



Supplemental Materials

for

Using PCR to Target Misconceptions about Gene Expression

Leslie K. Wright* and Dina L. Newman

*Thomas H. Gosnell School of Life Sciences, Rochester Institute of Technology,
Rochester, NY 14623*

Table of Contents

(Total pages 24)

Appendix 1: Student lab protocol

Appendix 2: Instructor notes

Appendix 3: Pre- and postlab assessment questions

Appendix 4: Reflection activity

*Corresponding author. Mailing address: School of Life Sciences, Rochester Institute of Technology, 85 Lomb Memorial Drive, Rochester, NY 14623-5608. Phone/Fax: 585-475-4449. E-mail: lkwsj@rit.edu.

©2013 Author(s). Published by the American Society for Microbiology. This is an Open Access article distributed under the terms of the a Creative Commons Attribution – Noncommercial – Share Alike 3.0 Unported License (<http://creativecommons.org/licenses/by-nc-sa/3.0/>), which permits unrestricted non-commercial use and distribution, provided the original work is properly cited.

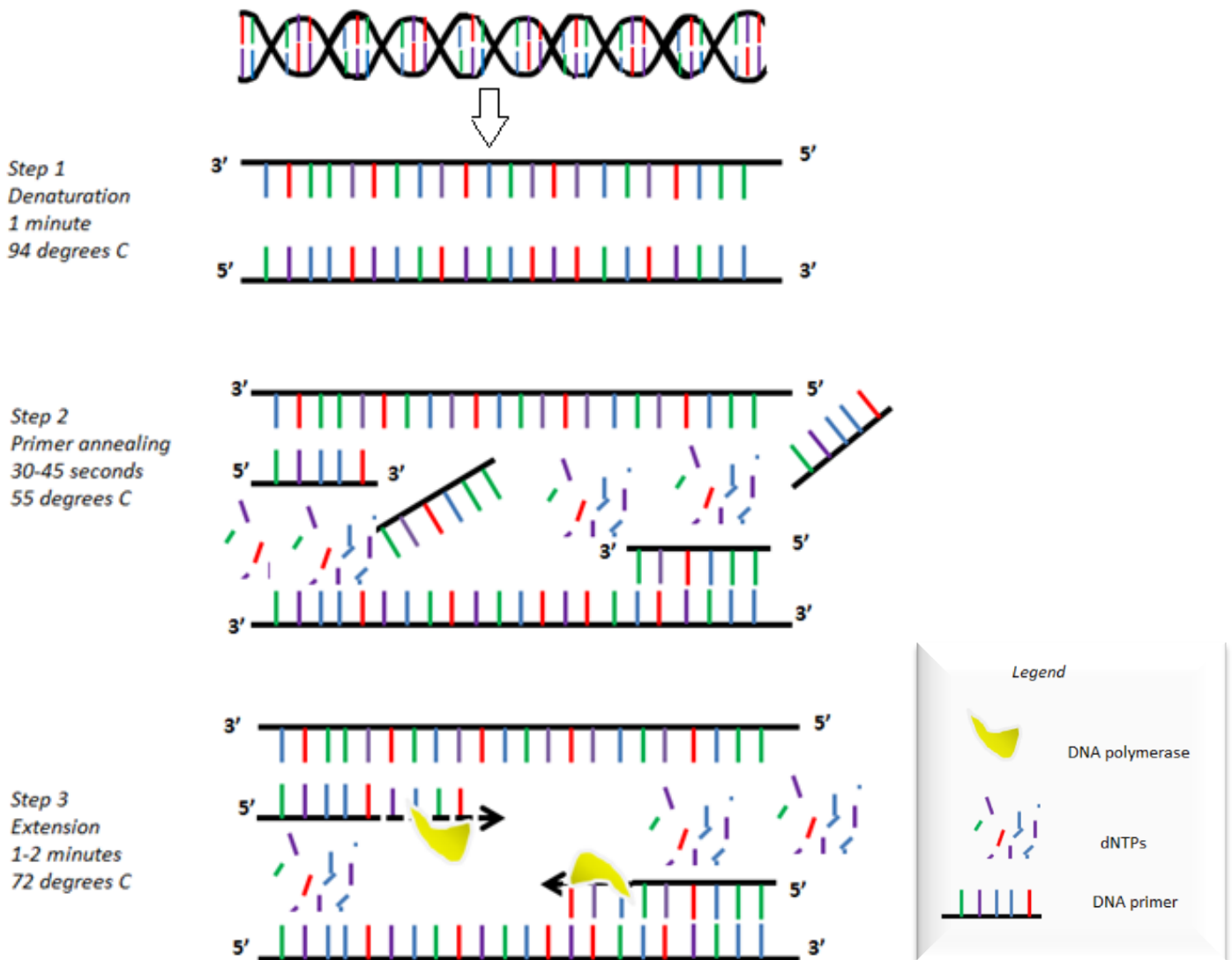
Appendix 1: Student lab protocol

Name _____

PCR: *in vitro* DNA replication of a specific target sequence

The polymerase chain reaction (PCR) is a molecular technique used to amplify a specific region of DNA, yielding millions of copies of the desired fragment. It is based on the natural process of DNA replication using DNA polymerase, but it is much simpler and only replicates a small target section of DNA instead of the whole genome. Since that section is replicated many times over, it is an “amplification” process, where lots of copies of *just the target region* are generated.

The key to PCR is that we use a **thermostable** DNA polymerase: one that works at high temperatures and is not inactivated by exposure to heat (it survives at the boiling temperature of water). This DNA polymerase was originally isolated from a bacterium that lives in hot springs, called *Thermus aquaticus*, and the enzyme is called **Taq polymerase**.



Using the schematic diagram of PCR to help you, see if you can explain what is happening at each major step:

Step 1 is the “denaturation” step.

What’s happening here?

What kinds of bonds are being broken/formed?

Why is 94°C an appropriate temperature for this step?

Step 2 is the “annealing” step.

What’s happening here?

What kinds of bonds are being broken/formed?

Why is 55°C an appropriate temperature for this step?

The optimal annealing temperature depends on the GC content of the primers. Explain why.

Step 3 is the “extension” step.

What’s happening here?

What kinds of bonds are being broken/formed?

Why is 72°C used for this step?

The optimal amount of time needed for the elongation step depends on the length of the product. Explain.

Steps 1-3 are repeated 30-40 times. Why?

Why wouldn't this work with a DNA polymerase isolated from *E. coli*?

Is there a replication fork when you do replication *in vitro* like this? Explain.

Why are the following not needed in a PCR reaction while they are needed for DNA replication in a cell?

Helicase

Topoisomerase

Primase

rNTPs

RNase

DNA ligase

What provides the specificity of this reaction? In other words, how does it allow you to just amplify a particular gene or section of DNA instead of the whole genome?

Next week we are going to use PCR to amplify a portion of the GFP gene from different samples of bacteria that you transformed today.

PCR primers have been designed to amplify a 714 base-pair region of the GFP gene from its coding sequence. That sequence is underlined below. Remember, you are only looking at the coding strand of the DNA.

GAGATATACATATGGCTAGCAAAGGAGAAGAAGCTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGAT
GTTAATGGGCACAAATTTTCTGTTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAATTTAT
TTGCACTACTGGAAAACCTACCTGTTCCATGGCCAACACTTGTCACTACTTTTCTTATGGTGTTC AATGCTTTTCCCGTTATCCGGATC
ATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTAC
AAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACAT
TCTCGGACACAAACTCGAGTACAAC TATAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCA
AAATTCGCCACAACATTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTA
CCAGACAACCATTACCTGTTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAAC
TGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAATAATGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGA

The forward primer includes the first 22 bp of the *amplified* sequence. Write the sequence of the FORWARD primer for GFP: (remember, DNA sequence is always written 5' to 3')

The reverse primer includes the last 22 bp of the *amplified* sequence. Write the sequence of the REVERSE primer for GFP: (remember, DNA sequence is always written 5' to 3')

Answer Key to Embedded Questions in Protocol

Step 1 is the “denaturation” step.

What’s happening here?

Double-stranded DNA is being denatured. The Hydrogen bonds are being broken by heat to separate the two strands.

What kinds of bonds are being broken/formed?

Hydrogen bonds are being broken.

Why is 94°C an appropriate temperature for this step?

Hydrogen bonds are fairly weak and will break at that temperature. Covalent bonds, such as the ones that hold together the backbone of DNA require much more energy to break and will remain intact.

Step 2 is the “annealing” step.

What’s happening here?

The primers are bonding with complementary bases on the single-stranded template DNA.

What kinds of bonds are being broken/formed?

New Hydrogen bonds are being formed between the bases of the primers and the complementary bases of the template.

Why is 55°C an appropriate temperature for this step?

This is a temperature that facilitates hydrogen bonding of small stretches of DNA but is not low enough to allow the entire template to re-anneal.

The optimal annealing temperature depends on the GC content of the primers. Explain why.

Since A-T pairs have two Hydrogen bonds while G-C pairs have three Hydrogen bonds, they take less energy to break. Thus the higher the GC content, the more energy (heat) it takes to separate the strands, and the higher the temperature at which it will anneal.

Step 3 is the “extension” step.

What’s happening here?

DNA is being synthesized, starting from each primer and growing in a 5' → 3' direction.

What kinds of bonds are being broken/formed?

Phosphodiester bonds are being formed between nucleotides as they are added, and hydrogen bonds are being formed between the new nucleotides and the template.

Why is 72°C used for this step?

This is the optimal temperature for Taq polymerase activity (the enzyme that catalyzes the DNA synthesis reaction).

The optimal amount of time needed for the elongation step depends on the length of the product. Explain.

The longer the product, the more time it takes to synthesize the new DNA strand.

Steps 1-3 are repeated 30-40 times. **Why?**

Once new DNA is synthesized, it can be used as a template for the next reaction. PCR is effective because with every cycle the amount of DNA synthesized is doubled (it is an exponential reaction). This allows a very large amount of a small stretch of DNA to be generated.

Why wouldn't this work with a DNA polymerase isolated from *E. coli*?

**E. coli*'s normal environment is 37°C, so all its enzymes are optimized for that temperature. If you heated *E. coli* DNA Polymerase up to a high enough temperature to denature DNA, it would also denature the enzyme, permanently destroying its activity. You could do it, but you would have to add more enzyme with every cycle.*

Is there a replication fork when you do replication *in vitro* like this? Explain.

No. The strands of the DNA template are completely separated during PCR by heat, unlike DNA replication in a cell, where only the region of DNA where the polymerase is working is denatured.

Why are the following not needed in a PCR reaction while they are needed for DNA replication in a cell?

Helicase: *DNA is denatured by heat, not enzymes.*

Topoisomerase: *DNA is completely denatured, so no positive supercoils are formed during DNA synthesis that need to be relieved*

Primase: DNA primers are synthesized separately and added to the reaction. Thus there is no need to make RNA primers.

rNTPs: Since RNA primers are not being synthesized, there is no need for ribonucleotides.

RNase: Since DNA rather than RNA primers are used, there is no need to get rid of the RNA

DNA ligase: There is no discontinuous replication so pieces do not need to be ligated together.

What provides the specificity of this reaction? In other words, how does it allow you to just amplify a particular gene or section of DNA instead of the whole genome?

The primers bracket the region of interest. Only the region between the two primers will be amplified.

The forward primer includes the first 22 bp of the *amplified* sequence. Write the sequence of the FORWARD primer for GFP: (remember, DNA sequence is always written 5' to 3')

ATGGCTAGCAAAGGAGAAGAAC

The reverse primer includes the last 22 bp of the *amplified* sequence. Write the sequence of the REVERSE primer for GFP: (remember, DNA sequence is always written 5' to 3')

TAGAGCTCATCCATGCCATGTG

Genetic Analysis of *E. coli* containing pGLO

Review

Last week you transformed *E. coli* with the pGLO plasmid (Bio-Rad) and streaked out transformed and non-transformed colonies on different types of media.

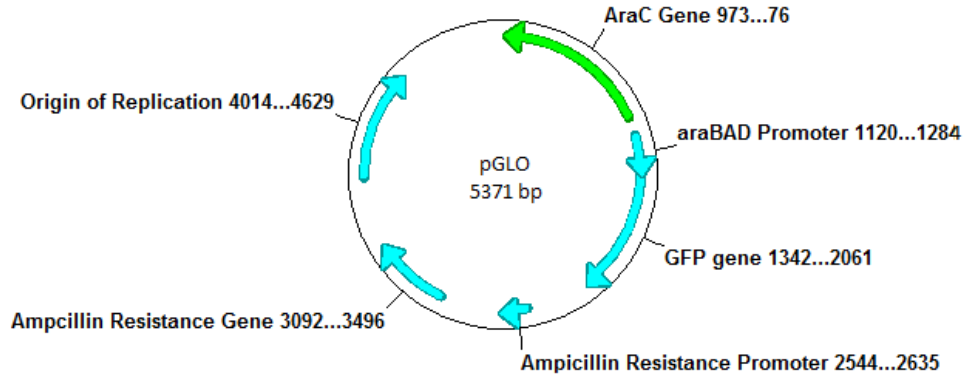


Figure 1. Simplified plasmid map of pGLO depicting the origin of replication, araBAD inducible promoter, GFP and Ampicillin Resistance genes. Figure was created using A Plasmid Editor tool (<http://biologylabs.utah.edu/jorgensen/wayned/ape/>).

pGLO is an “inducible” plasmid. As demonstrated by Figure 2, the GFP gene is only transcribed when arabinose (the inducer) is present in the media.

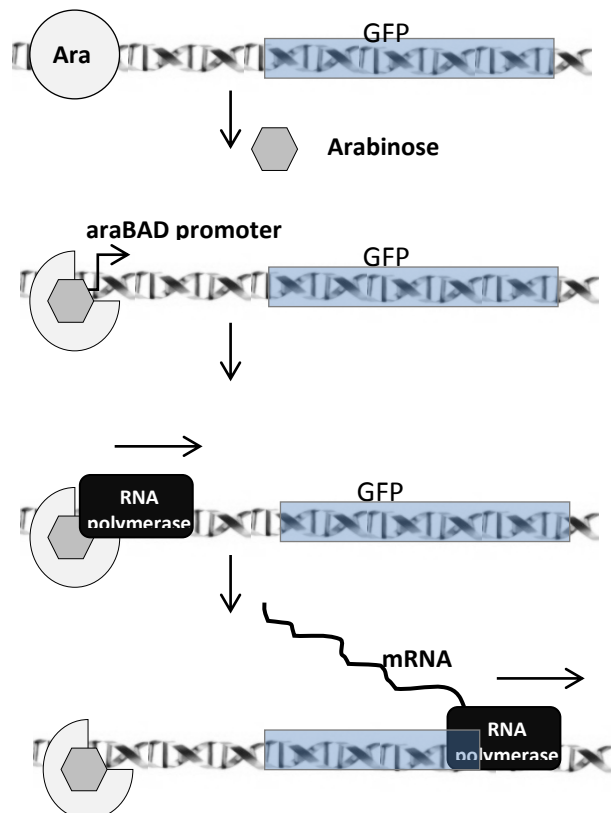


Figure 2. The GFP gene is transcribed when arabinose activates the AraC transcription factor.

Today you are going to do PCR, which you learned about last week, using the DNA contained in the bacteria you transformed!

Methods: It's time for you to write a detailed protocol. You have the skills to do so! Here are the basic facts:

- Taq polymerase must be kept on ice **at all times** and **should be added LAST to reaction tubes**.
- PCR tubes, as reagents are added, **should be kept on ice** (with lids closed between addition of reagents).
- Each pair of students will set up three reactions. The same set of primers will be used to amplify target DNA from the bacteria you transformed last week:
 - *E. coli* grown on LB plate
 - *E. coli* + pGLO grown on LB + amp
 - *E. coli* + pGLO grown on LB + amp + ara

After all reagents are added to reaction tubes, they should get thoroughly (but quickly) mixed and reaction must be spun down.

The reagents needed in a PCR reaction are:

Reagent	Stock concentration	Final concentration
DNA template	*	*
Buffer (containing Mg ²⁺)	10X	1X
dNTPS	10 mM	0.2 mM
Forward primer	50 uM	1.0 uM
Reverse primer	50 uM	1.0 uM
Taq polymerase	5 units/uL	5 units

**** In this case we are going to use a bacterial colony re-suspended in water rather than purified DNA.**

The first thing you will have to do is to figure out **the volume of each reagent for each 50ul reaction**. Most of the volumes can be calculated using the $c_1v_1=c_2v_2$ formula that you are familiar with. Note that 10X means "10 times the working concentration."

Reagent	Final concentration	Volume needed (per reaction)
Bacterial DNA	1 bacterial colony suspended in 10 uL of sterile dH ₂ O*	10 uL
Buffer (containing Mg ²⁺)		
dNTPS		
GFP forward primer		
GFP reverse primer		
Taq polymerase		
Sterile dH ₂ O**		

*Scrape a small amount of bacteria from your plate with a sterile toothpick and swirl it around in 10 uL of sterile dH₂O directly in your reaction tube. Don't take a huge glob—you don't need much. It's OK if it's not even visible in the solution.

**This is used to bring the final volume up to a total of 50 uL.

It's not efficient to set up three separate reactions pipetting in each individual reagent. Instead, make a "master mix" that contains all of the reagents (**EXCEPT FOR THE BACTERIA**) at a 3.1X volume (it's a 3.1X master mix to give you a little leeway on pipetting). This also ensures that you didn't make a mistake with just one tube—all tubes will have the same amounts of all reagents (assuming you mixed it properly before aliquoting).

For example:

If you need to use 5.0 ul of a particular reagent (the 1X) volume, then you would need

$$3.1 \times 5.0 \text{ ul} = 15.5 \text{ ul}$$

Calculate how much of each reagent would be used in a 3.1X 'master mix'. Notice the only reagent missing from the list is the template. That makes sense....you wouldn't want to add all three templates together.

	Volume for a 1X reaction	Volume for a 3.1 X reaction
Sterile dH ₂ O		
Buffer		
dNTPs		
Forward GFP primer		
Reverse GFP primer		
Taq DNA polymerase		

The final reaction volume (per reaction) is 50 ul. Remember that you have already made your bacterial suspensions in 10 ul of water, which is part of the total.

How much master mix will get added to each reaction tube? _____

You will have to make one master mix (3.1X) for your PCR. You might want to label your PCR tubes "1, 2, and 3" including your initials. Devise a labeling scheme such as:

Sample Tube #	Template
1	wild type <i>E. coli</i> (grown on LB)
2	<i>E. coli</i> + pGLO (grown on LB + amp)
3	<i>E. coli</i> + pGLO (grown LB + amp + ara)

**** You CANNOT label your tubes with "1-3" UNLESS you have a table, similar to this one, in your notebook. Write down which sample is in which tube so you remember! ****

Here are the PCR conditions used in the thermal cycler (protocol optimized for the GFP primers):

95° C	2 minutes	
95° C	30 seconds	} repeated 30X
59° C	30 seconds	
72° C	1 minute	
72° C	5 minutes	
4° C	hold	

To Summarize

- Write a detailed protocol in your lab notebook.
- Check it with the instructor before you proceed.
- After your reactions are set up in labeled tubes (make sure your initials or names are on the tubes also) place them on ice. The instructor will put all of the reactions at the same time.
- After the thermocycler completes the program, tubes will be moved to the freezer and stored until next week.
- You will run these PCR products on a gel next week.

Appendix 2: Instructor Notes

The following reagents are needed for completion of Part I: Bacterial Transformation with pGLO plasmid:

Reagents	Amount per tube	Amount required per Laboratory Section (12 pairs of students)
Plates of <i>E. coli</i> strain HB101 K-12 (streaked the day before)	--	4 plates
LB broth	1 ml	12 tubes
pGLO plasmid DNA, 100ng/ul	12 ul	12 tubes
50 mM, pH 6.1 CaCl ₂ solution (or "Transformation Solution" from Bio-Rad)	750 ul	12 tubes

Plates	Number of plates per experiment	Amount required per Laboratory Section (12 pairs of students)
LB plates	2	24 plates
LB + ampicillin (100 ug/ul) plates	2	24 plates
LB + ampicillin (100 ug/ul) + L- arabinose (0.3% w/v) plates	1	12 plates

The following reagents are needed for completion of part II: PCR amplification of GFP:

Reagent	Volume per tube	Amount required per Laboratory Section (12 pairs of students)
dNTPs (10 mM)	8 ul	12 tubes
Forward GFP primer (50 uM) 5'ATGGCTAGCAAAGGAGAAGAAC	5 ul	12 tubes
Reverse GFP primer (50 uM) 5'GTAGAGCTCATCCATGCCATGTG	5 ul	12 tubes
Sterile distilled water	500 uL	12 tubes
10X Taq Buffer (New England Biolabs)	20ul	12 tubes
Taq polymerase (New England Biolabs)	3.5 ul	12 tubes

A Thermal-cycler and PCR suitable sterile tubes are also required for this laboratory.

The following reagents are needed for completion of part III: Gel Electrophoresis of PCR products:

- Agarose powder
- LB Running Buffer (Faster Better Media LLC) or other buffer suitable for running agarose gels
- Loading dye (from Faster Better Media LLC) or other loading dye suitable for running agarose gels
- DNA ladder (such as 100 bp ladder from New England BioLabs)
- GelRed Nucleic Acid Stain (Biotium) or Ethidium Bromide
- Gel electrophoresis boxes, power supplies, and a gel imaging system

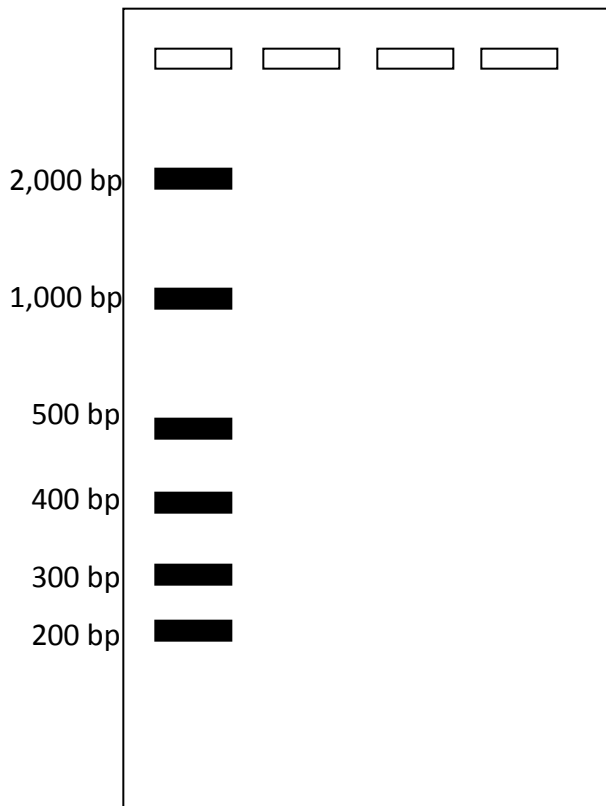
Appendix 3: Pre- and Postlab Assessment Questions

Pre-lab Assessment (Learning objectives #1 and #2) - Use after completion of PCR but before results are visualized using gel electrophoresis.

This post-lab quiz will be graded for effort. We want you to start thinking about your results for next week!

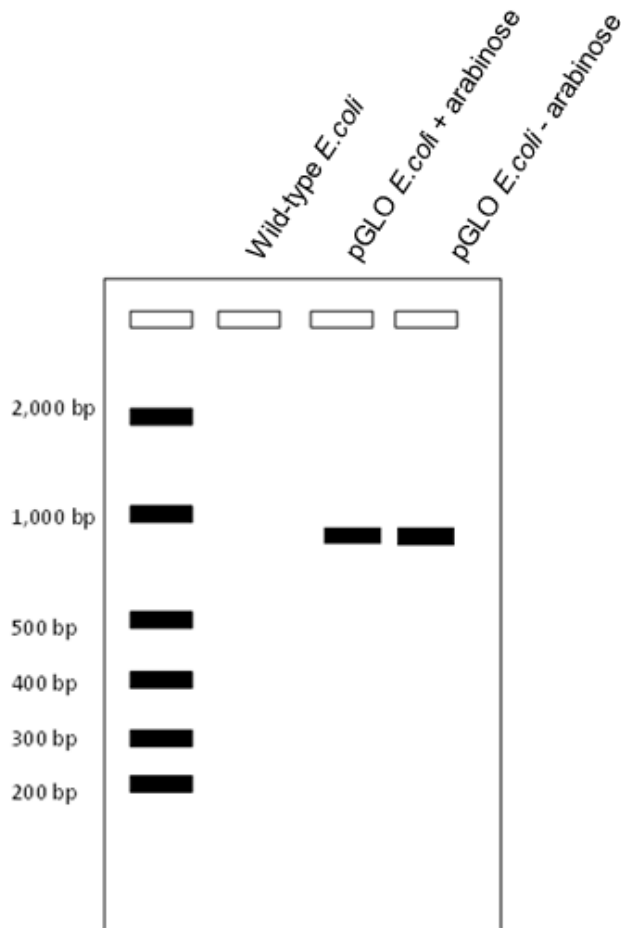
Predict the results from your PCR amplification of GFP by sketching/explaining on the figure below. You attempted to amplify a portion of GFP (green fluorescent protein) gene from three different DNA samples:

1. *E. coli* wild type grown in LB
2. *E. coli* + pGLO grown in LB + amp
3. *E. coli* + pGLO grown in LB + amp + arabinose



The GFP PCR product is 714 bp. Sketch the results you expect to see after your run your PCR samples on an agarose gel next week. Label the gel appropriately and explain your reasoning.

Answer Key: Students should be graded for effort, not correctness. An example of a correct gel diagram and explanation are provided below.



The 714 bp DNA fragment should be amplified from pGLO-transformed *E. coli* in the presence or absence of arabinose because these bacteria contain the pGLO plasmid, which contains the GFP gene.

The 714 bp DNA fragment will not be amplified from wild-type *E. coli* because it does not contain the pGLO plasmid and GFP is not a bacterial gene.

Post-lab Assessment #1 (Learning objective #1)

You are interested in a gene called synapsin-1, which is expressed by neural cells. The sequence for this gene is 100% identical in humans and mouse. You have designed a PCR assay to amplify a 1000 bp fragment of synapsin-1. Assuming all conditions have been optimized, if you were to test DNA from human neural tissue, human kidney tissue, mouse neural tissue and mouse kidney tissue using your PCR assay you would expect DNA electrophoresis results to look like:

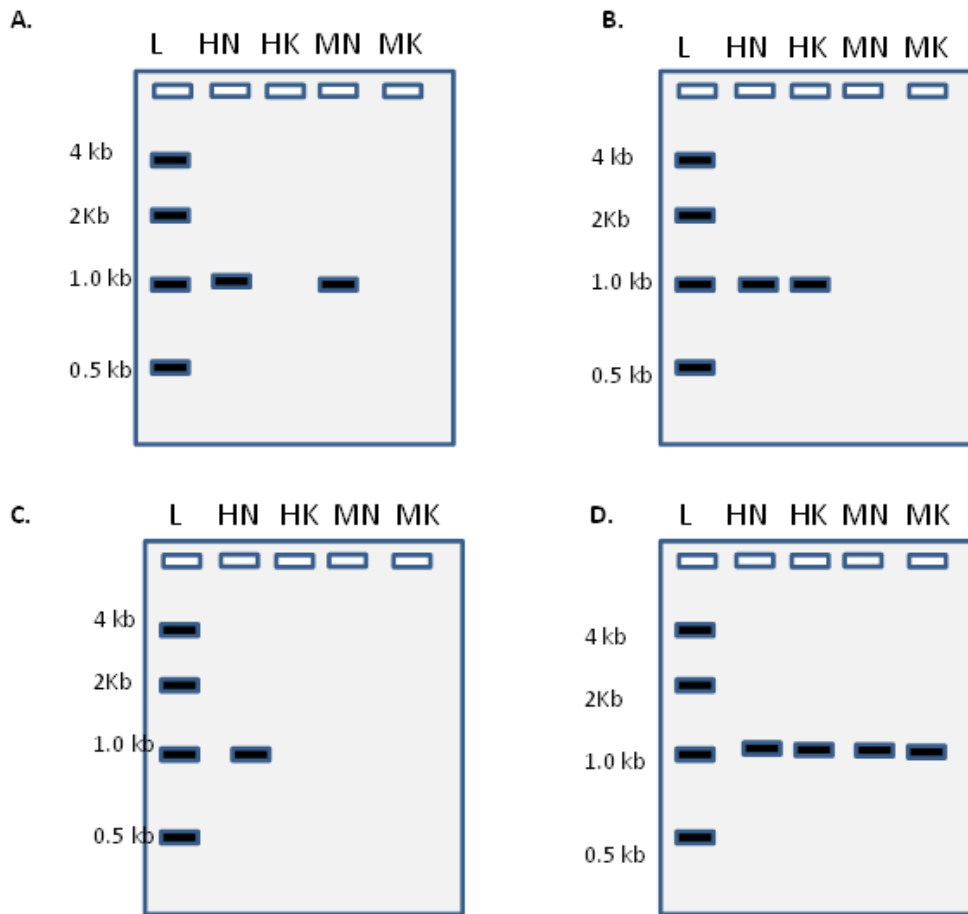
L = DNA ladder

HN = human neural tissue DNA

HK = human kidney tissue DNA

MN = mouse neural tissue DNA

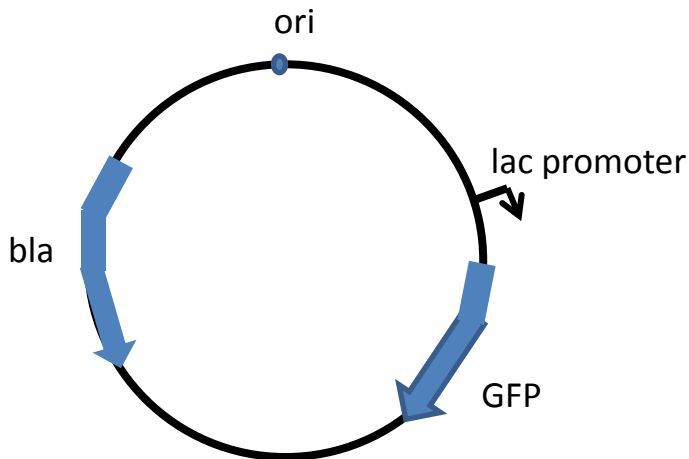
MK = mouse kidney tissue DNA



Answer = D

Alternative Post-lab Assessment (Learning objective #1) – this question assumes that students have studied the specifics of the lac operon.

Imagine that you transformed wild type *E. coli* with the following plasmid. Note that GFP is expressed under control of the lac promoter in this construct. Also, wild type *E. coli* contains the genes for CAP and lac repressor in its genome.



- a) If the transformed cells were maintained in media containing ampicillin (amp), which of the following would allow for PCR amplification of the GFP gene? (write +++, + or – next to each)
- _____ Media containing amp + glucose
 - _____ Media containing amp + lactose
 - _____ Media containing amp + both glucose and lactose
 - _____ Media containing amp but neither glucose nor lactose
- b) If the transformed cells were maintained in media containing amp, which of the following plates would contain green glowing colonies under UV light? (write +++, + or – next to each)
- _____ Media containing amp + glucose
 - _____ Media containing amp + lactose
 - _____ Media containing amp + both glucose and lactose
 - _____ Media containing amp but neither glucose nor lactose
- c) Explain why the answers to a and b are different, or why they are the same.

Answer Key for Alternative Assessment (Learning objective #1)

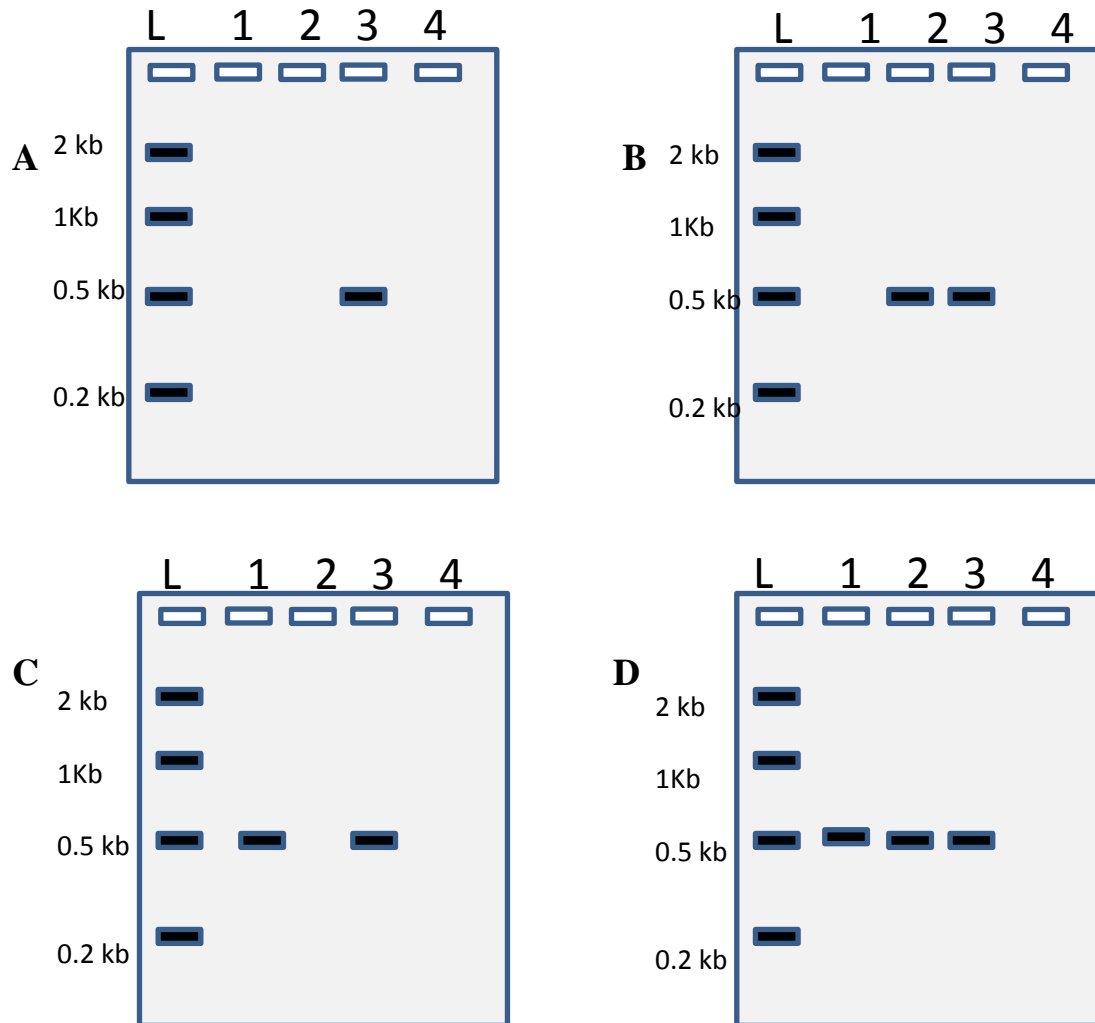
- a) If the transformed cells were maintained in media containing ampicillin (amp), which of the following would allow for PCR amplification of the GFP gene? (write +++, + or – next to each)
(NOTE: “+++” is considered correct because a strong PCR band would be amplified in each situation. You may choose to give credit for “+” instead)
- +++ Media containing amp + glucose
+++ Media containing amp + lactose
+++ Media containing amp + both glucose and lactose
+++ Media containing amp but neither glucose nor lactose
- b) If the transformed cells were maintained in media containing amp, which of the following plates would contain green glowing colonies under UV light? (write +++, + or – next to each)
- Media containing amp + glucose
+++ Media containing amp + lactose
+ Media containing amp + both glucose and lactose
-- Media containing amp but neither glucose nor lactose
- c) Explain why the answers to a and b are different, or why they are the same.

The answers are different because in (a) the questions refer to the presence of the GFP gene (DNA) which is present in the cells of *E.coli* during all of the experimental conditions where (b) is asking about the level of GFP protein produced in each condition.

Post-lab Assessment (Learning objective #2)

You have engineered a plasmid that contains the gene for GFP (green fluorescent protein) under control of the inducible lac operon promoter. You then isolate total DNA from transformed *E. coli* (growing in + or – lactose medium) as well as from wild-type *E. coli*.

If you were to use all three DNA samples as template in a PCR reaction to amplify a 500 bp fragment of the GFP gene you would imagine your resulting agarose gel to look like: (lane 1 = DNA from wild-type *E. coli*, 2 = DNA from uninduced transformed *E. coli* and 3 = DNA from induced transformed *E. coli*)

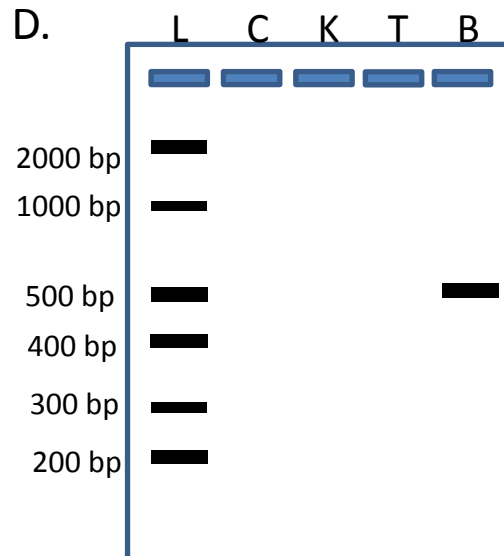
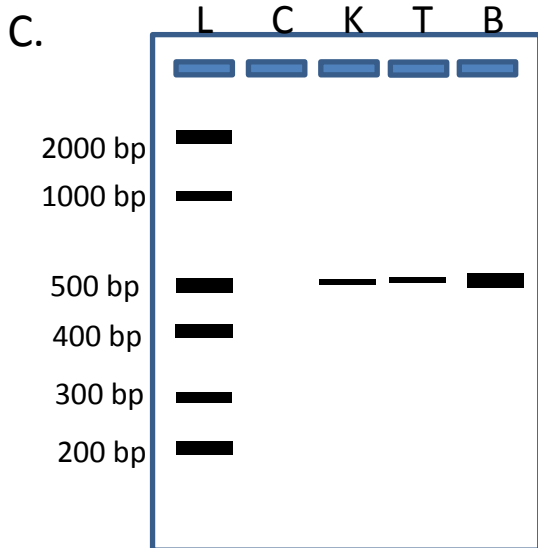
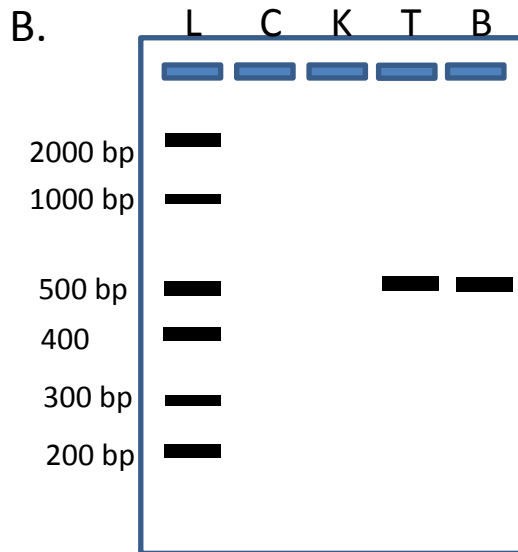
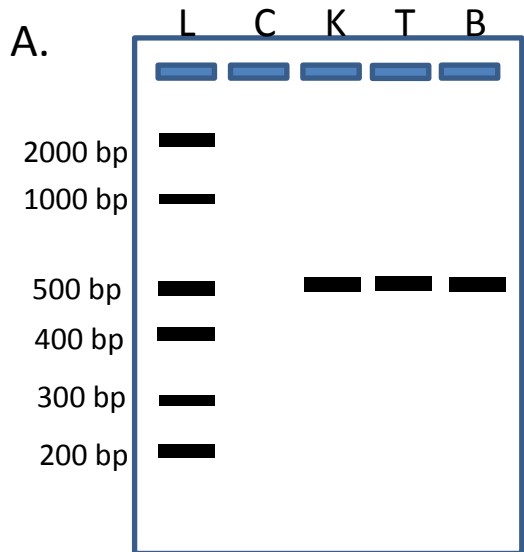


Answer = B

Alternative Post-lab Assessment (Learning objective #2)

You are studying a neuron-specific protein called *Sec1*. You have designed PCR primers to amplify a 500 bp fragment of the *Sec1* gene from various DNA sources. Which of the gels below would you predict? (L = ladder, C = control of no DNA, K = kidney, T = testis, B = brain)

Answer = A

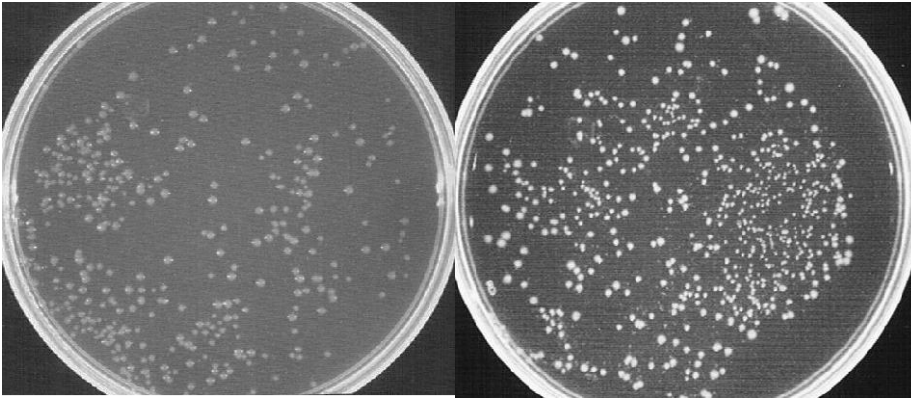


Post-lab Assessment #3 (Learning objective #3)

In the photo below, examine the results of *E. coli* after transformation with pGLO DNA.

A. LB + amp

B. LB + amp + ara



A higher level of Green Fluorescent Protein (GFP) is being expressed on which plate? Explain.

True or False? *E. coli* on both plates contain the genetic instructions for producing Green Fluorescent Protein. Explain.

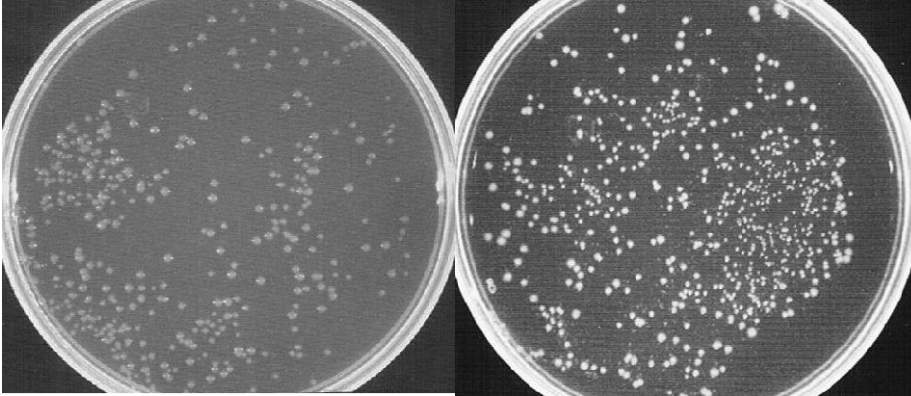
Post-lab Assessment #3 (Learning objective #3)

Answer Key

In the photo below, examine the results of *E. coli* after transformation with pGLO DNA.

B. LB + amp

B. LB + amp + ara



A higher level of Green Fluorescent Protein (GFP) is being expressed on which plate? Explain.

Answer: The plate containing arabinose (ara) contains a higher level of GFP protein because the expression of the GFP gene is induced by the arabinose. The expression of the GFP gene results in GFP protein which glows under UV light.

True or False? *E. coli* on both plates contain the genetic instructions for producing Green Fluorescent Protein. Explain.

Answer: True. Both *E. coli* strains have been transformed with the pGLO plasmid which contains the gene for GFP. *E. coli* will not express the GFP gene, however, unless the arabinose inducer has been added to the media.

Appendix 4: Reflection Activity

Name _____

Please complete and hand in to your instructor before leaving the lab today!

1. Place a copy of your gel image with your PCR results. Make sure all lanes are labeled and provide an appropriate figure legend below the gel.

2. Do you see a “band” around 714 bp? What does this represent?

ANSWER: This is the DNA that was amplified. It is the size of the distance from the 5' end of the forward primer to the 5' end of the reverse primer.

3. Do you see any other bands on the gel (discounting the DNA ladder)? If so, what are they?

ANSWER: Students may see primer-dimer or some background smearing.

4. Do you see any genomic or plasmid DNA on the gel? If not, why?

ANSWER: There should not be any genomic or plasmid DNA visible. This is because it is very low in copy number (concentration) compared to the amount of DNA synthesized during the reaction.

5. From which samples were you able to amplify the GFP gene? What is the size of this band in each lane in which it is present?

ANSWER: Students should see the same 714 bp band in the lanes from E. coli + pGLO both with and without arabinose. They should not see it in the lane of wild type E. coli (no pGLO).

ANSWER KEY to Post-lab Reflection:

1. *Gel will look something like this:*

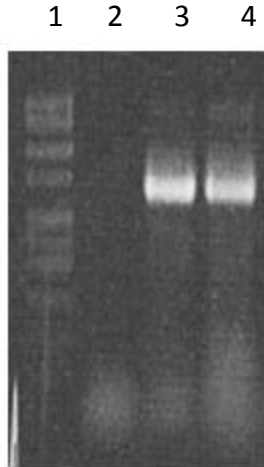


Figure Legend: PCR amplification of a fragment of the GFP gene from E. coli transformed with pGLO. Lane 1: ladder; Lane 2: wild type E. coli without pGLO; Lane 3: E. coli + pGLO grown in absence of arabinose; Lane 4: E. coli + pGLO grown in presence of arabinose.

2. *This is the DNA that was amplified. It is the size of the distance from the 5' end of the forward primer to the 5' end of the reverse primer.*
3. *Students may see primer-dimer or some background smearing.*
4. *There should not be any genomic or plasmid DNA visible. This is because it is very low in copy number (concentration) compared to the amount of DNA synthesized during the reaction.*
5. *Students should see the same 714 bp band in the lanes from E. coli + pGLO both with and without arabinose. They should not see it in the lane of wild type E. coli (no pGLO).*