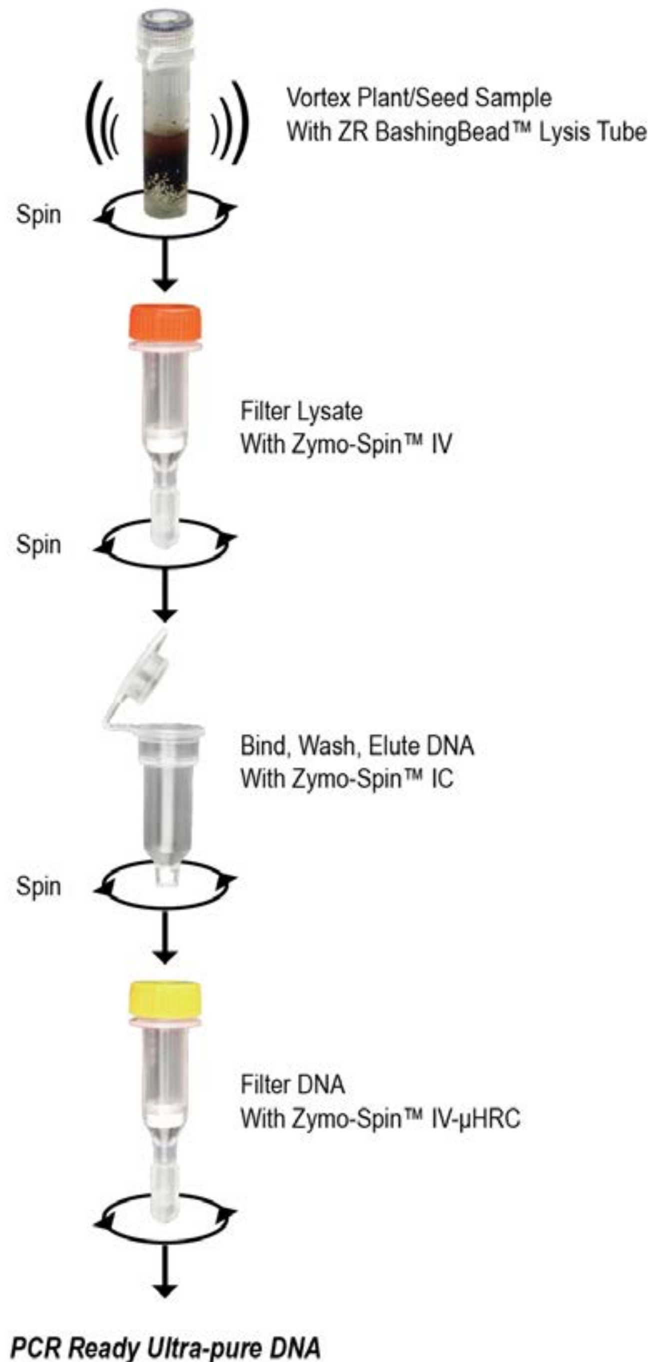
Inspect each seedling that you select for your DNA prep and record basic phenotype info concerning cotyledons, true leaves, root system, etc

Gently pull seedling from agar plate with forceps and use a kimwipe to brush the agar from each seedling

Remove the seed coat if it is still clinging to plant tissue

Pull leaves and roots off seedling and use for prep

Take your time here, and don't cross-contaminate seedling material



The **ZR P_l_a_n_t_/_S_e_e_d_ DNA_ _M_i_c_r_o_P_r_e_p_™** is designed for the simple, rapid isolation of inhibitor-free, PCR-quality DNA from a variety of plant sample sources including leaves, stems, buds, flowers, fruit, seeds, etc.

1. Plant samples (≤150 mg each) are added directly to a **Bashing Bead Lysis Tube** and rapidly and efficiently lysed by bead beating without the use of organic denaturants or proteinases.
2. Polysaccharides and polyphenols/tannins are removed from the DNA using our ***Fast-Spin*** column and **Zymo-Spin IV Column** technologies, respectively.
3. The eluted DNA is ideal for downstream molecular-based applications including PCR, arrays, etc.
4. A schematic of the procedure is shown to the left.

NOTE: tubes and columns are in bags at the front of the room. CHECK BAG LABELS CAREFULLY.

NOTES:

We will not be using beta-mercaptoethanol (see first sentence under protocol)
DON'T THROW anything out until you have completed the prep.
Step 1: Each student will prep DNA from four seedlings. At the beginning of lab, check chalkboard for info on selecting the seedlings for the DNA preps
Step 2: Vortex for tubes one at a time for about 2 minutes.
Step 5,6,7: Note the Binding Buffer plus filtrate mixture generated in step 5 will be loaded onto column in two steps – 800ul per each step.

Protocol

For optimal performance, add beta-mercaptoethanol (user supplied) to the **Plant/Seed DNA Binding Buffer** to a final dilution of 0.5%(v/v) i.e., 500 µl per 100 ml.

Before Starting: **Zymo-Spin™ IV-µHRC Spin Filters** (*yellow tops*) need to be prepared prior to use by: 1) snapping off the base, 2), inserting into a **Collection Tube**, and 3), spinning in a microcentrifuge at exactly 8,000 x *g* for 1 minute.

1. Add up to 150 mg of finely cut plant or seed sample to a **ZR BashingBead™ Lysis Tube**. Add 750 µl **Lysis Solution** to the tube.
2. Secure in a bead beater fitted with a 2 ml tube holder assembly (e.g., Disruptor Genie™) and process at maximum speed for 10 minutes.

   Processing times may be as little as 40 seconds when using high-speed cell disrupters (e.g., the portable ***Xpedition™* Sample Processor**, page 6, FastPrep®-24, or similar). See manufacturer's literature for operating information.
3. Centrifuge the **ZR BashingBead™ Lysis Tube** in a microcentrifuge at ≥10,000 x *g* for 1 minute.
4. Transfer up to 400 µl supernatant to a **Zymo-Spin™ IV Spin Filter** (orange top) in a **Collection Tube** and centrifuge at 7,000 rpm (~7,000 x *g*) for 1 minute.
5. Add 1,200 µl of **Plant/Seed DNA Binding Buffer** to the filtrate in the **Collection Tube** from Step 4 and mix.
6. Transfer 800 µl of the mixture from Step 5 to a **Zymo-Spin™ IC Column** in a **Collection Tube** and centrifuge at 10,000 x *g* for 1 minute.
7. Discard the flow through from the **Collection Tube** and repeat Step 6.
8. Add 200 µl **DNA Pre-Wash Buffer** to the **Zymo-Spin™ IC Column** in a new **Collection Tube** and centrifuge at 10,000 x *g* for 1 minute.
9. Add 500 µl **Plant/Seed DNA Wash Buffer** to the **Zymo-Spin™ IC Column** and centrifuge at 10,000 x *g* for 1 minute.
10. Transfer the **Zymo-Spin™ IC Column** to a clean 1.5 ml microcentrifuge tube and add 20 µl (10 µl minimum) **DNA Elution Buffer** directly to the column matrix. Centrifuge at 10,000 x *g* for 30 seconds to elute the DNA.
11. Transfer the eluted DNA from Step 10 to a prepared **Zymo-Spin™ IV-µHRC Spin Filter** (yellow top) (see above) in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 8,000 x *g* for 1 minute. The filtered DNA is now suitable for PCR and other downstream applications.
12. After setting up PCRs, label preps according to instructions on board and store in cardboard box at front of lab.

If the HRC matrix is <u>dry</u>, add 200-400 µl water prior to prepping the filter.

Cap tube tightly to prevent leakage. (screw cap)

Dried samples may be hydrated in water prior to processing to improve DNA extraction efficiency.

Alternatively, a standard bench top vortex can be used although the overall yield of DNA may be lower.

g vs rpm?

Snap off the base of the **Zymo-Spin™ IV Spin Filter** prior to use.

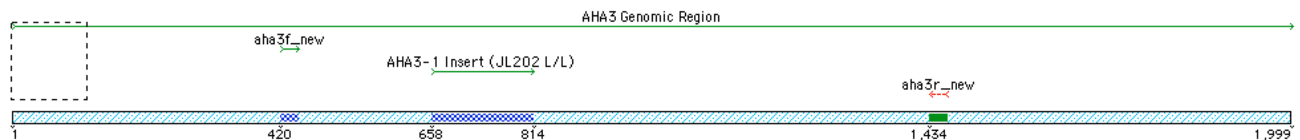
The **Zymo-Spin™ IC Column** has a maximum capacity of 800 µl.

PCR Genotyping can take any of a number of approaches to determine the genetic make-up of an individual. In this experiment, we will be using PCR to identify the genotype of plants that are segregating an ***'insertional mutation'***. To determine the function of aha-3, a H^+- ATPase primary transport gene, a foreign 6.2 kb DNA fragment was inserted into the coding sequence. We will use PCR to identify the presence of this insert and to determine whether the plant is homozygous for the wild-type allele, heterozygous or homozygous mutant.

To determine genotype at the aha 3 locus, **each genomic DNA preparation will be amplified with two different primer pairs in two separate PCR reactions.** See map of primer locations below.

Primer Pairs See also next page
Aha3f and a3-3R (aka Aha3r): amplify wildtype allele only (why?)
Aha3f and jl202: amplify mutant allele only (why?)

Primer	# bases	Sequence
aha3f	29	CAC AAA GGA CTT TAC ACG GTC TTC AGA AC
jl202	29	CAT TTT ATA ATA ACG CTG CGG ACA TCT AC
a3-3R	29	GTC GTG GTG TGA AGA TTT ACA ACA GAT TG

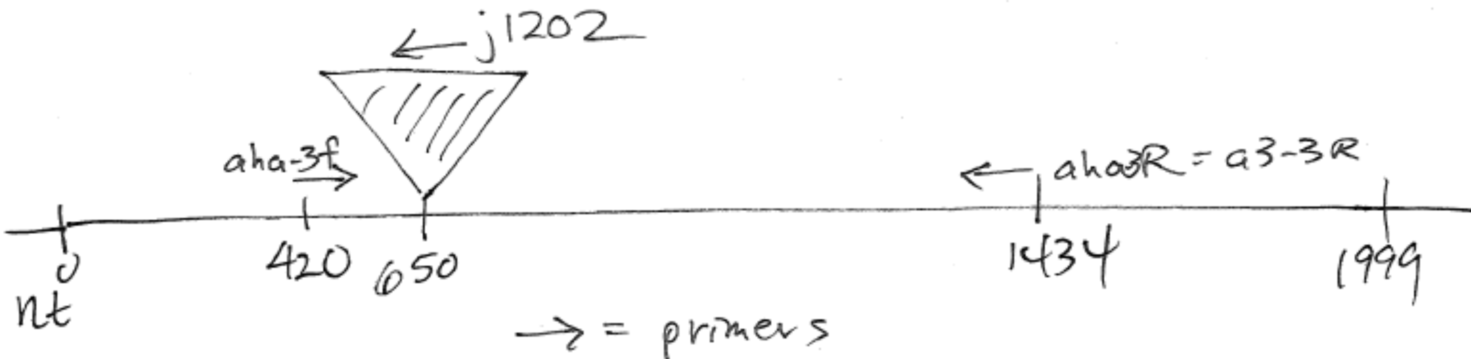


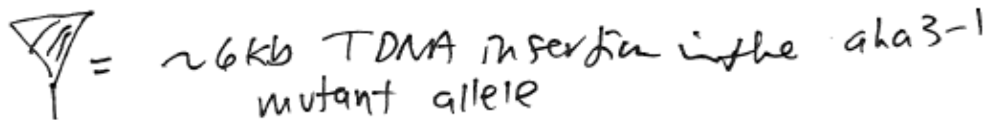
Aha3-1: a single TDNA insert is about 6kb. The aha3 mutant allele has at least two tandem inserts oriented in opposite orientations.

Each student will set up 14 PCR reactions:

- 4 experimental: template DNA from F2 seedlings
- 1 positive control: template DNA from known WT
- 1 positive control: template DNA from known aha3/+ het
- 1 negative control: no template DNA added to reaction

Remember that each template will be amplified with two different primer pairs in two separate reactions





5’ end of JI202: nt 814

5’ end of aha-3f : nt 420

5’ end of a3-3R: nt 1434

Setting up PCR Reactions: ***Check chalkboard for late-breaking information on the PCR reactions and/or changes in the protocol***

PCR General Procedure

1. The recipe for the master mixes is below. ***They will be premixed for you.***
2. Label 2 tubes per DNA prep. Label each tube in two different places as the labels tend to rub off. Use an appropriate code and carefully record identity of each tube in your notebook. **TAKE YOUR TiME HERE.** If one tube in the strip will be empty, use it for detailed labeling.
3. Add 23 µl of the appropriate master mix to each PCR tube.
4. Add 2 µl of the appropriate DNA to each PCR reaction. Be sure that you can see liquid in the pipet tip – 2µl are easy to see.
5. Carefully seal each tube with its cap. Leave on ice until placed in the thermocycler (see next page).
6. Don't discard your DNA preps. Your instructor will collect them

PCR master mix Recipe

Fill in blanks in this table and tape it into your lab notebook

Master Mix 1: primers aha3f and a3-3R
Master Mix 2: primers aha3f and jl202

Reagent (Stock conc.)	vol per 100 Rxns	vol per one 25 µl reaction: 23µl of master mix + 2µl DNA	final conc
H_2O (to make 23 µl per reaction)		14.3 µl	
10X Taq Buffer		2.5	
dNTP's (10 mM)		2.0	
$MgCl_2$ (25mM)		1.5	
primer 1 (10 µM) aha3f		1.25	
primer 2 (10 µM) either a3-3R or jl202		1.25	
Taq polymerase 5U/µl		0.2 µl	

PLEASE note the position of your tubes after you place them in the thermocycler

The thermocycler will be programmed as follows:

Step	Time/temp	Purpose? What is happening at this step?
1	5 min 94ºC	
2	1 min 90ºC	
3	1 min 60ºC	
4	1 min 72ºC	
5	Repeat steps 2-4 39 more times	
6	10 min 72ºC	
7	Hold 4ºC	

For each genotype indicate whether or not a product is expected

Genotype	**Primer Pairs**	
	aha3f + jl202 *predicted size?*	aha3f + a3-3R *predicted size?*
+/+		
+/ *aha3-1*		
aha3-1/aha3-1		

Agarose Gel Electrophoresis of PCR products

Gels: 1% agarose cast in 1X SB (Sodium Borate Electrophoresis buffer pH 8.5) with Ethidium Bromide added to a final concentration of 0.5 ug/ml

Tank Buffer: 1X SB buffer

PCR products will be combined with Gel Loading Dye (stock is at 6X) before loading. The Gel Loading Dye contains two tracking dyes (see below) as well as sucrose (40% in the 6X stock) which ***increases the density of your sample so it falls into the wells in the gel.*** Final concentration of the Gel Loading Dye should be 1X. Each well in your gel will accommodate about 8ul of sample. Check with your instructor concerning specifics about combining the loading dye with the PCR reaction.

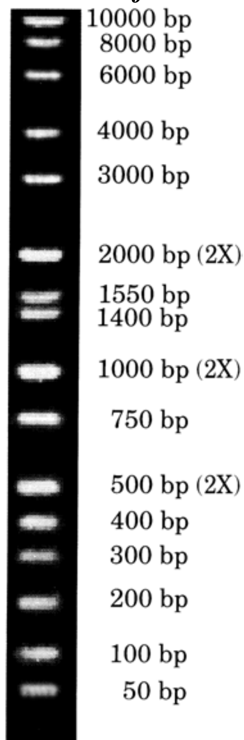
Tracking Dyes: Bromophenol Blue and Xylene Cyanol

Compare info below with size of PCR products – how far down the gel should we allow our tracking dyes to move?

Migration of loading dyes in different agarose concentrations

Agarose concentration, %	Xylene cyanol FF	Bromophenol blue	Orange G
0.7-1.7	~4000bp	~300bp	~50bp
2.5-3.0	~800bp	~100bp	~30bp

Load 5ul of HI LO size standards (which are already mixed with gel loading buffer)**.**



HI-LO SIZE STANDARDS:
When identifying Hi-Lo bands on your gels, be sure to use the 1.4 kb -1.55 kb doublet for orientation.

Do not try to count bands from the top or bottom of the gel.