Lab Recap: PCRG Mockups

Analysis Part I

Purpose: To use your PCR gel to approximate the size of your duckweed insert and to analyze problems in your prep.

Vocabulary:

Standard - The thing you added to the first gel lane. It contains DNA fragments of known lengths, which you will use to find out the length of your unknown duckweed DNA fragments.

Insert - The part of the plasmid vector that contains your duckweed DNA.

Vector - The circular ring of DNA that was initially in the bacteria you picked on the first day. This ring contains a variety of selectable markers, along with your duckweed DNA insert.

Primer - Short strands of DNA that provide a starting point for replication. You added these to your PCR tubes at the end of the PCR lab. These are required for the PCR reaction to happen.

Contamination - Picking two bacterial colonies, resulting in two separate duckweed inserts. A contaminated clone cannot be published. If your prep is contaminated, it will be very evident in the PCR gels because you will see two distinct bands.

Mock-up - Formal analysis of a gel.

Up until this point.

In the ON lab, you picked a colony of bacteria and added it to a tube to grow overnight. That bacteria contained a vector (a circular ring of DNA). That vector contained the duckweed DNA insert. In the PCR lab, you set up a PCR reaction using some of the bacteria you grew. The PCR reaction amplified the duckweed DNA insert region of your vector (plus a few extra bases). In the PCRG lab, you ran your PCR samples through a gel. Now you will be mocking up your gels, or formally analyzing them.

What you will be doing

1. Downloading the PCRG Mockup Template (Download File).
2. Following the directions on the powerpoint.
3. Inputting your data into the clone report sheet. If you cannot determine the size of your insert (i.e. contamination or lab error) you must put N/A in the box (exception: if it is contamination, put contam.). You also must COMMENT your explanation on the clone report sheet (insert→comment) if you cannot determine the size of your insert.
    a. How can you determine what problems occurred? We have provided a troubleshooting list to make your lives easier. It is imperative that you read it sequentially.

Things to understand before you start reading the troubleshooting

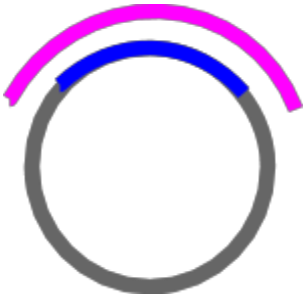
What does the brightness of the bands mean?

- The brighter the band, the more the amount of DNA present.
    - Recall that we can see the bands on the gels when we expose them to UV light because we added stain to the gels, which adds radioactive tags to DNA.
    - Those tags glow under UV light. So, if you have a lot of DNA, that means that you have a lot of places that the stain can add those radioactive tags to--so more glowing! A lot of DNA is a good thing!
- What if my band is faint?
    - A faint band COULD mean that you don't have a lot of DNA
        - You could have not added enough bacteria to your dilute culture, so less DNA could be amplified→less DNA in your PCR tube→lighter band
        - You could have not closed your PCR tube properly in the PCR lab. If this is the case, you would have noticed that your PCR tube had less liquid in it than the other tubes in your group.
    - A faint band could ALSO mean that you messed up with loading your gel or with adding your loading dye. If you think this is the case, review the gel loading or loading dye mistakes section at the end of this file.

Why do we subtract 200?

Refer to the figure on the right.

- The gray circle represents the vector.
- The blue line represents the duckweed insert.
- When we use PCR to amplify that insert, we actually amplify the pink region--the insert plus a few extra bases from the vector on the outside.
- Therefore, the PCR fragments in our gel contain a lot of copies of our duckweed attached to some vector nucleotides. Since those vector bases that are amplified amount to about 200 base pairs, you should subtract 200 base pairs from whatever size you determine when you match up your PCR fragment to your standard.



Why might there be smearing on my gel or why is my lane brighter than other lanes?

- In most cases, smearing or bright lanes just means that you have a lot of DNA which is generally a good thing because it means PCR worked well
- In some cases, smearing means that the gel was not made properly (some agarose was not melted)--but that's unlikely because we're pretty good with making gels.
- As long as smearing does not interfere with determining the size, don't worry about it

What are the primers and where can I see them?

- Again, primers are the short strands of DNA that you added to your PCR tube because they provide a starting point for replication.

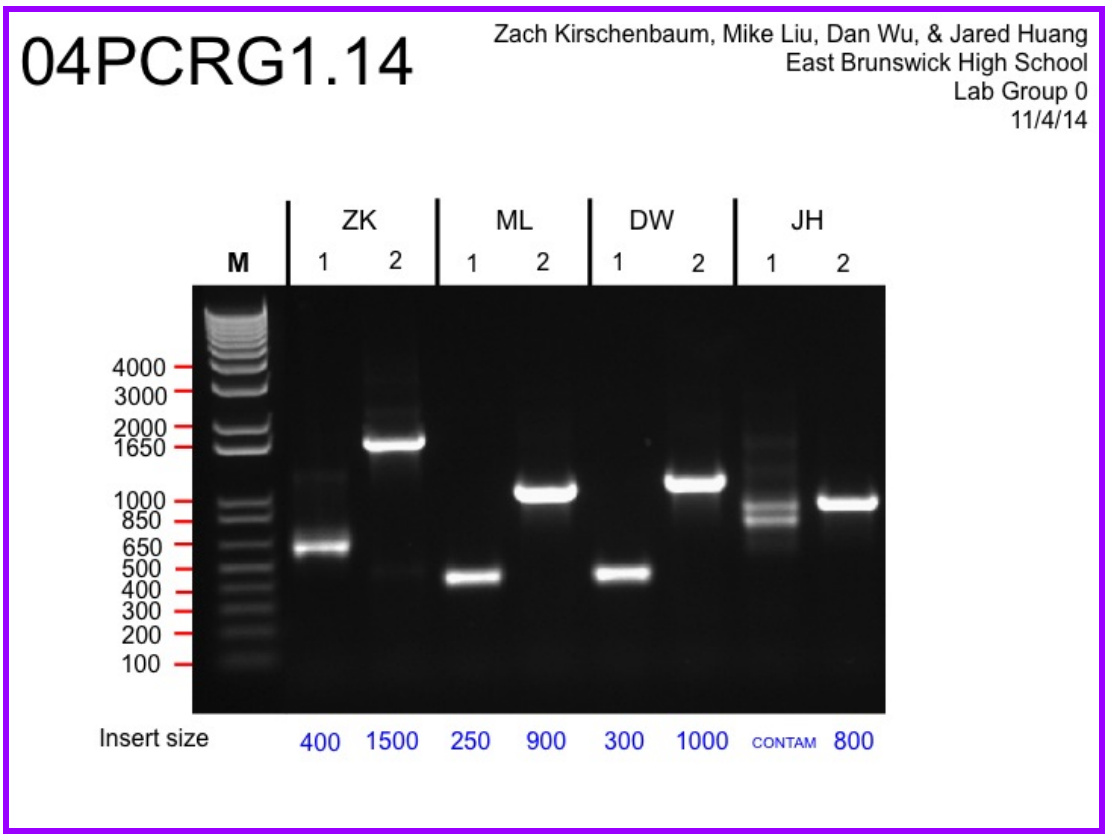
- Since the primer length is about about 40 base pairs, you should see relatively bright primer bands toward the bottom of your gel.
- So when we refer to non-primer bands, we mean bands other than those primer bands at the bottom

Why does the top of the gel look brighter than the bottom?

- The stain, which makes the bands visible when we flash UV light on the gel, actually has a net positive charge
- DNA (negatively charged) migrates down the gel because the top is negative and the bottom is positive
- Stain (positively charged) somewhat migrates up the gel away from the positive and toward the negative
- Since the stain migrates up, there will be less stain at the bottom so the bands will be lighter

How do I use the standard to approximate the size of my insert? (refer to figure below)

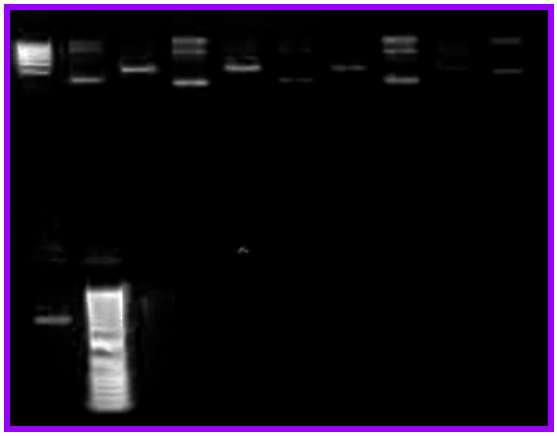
- Align your standard on the left
- Draw an imaginary line from your unknown band to the standard lane
- Subtract 200 from the size predicted by the standard (why? see above)
- Example: 04ZK1.14 matches up to about 600 on the standard lane. 600-200=400. The insert is approximately 400 base pairs (400bp). Try and do the rest on your own!



PCRG Mockup Troubleshooting

*Read this **sequentially**. Imagine you are in computer science class and reading if statements. If you made a mistake, put N/A in the mockup. Explain the mistake in a comment on the CRS.*

1. Do you have normal bands in your marker/standard lane?
    a. If yes, continue. There were no problems with your gel or how it ran.
    b. If no, then that means that there was an issue with the gel itself or how it ran. Gel issues could be:
        i. We forgot to add stain to your gel when we made your gel. Since the stain allows us to see the bands on the gel when we shine UV light on the gel, no stain means that no bands will be visible.
        ii. You did not run your gel properly. You should have ran you gel with a negative/black electrode at the top and a positive/red electrode at the bottom so that the negatively charged DNA moves down the gel. If you reversed the electrodes, then the gels would have ran in the reverse direction. Therefore, the fragments would have ran up, off the gel, and into the buffer. If the fragments are not on the gel, then you will not be able to see them. Lab group 2 made that mistake in 2012. The top of the gel is pictured to the right. Notice how the standard from the bottom of the gel migrated to the top of the gel.

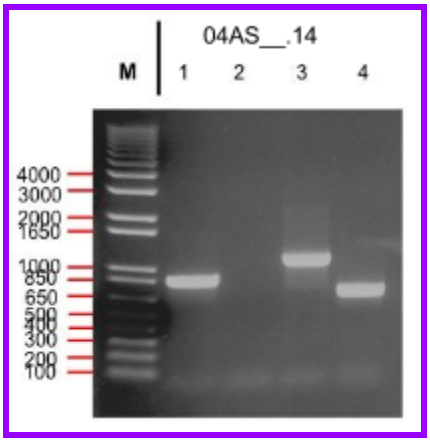


        iii. More of a problem with your ability to read labels: You forgot to load standard or loaded loading dye instead of standard

2. Do you see the primer bands at the bottom of your unknown lanes (even if they are very faint)?
    a. If yes, continue. You remembered to add primers.
    b. If no, that means that you made one of the following mistakes:
        i. Your group forgot to add primers to the 5X primer mix. This could have occurred only if everyone in your group does not have primer bands.
        ii. You forgot to add the primer mix to your PCR tube. This could have occurred only if other people in your group had visible primer bands.
        iii. You did not close your PCR tube properly in the PCR lab. If this is the case, you would have noticed that your PCR tube had less liquid in it before you loaded your gel, compared to other tubes in your group.
        iv. You made a gel loading or loading dye mistake (so you did not get enough DNA in the well). See last page for what that could have been.

3. Is there at least one person in the class with a visible non-primer PCR band on their gel?
    a. If yes, continue. There were no issues with the PCR machine.
    b. If no, then that means that one of your co-presidents or Ms. Sanelli did not set the thermocycler (the PCR machine) correctly. Or, there was a problem with the PCR machine. This is very unlikely because your co-presidents and especially Ms. Sanelli are ***Flawless and would never make that mistake.

4. Do you have any non-primer bands in your unknown lanes?
    a. If yes, continue. That means that PCR worked.
    b. If no, then that means that you made one of the following mistakes:
        i. You did not add bacteria to your dilute culture tube. Therefore, when you added the dilute culture to the PCR tube, there was no bacteria in the PCR tube. Therefore, you did NOT provide template DNA so the primers did NOT have template DNA to anneal to, the taq polymerase did NOT extend any new DNA strands, and your PCR reactions did NOT work. If PCR reactions didn't work, then you did not amplify your insert and you will not see any bands representing your PCR sample. Bands=DNA, and if you have no DNA, that means no bands. Remember you added very little bacteria to your dilute culture, so there is a pretty good chance that you pipetted incorrectly because it's really hard to handle small amounts with pipets. Clone 04AS2.14 on the right indicates this mistake.



        ii. You did not pick a colony in the ON lab or your colony did not reach your culture tube. If your culture tube looked clear when you got it back the day after the ON lab, that means you did not have any bacteria and you did not pick a colony. If your culture tube looked cloudy, then this mistake can be ruled out because you know that you did pick a colony.
        iii. There was something wrong with your PCR bead. If your bead did NOT look like a sphere (i.e. it was cracked or leaned on the side of the tube), then that means the enzymes inside the tube did NOT function properly. Therefore, PCR reactions did NOT work and you don't see any non-primer bands in the unknown lanes because bands = DNA.
        iv. Gel loading or loading dye mistake. See end of this file for details.

5. Do you have only ONE non-primer band in your unknown lane?
    a. If yes, continue.
    b. If no, the following problems/events could have occurred:

i. IF they are the same intensity/brightness, your sample is contaminated. That means that for whatever reason, you picked more than one bacterial colony in the ON lab. Therefore, you had two separate plasmid vectors and amplified two separate duckweed inserts. Therefore, you will see two separate bands of two separate sizes. The bands will most likely be visible, but not very bright because two inserts means more DNA to amplify/replicate, so the primer/enzyme to DNA ratio goes down, slowing down the PCR rate and lowering the amount of DNA produced at the end (dimming the brightness of the band). Refer to the figure on page 3. 04JH1.14 is contaminated.

ii. IF they are not the same intensity, then you actually do not have any problems!!! Use the brighter one to calculate your insert size. Sometimes primers can anneal to themselves and nucleotides get added, or they can anneal to random sections of the plasmid vector (not the just the region that contains the insert). In both cases, you will get faint bands of different sizes. This is random so don't worry about this. If you can't distinguish differences in intensity, call someone over.

iii. IF there are A LOT of bands and it looks a lot like your standard lane, then you added standard to your PCR samples instead of loading dye. Shame on you--you just wa$ted a lot of money.

6. If you've reached this point then that means you didn't make any mistakes! GJ! Now all you need to do is match up your band to the standard. Then, subtract 200 from what the standard tells you. Great work--you are almost at the level of ZK and BP. One day you might even be at the same level as the legendary AS!

***End of troubleshooting**

Gel loading or loading dye mistakes

All of these mistakes mean that you did not properly get DNA into your well. The biggest sign of this is that you do not see the dark blue DNA in the well after you pipet.

- You punctured the gel when you were pipetting. That is something that you would have noticed because you would have realized that you were trying to release liquid but nothing was initially coming out.
- You missed the well when you were releasing your PCR samples into the well. For example, you could have released when you were not inside the well, therefore the DNA floated away into your buffer.
- You released too quickly, so the DNA bounced out of your wells and floated away out of the wells and into the buffer.
- You did not add any or enough loading dye. Since loading dye allows your samples to stay into the well without floating away, if you do not have enough loading dye, then your samples will simply float away. If your samples were dark blue before you loaded, then this is probably not the problem.