Molecular Biology for Secondary Classrooms: MBSC

Educator and Student Materials

www.unl.mbsc.wordpress.com

Program Coordinator: Sarah D. Zulkoski-Benson
Nebraska EPSCoR
www.epscor.unl.edu

Contents

IMPORTANT INFORMATION!!!
PLEASE READ!!!

Module Notes (That will, hopefully, make everything run more smoothly!)

Group Assignments:

I recommend assigning groups ahead of time. Give each group a number and keep the information posted for the duration of the labs. I have seen teachers post a paper list in the classroom or tape strips of paper with the information down on the lab tables.

"Over Loading" tubes:

For teachers with multiple classes, there are a few steps we can save time on labeling tubes and aliquoting. In the RED and PCR labs, the gel electrophoresis days involve the use of a **Standard (or Marker)** and **Loading Dye**. Instead of labeling and aliquoting multiple tubes for multiple classes, multiply the amount specified in the directions by the number of classes you have, and aliquot that amount. Store those tubes on ice between classes. This will save some preparation time and in distributing materials during the labs.

Hot Water Baths:

The Bio-Rad hot water baths have been prone to blowing fuses in the past year. I will try to ensure a set of fuses are with the water baths. The fuses are easy to replace by pulling out the fuse holder at the base of the outlet on the back of the water bath. Ensure you are using a trusted outlet to plug in the water bath and also make sure there is water in the basin before plugging in the bath and/or turning it on. If you use the fuses in your equipment set, please let me know so I can replace them.

Ice:

If you only have ice cubes available for lab, be sure to add water so the foam racks can float and reagents are exposed to the ice.

Trash It or Keep It?

Please do not throw away the following materials. Package them up with the equipment to go to the next school.

- Blue, Bio-Rad staining trays
- Foam racks (unless they get melted in a boiling water bath)

You may keep the following materials upon completing the labs.

- Extra tubes
- Extra consumables (buffer, Fast Blast, reagents; just be sure to store according to directions)

Please let me know of any particular problems with prep. instructions or procedures so I can make changes.

Sarah Zulkoski-Benson • w.(402) 472-8946, h.(402)327-8364 • szulkoski-benson2@unl.edu

Reagent Checklist

In order to keep reagent costs down, please use all reagents as directed. Many of the reagents, especially enzymes, are provided in quantities very close to the volume needed for 8 groups or 32 students. Also, if you are using modules with multiple classes, please conserve reagents when possible. When volume allows, use all of the reagent from one kit before using reagents from the second. For example, the 50x TAE buffer is provided in a quantity that far exceeds what is actually needed. If you are using the buffer for 2 or more classes, use up the buffer from one kit before opening new reagents.

Please mark bottle or vials that may be returned with the date they were opened using a permanent marker.

The following chart lists the components of each module, quantities, and proper storage temperature. The chart also indicates which items should be returned to the Science Outreach Program if possible.

Reagent	Refrigerator	Freezer	Room Temp	Notes
pGlo Bacterial Transformation				
E. coli HB101 K-12, lyophilized			X	
Plasmid (pGLO), lyophilized, 20ug			X	
Ampicillin, lyophilized, 30 mg			X	
Arabinose, lyophilized, 600 mg			X	
Transformation solution (50 mM $CaCl_2$)			X	
LB nutrient broth, sterile			X	
LB nutrient agar powder			X	
PV92 PCR				
PV92 Controls, +/+, -/-, +/-		X		
PCR Master Mix		X		
Yellow Primer Mix		X		
EZ Load molecular mass ruler		X		
0.9% Saline Solution		X		
20X SB buffer (and 1X)			X	
Agarose powder			X	
Fast Blast DNA stain, 500x (and 1X)			X	
Instagene Matrix	X			
PV92 XC loading dye	X			
Restriction Enzyme Digest and Analysis				
HindIII lambda digest Marker		X		
Restriction Enzymes		X		
Restriction buffer		X		
Lambda DNA, uncut		X		
Sample loading dye	X			
Fast Blast DNA stain, 500x (and 1X)			X	
Agarose powder			X	
50x TAE buffer (and 1X)			**X**	

Reagent Charts for Agarose Gels and Buffers

Step 1: Determine number of groups per class and number of classes. This determines the amount of gel systems and gels.
Step 2: Prepare Buffer according to chart.
Step 3: Prepare Agarose according to chart.
Step 4: Add 500X Fast Blast to Buffer, AFTER making gels, according to chart or use Fast Blast Calculations.
Step 5: Add 500X Fast Blast to melted agarose, AFTER agar has cooled below 60°C, according to chart or use Fast Blast Calculations.
***Full instructions are in the teacher preparation sections.

Agarose Gels for Restriction Enzyme Digest				
Number of Gels	**Volume of 1% Agarose Needed**	**Agar Powder**	**1X Buffer***	**Volume of 500X Fast Blast to add AFTER agar has been dissolved and cooled below 60°C****
(units)	**(ml)**	**(g)**	**(ml)**	**(uL)**
8	240	2.4	240.0	158.4
16	480	4.8	480.0	316.8
24	720	7.2	720.0	475.2
32	1280	12.8	1280.0	844.8

*1X Buffer is TAE for Restriction Enzyme Digest and SB for PCR
**Fast Blast Calculation for Agarose: 33uL 500X Fast Blast per 50ml liquid agarose

Restriction Enzyme Digest: Gel Electrophoresis					
Number of Gel Systems	**Total Volume of 1X TAE Buffer Needed***	**50X TAE stock**	**Distilled Water**	**Volume of 500X Fast Blast to add AFTER using appropriate amount to make Agar****	****Volume of the 1X TAE needed for gels*
(units)	**(ml)**	**(ml)**	**(ml)**	**(uL)**	*(ml)*
8	3000.0	60.0	2940.0	1786.7	*320.0*
16	3000.0	60.0	2940.0	1573.3	*640.0*
24	4000.0	80.0	3920.0	2026.7	*960.0*
32	6000.0	120.0	5880.0	3146.7	*1280.0*

*Buffer can be re-used between classes within the same lab
**Fast Blast Calculation for Buffer: 200uL 500X Fast Blast per 300ml Buffer

Agarose Gels for PCR				
Number of Gels	**Volume of 1% Agarose Needed**	**Agar Powder**	**1X Buffer***	**Volume of 500X Fast Blast to add AFTER agar has been dissolved and cooled below 60°C****
(units)	**(ml)**	**(g)**	**(ml)**	**(uL)**
8	320	3.2	320.0	211.2
16	640	6.4	640.0	422.4
24	960	9.6	960.0	633.6
32	1280	12.8	1280.0	844.8

PCR: Gel Electrophoresis					
Number of Gel Systems	**Total Volume of 1X SB Buffer Needed***	**20X SB stock**	**Distilled Water**	**Volume of 500X Fast Blast to add AFTER using appropriate amount to make Agar****	****Volume of the 1X SB needed for gels*
(units)	**(ml)**	**(ml)**	**(ml)**	**(uL)**	*(ml)*
8	3000.0	150.0	2850.0	1786.7	*320.0*
16	3000.0	150.0	2850.0	1573.3	*640.0*
24	4000.0	200.0	3800.0	2026.7	*960.0*
32	6000.0	300.0	5700.0	3146.7	*1280.0*

Instructor Preparation Checklist

pGlo Bacterial Transformation	**# of Days in Advance:**	**Minimum**	**Maximum**
☐ Prepare LB agar		3	14
☐ Prepare arabinose and ampicillin		During agar preparation	
☐ Mark plates		During agar preparation	
☐ Pour LB nutrient agar plates (LB, LB/amp, LB/amp/ara)		Immediately after agar preparation	
☐ Rehydrate E.coli		24 hours	36 hours
☐ Streak starter plates		24 hours	36 hours
☐ Prepare pGLO plasmid		24 hours	36 hours
☐ Prepare Tubes and Aliquot Reagents o +pGlo o –pGlo o LB		1	14
☐ Set up Student Work Stations for Transformation		Day of Transformation	
☐ Set up teacher station for Transformation		Day of Transformation	
☐ Turn on 42°C water bath		Day of Transformation	
☐ Turn on 37°C incubator		Day of Transformation	
☐ Set up Student Work Stations for Results		Day of Results	

New! Fast Digest and Student's Choice	**# of Days in Advance:**	**Minimum**	**Maximum**
☐ Prepare TAE electrophoresis buffer		1	14
☐ Prepare and Cast agarose gels		1	7
☐ Prepare Fast Blast DNA stain		1	14
☐ Prepare Tubes and Aliquot Reagents			
o Student Enzymes		1	2
o Restriction buffer		1	14
o Lambda DNA			
o Student digest (reaction) tubes		1	14
o Sample loading dye		1	14
o Gene Ladder DNA Marker		1	14
☐ Set up Student Work Stations for Digestion		Day of Lab	
☐ Turn on 37°C water bath		Day of Lab	
☐ Turn on 65°C water bath		Day of Lab	
☐ Set up gel electrophoresis stations		Day of Lab	

PV92 PCR	# of Days in Advance:	Minimum	Maximum
☐ Prepare SB electrophoresis buffer		1	14
☐ Prepare and Cast agarose gels		1	7
☐ Prepare Fast Blast DNA stain		1	14
☐ Prepare Tubes and Aliquot Reagents o InstaGene Matrix o Controls o DNA Standard o Loading Dye o Saline Solution		1	14
☐ Set up Student Work Stations for Day 1, DNA Extraction		Day of DNA Extraction	
☐ Turn on 56°C water bath and prepare boiling water bath(s)		Day of DNA Extraction	
☐ Set up Student Work Stations for Day 2, Amplification		Day of Amplification	
☐ Set up PCR Thermal Cycler and Program o Make copy of PCR Template for Loading Student Samples		Day of Amplification	
☐ Prepare complete Master Mix and aliquot		Immediately before Amplification Lab	
☐ Add complete Master Mix to controls		During Amplification Lab	
☐ Set up Student Work Stations for Day 3, Gel Electrophoresis		Day of Electrophoresis	
☐ Add Loading Dye to controls		Day of Electrophoresis or During Lab	
☐ Set up Student Electrophoresis Stations for Day 3		Day of Electrophoresis	

Materials and Equipment

PCR Thermal Cycler

Water Bath

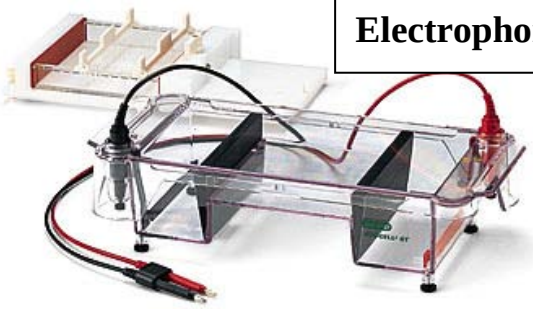
UV Illuminator

Power Supply

Incubator

Mini Centrifuge

Horizontal Gel Electrophoresis

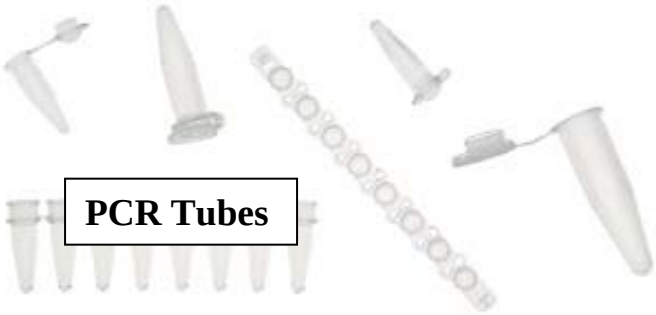


Micropipettes

Vertical Protein Gel Electrophoresis

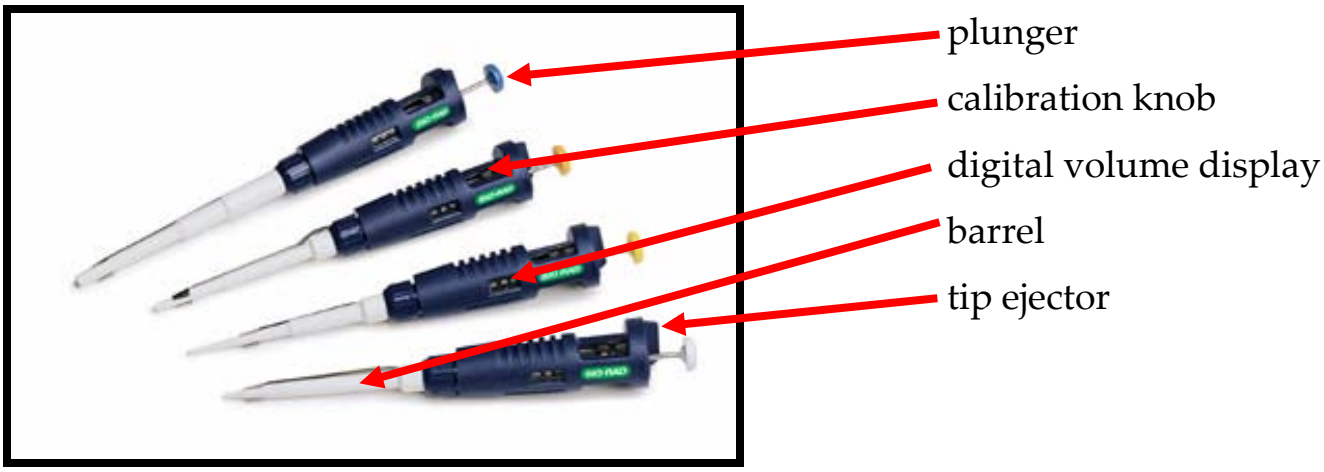
Screw Cap Tubes

PCR Tubes



Micro Test Tubes

Anatomy of a Digital Micropipettor



How to Operate a Digital Micropipettor

1. Hold the micropipettor in one hand, slowly turn the calibration knob about one-third a turn above the desired setting and then slowly down until the required volume is on the micrometer. **Always dial down** to the desired volume. **Always monitor** the digital display while adjusting the knob.

Attach a new disposable tip to the pipette shaft, pressing firmly to ensure an airtight seal. **DO NOT** jam the barrel into the pipet tips repeatedly!!! Use fresh tip for every sample of a different composition.

2. Depress the plunger to the first stop. This part of the stroke will achieve the desired volume as set on the micrometer (see picture below).
3. Holding the Pipetman in one hand, immerse only the disposable tip into the sample liquid to a volume approximately one-quarter of the way up the tip.
4. Slowly release the plunger slowly to the up position. Never let the plunger snap up!
5. Wait a moment for the volume to fill the tip.

Push plunger to first stop. Keep tip out of fluid while plunger is depressed. **Note**: Always hold micropipettor vertically.

Keep plunger at first stop. Now insert tip into fluid (about 2-6 mm below top of liquid). stay clear of sides and bottom of container so you do not restrict uptake of your liquid sample.

Slowly release plunger to draw the correct amount of fluid into the tip.

7. Withdraw the tip from the sample liquid and place the tip against the sidewall of the tube you intend to transfer the liquid. Depress the plunger to its first stop slowly. Then press the plunger to its second stop to expel any residual liquid.

8. Carefully remove the micropipettor from the liquid while continuing to hold down the plunger.

Position tip so fluid will be deposited on the inside of the tube. Push the plunger to the first stop.	Continue to push the plunger to the second (**2**) stop. Fluid is released on to the inside of the tube	Remove the tip and **slowly** release the plunger. Spin the tube in a microcentrifuge to pool the fluid in the bottom of the tube.
		
		

9. Allow the plunger to return to the up position and then discard the tip by depressing the ejector bottom. Always use a fresh tip for each sample to prevent contamination.

10. When pipets are not in use, leave them undisturbed on the benchtop. Place the pipets where they will not be knocked off the bench top, onto the ground, which could cause considerable damage. Leaving your pipets undisturbed will ensure they are not unconsciously or inadvertently damaged.

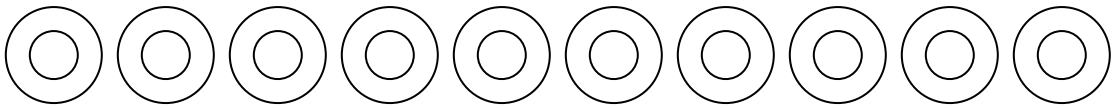
Micropipetting Practice Sheet

Pipetting Reminders:

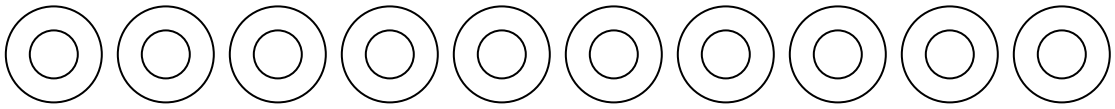
- ☑ **1st Stop** for **Uptake**
- ☑ **2nd Stop** for **Dispensing**
- ☑ Pipet should always remain vertical...**plunger UP**!!!
- ☑ **DO NOT** adjust beyond recommended volumes…Double Check for **appropriate volume**!!!
- ☑ **DO NOT** contaminate the barrel…Always **use a pipet tip** for dispensing samples!!!

Use a **p20**, range **2uL-20uL**, to pipet the following volumes into the spaces provided. Check for **drop size similarity** to determine consistency and accuracy. Practice until you feel comfortable handling the pipet.

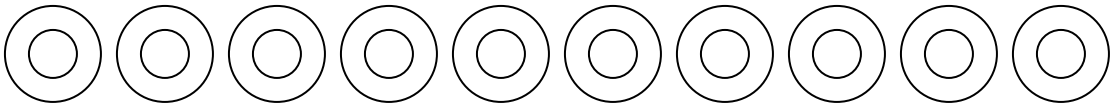
p20,2ul
0
2
0



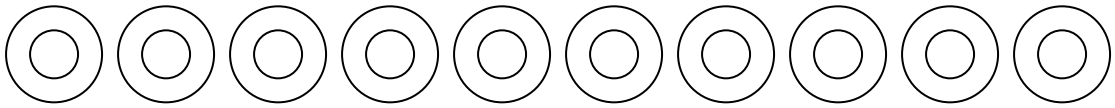
p20,4ul
0
4
0



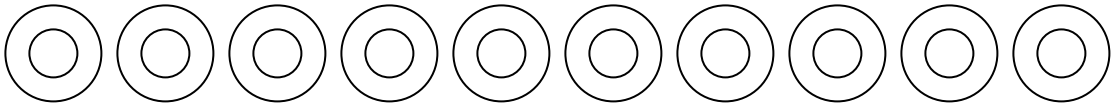
p20,6ul
0
6
0



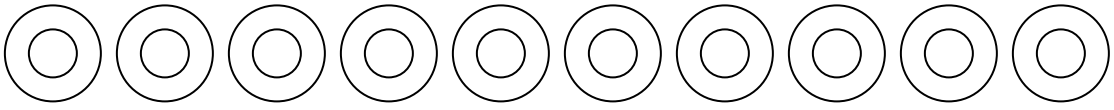
p20,10ul
1
0
0



p20,20ul
2
0
0



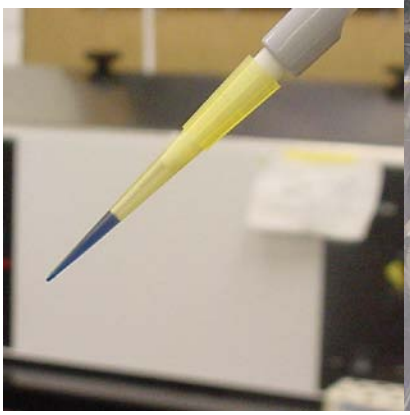
p200, 150ul
1
5
0

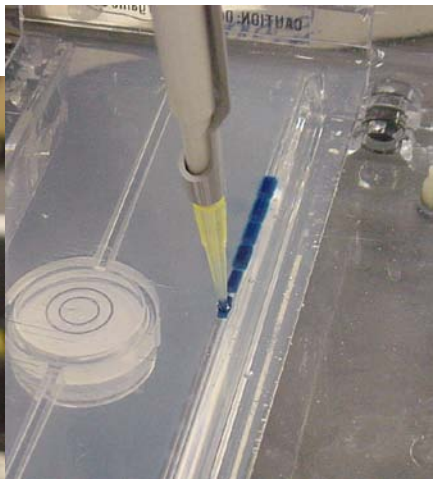


Loading agarose gels (from http://www.cofc.edu/~delliss/virtuallabbook/LoadingGel/LoadingGel.html)
Loading gels is a necessary skill in molecular biology. The first step is checking the sample before loading, as shown below:

After loading sample into the pipette tip, check carefully for bubbles. Make sure that there is no air at the end of the tip. This can create bubbles, which might "puff" the DNA out of the well.

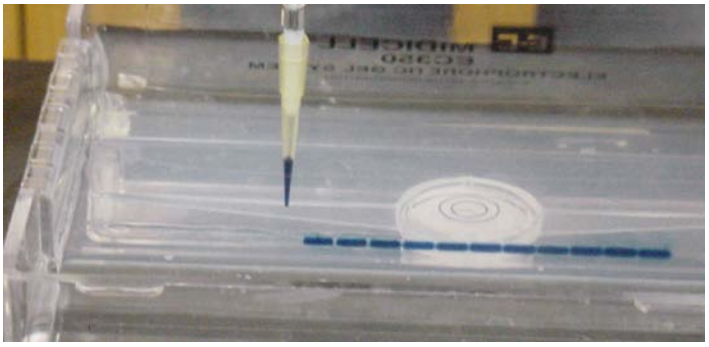
The sample is blue because of the loading/tracking dye mixed in with the DNA solution. This helps us see the solution when loading into the gel, and also helps follow the progress of the gel as it is run.



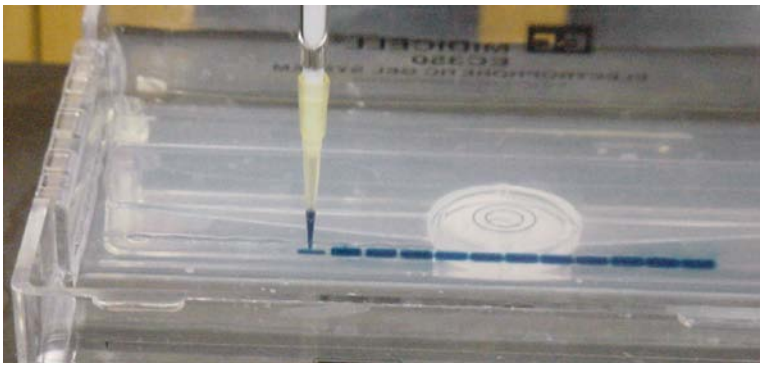


When loading a gel, support the hand holding the pipettor and help guide the tip into the well. This will help keep the tip steady. This series of three photos show the process of loading a sample into a gel well:

- ☑ Place the pipette tip into the buffer and above the gel well.
- ☑ **DO NOT** put the tip too deeply into the gel well because you can puncture through the well and the sample will leak out

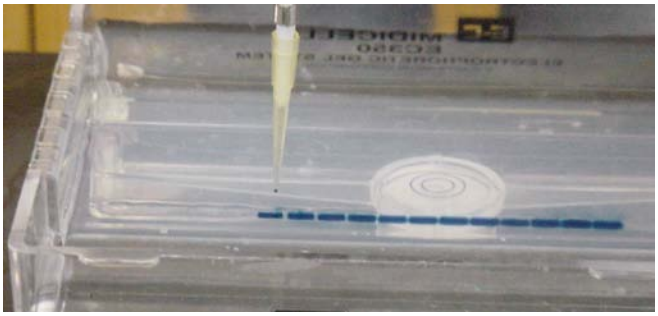


- ☑ Slowly dispense the sample into the well.
- ☑ Push the pipette plunger down to the first stop.
- ☑ **DO NOT** push past the first stop because you will create a bubble.
- ☑ Remove the pipette tip out of the gel, **THEN** release the plunger
- ☑ **DO NOT** release the plunger until the tip is out of the gel.



Notice that only a small amount of sample remains in the tip, not enough to matter.

Eject the tip into a waste container and obtain a new pipet tip for each sample.



Procedures and Classroom Materials
Adapted from Biotechnology Explorer™ Program,
Bio-Rad Laboratories

Instructor Materials

pGlo Teacher Preparation Guide

Agar Plates: 3-14 Days in Advance (Necessary items and equipment are in bold)

1. Prepare LB (nutrient) agar (autoclave-free)

The agar plates should be prepared at least three days before the student experiment is performed. They should be left out at room temperature for two days and then refrigerated until they are to be used. The two days on the benchtop allows the agar to dry out (cure) sufficiently to readily take up the liquid transformation solution in student lesson 2.

Agar Preparation:

- ✓ Add 500 ml of **distilled water** to a **1 L or larger Erlenmeyer flask**. Add the entire contents of the **LB nutrient agar packet**. Swirl the flask to dissolve the agar, and heat to boiling in a **microwave or on a hot plate**. Repeat heating and swirling about three times until all the agar is dissolved (no more clear specks swirl around), but be careful to allow the flask to cool a little before swirling so that the hot medium does not boil over onto your hand.
- ✓ When all the agar is dissolved, allow the LB nutrient agar to cool so that the outside of the flask is just comfortable to hold (50°C). The bottom of the flask can be submerged in a cool water bath to lower the temperature more quickly, but monitor closely so the agar does not begin to solidify.
- ✓ While the agar is cooling, label the plates and prepare the arabinose and ampicillin as outlined below. Be careful not to let the agar cool so much that it begins to solidify.

2. Prepare arabinose and ampicillin:

- ✓ With a new **sterile pipet**, add 3 ml of **transformation solution** directly to the **vial of lyophilized arabinose**. Mix the vial; a vortexer helps. (Transformation solution is being used here because it is a handy sterile solution. Sterile water would work just as well.)
- ✓ With a new **sterile pipet**, add 3 ml of **transformation solution** directly to the **vial of lyophilized ampicillin**.

Notes:

- ➢ Excessive heat (>50°C) will destroy the ampicillin and the arabinose, but the nutrient agar solidifies at 27°C so one must carefully monitor the cooling of the agar and then pour the plates from start to finish without interruption.
- ➢ Arabinose requires *at least* 10 minutes to dissolve—be patient.
- ➢ After the plates are poured do not disturb them until the agar has solidified.
- ➢ Pour excess agar in the garbage, not the sink.

3. Mark plates

- ✓ The **40 supplied agar plates** should be marked with a **permanent marker** on the bottom close to the edge. Label 16 plates LB**,** 16 plates LB/amp, and 8 plates LB/amp/ara.

4. Pour LB nutrient agar plates (LB, LB/amp, LB/amp/ara): 3 Steps

- ✓ First, pour LB nutrient agar into the 16 plates that are labeled LB. Stack the empty plates 4 to 8 high and starting with the bottom plate lift the lid and the upper plates straight up and to the side with one hand and pour the LB nutrient agar with the other. Fill the plate about one-third to one-half (~12 ml) with agar, replace the lid and continue up the stack. Let the plates cool in this stacked configuration.
- ✓ Second, add the **hydrated ampicillin** to the remaining LB nutrient agar. Swirl briefly to mix. Pour into the 16 plates that are labeled as **LB/amp** using the technique utilized above.
- ✓ Third, add the **hydrated arabinose** to the remaining LB nutrient agar containing ampicillin. Swirl briefly to mix and pour into the 8 plates labeled as **LB/amp/ara** using the technique utilized above.

5. Plate storage

After the plates have cured for two days at room temperature or they can be used or stacked up twenty high and the plastic sleeve bag slipped back down over them. The stack is then inverted, the bag taped closed, and the plates stored upside-down in a refrigerator until used.

E.coli Starter Plates and pGlo plasmid: 24-36 Hours in Advance (Necessary items and equipment are in bold)

1. Rehydrate *E.coli* bacteria

- ✓ Using a **sterile pipet**, rehydrate the **lyophilized *E. coli* HB 101** by adding 250 µl of **Transformation solution** directly to the vial. Recap the vial and allow the cell suspension to stand at room temperature for ***5 minutes***. Then shake gently to mix (do not vortex) before streaking on **LB** starter plates. Store the rehydrated bacteria in the refrigerator until used (within 24 hours for best results, no longer than 3 days).

2. Streak starter plates to produce single bacterial colonies on agar plates

Each lab team will need their own starter plate (recipient culture) as a source of cells for transformation. This kit contains sufficient material to outfit eight complete student workstations. LB plates should be streaked for single colonies and incubated at 37°C for 24–36 hours before the transformation activity is planned. The purpose of streaking is to generate single colonies from a concentrated suspension of bacteria. A minute amount of the bacterial suspension goes a long way. Under favorable conditions, one cell multiplies to become millions of genetically identical cells in just 24 hours. There are millions of individual bacteria in a single 1 mm bacterial colony.

Using the rehydrated *E. coli* you prepared in the last step and eight **LB** agar plates (prepared in step one), streak one starter plate for each of your student teams.

- ✓ Insert a **sterile inoculation loop** into the **rehydrated bacterial culture**. Tilt the vial and submerge the loop in the solution. You should see a film of bacterial suspension across the loop. Go back and forth with the loop about a dozen times at one "edge" of the plate, until the film of bacteria on the loop is gone. Lift the loop off the agar surface then drag it across the area that was just inoculated and continue streaking the bacteria across the rest of the plate's surface. The goal is to use as much of the surface area of the plate as possible.
- ✓ Use the same inoculation loop for all plates. When you are finished with each plate, cover it immediately to avoid contamination.
- ✓ Place the plates upside down inside the **incubator** , for at least 24 hours, at **37°C**. Use for transformation within 24–36 hours. **DO NOT REFRIGERATE BEFORE USE.**
- ✓ *E. coli* forms off-white colonies that are uniformly circular with smooth edges. Avoid using plates with contaminant colonies.

3. Prepare pGLO plasmid

Using a **new sterile pipet** add 250 µl of **transformation solution** into the **vial of lyophilized pGLO plasmid DNA**. Note that the quantity of DNA is so small that the vial may appear empty. If possible, store the hydrated DNA in a **refrigerator**.

Reagent Preparation: Day of Transformation or up to 14 days in advance

1. Prepare Tubes and Aliquot Reagents

- ✓ Label **8, green, micro test tubes** + pGlo
- ✓ Label **8, clear, micro test tubes** –pGlo
- ✓ Using a **sterile pipet** or a **p1000 micropipettor**, pipet 250ul of **transformation solution** into all of the **+ pGlo** and **–pGlo** micro test tubes. Refrigerate until distributed to students.
- ✓ Label **8, micro test tubes (same color)** LB
- ✓ Using a **sterile pipet** or a **p1000 micropipettor**, pipet 1ml of **LB Nutrient Broth** into all of the **LB** micro test tubes. Refrigerate until distributed to students.

Set up Student Work Stations, Teacher Station, and necessary equipment: Day of Transformation

1. Each station should have the following materials

- ✓ Foam test **tube rack**
- ✓ Styrofoam **cup with ice** (Test tubes listed below go in foam rack, on ice) *Alternatively, have one, large ice bath for all students to use.
- ✓ One green micro test tube labeled, **+ pGlo**
- ✓ One clear micro test tube labeled, **–pGlo**
- ✓ One test tube labeled, **LB**
- ✓ One E.coli **starter plate**
- ✓ 4 **genetic selection plates** (1 LB, 2 LB/Amp, 1 LB/Amp/Ara)
- ✓ One packet of **sterile loops**
- ✓ Six **sterile pipets**, individually wrapped
- ✓ One **permanent marker**
- ✓ Two strips of **lab tape or masking/scotch tape**
- ✓ Student **lab procedure**
- ✓ Waste beaker or tray

2. Set up teacher station

- ✓ pGlo **plasmid vial** on ice
- ✓ packet of **sterile loops**

3. Turn on water bath

- ✓ Set **water bath** to 42°C and turn on. Leave lid off. Monitor temperature for necessary adjustments.

4. Turn on incubator

- ✓ Set incubator to 37°C and turn on. Monitor temperature for necessary adjustments.

Day of Results: Necessary items and equipment are in bold

1. Set up Student Work Stations: Each station should have the following materials

- ✓ Hand-held, short-wave UV light
- ✓ 4 genetic selection plates
- ✓ E. coli starter plate
- ✓ Worksheets, if needed
- ✓ Permanent marker (small tip if available)

2. Disposal of waste from Day 1 and agar plates

- ✓ Submerge all tubes, pipettes, and loops in a 10% bleach solution for 2 minutes. Bag and discard in trash.
- ✓ Submerge all agar plates (open) and lids in a 10% bleach solution for 10 minutes. Double bag and discard in trash. **Agar plates can be stored in the refrigerator for up to a week if needed for GFP purification or for pictures.**

Procedure

pGlo Bacterial Transformation Procedure WITH INSTRUCTOR NOTES

1. At your group's lab station, check to make sure you have the following materials:

   Test tube labeled, **+pGlo**, containing **250 µl** of **transformation solution** ($CaC1_2$)
   Test tube labeled, **-pGlo**, containing **250 µl** of **transformation solution** ($CaC1_2$)
   Test tube labeled, **LB,** containing 1ml of **LB Nutrient Broth**
   ***Label the cap of each tube with your group's number.**
   One E.coli starter plate (with colonies of E.coli growing on it)
   4 genetic selection plates (1 LB, 2 LB/Amp, 1 LB/Amp/Ara)
   1 packet of sterile loops
   6 sterile pipets in individual packages

In order to minimize contamination from exposure to the air, ensure all tubes and plates are closed when not in use. When tubes and plates are in use, make sure they are only open for a short time. For plates, only lift the lids enough to insert loops or pipets. Do not completely remove lids and place on the counter-top. This will expose the entire plate to possible contamination.

2. Label the cap of each tube (+, -, and LB) with your group's number.
3. Use a sterile loop to pick up a single colony of bacteria from your E.coli starter plate.

In order to avoid contamination, only remove the loop from the package right before you use it and do not touch the loop to any other surfaces besides the starter plate.

4. Pick up the **+pGLO tube** and place the loop into the transformation solution at the bottom of the tube. Spin the loop between your index finger and thumb until the entire colony is dispersed in the transformation solution (with no floating chunks). Place the tube back in the tube rack in the ice.
5. Using a **new sterile loop**, repeat steps 2 and 3 for the **-pGLO tube**.
6. Take your **+pGLO tube** to your instructor to receive the pGlo plasmid. Immerse a **new sterile loop** into the **plasmid DNA stock tube**. Withdraw a loopful. *The loop should have a film on it, as if you were blowing bubbles.* Mix the loopful into the cell suspension of the **+pGLO tube**. Close the tube and return it to the rack on ice. Close the **-pGLO** tube. **Do not add plasmid DNA to the -pGLO tube.**
7. Incubate the tubes on ice for 10 minutes. Push the tubes all the way down in the rack so the bottoms of the tubes make contact with the ice. **If you do not have crushed ice, make an ice *bath* so that the tubes reach the correct temperature.**
8. While the tubes are sitting on ice, label your four agar plates **on the bottom**. Follow the picture below. *Label your plates as close to the edge as possible so that you can view your results.*

Turn Over

9. **Heat shock.** Using the foam rack as a holder, transfer both the (+) pGLO and (-) pGLO tubes into the water bath, set at 42 °C, **for exactly 50 seconds**.

10. When the 50 seconds are done, incubate tubes on ice for **2 minutes**.

11. Place the foam tube rack on the bench top. Open a new sterile pipet and add **250 µl of LB nutrient broth** to the **+pGLO tube**. ***250 µl is the 3rd section up on the pipet.***

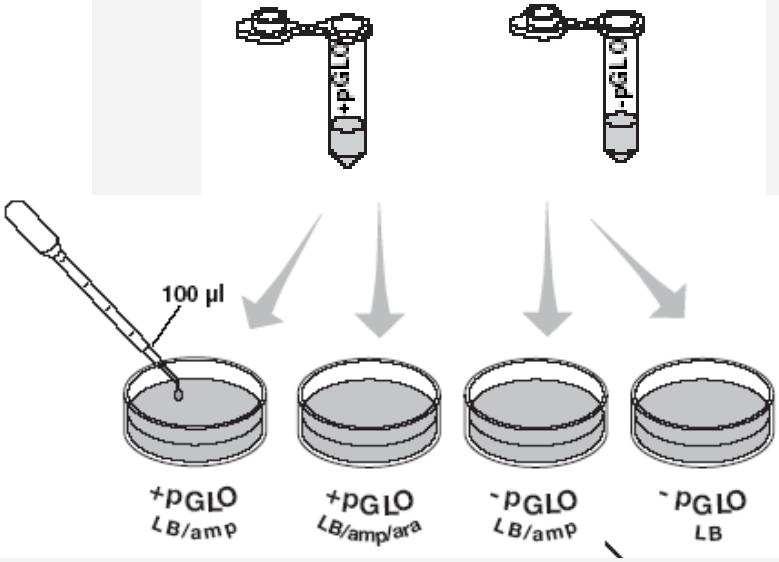
In order to avoid contamination, open the pipet package from the top (near the bulb) and do not touch the pipet tip to any other surfaces.

12. Repeat step 10 with a new sterile pipet for the **-pGLO tube**.

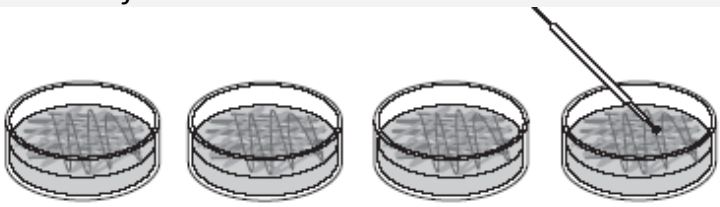
13. Incubate the tubes for 10 minutes at room temperature on the bench top.

14. After the 10 minutes incubation, **flick the closed tubes with your finger to mix**.

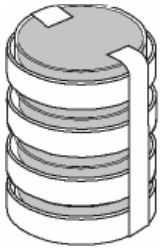
15. Using a new sterile pipet for each tube, **pipet 100 µl of the transformation (+pGlo) and control (-pGlo)** onto the correct plates. ***100 µl is the second section up on the pipet.*** *Make sure to pipet the suspensions onto the agar surface of the plates, NOT onto the lids of the plates.*



16. Use a new sterile loop for each plate. Spread the suspensions evenly around the surface of the agar by skimming the flat surface of the loop back and forth across the surface. *DO NOT dig the loops into the surface of the agar and create marks on the surface.*



17. Stack up your plates and tape them together. Put your group number and class period on the bottom of the stack and place the stack upside down in the 37°C incubator until the next day. Discard all tubes in your waste beaker. Leave your E.coli starter plate at your station. *Wash your hands thoroughly before leaving class.*



pGlo Bacterial Transformation Procedure

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   Test tube labeled, **+pGlo**, containing **250 µl** of **transformation solution** ($CaC1_2$)

   Test tube labeled, **-pGlo,** containing **250 µl** of **transformation solution** ($CaC1_2$)

   Test tube labeled, **LB,** containing 1ml of **LB Nutrient Broth**

   *****Label the cap of each tube with your group's number.**

   One E.coli starter plate (with colonies of E.coli growing on it)

   4 genetic selection plates (1 LB, 2 LB/Amp, 1 LB/Amp/Ara)

   1 packet of sterile loops

   6 sterile pipets in individual packages

In order to minimize contamination from exposure to the air, ensure all tubes and plates are closed when not in use. When tubes and plates are in use, make sure they are only open for a short time. For plates, only lift the lids enough to insert loops or pipets. Do not completely remove lids and place on the counter-top. This will expose the entire plate to possible contamination.

2. Label the cap of each tube (+, -, and LB) with your group's number.

3. Use a sterile loop to pick up a single colony of bacteria from your E.coli starter plate.

   In order to avoid contamination, only remove the loop from the package right before you use it and do not touch the loop to any other surfaces besides the starter plate.

4. Pick up the **+pGLO tube** and place the loop into the transformation solution at the bottom of the tube. Spin the loop between your index finger and thumb until the entire colony is dispersed in the transformation solution (with no floating chunks). Place the tube back in the tube rack in the ice.
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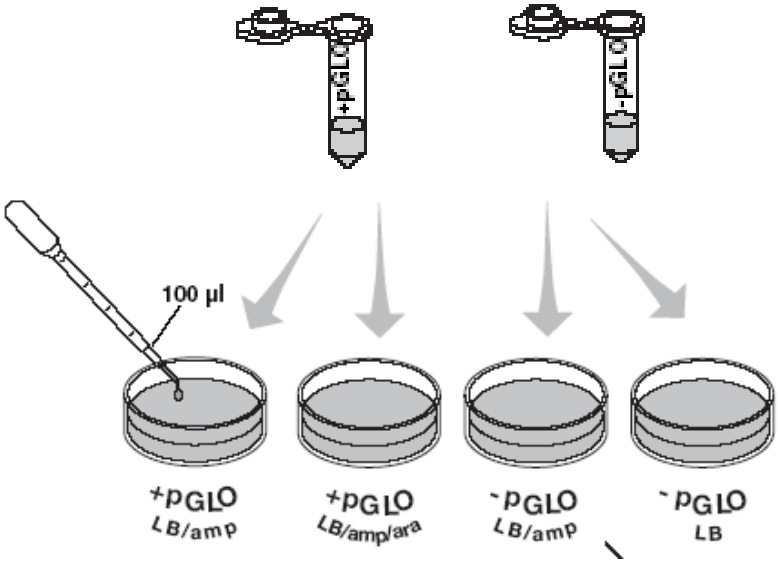

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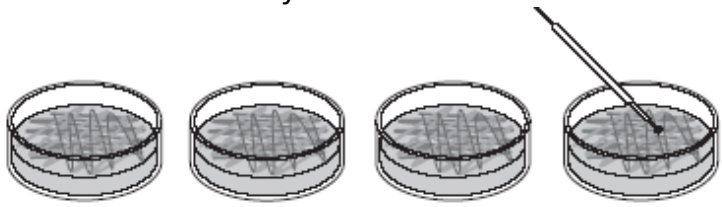

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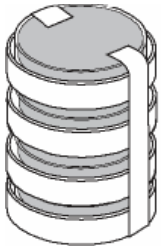


Student Materials

pGlo Introduction to Transformation and Pre-Lab Observations

Genetic transformation is taking genes from one organism and putting them in another. A gene is a piece of DNA that gives the instructions for making a protein. This protein gives an organism a certain trait. A gene is inserted into an organism in order to change the organism's trait. Genetic transformation is used in many areas of biotechnology. In agriculture, genes coding for traits such as frost, pest, or spoilage resistance can be inserted into plants. In medicine, gene therapy treats diseases caused by defective genes by inserting healthy copies of the defective gene in a sick person's cells.

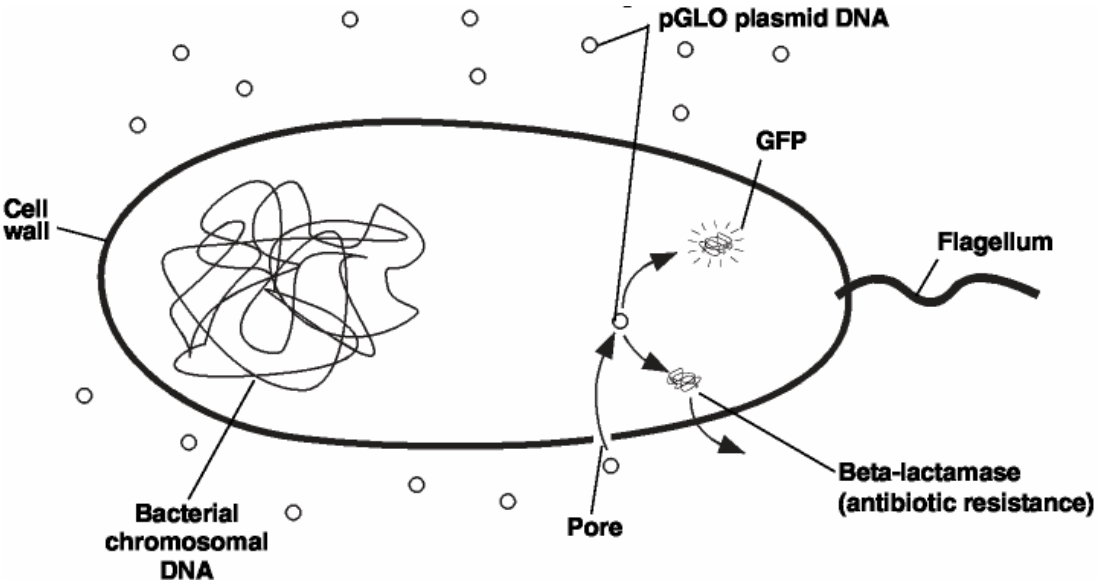
You will transform bacteria with a gene that codes for Green Fluorescent Protein (GFP). The real-life source of this gene is a jellyfish. GFP causes certain areas of the jellyfish to glow in the dark. After you transform the bacteria, they will express their new jellyfish gene and produce the fluorescent protein. It causes them to glow a brilliant green color under ultraviolet light.

You will learn about the process of moving genes from one organism to another with the aid of a plasmid, a small circular piece of DNA. Bacteria have one large chromosome and one or more plasmids. Plasmids usually contain genes for traits that may help the bacteria survive. In nature, bacteria can transfer plasmids back and forth allowing them to share these beneficial genes. This process allows bacteria to adapt to new environments.

Bio-Rad's pGLO plasmid has three special genes: one for GFP, a gene for antibiotic resistance, and a gene regulation system. This system can be used to control when the bacteria produce fluorescent protein. The gene for GFP can be switched on in transformed cells by adding the sugar arabinose to the cells' food source. Transformed cells will appear white on plates not containing arabinose, and fluorescent green when arabinose is included in the nutrient agar medium. Also, we can test that cells have been transformed with pGLO DNA by growing them on antibiotic plates.

The Genes

Genetic transformation involves the insertion of some new DNA into the E. coli cells. Bacteria have one large chromosome and one or more plasmids. Plasmids usually contain genes for traits that may help the bacteria survive. Scientists can use a process called genetic engineering to insert genes coding for new traits into a plasmid. In this lab, the pGLO plasmid has the GFP gene that codes for the green fluorescent protein and a gene that codes for a protein that gives the bacteria resistance to an antibiotic. The pGlo plasmid can then be used to transform bacteria to give them this new trait.



The Act of Transformation

This transformation procedure involves three main steps. These steps are intended to insert the plasmid DNA into the E. coli cells and provide an environment for the cells to produce their newly acquired genes.

To move the pGLO plasmid DNA through the cell membrane you will:

1. Use a **transformation solution** of $CaCl_2$ (calcium chloride) to make cells **competent**
2. Carry out a procedure referred to as **heat shock** so bacteria can take in the plasmid

For transformed cells to grow in the presence of ampicillin you must:

3. Provide them with nutrients and a short **incubation period** to begin expressing their newly acquired genes

Competent Cells

Bacterial cells must be in a particular state before they can be transformed. This state is referred to as *competency*. This state can be achieved naturally in some species of bacteria when levels of nutrients and oxygen are low. E.coli, the organism on which most current research is performed, must be artificially induced to make it competent. Competent E. coli cells are very fragile and must be treated carefully.

Transformation Solution: Calcium Chloride $CaCl_2$

The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment should be followed by heat.

Heat Shock Treatment

When E.coli are subjected to 42°C heat, a set of genes are expressed which aid the bacteria in surviving at that temperature. This set of genes is called the heat shock genes. The heat shock step is necessary for the uptake of DNA. At temperatures above 42°C, the bacteria's ability to uptake DNA is lowered, and at extreme temperatures the bacteria will die.

Incubation

After the heat shock step, intact plasmid DNA molecules replicate in bacterial host cells. To help the bacterial cells recover from the heat shock, the cells are briefly incubated in LB Nutrient Broth, a solution that provides nutrients for the bacteria. As the cells recover, plasmid genes are expressed, including those that allow the replication of plasmids which will end up in new, dividing bacterial cells.

Genetic Regulation

Our bodies contain thousands of different proteins which perform many different jobs. Digestive enzymes are proteins; some of the hormone signals that run through our bodies and the antibodies protecting us from disease are proteins. The information for assembling a protein is carried in our DNA. The section of DNA which contains the code for making a protein is called a gene. There are over 30,000–100,000 genes in the human genome. Each gene codes for a unique protein: one gene, one protein. The gene that codes for a digestive enzyme in your mouth is different from one that codes for an antibody or the pigment that colors your eyes.

Organisms regulate expression of their genes and ultimately the amounts and kinds of proteins present within their cells for many reasons, including developmental changes, cellular specialization, and adaptation to the environment. Gene regulation not only allows for adaptation to differing conditions, but also prevents wasteful overproduction of unneeded proteins which would put the organism at a competitive disadvantage. The genes involved in the transport and breakdown (catabolism) of food are good examples of highly regulated genes. For example, the sugar arabinose is both a source of energy and a source of carbon. *E. coli* bacteria produce three enzymes (proteins) needed to digest arabinose as a food source. The genes which code for these enzymes are not expressed when arabinose is absent, but they are expressed when arabinose is present in their environment. How is this so?

Regulation of the expression of proteins often occurs at the level of transcription from DNA into RNA.

This regulation takes place at a very specific location on the DNA template, called a promoter, where RNA polymerase sits down on the DNA and begins transcription of the gene. In bacteria, groups of related genes are often clustered together and transcribed into RNA from one promoter. These clusters of genes controlled by a single promoter are called operons. The three genes *(araB, araA* and *araD)* that code for three digestive enzymes involved in the breakdown of arabinose are clustered together in what is known as the arabinose operon. When arabinose is present in the environment, bacteria take it up. Once inside, the arabinose interacts directly with arabinose operon and the interaction causes the transcription of the three digestive enzyme genes. When the three enzymes are produced, they break down arabinose, and eventually the arabinose runs out. In the absence of arabinose the transcription is shut off.

The DNA code of the pGLO plasmid has been engineered to incorporate aspects of the arabinose operon. The genes which code for break down of arabinose, *araB, A* and *D,* have been replaced by the single gene which codes for GFP. Therefore, in the presence of arabinose, GFP is produced. Cells fluoresce brilliant green as they produce more and more GFP. In the absence of arabinose, GFP gene is not transcribed. When GFP is not made, bacteria colonies will appear to have a wild-type (natural) phenotype—of white colonies with no fluorescence.

Genetic Selection

Not all cells will have the plasmid and not all newly produced plasmids will end up in new bacteria cells. So, it is necessary to select for bacterial cells which contain the plasmid. This is commonly performed with antibiotic selection. Some E.coli strains cannot grow in the presence of common antibiotics like ampicillin. Plasmids used for the cloning and manipulation of DNA have been engineered to contain the genes for antibiotic resistance. Thus, if the bacterial transformation is plated onto media containing ampicillin, only bacteria which have the plasmid DNA will have the ability to metabolize ampicillin and form colonies. In this way, bacterial cells containing plasmid DNA are selected.

Pre-Lab Observations

The goal of genetic transformation is to change an organism's traits (phenotype). Before any change in the phenotype of an organism can be seen, a good examination of its natural phenotype must be made. Look at the colonies of E. coli on your starter plates. The following pre-lab observations of E. coli will provide basic data to make reference to when attempting to determine if any genetic transformation has occurred.

Use the following list of traits to help you describe what you see:

(Use a separate sheet of paper to record your observations. Include your name, group number, and the headings below on the paper.)

a) Number of colonies:

b) Size of colonies (in millimeters):

1) the largest colony:

2) the smallest colony:

3) the majority of colonies:

c) Color of the colonies:

d) Distribution of the colonies on the plate:

e) Visible appearance when viewed with ultraviolet (UV) light:

pGlo Bacterial Transformation Power Point Follow-up Questions (Pre-lab evaluation)

1. What is the purpose of this lab? What are we trying to do?

2. What is the original source of green fluorescence protein?

3. Write a short definition of the following terms:
   a. Bacterial Transformation:

   b. Plasmid:

   c. Gene Regulation:

   d. Genetic Selection:

4. What is the purpose of the following solutions?
   a. Calcium Chloride ($CaCl_2$):

   b. LB Nutrient Broth:

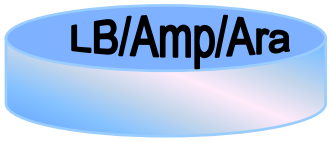
5. What is happening to the bacteria we are trying to transform in the following steps?

   a. Heat Shock Treatment:

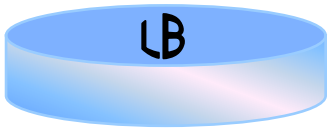
   b. Incubation in LB Nutrient Broth:

6. Normal, non-transformed, E.coli bacteria are white, use arabinose (Ara) as a food source, and cannot live when exposed to antibiotics like ampicillin (amp). What do you expect to see if E.coli is grown under the following conditions? Will there be **growth** or **no growth**?

LB Nutrient Agar + Ampicillin



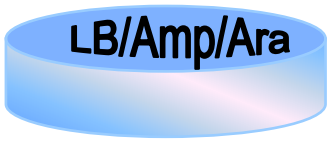
LB Nutrient Agar + Ampicillin + Arabinose



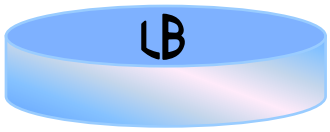
LB Nutrient Agar

7. What do you expect to see if E.coli bacteria is transformed so that it produces a green fluorescence protein in the presence of arabinose and the E.coli is now resistant to ampicillin? Will there be **growth** or **no growth**? Will there be any **glowing** bacteria?

LB Nutrient Agar + Ampicillin



LB Nutrient Agar + Ampicillin + Arabinose



LB Nutrient Agar

pGlo Bacterial Transformation Observations and Results

Before collecting data and analyzing your results, answer the following questions.

1. Which plate do you expect to find bacteria most like the original E.coli colonies you observed? Explain why you predict this outcome.

2. On which plate or plates would you expect to find genetically transformed bacteria? Explain your predictions.

3. Which plates should be compared to determine if any genetic transformation has occurred?

4. Which plate or plates would be considered control plates in this lab? Why are they called control plates?

5. At the time you spread your transformed bacterial cells on the LB/amp/ara plate, how many individual cells were on the plate? Were the cells visible?

If the number of colonies you end up with is the same as the number of cells originally on the plate, how many cells are there in each colony after 24 hours of growth? The following equation shows the number of cells per colony.

Colonies are grown for 24 hours and there are 60 minutes/hour. E.coli bacteria double every 40 minutes.

This means: Colonies = (24 hrs) X (60 min/hr) X (1 doubling/40 min) = 36 doublings

Number of cells per colony (starting with one cell) = $2^{36} = 6.8 \times 10^{10}$

There are almost 70 billion cells per colony!!!

Review your predictions from the Pre-Lab worksheet for bacterial growth. What are your actual results? Under each plate below, list your observations. (If you did not end up with any glowing bacteria, indicate this in your observations, by placing an "X" over the plates below. Fill in the observations by using another group's results.)

Pattern of growth: (no growth, single colonies, or lawn)	_____	_____	_____	_____
Number of colonies:	_____	_____	_____	_____
Average size of colonies (mm):	_____	_____	_____	_____
Color of colonies under *normal lighting*:	_____	_____	_____	_____
Color of colonies under *UV light*:	_____	_____	_____	_____
Additional Notes:	_____	_____	_____	_____
	_____	_____	_____	_____
	_____	_____	_____	_____

6. Which traits that you initially observed for E.coli **did not** change?

7. Which traits that you initially observed for E.coli **did** change after performing the procedure?

pGlo Bacterial Transformation Analysis and Review

Observe your four plates and answer the following questions.

1. Is there E.coli growing on the LB plates that do not contain ampicillin (amp) or arabinose (ara)? How are they able to grow on these plates?

2. Explain if you can tell if these bacteria are resistant to ampicillin just by looking at them?

3. How would you change the bacteria's environment to test if the bacteria are resistant to ampicillin?

4. What two factors must be present in the bacteria's environment for you to see the green color? (Hint: one factor is in the plate and the other factor is in how you look at the bacteria).

5. What are the two environmental factors you listed above doing to cause the genetically transformed bacteria to turn green?

6. What are the advantages for an organism that can turn on or turn off particular genes in response to certain conditions?

How successful was your Bacterial Transformation?

1. What evidence is there to show that your transformation worked?

2. From the results that you obtained, how can you prove that the changes that occurred were due to the procedure that you performed?

3. Think about what you saw when you viewed the plasmid DNA under UV light before adding it to your E.coli. What were your observations?

4. After performing the bacterial transformation, what sources of GFP can be eliminated?

5. After deciding what sources can be eliminated, what can be concluded about the actual source of the green fluorescent protein?

Calculating Transformation Efficiency

You can determine if you were successful at transforming E.coli bacteria just by observing your plates. Scientists often need more precise data about how successful a procedure was, so they use calculations like *transformation efficiency*. Transformation efficiency is a *quantitative value*, or one that can be measured, that describes how effective you were at getting a plasmid into bacteria. The number you end up with represents the number of transformed colonies produced per microgram (µg) of DNA added.

This particular procedure has been determined to have a transformation efficiency between 8.0×10^2 and 7.0×10^3, which equals between 128 and 1,120 transformed colonies.

Find the number of transformed colonies from your Observation and Results worksheet.

1. Write that number here. _____
2. Was your transformation efficiency within the range listed above?
3. If you want to calculate the numerical value, complete the following equation:

Transformation Efficiency = $\frac{\text{Number of transformed colonies}}{\text{Amount of pGlo DNA spread on plates (µg)}}$

= $\frac{\text{Your number of transformed colonies}}{.16\ \mu g}$

= _____ tranformants/µg

Some classes will 100% transformation results, which means all groups will have green, glowing bacteria. Most classes will have some groups that do not end up with glowing bacteria. There are many factors that can influence the results. Look over the list below and discuss with your group if you think any of these factors influenced your results and circle them. Write a short reason why you selected these factors.

A. Refrigeration of cultured E.coli plates

B. Size of colony originally suspended in the CaCl2

C. Amount of plasmid added

D. Time on ice allowing plasmid to come in contact with cells

E. Heat Shock Treatment

F. Diligence in going directly from ice to 42oC back to ice

G. Amount of LB broth added

H. Recovery time in LB broth

I. Amount of cells spread on plates

J. Spreading transformants and controls

Discuss your results with the rest of the class. You can see how important proper technique and following procedure are in obtaining good results.

pGlo Bacterial Transformation, The Short Version

Genetic transformation is taking genes from one organism and putting them in another. A gene is a piece of DNA that gives the instructions for making a protein. This protein gives an organism a certain trait. A gene is inserted into an organism in order to change the organism's trait.

You will learn about the process of moving genes from one organism to another with the aid of a **plasmid**, a small circular piece of DNA. Bacteria have one large chromosome and one or more plasmids. Plasmids usually contain genes for traits that may help the bacteria survive. In nature, bacteria can transfer plasmids back and forth allowing them to share these beneficial genes. This process allows bacteria to adapt to new environments.

Bio-Rad's **pGLO plasmid** has three special genes: one for **green fluorescent protein**, a gene for **antibiotic resistance**, and a **gene regulation system**. This system can be used to control when the bacteria produce fluorescent protein. The gene for GFP can be switched on in transformed cells by adding the sugar **arabinose** to the cells' food source. Transformed cells will appear white on plates not containing arabinose, and fluorescent green when arabinose is included in the nutrient agar medium. Also, we can test that cells have been transformed with pGLO DNA by growing them on plates containing the **antibiotic, ampicillin**.

To move the pGLO plasmid DNA through the cell membrane you will:

1. Use a **transformation solution** of $CaCl_2$ (calcium chloride) to make cells **competent**
2. Carry out a procedure referred to as **heat shock** so bacteria can take in the plasmid

For transformed cells to grow in the presence of ampicillin you must:

Provide them with nutrients and a short **incubation period** to begin expressing their newly acquired genes

pGlo Bacterial Transformation: Observation of Results

Review your predictions from the Pre-Lab worksheet for bacterial growth. What are your actual results? Under each plate below, list your observations. (If you did not end up with any glowing bacteria, indicate this in your observations, by placing an "X" over the plates below. Fill in the observations by using another group's results.)

Pattern of growth: (no growth, single colonies, or lawn)	_____	_____	_____	_____
Average size of colonies (mm):	_____	_____	_____	_____
Color of colonies under *normal lighting*:	_____	_____	_____	_____
Color of colonies under *UV light*:	_____	_____	_____	_____
Number of colonies:	_____	_____	_____	_____
Additional Notes:	_____	_____	_____	_____
	_____	_____	_____	_____
	_____	_____	_____	_____

Which traits that you initially observed for E.coli **did not** change?

Which traits that you initially observed for E.coli **did** change after performing the procedure?

Modified Student Materials

pGlo Bacterial Transformation Power Point Follow-up Questions (Pre-lab evaluation)

1. What is the purpose of this lab? What are we trying to do?

2. What is the original source of green fluorescence protein?
    a. Ultra-violet Light
    b. Humans
    c. Jellyfish
    d. Fireflies

3. Complete the following sentences using the terms listed:

Bacterial Transformation **Calcium Chloride ($CaCl_2$)**
LB Nutrient Broth **Genetic Selection**
Plasmid **Heat Shock Treatment**
Genetic Regulation

a. A _____ is a small, circular piece of DNA that can multiply on its own.

b. _____ is a sterile solution that makes cells able to take in a plasmid (competent).

b. Bacteria can be grown under different conditions to determine what traits they have by using _____.

c. _____ is the process of moving genes from one organism to another.

d. _____ is a mixture that allows cells to grow and produce gene products (proteins).

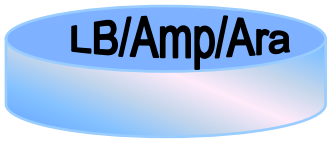
e. Competent cells take in plasmids during _____ in a transformation procedure.

f. Organisms can control the kinds and amounts of proteins they produce because of the process of _____.

4. Normal, non-transformed, E.coli bacteria are **white**, **use arabinose** (Ara) as a food source, and **cannot live when exposed to antibiotics** like ampicillin (amp). What will you see if E.coli is grown under the following conditions? Will there be **growth** or **no growth**? *Circle your answer below for each plate.*

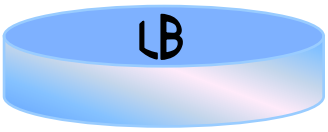
LB Nutrient Agar + Ampicillin

growth **no growth**



LB Nutrient Agar + Ampicillin + Arabinose

growth **no growth**



LB Nutrient Agar

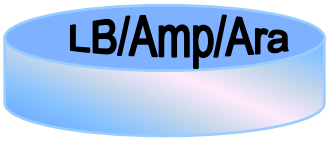
growth **no growth**

5. What do you expect to see if E.coli bacteria is transformed so that it **produces a green fluorescence protein** in the **presence of arabinose** and the E.coli is now **resistant to ampicillin**? Will there be **growth** or **no growth**? Will there be any **glowing** bacteria? *Circle your answer below for each plate.*

LB Nutrient Agar + Ampicillin

growth **no growth**

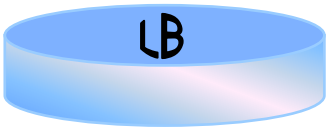
glowing **not glowing**



LB Nutrient Agar + Ampicillin + Arabinose

growth **no growth**

glowing **not glowing**



LB Nutrient Agar

growth **no growth**

glowing **not glowing**

pGlo Bacterial Transformation Observations and Results

Before collecting data and analyzing your results, answer the following questions.

1. Which plate do you expect to find bacteria most like the original E.coli colonies you observed?

   +pGlo, LB/amp Explain your prediction:

   +pGlo, LB/amp/ara

   –pGlo, LB/amp

   –pGlo, LB

2. On which plate or plates would you expect to find genetically transformed bacteria?

   +pGlo, LB/amp Explain your prediction:

   +pGlo, LB/amp/ara

   –pGlo, LB/amp

   –pGlo, LB

3. Which plates should be compared to determine if any genetic transformation has occurred?

   +pGlo, LB/amp Why?

   +pGlo, LB/amp/ara

   –pGlo, LB/amp

   –pGlo, LB

4. Which plate or plates would be considered control plates in this lab?

   +pGlo, LB/amp Why?

   +pGlo, LB/amp/ara

   –pGlo, LB/amp

   –pGlo, LB

5. At the time you spread your transformed bacterial cells on the LB/amp/ara plate, how many individual cells were on the plate? Were the cells visible?

If the number of colonies you end up with is the same as the number of cells originally on the plate, how many cells are there in each colony after 24 hours of growth? The following equation shows the number of cells per colony.

Colonies are grown for 24 hours and there are 60 minutes/hour. E.coli bacteria double every 40 minutes.

This means: Colonies = (24 hrs) X (60 min/hr) X (1 doubling/40 min) = 36 doublings

Number of cells per colony (starting with one cell) = $2^{36} = 6.8 \times 10^{10}$

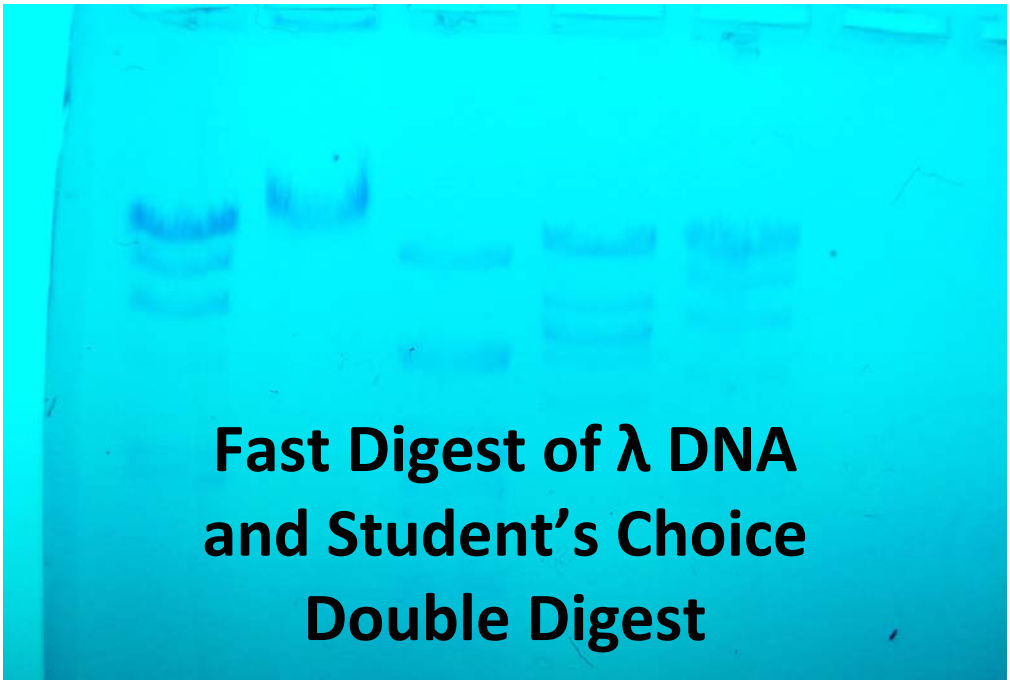
There are almost 70 billion cells per colony!!!

Review your predictions from the Pre-Lab worksheet for bacterial growth. What are your actual results? Under each plate below, list your observations. (If you did not end up with any glowing bacteria, indicate this in your observations, by placing an "X" over the plates below. Fill in the observations by using another group's results.)

Pattern of growth: (no growth, single colonies, or lawn)	_____	_____	_____	_____
Average size of colonies (mm):	_____	_____	_____	_____
Color of colonies under *normal lighting*:	_____	_____	_____	_____
Color of colonies under *UV light*:	_____	_____	_____	_____
Number of colonies:	_____	_____	_____	_____
Additional Notes:	_____	_____	_____	_____
	_____	_____	_____	_____
	_____	_____	_____	_____

6. Which traits that you initially observed for E.coli **did not** change?

7. Which traits that you initially observed for E.coli **did** change after performing the procedure?



Procedures and Classroom Materials
Adapted from Biotechnology Explorer™ Program of
Bio-Rad Laboratories

Instructor Materials

Restriction Enzyme Digestion and Analysis of Lambda DNA Teacher Preparation Guide

TAE Buffer, Agarose Gel, and DNA Stain Preparation: 1 to 7 days in advance of Day 2 (Gel Electrophoresis)

*****A reagent chart for agarose gels and buffers is located at the beginning of this notebook for additional preparation support.**

Preparation Steps:

A. Prepare buffer. (Step 1)
B. Remove appropriate amount of buffer for gels. (**Consult the reagent chart**)
C. Add appropriate amount of Fast Blast to remaining buffer. (Step 1)
D. Add appropriate amount of agar powder to reserved buffer and melt agar. (Step 2)
E. Add Fast Blast to melted agar and pour gels. (Step 2 and 3)
F. Prepare Fast Blast. (Step 4)

1. Prepare electrophoresis buffer ***NEW Protocol for IN-GEL staining!!!

The buffer is provided as a 50x concentrated solution. A 1x TAE buffer solution is needed to make the agarose gels and for the electrophoresis chambers. Three liters of 1x TAE buffer will run 8 electrophoresis chambers and pour 8 agarose gels.

- ✓ Add **60ml of concentrate** to 2.94L of **distilled water**.
- ✓ This solution can be stored at room temperature for over a week until needed. Ensure the container is covered properly to avoid contamination.
- ✓ For **IN-GEL STAINING,** add 200uL 500x Fast Blast stain per 300mL of 1x TAE buffer **AFTER** preparing agarose gels. You will add approximately 1.787ml (1787uL) 500x Fast Blast stain to the 2680mL 1x TAE buffer left after making agarose gels.

Preparing buffer in a one gallon jug of distilled water:

- ✓ **One gallon** of distilled water from the store is equal to **3790ml**. To make 3L of 1x TAE buffer in the jug, remove 850mL water from the jug (790ml + 60ml), then add 60ml of 50x TAE buffer to the jug and mix well. Remove the amount needed to make agarose gels and add the appropriate amount of 500x Fast Blast stain for in-gel staining.

2. Prepare agarose gels ***NEW Protocol for IN-GEL staining!!!

- ✓ To make the recommended 1% agarose solution for 8 gels (320ml of molten agarose), add 3.2g of **agarose powder** to 320ml of **1x TAE buffer** in a 1L flask or bottle.
- ✓ Swirl briefly to mix
- ✓ Heat to boiling on a hot plate or in a microwave until the agarose is completely dissolved
- ✓ Monitor the boiling carefully to ensure the solution does not boil over and use protective gloves to handle the container
- ✓ Allow the molten gel to cool to at least 60°C before casting gels (you should be able to comfortably hold the container in your hand)
- ✓ For **IN-GEL STAINING,** add 33uL of 500x Fast Blast stain per 50mL of molten agarose (211uL of Fast Blast for 320mL molten agarose). Mix by swirling gently.

3. Cast Agarose Gels

- ✓ Set up the appropriate number of **gel electrophoresis stations with gels trays, casting gates, and 8-well combs**
- ✓ Pour **molten agarose** (cooled to at least 60°C) into the casting area so the agarose reaches at least half-way up the comb. Thinner gels will have a quicker running time, but students may be more likely to puncture the bottom of the wells. Remove any bubbles by pricking them with a pipet tip.
- ✓ Allow the gel to solidify at room temperature for 10 to 20 minutes
- ✓ Carefully remove the comb and casting gates
- ✓ Gels can be stored in a refrigerator (wrapped in platic wrap in an air-tight container) for up to week prior to use
- ✓ Prepare as many gels as needed using this method

4. Prepare Fast Blast DNA stain **NEW Protocol for IN-GEL staining!!!

Fast Blast DNA stain is provided in a 500x concentrate. A 1x concentration is necessary for overnight staining. 120ml of 1x concentrate is needed per staining tray (two gels per tray). **If using IN-GEL staining, it may still be necessary to stain the gels overnight in a 1x stain for best resolution of DNA.**

- ✓ Add 1ml of **500x Fast Blast stain** to 499mL of **distilled water**. Cover and store at room temperature until ready for use.

Prepare Tubes and Aliquot Reagents: Up to 2 days in advance of lab

1. **Prepare restriction enzymes**
   - ✓ Remove restriction enzyme stock tubes from the freezer (H, P, E, and B**)** and place ***on ice*** for 5 minutes***.***
   - ✓ Centrifuge tubes for 10 seconds, then return to ice.
2. **Aliquot restriction enzymes (student stock tubes)**
   - ✓ Label **4 sets of 8, clear micro test tubes** H, P, E, and B **in red marker** and separate the sets into separate foam racks for aliquoting***.***
   - ✓ Use a **p20 micropipet** to aliquot 5 µl of each enzyme (1 ug/ul stock concentration) into the appropriate eight, clear micro test tubes. As you aliquot, each set of tubes should remain on ice and be placed in the freezer immediately after aliquoting.

Notes:
- ➢ **Restriction enzymes are temperature sensitive and must be kept on ice at all times!!!**
- ➢ Store enzymes, and all other regents, in the freezer until they are ready to be used

3. **Thaw** Restriction Buffer, Lambda DNA, Loading Dye, and Marker at room temperature for 5 to 10 minutes, **then spin** tubes for 10 seconds and place on ice while aliquoting.
4. **Aliquot restriction buffer**
   - ✓ Label **8, micro test tubes** "RB".
   - ✓ Aliquot 30 µl of **restriction buffer** (2.5X stock concentration) into the **RB tubes** and place tubes in freezer
5. **Aliquot Lambda DNA**
   - ✓ Label **8, micro test tubes** "DNA".
   - ✓ Aliquot 25 µl of **lambda DNA** (.5 ug/ul stock concentration) into the **DNA tubes** and place tubes in freezer.
6. **Prepare student digest tubes (student reaction tubes)**
   - ✓ Label **6 sets of 8, colored test tubes** with L, P, E, H, B, and DD **in black marker** and organize into foam racks (8 sets containing one of each tube).

7. Aliquot sample loading dye.
- ✓ Label **8 micro test** tubes "LD"
- ✓ Aliquot 15 µl of **sample loading dye** (6X stock concentration) into each tube and place in freezer.

8. Aliquot Gene Ruler.
- ✓ Label 8 micro test tubes "M" and centrifuge **stock tube**.
- ✓ Aliquot 12 µl of the DNA marker (already contains loading dye) in the test tubes labeled **"M"** and place in freezer.

Set up Student Work Stations and necessary equipment, Day of Lab

1. Each station should have the following materials
- ✓ Three **foam racks** per group, containing the following tubes:
- ✓ One micro test tube of **each enzyme mixture, H, P, E, and B**
- ✓ One micro test tube of **restriction buffer, RB**
- ✓ One micro test tube of **lambda DNA, DNA**
- ✓ One set of **student digest tubes, L, P, E, H, B, and DD**
- ✓ One micro test tube of **DNA marker, M**
- ✓ One micro test tube of **loading dye, LD**
- ✓ One box of **pipet tips**
- ✓ p20 **micropipet**
- ✓ Styrofoam **cup of ice**
- ✓ **4 mini-centrifuges,** shared between groups
- ✓ One **permanent marker**
- ✓ One **waste beaker or bin**
- ✓ Student **lab procedure**

2. Turn on water baths

- ✓ Set a **water bath** to 37°C and turn on. Leave lid off. Set a second **water bath** to 65°C and turn on. Monitor temperature for necessary adjustments. If you do not have two water baths, keep some boiling or near boiling water near the 37°C water bath and adjust the temperature up to 65°C as soon as all students are done incubating their digests. Be sure to turn off water baths and replace lids when the lab is completed.

3. Set up gel electrophoresis stations

- ✓ Set up the appropriate number of stations allowing room for students to work
- ✓ Place a **gel tray with gel** at each station
- ✓ Fill each chamber will the appropriate amount of **1x TAE buffer**
- ✓ For every two stations, provide one **staining tray** and one small flask of **1x Fast Blast stain** (at least 120ml)

Procedure

NEW! Fast Digest of Lambda DNA Procedure with Students' Choice Double Digest

1. At your group's lab station, check to make sure you have the following materials:
   - 4 clear tubes of enzyme solutions labeled in **red marker, P, E, H, and B** (5ul each)
   - one tube of lambda DNA labeled, **DNA** (25ul)
   - one tube of restriction buffer labeled, **RB** (30ul)
   - six, *empty*, color coded tubes labeled, **L, P, E, H, B, and DD** (These tubes are your REACTION tubes. You will add your enzyme solutions and DNA to these tubes.)
   - one tube of loading dye, **LD** (15ul)
   - one tube of DNA marker, **M** (10ul)

   Keep all the stock solutions on ice.

2. Spin all reagent tubes for 5 seconds in a mini-centrifuge.

3. Using a **p20 pipet** and a fresh tip for each **COLUMN**, pipet the reagents into each tube according to the table below:

	1 tip	1 tip	1 tip	1 tip	1 tip	1 tip
Tube	**DNA**	**RB, Buffer**	**P, PstI**	**E, EcoRI**	**H, HindIII**	**B, BamHI**
L	**4 ul**	**6 ul**	-	-	-	-
P	**4 ul**	**4 ul**	**2 ul**	-	-	-
E	**4 ul**	**4 ul**	-	**2 ul**	-	-
H	**4 ul**	**4 ul**	-	-	**2 ul**	-
B	**4 ul**	**4 ul**	-	-	-	**2 ul**
DD	**4 ul**	**4 ul**	*	*	*	*

p20, 4ul	p20, 2ul
0	0
4	2
0	0

*Choose 2 enzymes for a double digest and add **2 ul** of each to the DNA and Buffer

4. Mix the tubes gently by flicking the tube with your finger. *Adequate mixing is required for optimal digestion, but mixing by shaking violently or vortexing might damage the enzymes.* Spin the tubes in the mini-centrifuge for 3 seconds to collect all liquid in the bottom of the tubes.

5. Place the tubes (L, P, E, and H) in a foam rack and **label the lids** of the tubes with your group number.

6. Place the tubes in a 37°C water bath and incubate for 5 minutes. You may discard the tubes labeled DNA, RB, and the four tubes labeled in red.

7. After the incubation, retrieve your group's digested DNA samples, the tube of sample loading dye, **LD**, and the tube of DNA marker**, M**. Spin all tubes in a mini-centrifuge for 5 seconds.

8. Set a p20 to 2ul.Using **a new tip for each tube**, add **2 ul** of **sample loading dye, LD,** into each tube (L, P, E, H, B, and DD). Mix the contents by flicking the tube with your finger. Collect the sample at the bottom of the tube by tapping gently on the table.

9. Spin the tubes labeled L, P, E, H, B, and DD for 3 seconds in a mini-centrifuge and place in a foam rack.

10. Heat your digested DNA samples in a 65°C water bath for 5 minutes. **Do not heat** the DNA Marker tube labeled "M".

11. While the samples are heating, move your materials to a gel electrophoresis station. Make sure there is a gel in the tank with the wells near the black electrode and that the tank is filled with buffer.

12. After the samples are done heating, use the **p20 pipet** and **fresh tips for each sample** to load each sample into separate wells in the agarose gel in the following order (**left to right**):

Lane	1	2	3	4	5	6	7
Sample	M	L	P	E	H	B	DD
Volume	10ul	12ul	12ul	12ul	12ul	12ul	12ul

p20, 8ul	p20,12ul
1	1
0	2
0	0

13. Place the lid on the electrophoresis chamber. Connect the leads to the power supply (red to red, black to black). Use a small piece of tape to label the corner of your chamber with your group's number (or initials).

14. Wait until all of the groups at your station are ready, then run the gel at 100V for 30 minutes.

15. While the gel is running, obtain a plastic staining tray to share with the lab group next to you. Label the tray with your period and group number (or initials).

16. After 30 minutes, turn off the power and remove the top of the chamber.

17. Carefully remove the tray holding the gel from the gel box. **The gel is very slippery!!!** Slide the gel into the **plastic staining tray.**

18. Add 120 ml of **1x Fast Blast DNA stain** to the staining tray. The stain should just cover the gels.

19. Let the gels stain overnight.

20. You may discard your test tubes. *Wash your hands thoroughly before leaving class.*

Student Materials

Introduction to Restriction Enzyme Digestion and Analysis of Lambda DNA

Restriction Analysis — Links to Biotechnology

The techniques introduced in this exercise form the basis of **recombinant DNA technology techniques**, **DNA fingerprinting**, and **forensic DNA analysis**.

This kit introduces students to some important principles of **genetic engineering**. Specifically, the functions of restriction enzymes and their use as molecular biology tools will be stressed. Using agarose gel electrophoresis, students will examine the digestion patterns, analyze the migration distances, and determine the sizes of unknown DNA fragments.

Restriction enzymes were a catalyst for the molecular biology revolution, and now hundreds of such enzymes are known. In this investigation, the restriction enzymes *Eco*RI, *Pst*I, and *Hin*dIII will be used to digest bacteriophage lambda DNA. Gel electrophoresis will be employed to separate the resulting DNA fragments, and a nontoxic blue dye (Fast Blast™ DNA stain) will be used to stain the DNA fragments for visualization.

Student Objectives

- Understand the use of restriction enzymes as biotechnology tools
- Become familiar with principles and techniques of agarose gel electrophoresis
- Estimate DNA fragments sizes from agarose gel data

Background

DNA splicing, the cutting and linking of DNA molecules, is one of the basic tools of modern biotechnology. The basic concept behind DNA splicing is to remove a functional DNA fragment — let's say a gene — from one organism and to combine it with the DNA of another organism in order to study how the gene works. The desired result of gene splicing is for the recipient organism to carry out the genetic instructions provided by its newly acquired gene. For example, certain plants can be given the genes for resistance to pests or disease, and in a few cases to date, functional genes have been given to people with nonfunctional genes, such as those who have a genetic disease like cystic fibrosis.

Restriction Enzymes

The ability to **cut and paste**, **or cleave and ligate**, a functional piece of DNA predictably and precisely is what enables biotechnologists to recombine DNA molecules. This is termed recombinant DNA technology. The first step in DNA splicing is to locate a specific gene of interest on a chromosome. A restriction enzyme is then used to cut out the targeted gene from the rest of the chromosome. This same enzyme is also used to cut the DNA of the recipient into which the fragment will be inserted.

Restriction enzymes are proteins that cut DNA at specific sites. Restriction enzymes, also known as **restriction endonucleases**, recognize specific sequences of DNA base pairs and cut, or chemically separate, DNA at that specific arrangement of base pairs. They were first identified in and isolated from bacteria that use them as a natural defense mechanism to cut up the invading DNA of bacteriophages — viruses that infect bacteria. Any foreign DNA encountering a restriction enzyme will be digested, or cut into many fragments, and rendered ineffective. These enzymes in bacteria make up the first biological immune system. There are thousands of restriction enzymes, and each is named after the bacterium from which it is isolated. For example:

***Eco*RI** = The first restriction enzyme isolated from *Escherichia coli* bacteria

***Hin*dIII** = The third restriction enzyme isolated from *Haemophilus influenzae* bacteria

***Pst*I** = The first restriction enzyme isolated from *Providencia stuarti* bacteria

Each restriction enzyme recognizes a specific nucleotide sequence in the DNA, called a **restriction site**, and cuts the DNA molecule at only that specific sequence. Many restriction enzymes leave a short length of unpaired bases, called a **"sticky" end**, at the DNA site where they cut, whereas other restriction enzymes make a cut across both strands creating double-stranded DNA fragments with **"blunt" ends**. In general, restriction sites are **palindromic**, meaning the sequence of bases reads the same forwards as it does backwards on the opposite DNA strand. Here are some examples of restriction enzymes and the sequences they recognize:

Lambda Phage DNA

Lambda DNA is the genomic DNA of a bacterial virus, or **bacteriophage** (phage), which attacks bacteria by inserting its nucleic acid into the host bacterial cell. Lambda is a phage that replicates rapidly inside host cells until the cells burst and release more phages to carry out the same infection process in other bacterial host cells. Bacteriophage lambda is harmless to man and other eukaryotic organisms, and therefore makes an excellent source of DNA for experimental study.

In this investigation, students observe the effects of three restriction enzymes on lambda DNA. Since the lambda genome is approximately 48,000 base pairs, each restriction enzyme will cut the DNA several times and generate restriction fragments of different sizes. In this activity, three separate samples of lambda DNA will be cut using three different restriction enzymes, and one sample will remain undigested. Each sample produces DNA fragments whose sizes can be estimated when run on an agarose gel using electrophoresis.

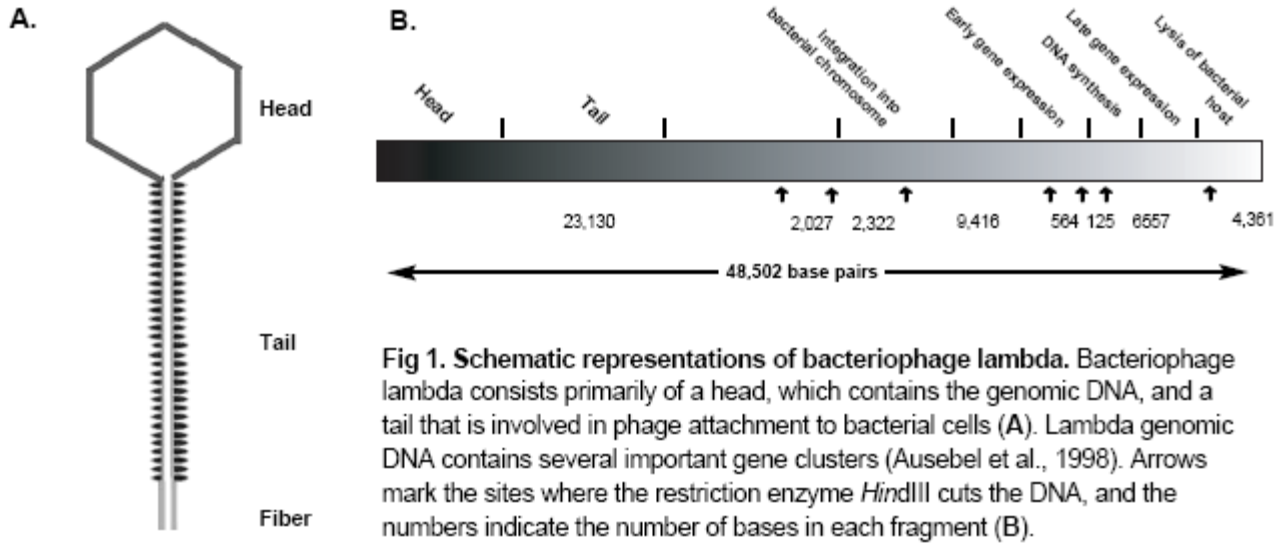


Fig 1. Schematic representations of bacteriophage lambda. Bacteriophage lambda consists primarily of a head, which contains the genomic DNA, and a tail that is involved in phage attachment to bacterial cells (**A**). Lambda genomic DNA contains several important gene clusters (Ausebel et al., 1998). Arrows mark the sites where the restriction enzyme *Hin*dIII cuts the DNA, and the numbers indicate the number of bases in each fragment (B).

Electrophoretic Analysis of Restriction Fragments

A restriction enzyme acts like molecular scissors, making cuts at the specific sequence of base pairs that it recognizes. The three-dimensional structure or shape of a restriction enzyme allows it to fit perfectly in the groove formed by the two strands of a DNA molecule. When attached to the DNA, the enzyme slides along the double helix until it recognizes a specific sequence of base pairs which signals the enzyme to stop sliding. The enzyme then chemically separates, or cuts, the DNA molecule at that site — called a restriction site.

If a specific restriction site occurs in more than one location on a DNA molecule, a restriction enzyme will make a cut at each of those sites, resulting in multiple fragments of DNA. Therefore, if a given piece of linear DNA is cut with a restriction enzyme whose specific recognition sequence is found at five different locations on the DNA molecule, the result will be six fragments of different lengths. The length of each fragment will depend upon the location of restriction sites on the DNA molecule.

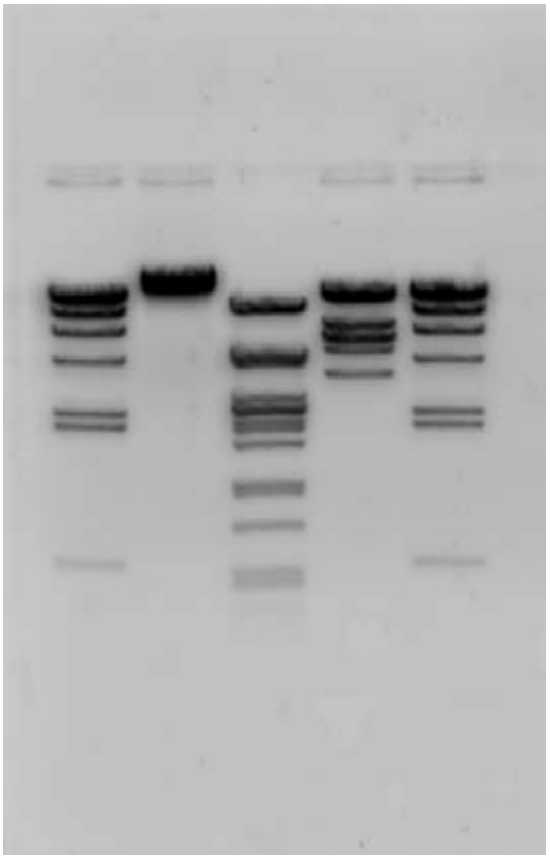
A DNA fragment that has been cut with restriction enzymes can be separated using a process known as **agarose gel electrophoresis.** The term electrophoresis means to *carry with electricity.* Agarose gel electrophoresis separates DNA fragments by size. DNA fragments are loaded into an agarose gel slab, which is placed into a chamber filled with a conductive buffer solution. A direct current is passed between wire electrodes at each end of the chamber. Since DNA fragments are negatively charged, they will be drawn toward the positive pole (anode) when placed in an electric field. The matrix of the agarose gel acts as a molecular sieve through which smaller DNA fragments can move more easily than larger ones. Therefore, the rate at which a DNA fragment migrates through the gel is inversely proportional to its size in base pairs. Over a period of time, smaller DNA fragments will travel farther than larger ones. Fragments of the same size stay together and migrate in single bands of DNA. These bands will be seen in the gel after the DNA is stained.

An analogous situation is one where all the desks and chairs in the classroom have been randomly pushed together. An individual student can wind his/her way through the maze quickly and with little difficulty, whereas a string of four students would require more time and have difficulty working their way through the maze.

Making DNA Visible

DNA is colorless so DNA fragments in the gel cannot be seen during electrophoresis. A loading dye containing two blue dyes is added to the DNA solution. The loading dye does not stain the DNA itself but makes it easier to load the gels and monitor the progress of the DNA electrophoresis. The dye fronts migrate toward the positive end of the gel, just like the DNA fragments. The "faster" dye co-migrates with DNA fragments of approximately 500 bp, while the "slower" dye co-migrates with DNA fragments approximately 5 kb in size. Staining the DNA pinpoints its location on the gel. When the gel is immersed in Fast Blast DNA stain, the stain molecules attach to the DNA trapped in the agarose gel. When the bands are visible, your students can compare the DNA restriction patterns of the different samples of DNA.

The band patterns that will be obtained by your students following electrophoresis of DNA samples that have been digested using three different restriction digestion enzymes are shown in Figure 2. By convention, the lanes are numbered from the top left. Notice that each restriction enzyme produces a unique banding pattern in each lane. The relative size of fragments in each band can be determined by measuring how far each band has traveled from its origin. Since the fragment sizes are known for the *Hin*dIII lambda digest, this sample will function as a DNA standard or marker.



Electrophoresis of lambda DNA digested using three different restriction enzymes. Lane 1, DNA markers (*Hin*dIII lambda digest); lane 2, uncut lambda DNA; lane 3, lambda DNA digested with *Pst*I; lane 4, lambda DNA digested with *Eco*RI; lane 5, lambda DNA digested with *Hin*dIII.

Restriction Enzyme Digestion and Analysis of Lambda DNA Power Point Follow-up Questions (Pre-lab evaluation)

1. What is the purpose of this lab? What are we trying to do?

2. What are restriction enzymes? What do they do?

3. What is a palindromic sequence?

4. Using the symbols for the four DNA bases (A, C, T, G), write an example of a palindromic sequence.

5. Why are restriction enzyme digestions of DNA incubated at 37°C?

6. Why do we use lambda phage DNA for this laboratory?

7. How does agarose electrophoresis separate DNA fragments?

8. Why do smaller fragments move faster than larger fragments?

9. What is the purpose of the DNA marker in Restriction Enzyme Digestion?

Fast Digest of Lambda DNA and Student's Choice Double Digest: Analysis of Results

Simulating the effects of four restriction enzymes and one double digest on Lambda DNA.

You will use a computer program from the National Center for Biotechnology Education which allows you to download a document showing the entire Lambda genome. This enables you to simulate theoretically the same restrictions which you are carrying out practically with the Lambda protocol.

Download the Lambda Sequence:

1. At your computer, open you web browser and type in the following address:

   http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/simulation.html

2. Below the bulleted items at the right of this web page, there are 3 items that can be downloaded named, **UNCUT, MASTER, and SIMULATE**. Click on the **UNCUT** document to download it to you computer desktop. This is the Lambda sequence you will "cut" using different restriction enzymes.
3. Hide your web browser page (do not close it in case you need to retrieve information from it again) and open the **UNCUT** file you just downloaded to the desktop.
4. Browse through the genome. Each base listed is counted as one character, so under the **Tools** menu select the **Word Count…** option. Determine the number of base pairs in the Lambda genome from the **Characters** column. Use this same method to determine the size of fragments when "cutting" the sequence using various restriction enzymes.

   The Lambda base sequence may be cut at various points with restriction enzymes. As we have seen, each enzyme cuts within a particular base sequence. For example, EcoRI cuts between the A and the G in the six base sequence GAATTC and nowhere else. This usually shown as **G/AATTC or G^AATTC**. Other restriction sites are BamHI - G/GATCC and HindIII - A/AGCTT.

   You can use the NCBE document of the Lambda base sequence to find the restriction sites of these three enzymes - and indeed any other enzymes for which you know the restriction sites.

   You can then 'cut the genome' and count the number of fragments and the lengths of each of these fragments. These can later be compared with the results of your gel electrophoresis runs from the Lambda protocol.

Restriction Enzyme Simulation:

5. Place the cursor at the beginning of the base sequence

6. Open the **EDIT** window and select **REPLACE**
7. In answer to the question 'Find what ?' type in **gaattc**
8. Move the cursor to **'Replace with ?'** and type in **G^pAATTC**. This cuts the base sequence at all the **EcoRI** restriction sites and puts a **paragraph break (^p)** at each point where the sequence has been cut. It also converts the recognition sequence of six bases to upper case letters so that you can easily see them
9. Select **'Replace all'**
10. Choose **'OK'** and then choose **'close'**. Your Lambda DNA sequence has now been "cut" by the restriction enzyme EcoRI.
11. Highlight the first 'paragraph' of the genome **(in reality the first fragment resulting from restriction)** by double clicking anywhere in the margin alongside the first few lines.

12. Open the **Tools** window and select **Word Count…**. Note the number of characters **(the number of bases)** in this fragment and enter this number into the EcoRI Fragment 1 row below:

EcoRI: G^pAATTC	
Fragment	**Size (base pairs)**
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

PstI: CTGCA^pG	
Fragment	**Size (base pairs)**
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

HindIII: A^pAGCTT	
Fragment	**Size (base pairs)**
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

BamHI: G^pGATCC	
Fragment	**Size (base pairs)**
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

13. Now select **'cancel'**

14. Move the cursor to the start of the next paragraph, highlight as before, and repeat - noting the number of characters **(bases)** in the appropriate row on the EcoRI table.

15. Repeat this process until you reach the end of the genome.

! ! ! QUESTION ! ! !

Is the order you recorded the fragment sizes the same order you will obtain from your gel electrophoresis lab? Why or why not?

16. Now, arrange the fragments resulting from the restriction by EcoRI in order of **size**, starting with the **largest**. Enter these in the table below:

EcoRI: G^AATTC	
Fragment	**Size (base pairs)**
largest	
smallest	

PstI: CTGCA^G	
Fragment	**Size (base pairs)**
largest	
smallest	

HindIII: A^AGCTT	
Fragment	**Size (base pairs)**
largest	
smallest	

BamHI: G^pGATCC	
Fragment	**Size (base pairs)**
Largest	
smallest	

17. Open the **EDIT** window and select **UNDO REPLACE ALL**

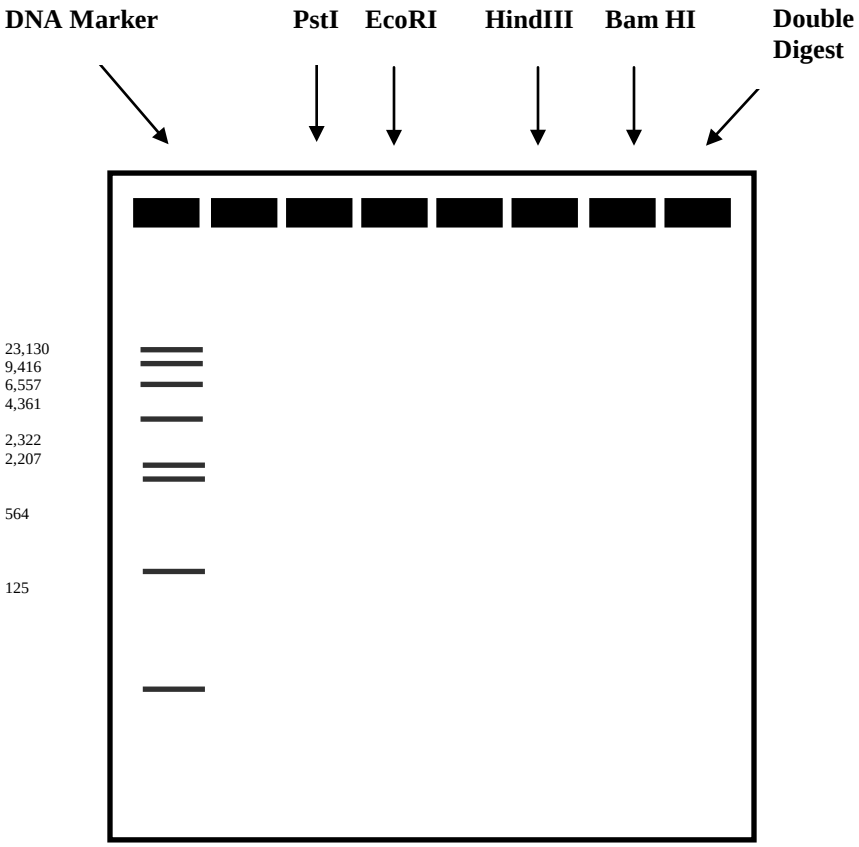
18. Repeat the whole process for **PstI, HindIII**,and **BamHI** in each case entering the fragment sizes in the appropriate tables. *The restriction site and where the enzyme cuts the DNA is listed on the table for each enzyme.*

19. Choose two restriction enzymes from above to complete a double digest (you will actually do this double digest in lab). Determine the number of fragments you would see if the lambda DNA was digest with both restriction enzymes *simultaneously*. Hint: once you use the find/replace mechanism with one enzyme, don't undo the changes before "digesting" with a second enzyme. Now write down some of the fragment sizes you will see and what order they will appear in on your gel.

Enzymes:	
Fragment	**Size (base pairs)**
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

Enzymes:	
Fragment	**Size (base pairs)**
Largest	
smallest	

20. On the gel representation below, you see the banding pattern and fragment sizes of a DNA Marker. Based on the data you collected above, draw what you would expect your gel to look like.



You can “cut” the lambda genome with additional enzymes using a comprehensive list of restriction enzymes. Use the following website to select various restriction enzymes and their corresponding restriction site to explore the effects of different enzymes.

http://internalmed.wustl.edu/divisions/enzymes/Re2list.html

For example, the restriction enzyme **Afa I** recognizes the sequence **GT^AC**. This restriction enzyme cuts the Lambda genome into ***114 fragments***! The restriction enzyme **Pme I** recognizes the sequence, **GTTT^AAAC,** and only cuts the Lambda genome into 3 fragments.

List a few of the various enzymes and number of fragments for each you explored:

Modified Student Materials

Restriction Enzyme Digestion and Analysis of Lambda DNA Power Point Follow-up Questions (Pre-lab evaluation)

1. What is the purpose of this lab? What are we trying to do?

2. Complete the following sentences with the correct term from the list below.

Palindromic Sequence	**Agarose Electrophoresis**
Lambda Phage DNA	**DNA Marker**
Restriction Enzymes	

   a. _____ are proteins that cut DNA at specific sites.

   b. A sequence of double stranded DNA is a _____ if it reads the same on the first strand from left to right as is does on the second strand from right to left.

   c. DNA fragments are separated by size in a porous material using electrical current in the process of _____.

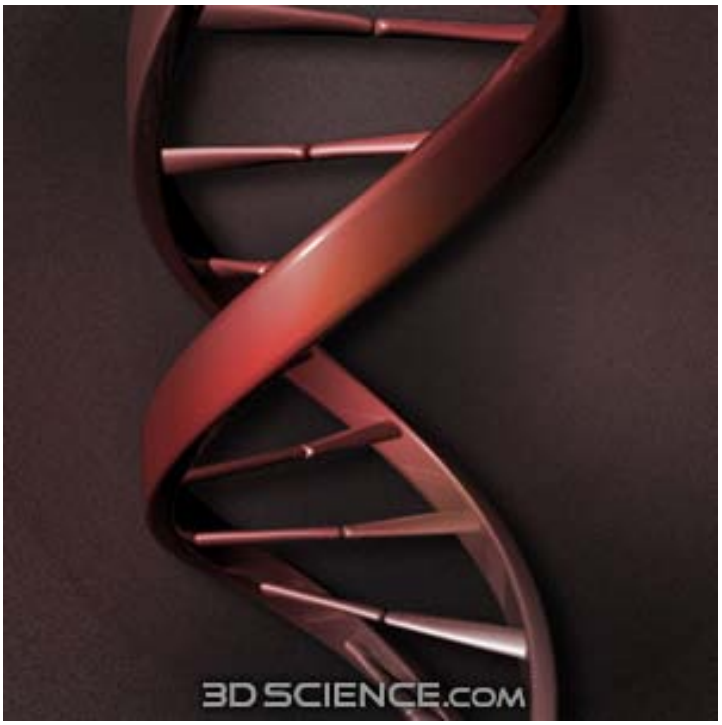
   d. A _____ a mixture of DNA fragments of known lengths.

   e. The genomic DNA of a bacterial virus, _____, is harmless to man and replicates rapidly inside its host.

3. Why are restriction enzyme digestions of DNA incubated at 37°C?

4. Why do we need to use a DNA marker during a restriction enzyme digestion?

5. Why do smaller DNA fragments move faster than larger fragments through agarose gel?



Polymerase Chain Reaction: Alu Insert on locus PV92 Chromosome 16

Procedures and Classroom Materials
Adapted from Biotechnology Explorer™ Program of
Bio-Rad Laboratories

Instructor Materials

PV92 PCR Teacher Preparation Guide

SB Buffer, Agarose Gel, and DNA Stain Preparation: 1 to 7 days in advance of Day 3 (Gel Electrophoresis)

*****A reagent chart for agarose gels and buffers is located at the beginning of this notebook.**

Preparation Steps:

A. Prepare buffer. (Step 1)
B. Remove appropriate amount of buffer for gels. (**Consult the reagent chart**)
C. Add appropriate amount of Fast Blast to remaining buffer. (Step 1)
D. Add appropriate amount of agar powder to reserved buffer and melt agar. (Step 2)
E. Add Fast Blast to melted agar and pour gels. (Step 2 and 3)
F. Prepare Fast Blast. (Step 4)

1. **Prepare electrophoresis buffer ***NEW Protocol using Sodium Borate buffer and IN-GEL staining!!!**
   The buffer is provided as a 20x concentrated solution. A 1x SB buffer solution is needed to make the agarose gels and for the electrophoresis chambers. Three liters of 1x SB buffer will run 8 electrophoresis chambers and pour 8 agarose gels.
   - ✓ Add **150ml of concentrate** to 2.85L of **distilled water**.
   - ✓ This solution can be stored at room temperature for over a week until needed. Ensure the container is covered properly to avoid contamination.
   - ✓ For **IN-GEL STAINING,** add 200uL 500x Fast Blast stain per 300mL of 1x SB buffer **AFTER** preparing agarose gels. You will add approximately 1.8ml (1800uL) 500x Fast Blast stain to the 2650mL 1x SB buffer left after making agarose gels.

Preparing buffer in a one gallon jug of distilled water:

- ✓ **One gallon** of distilled water from the store is equal to **3790ml**. To make 3L of 1x SB buffer in the jug, remove 940mL water from the jug (790ml + 150ml), then add 150ml of 20x SB buffer to the jug and mix well. Remove the amount needed to make agarose gels and add the appropriate amount of 500x Fast Blast stain for in-gel staining.

2. **Prepare agarose gels ***NEW Protocol using Sodium Borate buffer and IN-GEL staining!!!**
   - ✓ To make the recommended 1% agarose solution for 8 gels (350ml of molten agarose), add 3.2g of **agarose powder** to 320ml of **1x SB buffer** in a 1L flask or bottle.
   - ✓ Swirl briefly to mix
   - ✓ Heat to boiling on a hot plate or in a microwave until the agarose is completely dissolved
   - ✓ Monitor the boiling carefully to ensure the solution does not boil over and use protective gloves to handle the container
   - ✓ Allow the molten gel to cool to at least 60°C before casting gels (you should be able to comfortably hold the container in your hand)
   - ✓ For **IN-GEL STAINING,** add 33uL of 500x Fast Blast stain per 50mL of molten agarose (231uL of Fast Blast for 350mL molten agarose). Mix by swirling gently.

3. **Cast Agarose Gels**
   - ✓ Set up the appropriate number of **gel electrophoresis stations with casting materials**
   - ✓ Pour **molten agarose** (cooled to at least 60°C) into the casting area so the agarose reaches at least half-way up the comb. Thinner gels will have a quicker running time, but students may be more likely to puncture the bottom of the wells. Remove any bubbles by pricking them with a pipet tip.
   - ✓ Allow the gel to solidify at room temperature for 10 to 20 minutes
   - ✓ Carefully remove the comb and casting gates
   - ✓ Gels can be stored in a refrigerator (wrapped in plastic wrap in an air-tight container) for up to week prior to use
   - ✓ Prepare as many gels as needed using this method

4. **Prepare Fast Blast DNA stain ***NEW Protocol for IN-GEL staining!!!**
   Fast Blast DNA stain is provided in a 500x concentrate. A 1x concentration is necessary for overnight staining. 120ml of 1x concentrate is needed per staining tray (two gels per tray). **If using IN-GEL staining, it may still be necessary to stain the gels overnight in a 1x stain for best resolution of DNA.**

   Add 1ml of **500x Fast Blast stain** to 499mL of **distilled water**. Cover and store at room temperature until ready for use.

Prepare Tubes and Aliquot Reagents: Up to 14 days in advance of Day 1 (DNA Extraction)

1. Aliquot InstaGene Matrix

- ✓ Label **one screw cap tube, IM, on the side, for *each* student**. Remove caps from tubes.

Thoroughly mix the **InstaGene matrix** by gently shaking or vortexing the bottle several times to re-suspend the matrix. You must be sure that the matrix is well mixed when you aliquot it. The beads settle out of solution quickly, so gently remix the bottle several times during aliquotting.

- ✓ Using a p1000 pipet, aliquot 200µl of InstaGene matrix into each tube. Distribute one tube per student.
- ✓ Keep tubes **refrigerated** until they are distributed to lab groups.

2. Set up control PCR reactions

- ✓ Label **4 sets of 3 PCR tubes** +/+, -/-, and +/-. *You will have 12 tubes total for one class.* ***One set of controls shared between two student groups***.
- ✓ Remove **controls** from the freezer and allow to thaw for several minutes on the bench top, then centrifuge for a few seconds. **Keep on ice** while pipetting.
- ✓ Using a **p20 pipet**, aliquot 20ul of each **control (+/+, -/-, and +/-)** into the appropriate tubes. Be sure to switch pipet tips between controls. Controls should remain frozen if prepared prior to the laboratory.

3. Aliquot DNA size standards

- ✓ Label **8 micro test tubes**, **S**, for standard
- ✓ Aliquot 11ul of the **EZ load molecular mass ruler** into each tube

4. Aliquot sterile dH_2O

- ✓ Aliquot 100ul of **sterile dH_2O** into 8 clear micro test tubes
- ✓ Label **the 8 test tubes**, **H_2O**

5. Aliquot orange loading dye for SB buffer

- ✓ Label **8 micro test tubes**, **LD**, for loading dye
- ✓ Aliquot 50ul of **orange loading dye** into each tube

6. Prepare saline solution

- ✓ Prepare 500ml of a 0.9% saline solution by adding 4.5g of **noniodinated table salt, or NaCl**, to 500ml of **distilled water**. Mix until the salt goes into solution. Store in the refrigerator until day of DNA Extraction. ***Use a .5L bottle of drinking water for easy mixing and pouring.

Set up Student Work Stations and necessary equipment for Day 1, DNA Extraction: Day of Day 1.

1. Aliquot 10ml of the saline into **paper cups** for each student.

2. Set up Student Work Stations: Each station should have the following materials

- ✓ One **foam rack** *per group*
- ✓ One tube of Instagene Matrix per student
- ✓ One **clear micro test tube** *per student*
- ✓ **10ml saline** *per student*
- ✓ One **plastic transfer pipet** *per student*
- ✓ One **permanent marker**
- ✓ One box of **pipet tips**
- ✓ p200 **micropipettors**
- ✓ 4 mini-centrifuges
- ✓ One **waste beaker** or bin
- ✓ Student **lab procedure**

3. Turn on water baths

- ✓ Set a **water bath** to 56°C and turn on. Leave lid off. Monitor temperature for necessary adjustments.
- ✓ Heat two large beakers ½ full of water to boiling. Monitor for safety. (Only use 1 beaker for classes under 24 students).

Set up Student Work Stations and necessary equipment for Day 2 (Amplification): Day of Day 2

1. Set up student work stations: Each station should have the following materials

- ✓ One foam rack *per group*
- ✓ One PCR tube *per student*
- ✓ One permanent marker
- ✓ One box of pipet tips
- ✓ p20 and p200 micropipettors
- ✓ One tube of ddH_2O per group
- ✓ 4 mini-centrifuges
- ✓ One waste beaker or bin
- ✓ Student lab procedure

2. Set up PCR Thermal Cycler and program, if necessary.

- ✓ Remove Thermal Cycler from packaging, set on a countertop that allows for at least 6 inches of space on all sides of the cycler and plug in. Wait a few minutes for the home screen to appear. Follow the quick guide (following the instructor pages) for choosing a program or setting a new program. *The program saved in the thermal cycler you are using for this lab is named **pv92pcr.***
- ✓ The following is the appropriate program for this procedure:

The thermal cycler should be programmed for 3 steps in cycle 2, which will repeat 45 times. The final Cycle 3 insures that the final extension reaction goes to completion and all possible PCR products are made. The PCR reaction will take approximately 2.5 hours.

Cycle	Step	Function	Temperature	Time
1	Step 1	Pre-denaturation	94°C	2 minutes
	Repeat 1 time			
2	Step 1	Denature	94°C	30 seconds
	Step 2	Anneal	60°C	30 seconds
	Step 3	Extend	72°C	1 minute
	Repeat 45 times			
3	Step 1	Final extension	72°C	10 minutes
	Repeat 1 time			

- ✓ If you will be unable to remove the samples from the Thermal Cycler and move them to a refrigerator when the program is complete, choose the program named ***pv92pcr w/free***. You will be able to stop the program the following day to interrupt the refrigeration cycle.
- ✓ For efficiency, assign PCR wells to each student and post at the thermal cycler using the form available after these instructions.

Immediately before Amplification Lab: Necessary items and equipment are in bold

1. Prepare complete master mix and aliquot student tubes

Label **8 micro test tubes** for student master mix, **M**
Label **one micro test tube of a different color** for teacher's master mix, **M***
Using a **p1000 pipet**, aliquot 1,100ul of **Master Mix** into the teacher tube
Using a **p200 pipet**, aliquot 22ul of **yellow primer mix** into the teacher tube

*****If you only have 16 students, or less, mix 550ul of Master Mix and 11ul of yellow primer mix to conserve materials, or make further adjustment for class size. Each student only needs 20ul of complete master mix, so adjust aliquots according go group size.**

Tap tube to mix the complete master mix
Using a p200 pipet, aliquot **95ul** of the **complete master mix** into the **8 student master mix tubes** and distribute one to each group, **ON ICE**

2. Add complete master mix to controls.

Remove **controls** from the refrigerator if necessary and aliquot 20ul of complete master mix into each control tube. Use a new tip for each set of controls.
***Students can complete this step during the lab. Provide those groups with the left over master mix.
Keep controls on ice until ready to load into the thermal cycler. Run controls along with the student samples.

Set up Student Work Stations and necessary equipment for Day 3 (Gel Electrophoresis): Day of Day 3

1. **Prepare control samples (Cannot complete this step until after the controls have been amplified)**
   - ✓ Remove **controls** from the thermal cycler.
   - ✓ Add 10ul of **orange loading dye** to each amplified control. Keep the tubes at the teacher work station. Distribute the controls to student groups when they are loading their gels. Each set of controls is shared between two groups of students.
   - ✓ ***Students can complete this step during the lab. Provide extra loading dye for those groups.
2. **Set up gel electrophoresis stations**
   - ✓ Set up the appropriate number of stations allowing room for students to work
   - ✓ Place a **gel tray with gel** at each station
   - ✓ Fill each chamber will the appropriate amount of **1x SB buffer**
   - ✓ For every two stations, provide one **staining tray** and one small flask of **1x Fast Blast stain** (at least 120ml)
3. **Set up student work stations: Each station should have the following materials**
   - ✓ One **foam rack** per student
   - ✓ One micro test tube of **standard, S**
   - ✓ One micro test tube of **loading dye, LD**
   - ✓ One box of **pipet tips**
   - ✓ p20 and p200 **micropipettes**
   - ✓ **4 mini-centrifuges**
   - ✓ One **permanent marker**
   - ✓ One **waste beaker or bin**
   - ✓ Student **lab procedure**

MyCycler™ Thermal Cycler Quick Guide

Starting the Instrument. Plug in the MyCycler, and a quick diagnostic routine will be performed. Once this routine is completed, the Home screen is displayed. An LED on the front panel of the instrument glows to indicate that the MyCycler is on.

Putting the Instrument Into Standby Mode. When the instrument is on, hold down the Standby key on the front panel of the instrument for 3 seconds. If the instrument is in an idle state, Standby mode will be started. If a protocol is either running or has been edited without saving, you will be prompted to verify the selection prior to initiating Standby mode.

Note: Standby mode is a feature to reduce power consumption of an idle instrument. Just enough power is supplied to maintain microprocessor operation. To shut off the instrument completely, remove the electrical plug from the outlet.

Running a Stored Protocol

1. From the Home screen, press **F1-Protocol Library** to display the menu of available protocols.
2. Use the arrow keys to highlight the desired protocol, **PV92 PCR** (or **PV92 PCR w/ freeze** if leaving samples overnight) and press **Enter**.
3. Select **Run Protocol** from the selection box.
4. Confirm the selections in the **Run Setup** screen. Use the enter key and arrow keys to specify the temperature measurement mode (**Algorithmic**), volume of samples (**40ul**), and whether or not to include a hot start (**NO**) prior to running the protocol.
5. Place samples into the metal PCR tray, or place the green PCR tube holder onto the metal tray. Close the lid.
6. Press **F5-Begin Run**, and double check the screen to ensure the protocol is running and is correct.

Creating a New Protocol

1. From the Home screen, press **F2-Create** to display the menu of available protocol templates.
2. Choose a preprogrammed template protocol to edit, or select the **Custom** option from the selection menu; press **Enter**
   - Editable fields for the protocol may be changed using the arrow keys and alphanumeric keys
   - To add or delete a step or cycle, press **F4-Add/Del**
   - To choose increment or decrement time or temperature, put the cursor on the time or temperature field to be adjusted and press **F3-Option**
3. When you finish editing the protocol, press **F5-Done**
   - To run the edited protocol without saving the edits, choose **Run Protocol** from the selection box

   or
   - To save the edits prior to running the protocol, choose **Save Protocol** or **Save Protocol As…** from the selection box.

Once edits are saved, you may run the protocol from the Protocol Library

Editing a Stored Protocol

1. From the Home screen, press **F1-Protocol Library** to display the menu of available protocols.
2. Use the arrow keys to highlight the desired protocol, and press **Enter**.
3. Select **Edit Protocol** from the selection box
   - Editable fields for the protocol may be changed using the arrow keys and alphanumeric keys
   - To add or delete a step or cycle, press **F4-Add/Del**
   - To choose increment or decrement time or temperature, put the cursor on the time or temperature field to be adjusted and press **F3-Option**
4. When you finish editing the protocol, press **F5-Done**
   - To run the edited protocol without saving the edits, choose **Run Protocol** from the selection box

   or
   - To save the edits prior to running the protocol, choose **Save Protocol** or **Save Protocol As…** from the selection box.

Once edits are saved, you may run the protocol from the Protocol Library

Accessing the Dolan DNA Learning Center and Entering PCR Results

1. On your web browser, go to **www.bioservers.org/bioserver/**
2. Log in to Allele Server (the option in the middle) using the username, **szulkoski**, and the password, **aklein32**
3. Once you have logged in, follow the instructions provided in the pop-up window for using the Allele Server.
4. On the left side of the screen a list should contain a label for our group.
5. Add new data to the group by going under the button "**Manage Groups**".
6. Choose the "**Your groups**" option from the pull-down menu on the right.
7. Once the list of groups' names is on the screen, you can choose your particular group and edit it. Choose the **Edit Group** button to the right of your group's name.
8. Click on the **"Individuals" tab** to enter information on each student.
9. After all information has been added, you can then analyze the class data by selecting you group from the list and selecting "**Analyze**".
10. You can also compare you data with others' by using the "**Compare**" option.
11. Under the "**Manage Groups**" option, you can select from a wide range of population samples under the **"Reference"** option. Students can map the genetic frequencies and compare and hypothesis about the results.
12. To exit the Dolan DNA Learning Center, click on "back to registration".
13. The Dolan DNA Learning Center also gives tips for additional activities to use with this lab.

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PCR Template for Loading Student Samples

Use this chart to record where students load their samples. Make a mark between spaces to indicate the beginning/end of a group. It will make it much easier the next day when removing PCR tubes from the machine. Sometimes the heat from the PCR machine removes labels from the tubes.

Procedure

PV92/PCR Procedure WITH INSTRUCTOR NOTES

PV92/PCR Day 1: DNA Template Preparation

At your group's lab station, check to make sure you have the following materials:

Per student:
1 cup of saline solution
1 plastic transfer pipet
1 screw cap tube with Instagene Matrix
1 empty clear micro test tube

1. Label a clear micro test tube and a screw cap tube, on the lids, with your initials. **Write as dark, and legible as possible!**
2. Swish the saline **vigorously** for 60 seconds. Expel the saline back into the cup.
3. Transfer 1ml of your saline rinse into your **clear micro test tube** with a sterile plastic pipet.

Save students' cups and pipets until they have obtained a sufficient sized cell pellet.

4. Spin your tube in a balanced centrifuge for 2 minutes. Carefully remove the tube and check for a **match-head sized cell pellet** at the bottom of the tube.
5. Pour the saline out of the micro test tube back into your cup, being careful not to lose your cell pellet. Tap the tube (while it is upside-down) gently on edge of the cup to release the saline solution. It is OK for a *small* amount of saline **(1-2 mm above pellet)** to remain in the tube.

This is the limiting step in the lab! **Students should repeat the spinning until they have obtained a good sized cell pellet. ***Some students will have trouble obtaining a cell pellet at all due to very dense saline mouth rinse. Have these students add a little water to their saline rinse to thin it out, then re-spin.**

6. Re-suspend the pellet into the remaining saline solution by flicking the tube with your fingers. The saline should now appear cloudy.

Students must flick the tubes vigorously in order to ensure the cell pellets are dissolved and mixed in with the Instagene Matrix.

7. Set a **P-200** pipet to **200 µl**. Transfer all of the re-suspended cell pellet into your screw cap tube. This may take several tries.
8. Close the screw cap tube. Shake or flick the tube very well to mix the contents.
9. Incubate your group's tubes in a foam holder in the 56°C water bath for 10 minutes. At the 5 minute mark, remove the tubes briefly and ***gently*** shake, and return to the water bath.
10. Remove the tubes from the water bath and ***gently*** invert twice to mix. Place your screw cap tubes in the round tube holder in a boiling water bath (100°C) for 5 minutes.
11. Remove the tubes from the boiling water bath and ***gently*** invert once. This will re-suspend the contents of the tube.

While the instructions in steps 9-11 call for *gentle* shaking, it is important to use enough force to actually mix the sample. Just inverting the tubes will not always mix the sample due to the surface tension inside the tube.

12. Spin the tubes in a balanced centrifuge for 10 minutes. Place your group's tubes in a foam rack. Make sure your tubes have your group number, or use tape to label your foam rack. Your teacher will refrigerate your DNA for Day 2.

PV92/PCR Day 2: PCR WITH INSTRUCTOR NOTES

At your group's lab station, check to make sure you have the following materials:

Per student:	**Per group**:	one tube of yellow master mix labeled **M**
Your screw cap tube		one clear micro test tube with ddH_2O
one PCR tube		p20 and p200 pipettes

13. Obtain your screw cap tube and place it in a foam rack. Do not disturb the pellet in the bottom of the tube by shaking the tube or laying it on its side.

14. Label a **PCR tube** with your initials, on the cap. Write as dark, and legible as possible!

15. Set a **p20** to **18µl**. Pipet **18µl** of the ddH_2O into the bottom of your PCR tube.

16. Set the **p20** to **2µl**. Pipet **2µl** of the **DNA template, the liquid in your test tube,** into the bottom of your PCR tube.

Make sure NOT to transfer ANY beads from the InstaGene matrix into the PCR tube!!!

17. Obtain your group's tube containing yellow **Master Mix**. Pipet **20µl of the Master Mix** into the PCR tube. Cap the PCR tube tightly and flick to mix. The mixture should be yellow. Spin the tubes for ~3 seconds.

***Check all tubes for similar volume and color and for InstaGene.**

18. Place the PCR tube in the thermal cycler according to your instructor's directions. Your instructor will place the control reactions in the thermal cycler as well. The reactions will undergo 45 cycles of PCR amplification.

19. Save your group's capless tubes in a foam rack for Day 3. Discard all other tubes into your waste beaker. Wash your hands thoroughly before leaving class.

***Students can also add the Complete Master Mix to *controls* during step 15.**

***Use the PCR Template for Loading Student Samples when collecting PCR tubes. Labels are frequently lost during the PCR thermal cycle due to high temperatures.**

***Student samples and controls should be loaded into the green, PCR tube trays that come with the PCR Thermal Cycler. This tray is loaded directly onto the metal PCR tray by lining up the trays.**

***After loading the samples into the green PCR tube tray, check all student PCR samples for Instagene Matrix, volume, and sample color. It will be very easy to see if a student has transferred any Matrix into the tube as it will collect in the bottom of the tube very quickly. If Instagene Matrix is seen in the tube, give the student a second tube so they can repeat steps 13-15. Students who have transferred too little Master Mix will have a lighter yellow solution, unless it appears they have transferred too much DNA. Problems with volume and sample color can be addressed by having the student repeat steps 16 and 17.**

*** For those of you with more than one class completing the PCR Module, you may freeze your PCR samples immediately after each class completes Day 2, until all classes are done, then all of the samples can be loaded at the same time. Keep in mind that the PCR machine only handles 96 samples, which must include your controls. You may have to run one or two classes during the course of the day to ensure all samples are run on the same day.**

PV92/PCR Day 3: Electrophoresis WITH INSTRUCTOR NOTES

At your group's lab station, check to make sure you have the following materials:

a tube of loading dye labeled, **LD**
a tube of DNA standard labeled, **S**
p20 and p200 pipettes
3 PCR controls (to share between two groups)

20. Obtain **YOUR** PCR tube from the thermal cycler.

21. Set a **p20** to **10µl** and pipet **10µl** of the **loading dye, LD,** into your PCR tube and mix well by flicking the tube. Spin the tubes for ~3 seconds.

22. Move your group's materials to a gel electrophoresis station. Make sure there is a gel in the tank with the wells near the black electrode and that the tank is filled with buffer.

23. Using a p20 and p200 and a clean tip for each sample, load the samples into 8 wells of the gel in the following order:

Pipet Display

p20, 10ul
1
0
0

p200, 30ul
0
3
0

Lane	Sample	Volume
1	MMR (DNA standard), **S**	10 ul
2	Homozygous control +/+	10 ul
3	Homozygous control -/-	10 ul
4	Heterozygous control +/-	10 ul
5	Student 1	**30 ul**
6	Student 2	**30 ul**
7	Student 3	**30 ul**
8	Student 4	**30 ul**

24. Place the lid on the electrophoresis chamber. Connect the leads to the power supply (red to red, black to black). Use a piece of tape to label the corner of your chamber with your group's number and record who is student 1, 2, 3 or 4.

25. Wait until all of the groups at your station are ready, then run the gel at 200V for 15 minutes.

26. While the gel is running, obtain a plastic staining tray to share with the lab group next to you. Label the tray with your period and group number, with tape. *You will share a tray with another group (two gels per tray).*

27. After 15 minutes, turn off the power and remove the top of the chamber. Remove the piece of tape with your student initials and place it on your staining tray.

28. Carefully remove the tray holding the gel from the gel box. **The gel is very slippery!!!** Slide the gel into the **plastic staining tray.**

29. Add 120 ml of **1x Fast Blast DNA stain** to the staining tray. The stain should just cover the gels. Let the gels stain overnight.

30. Discard all tubes in your waste beaker. Wash your hands thoroughly before leaving class.

PV92/PCR Procedure

PV92/PCR Day 1: DNA Template Preparation

At your group's lab station, check to make sure you have the following materials:

Per student:
1 cup of saline solution
1 plastic transfer pipet
1 screw cap tube with Instagene Matrix
1 empty, clear micro test tube

1. Label a clear micro test tube and a screw cap tube, on the lids, with your initials. **Write as dark, and legible as possible!**
2. Swish the saline **vigorously** for 60 seconds. Expel the saline back into the cup.
3. Transfer 1ml of your saline rinse into your **clear micro test tube** with a sterile plastic pipet.
4. Spin your tube in a balanced centrifuge for 2 minutes. Carefully remove the tube and check for a **match-head sized cell pellet** at the bottom of the tube.
5. Pour the saline out of the micro test tube back into your cup, being careful not to lose your cell pellet. Tap the tube (while it is upside-down) gently on edge of the cup to release the saline solution. It is OK for a *small* amount of saline (1-2 mm above pellet) to remain in the tube.
6. Re-suspend the pellet into the remaining saline solution by flicking the tube with your fingers. The saline should now appear cloudy.
7. Set a **P-200** pipet to **200 µl**. Transfer all of the re-suspended cell pellet into your screw cap tube. This may take several tries.
8. Close the screw cap tube. Flick the tube vigorously to mix the contents.
9. Incubate your group's tubes in a foam holder in the 56°C water bath for 10 minutes. At the 5 minute mark, remove the tubes briefly and ***gently*** shake, and return to the water bath.
10. Remove the tubes from the water bath and ***gently*** invert twice to mix. Place your screw cap tubes in the round tube holder in a boiling water bath (100°C) for 5 minutes.
11. Remove the tubes from the boiling water bath and ***gently*** invert once. This will re-suspend the contents of the tube.
12. Spin the tubes in a balanced centrifuge for 10 minutes. Place your group's tubes in a foam rack. Make sure your tubes have your group number, or use tape to label your foam rack. Your teacher will refrigerate your DNA for Day 2.

PV92/PCR Day 2: PCR

At your group's lab station, check to make sure you have the following materials:

Per student:	**Per group**:	one tube of yellow master mix labeled **M**
Your screw cap tube		one clear micro test tube with ddH_2O
one PCR tube		p20 and p200 pipettes

13. Obtain your screw cap tube and place it in a foam rack. Do not disturb the pellet in the bottom of the tube by shaking the tube or laying it on its side.

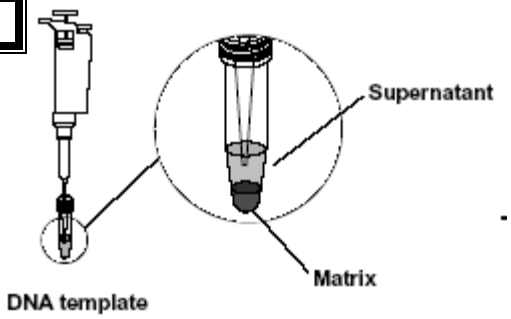
14. Label a **PCR tube** with your initials, on the cap. Write as dark, and legible as possible!

15. Set a **p20** to **18µl**. Pipet **18µl** of the ddH_2O into the bottom of your PCR tube.

16. Set the **p20** to **2µl**. Pipet **2µl** of the **DNA template, the liquid in your test tube,** into the bottom of your PCR tube.

p20, 18ul	p20, 2ul
1	0
8	2
0	0

Make sure NOT to transfer ANY beads from the InstaGene Matrix into the PCR tube!!!



17. Obtain your group's tube containing yellow **Master Mix**. Set the p20 pipet to **20µl**. Pipet **20µl of the Master Mix** into the PCR tube. Cap the PCR tube tightly and flick the tube to mix. The mixture should be yellow. **Spin the tubes for ~3 seconds** in a centrifuge.

p20, 20ul
2
0
0

18. Place the PCR tube in the thermal cycler according to your instructor's directions. Your instructor will place the control reactions in the thermal cycler as well. The reactions will undergo 45 cycles of PCR amplification.

19. Discard all other tubes into your waste beaker. Wash your hands thoroughly before leaving class.

PV92/PCR Day 3: Electrophoresis of Amplified Samples and Staining of Gels

At your group's lab station, check to make sure you have the following materials:

a tube of loading dye labeled, **LD**	p20 and p200 pipettes
a tube of DNA standard labeled, **S**	*3 PCR controls (to share between two groups)*

20. Obtain **YOUR** PCR tube from the thermal cycler.

21. Set a **p20** to **10µl** and pipet **10µl** of the **loading dye, LD,** into your PCR tube and mix well by flicking the tube vigorously. Spin the tubes for 3 seconds.

22. Move your group's materials to a gel electrophoresis station. Make sure there is a gel in the tank with the wells near the black electrode and that the tank is filled with buffer.

23. Using a **p20 and p200** and a clean tip for each sample, load the samples into 8 wells of the gel in the following order:

Lane	1	2	3	4	5	6	7	8
Sample	Standard	Homozygous control +/+	Homozygous control -/-	Heterozygous control +/-	Student 1	Student 2	Student 3	Student 4
Volume	10ul	10ul	10ul	10ul	**30ul**	**30ul**	**30ul**	**30ul**

24. Place the lid on the electrophoresis chamber. Connect the leads to the power supply (red to red, black to black). Use a piece of tape to label the corner of your chamber with your group's number and record who is student 1, 2, 3 or 4.

25. Wait until all of the groups at your station are ready, then run the gel at 200V for 15 minutes.

26. While the gel is running, obtain a plastic staining tray to share with the lab group next to you. Label the tray with your period and group number, with tape. *You will share a tray with another group (two gels per tray).*

27. After 15 minutes, turn off the power and remove the top of the chamber. Remove the piece of tape with your student initials and place it on your staining tray.

28. Carefully remove the tray holding the gel from the gel box. **The gel is very slippery!!!** Slide the gel into the **plastic staining tray.**

29. Add 120 ml of **1x Fast Blast DNA stain** to the staining tray. The stain should just cover the gels. Let the gels stain overnight.

30. Discard all tubes in your waste beaker. Wash your hands thoroughly before leaving class.

Student Materials

PV92/PCR Bioinformatics Power Point Follow-up Questions
(Pre-lab evaluation)

1. What is the purpose of this lab? What are we trying to do?

2. What is PCR and why is it important?

3. Label each step and describe what happens during the three steps in PCR.
    e. Heat to 94°C:

    f. Cool to 60°C:

    g. Warm to 72°C:

4. Write a short definition of the following terms as related to PCR:
    a. Template DNA:

    b. Primers:

    c. Nucleotides:

    d. Taq Polymerase:

5. What is the source of this laboratory's template DNA?

6. What are the three possible results for the allele we are amplifying?

7. What is the difference between and intron and an exon?

PV92/PCR Bioinformatics: Template Preparation
Read the following information and answer the questions to check for understanding.

To obtain DNA for use in the polymerase chain reaction (PCR) you will extract the DNA from your own living cells. It is interesting to note that DNA can be also extracted from mummies and fossilized dinosaur bones. In this lab activity, you will isolate DNA from epithelial cells that line the inside of your cheek. To do this, you will rinse your mouth with a saline (salt) solution, and collect the cells using a centrifuge. You will then boil the cells to rupture them and release the DNA they contain. To obtain pure DNA for PCR, you will use the following procedure:

1. Cheek cells are transferred to a micro test tube containing **InstaGene™ matrix**. This matrix is made up of negatively charged, microscopic beads that grab metal ions out of solution. These metal ions, such as Mg2+, are required as **catalysts or cofactors** for certain enzymes to function properly.
2. The cheek cells suspended in matrix are incubated at 56°C for 10 minutes. This pre-incubation step helps to soften plasma membranes and release clumps of cells from each other. The heat also inactivates enzymes, such as DNases, which can degrade the DNA template.
3. After this 10-minute incubation period, cells will be placed in a boiling (100°C) water bath for 5 minutes. Boiling ruptures the cells and releases DNA from their nuclei and all of their other cellular contents, including enzymes. Some enzymes are contained in the cheek-cell *lysosomes*. *Lysosomes* are sacs in the cytoplasm that contain powerful enzymes, such as **DNases**. **DNases** are used by cells to digest the DNA of invading viruses. When you rupture the cells, these DNases can digest the released DNA. However, when the cells are lysed in the presence of the **InstaGene™ matrix**, the cofactors are not available to the enzymes. This virtually blocks enzymatic degradation of the extracted DNA so you can use it as the template in your PCR reaction.
4. You will use the extracted genomic DNA as the target template for PCR amplification.

1. What is the purpose of the InstaGene Matrix during template preparation?

2. What would happen if you did not use the InstaGene Matrix?

3. What is needed from the cells you collect for PCR?

4. What structures must be broken to release DNA from a cell?

PV92/PCR Bioinformatics: PCR Amplification

Read the following information and answer the questions to check for understanding.

It is estimated that there are 30,000–50,000 individual genes in the human genome. The true power of PCR is the ability to target and make millions of copies of (or **amplify**) a specific piece of DNA (or gene) out of a complete genome. In this activity, you will amplify a region within your chromosome 16.

The recipe for a PCR amplification of DNA contains a simple mixture of ingredients. To replicate a piece of DNA, the reaction mixture, known as **Master Mix**, requires the following components:

1. **DNA template**: contains the intact sequence of DNA to be amplified
2. **Nucleotides**: a nitrogen base (A, T, G, and C), and sugar molecule, and a phosphate group→the basic subunit of DNA
3. **DNA polymerase**: an enzyme that assembles the nucleotides into a new DNA chain
4. **Magnesium ions**: a cofactor (catalyst) required by DNA polymerase to create the DNA chain
5. **Primers:** pieces of DNA complementary to the template that tell DNA polymerase exactly where to start making copies
6. **Buffer:** provides the optimum ionic environment and pH for the PCR reaction

The *template DNA* in this exercise is genomic DNA that was extracted from your cells.
The complete master mix contains Taq polymerase (a DNA polymerase), nucleotides, primers, magnesium ions, and buffer.

PCR makes use of the same basic processes that cells use to duplicate their DNA.

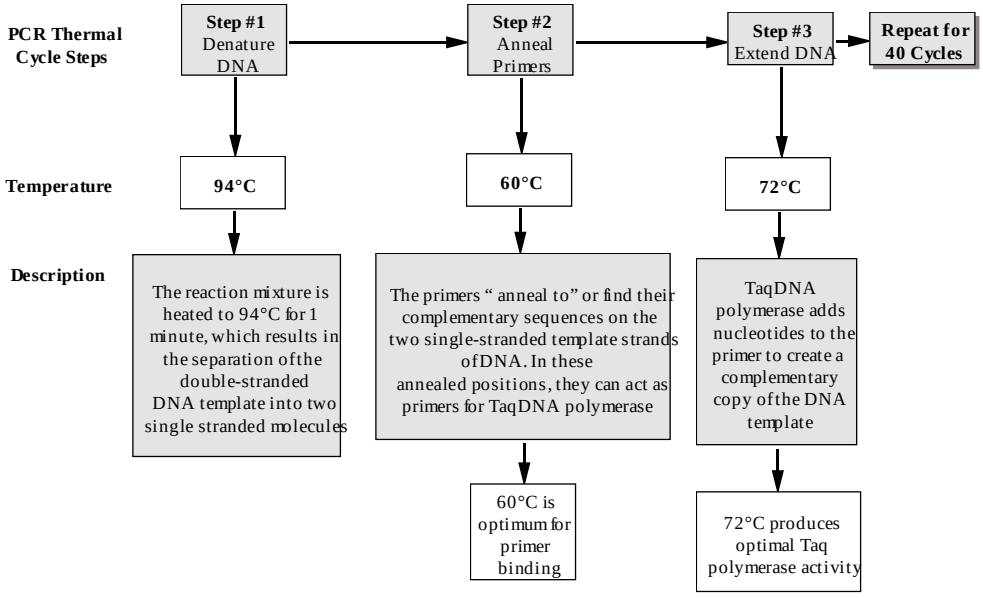
1. Complementary DNA strand hybridization

2. DNA strand synthesis via DNA polymerase

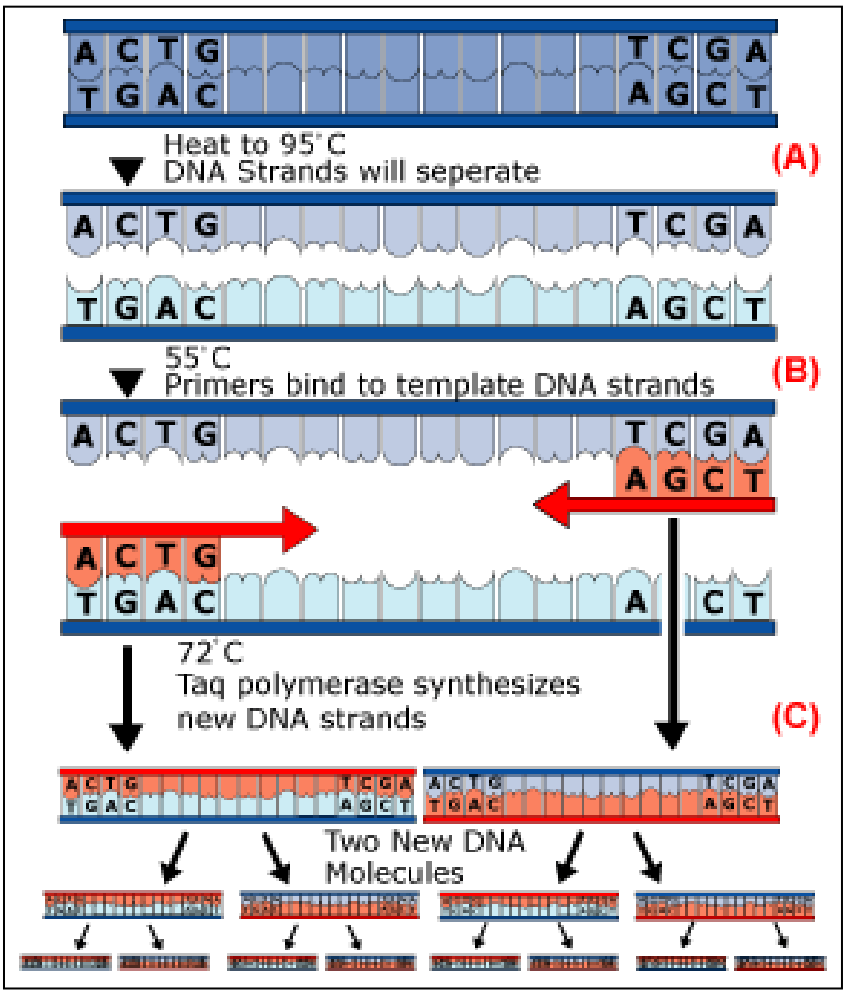
The two *DNA primers* provided in this kit are designed to flank a DNA sequence within your genome and provide the exact start signal for the DNA polymerase begin synthesizing (replicating) copies of that target DNA. The primers are two short single-stranded DNA molecules (23 bases long). One is complementary to a portion of one strand of the template and another that is complementary to a portion of the opposite strand. These primers anneal to the separated template strands and serve as starting points for DNA replication by Taq polymerase.

Taq polymerase extends the annealed primers by "reading" the template strand and synthesizing the complementary sequence. In this way, Taq polymerase replicates the two template DNA strands. This polymerase was isolated from a heat-stable bacterium (*Thermus aquaticus*) which in nature lives within high temperature steam vents such as those found in Yellowstone National Park. For this reason, these enzymes have evolved to withstand high temperatures (94°C) and can be used in the PCR reaction.

The PCR Thermal Cycle



When all the other components are combined under the right conditions, a copy of the original double-stranded template DNA molecule is made — doubling the number of template strands. Each time this cycle is repeated, copies are made from copies and the number of template strands doubles — from 2 to 4 to 8 to 16 and so on — until after 20 cycles there are 1,048,576 exact copies of the target sequence.



1. **Why is it necessary to have two primers for PCR amplification?**

2. **Why are all four nucleotides included in the master mix?**

3. **Which component of the master mix requires the Magnesium ions? Why?**

4. **What is the significance of the temperatures in each step of the PCR Thermal Cycle?**

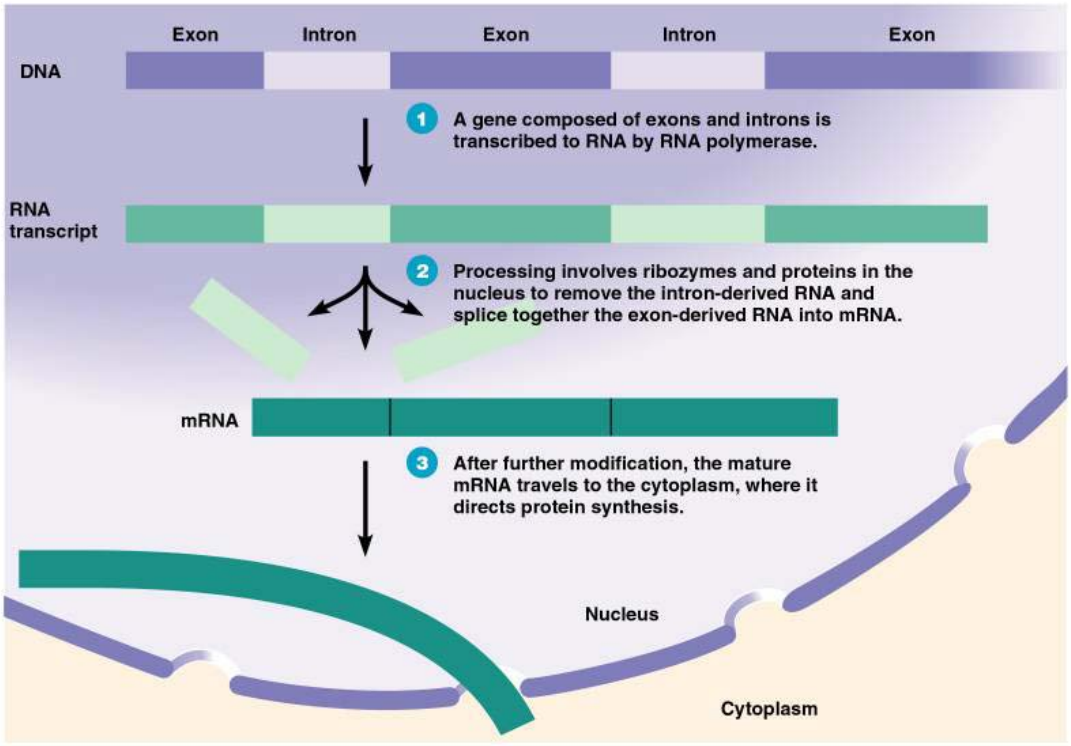
PV92/PCR Bioinformatics: Analysis of Results

Gel Electrophoresis of Amplified PCR Samples and Analysis of Results

What Can Genes and DNA Tell Us?

It is estimated that the 23 pairs, or 46 **chromosomes,** of the human genome (23 chromosomes come from the mother and the other 23 come from the father) contain approximately 30,000–50,000 **genes**. Each chromosome contains a series of specific genes. The larger chromosomes contain more DNA, and therefore more genes, compared to the smaller chromosomes. Each of the **homologous chromosome pairs** contains similar genes. ***Each gene holds the code for a particular protein***. The 30,000–50,000 genes only comprise 5% of the total chromosomal DNA. The other 95% **is noncoding** DNA. This noncoding DNA is scattered in blocks between working segments of genes and within genes, splitting them into segments. The exact function of the noncoding DNA is not known, although it is thought that noncoding DNA allows for the accumulation of mutations and variations in genomes.

When RNA is first **transcribed** from DNA, it contains both coding and noncoding sequences. While the RNA is still in the nucleus, the noncodong **introns** (in = stay with**in** the nucleus), are removed from the RNA while the **exons** (ex = **ex**it the nucleus) are spliced together to form the complete messenger RNA coding sequence for the protein (see Figure 10). This process is called **RNA splicing** and is carried out by specialized enzymes called **spliceosomes.**



Introns often vary in their size and sequence among individuals, while exons do not. This variation is thought to be the result of the accumulation of different mutations in DNA throughout evolution. These mutations in our noncoding DNA are silently passed on to our descendants; ***we do not notice them because they do not affect our phenotypes***. However, these differences in our DNA represent the molecular basis of DNA fingerprinting used in human identification and studies in population genetics.

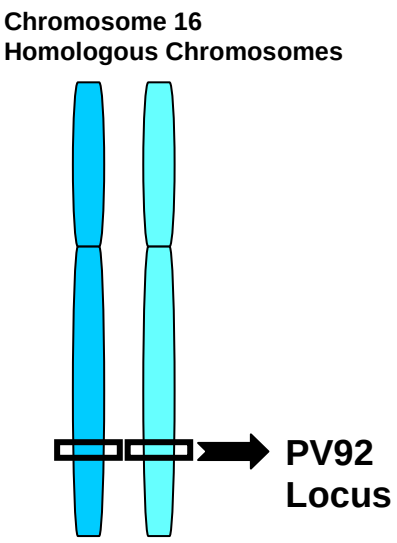
The Target Sequence: The human genome contains small repetitive DNA elements or sequences that have become randomly inserted into it over millions of years. One such repetitive element is called the **"Alu sequence"** (see Figure 11). This is a DNA sequence about **300 base pairs** long that is repeated almost 500,000 times throughout the human genome. The origin and function of these repeated sequences is not yet known.

Some of these Alu sequences have characteristics that make them very useful to geneticists. If an Alu sequence is present within the introns of certain genes, they can either be *associated with a disease* or be used to *estimate relatedness* among individuals. In this exercise, analysis of a single Alu repeat is used to estimate its frequency in the population and as a simple measure of molecular genetic variation — **with no reference to disease or relatedness among individuals.**

In this laboratory activity you will look at an Alu element in the PV92 region of chromosome 16. This particular Alu element is **dimorphic,** meaning that the element is present in some individuals and not others. Some people have the

insert in one copy of chromosome 16 **(one allele),** others may have the insert in both copies of chromosome 16 **(two alleles),** while some may not have the insert on either copy of the chromosome (see Figure 12). The presence or absence of this insert can be detected using PCR followed by agarose gel electrophoresis; we will designate the presence of the insert on a chromosome as **positive, +,** and the absence of the insert as **negative, -** .Since you are amplifying a region of DNA contained within an intron, the region of DNA is never really used in your body. ***So if you don't have it, don't worry.***

The primers in this kit are designed to bracket a sequence within the PV92 region that is approximately 600 base pairs long if the intron does not contain the Alu insertion, or approximately 900 base pairs long if Alu is present. This increase in size is due to the 300 base pair sequence contributed by the Alu insert. **When your PCR products are electrophoresed on an agarose gel, three distinct outcomes are possible.**

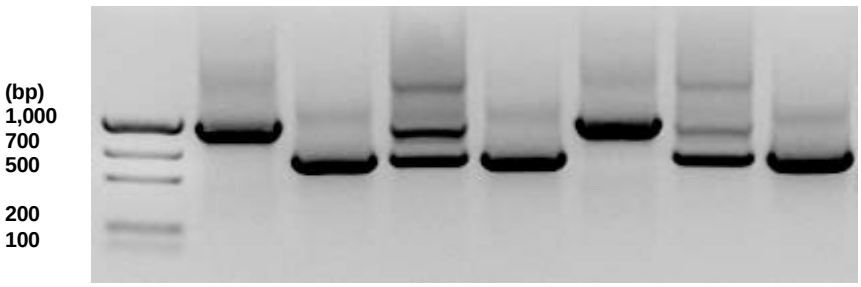


1. **Homozygous (+/+), both chromosomes contain Alu inserts**: each amplified PCR product will be 900 base pairs long. On a gel they will migrate at the same speed so there will be one band that corresponds to 900 base pairs.
2. **Homozygous (–/–), neither chromosome contains the insert**: each amplified PCR product will be 300 base pairs and they will migrate as one band that corresponds to 300 base pairs.
3. **Heterozygous (+/–), there is an Alu insert on one chromosome but not the other**: there will be one PCR product of 300 base pairs and one of 900 base pairs. The gel will reveal two bands for such a sample.

Electrophoresis separates DNA fragments according to their relative sizes (molecular weights). DNA fragments are loaded into an agarose gel slab, which is placed into a chamber filled with a conductive buffer solution. A direct current is passed between wire electrodes at each end of the chamber. DNA fragments are negatively charged, and when placed in an electric field will be drawn toward the positive pole and repelled by the negative pole. The matrix of the agarose gel acts as a molecular sieve through which smaller DNA fragments can move more easily than larger ones. Over a period of time, smaller fragments will travel farther than larger ones. Fragments of the same size stay together and migrate in what appears as a single "band" of DNA in the gel. In the sample gel below (Figure 13), PCR-amplified bands of 941 bp and 641 bp are separated based on their sizes.

Electrophoretic separation of DNA bands based on size.

Lane 1: EZ Load DNA molecular mass ruler, which contains 1,000 bp, 700 bp, 500 bp, 200 bp, and 100 bp fragments
Lanes 2 through 3: homozygous (+/+),homozygous (–/–), and heterozygous (+/–) controls
Lanes 5 and 8: homozygous (–/–) individuals with 641 bp fragments
Lane 6: homozygous (+/+) individual with 941 bp fragment
Lanes 7: heterozygous (+/–) individual with 941/641 bp fragments

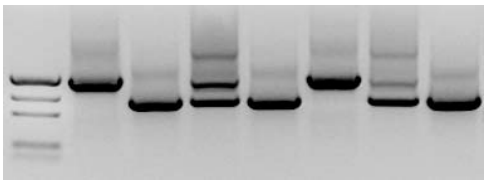


PV92/PCR Analysis of Results

1. Place your gel on a white background and record your results by making a diagram as follows:
    a. Place a clear, plastic sheet over the gel.
    b. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel.
    c. Remove the plastic sheet for later analysis.

2. Determine whether you are homozygous or heterozygous for the Alu insertion:

    a. First look at the **control samples** and note the migration patterns of the homozygous +/+, the homozygous –/–, and the heterozygous +/– samples (also refer to the example on page 51). You may notice that in the heterozygous sample the smaller 641 base pair band is more intense than the larger 941 bp band. This difference is due to the fact that the smaller fragment is amplified more efficiently than the larger fragment. Copies of the shorter fragment can be made at a faster rate than the bigger fragment, so more copies of the shorter fragment are created per cycle.



Example of a stained gel.

1. Explain the difference between an intron and an exon. Why is this important to your results?

2. Why do the two possible PCR products differ in size by 300 base pairs?

3. What is your genotype for the Alu insert in your PV92 region?

4. **Fill out the table below with your class.** What are the observed genotypic frequencies of +/+, +/-, or -/- in your class population?

Observed Class Genotypic Frequencies

Category		Number of genotypes per class	Frequency = # of genotypes/total
Homozygous (+/+)	p^2 =	_____	=_____
Heterozygous (+/ –)	pq =	_____	=_____
Homozygous (–/–)	q^2 =	_____	=_____
	Total =	_____	= 1

5. **Fill out the next table with your class.** What is the frequency of each allele in your overall class sample? Remember if you have 32 students in your class, the total number of alleles is two times that number, or 64.

Calculated Allelic Frequencies for the Class

Category		Number	Frequency (# of genotypes/total)
Total (+) alleles	p	_____	=_____
Total (-) alleles	q	_____	=_____
		Total alleles=_____	= 1.00

The following table represents data from a USA-wide random population study.

USA Genotypic Frequencies

Category		Number of genotypes per class	Frequency = # of genotypes/total
Homozygous (+/+)	p^2 =	2,422	= 0.2422
Heterozygous (+/ –)	pq =	5,528	= 0.5528
Homozygous (–/–)	q^2 =	2,050	= 0.2050
	Total =	10,000	= 1

USA Allelic Frequencies

Category		Number	Frequency (# of genotypes/total)
Total (+) alleles	p	10,372	=0.5186
Total (-) alleles	q	9,628	=0.4814
		Total alleles= 20,000	= 1.00

6. How does your actual class data for allelic frequencies compare with that of the random sampling of the USA population? Would you expect them to match? What reasons can you think of to explain the differences or similarities?

Modified Student Materials

PV92/PCR Bioinformatics Power Point Follow-up Questions
(Pre-lab evaluation)

1. What is the purpose of this lab? What are we trying to do?

2. What is PCR and why is it important?

3. Match the following steps in the PCR reaction with the correct process.

_____ Heat to 94°C	A. Annealing Step
_____ Cool to 60°C	B. Extension Step
_____ Warm to 72°C	C. Denaturation Step

4. Complete the following sentences using the terms listed:

Template DNA	**Taq Polymerase**	**Denaturation Step**
Primers	**Annealing Step**	
Nucleotides	**Extension Step**	

a. Primers find their complementary sequences on the two single-stranded DNA templates during the _____ in the PCR reaction.

b. _____ are made up of a nitrogen base, a sugar, and a phosphate group.

c. The short, single-stranded DNA molecules that anneal to the separated DNA template strands are called _____.

d. During the _____, DNA is separated into two single-strands of DNA.

e. A DNA template can be amplified at high temperatures because of the activity of _____.

f. In the PCR reaction, Taq polymerase is active and adds nucleotides to the primers to create a complementary copy of DNA during _____.

g. PCR gives scientists the ability to amplify _____, a specific piece of DNA out of a complete genome.

5. What is the source of this laboratory's template DNA?

6. If having a particular allele is **+** and not having the allele is **-**, and there are two chromosomes with the allele, what are the three possible results for the allele we are amplifying?

7. What is the difference between and intron and an exon?