Module IV: MAKING A GM OUT OF A GM

Background*:* Genetically modified organisms are a broad category that include bacteria, plants, animals, and fungi. We will use a genetically-modified plant (the Kaleidocell *Arabidopsis* plant that you used for your positive control in the GM lab) to make a genetically-modified bacteria. We will be combining several of the techniques you have learned this semester (PCR, Restriction digestion, ligation, gel electrophoresis) in order to accomplish this goal.

Flow chart

PCR of GFP with primers that include restriction sites (Week 1)

Gel electrophoresis to confirm amplification worked (Week 2)

Restriction digestion of PCR product **and** pUC19 plasmid (Week 3)

Ligation of digested PCR product (insert) into pUC19 plasmid (vector) (Week 3)

Transformation of recombinant plasmid into *E. coli* DH5 alpha cells (Week 4)

Selection and observation of GFP fluorescence using 488 laser light. (Week 5)

Week 1: Amplify GFP gene using primers that include restriction sites

First step of the is protocol would be to extract your template DNA from a genetically-modified plant that contains GFP. This needs to be a much cleaner DNA extraction then what we did for the LAMP reactions because we need a lot of template to get enough product for cloning later. I have done the DNA extraction step for you. I used a kit called PureLink™ Plant Total DNA Purification kit (Invitrogen) but need to explain the chemistry involved.

Attached to this module is the kit protocol. Steps in the protocol will be explained below.

1-2. The first and second steps are similar to what you have already done to extract plant DNA before – you broke up the plant by grinding it in a solution. In the case of the kit, the R2 solution is a detergent (not just water like what you used). This detergent called CTAB, is a mild hydrophobic compound that will aid

lysing of the plant cell membrane, assuming you have already removed the cell wall by mechanical disruption (breaking it open by grinding the plant tissue).

3. SDS is a strong ionic detergent and will help break down proteins in the cell membrane. RNAse A will destroy any RNA.

4. Then heat at 55 ^{o}C for 15 minutes to ensure complete lysis of the cell membrane. The combination of the heat, CTAB detergent, SDS, and RNAse A means that at this point, the plant cell should have most of the proteins denatured, RNA destroyed, lipids dissipated, and only sugars and DNA left to deal with. We just need to separate this 'debris' from the DNA and the rest is simply washes.

5. Centrifuge to separate debris from DNA. The supernatant contains the DNA.

6. Next is to add the Precipitation buffer (N2) which precipitates out the proteins.

7. Centrifuge to remove precipitated proteins. DNA is left in the supernatant.

8. Binding buffer B4 (isopropanol plus salts) is added to the DNA which is then added to a Purelink™ spin column to precipitate the DNA onto the membrane that is part of the column. Alcohol is an amphipathic molecule and will remove all water molecules away from DNA – precipitating it.

The rest of the protocol is a series of washes with 70% or higher alcohol (ethanol) to remove extra salts. The final step of the protocol is the addition of an elution buffer, a buffer with a specific pH (pH 7.5 – 8.0) that will release the DNA from the membrane in the column. The final product is DNA in the elution buffer in a clean 1.5 ml tube. T**his is the DNA template I have provided for you.** The procedure from this kit should sound very similar to what was done for the extraction of human DNA; a few additional reagents and centrifugation steps added, and a step for breaking down the plant cell wall, but the rest of the protocol is essentially the same.

What will you do?

In Lab

Template of DNA extracted from Kaleidocell *Arabidopsis* plants will be provided to you. You need to set up the following PCR reaction per a group. Please note that we always include a no template control in all PCR reactions to ensure that amplified product is the result of the template added and not anything else in the reaction mix.

PCR X 2

2X PCR Master mix (PCR MM tube)	25 µl
20 µM HGFP for	1.25 µl
20 µM EGFP rev	1.25 µl
Sterile dH20	18.5 µl

23 ul/ reaction + 2 ul of template (plant or water)

Bonus opportunity!!! +3 pts onto your final notebook check grade. In your last notebook check – calculate the final concentration of the PCR master mix and primers used in this PCR reaction. Note the final concentrations need to be per a reaction. Use the formula C1 V1 = C2 V2. I will be glad to help you with this but the easiest way to learn this equation is to use it. E.g. problem. I have 10 mM dNTP mix and use 0.5 ul in a single PCR reaction. C1 = 10 mM V1 = 0.5 ul, C2 is unknown, V2 = 25 ul PCR reaction. Solve for C2.

HGFPfor: 5' AAGCTTC ATGTCGAAGGGCGAGGAG 3'

EGFPrev: 5' GAATTC CTACTTGTACAGCTCGTCCA 3'

Note: the underline portion is the sequence of the restriction site attached to each primer. H = *Hin*d III restriction site added; E = *Eco*RI restriction site added.

PCR program:

95 °C 2 min

95 °C 1 min
58 °C 1 min
72 °C 5 min
] X 50 cycles

12 °C hold

Bioinformatics part

We need to start annotating the position of the primers and predict the size of the PCR product expected based on where the primers bind to the GFP gene region. All this information should be in your lab notebook.

1. Download the program ApE from the following website: https://jorgensen.biology.utah.edu/wayned/ape/
2. Copy and paste the GFP sequence below into ApE and save the file.
   ATGTCGAAGGGCGAGGAGCTGTTCACCGGCGTCGTCCCGATCCTGGTCGA
   GCTGGACGGTGACGTCAACGGCCACAAGTTCTCCGTCTCCGGCGAGGGTG
   AGGGCGACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC
   GGTAAGCTGCCGGTCCCGTGGCCGACCCTGGTCACCACCCTGACCTACGG
   CGTCCAGTGCTTCTCCCGCTACCCGGACCACATGAAGCGCCACGACTTCTT
   CAAGTCCGCCATGCCGGAGGGTTACGTCCAGGAGCGCACCATCTCCTTCAA
   GGACGACGGTAACTACAAGACGCGTGCCGAGGTCAAGTTCGAGGGCGACA
   CCCTGGTCAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGT

AACATCCTGGGCCACAAGCTGGAGTACAACTACAACTCCCACAACGTCTAC
ATCACCGCGGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGACCCG
CCACAACATCGAGGACGGTGGCGTCCAGCTAGCCGACCACTACCAGCAGA
ACACCCCGATCGGCGACGGCCCGGTCCTGCTGCCGGACAACCACTACCTG
TCCACCCAGTCCGCCCTGTCCAAGGACCCGAACGAGAAGCGCGACCACAT
GGTCCTGCTGGAGTTCGTCACCGCCGCCGGCATCACCCACGGCATGGACG
AGCTGTACAAGTAG

3. Using control F – find where the two primers we are using for our PCR reaction bind to this GFP sequence. Remember that the restriction sites are not going to match to the GFP sequence. You need to search first for the part of GFP that each primer will bind to. Once this region is highlighted in the input GFP sequence, click 'Feature', select 'New Feature' from the drop-down menu, type a name for the region in the box and click 'OK'. This will annotate the region that the primer binds to. Make sure to search for the rev comp for your reverse primer.
4. Now add the restriction sites to the regions that each primer binds to. You can do this by just typing in the nucleotides for each restriction site on the 5' end of where the primer will bind. Remember that your reverse primer will actually bind in the complimentary orientation – so you will need to add the restriction site to the 3' end of the reverse primer sequence. Add each restriction site as a 'New Feature'.
5. Save your file. Click the circle icon to see the annotation of your GFP sequence with the primers. Print this out and put in your notebook. From this annotation you should be able to get an idea of the size of PCR product you expect from the PCR reaction. Tell this to your lab partner and provide them with a 'predicted gel image' so they know what to look for next week.

Week 2: Gel electrophoresis to see if PCR worked. PCR Cleanup.

In Lab

1. You need to run your PCR products out on a gel to see if the reaction worked. Since we need to use these PCR products later we will **not** run all of the sample on the gel. ***Only run 5 ul of your PCR product on a gel. Add 1 ul of loading dye to 5 ul of PCR product in a new tube. DO NOT ADD LOADING DYE TO THE REST OF THE PCR PRODUCT.***

   You will use the same DNA ladder that we have been using all semester for your standard to determine if you got the expected size for your PCR product.

2. While the gel is running we need to clean-up the template PCR reaction (**Not the NTC reaction)**. This is to remove unused dNTPs, primer, etc. and separate it from your PCR product. We will use the GeneJet™ PCR Clean-up kit (Thermo Scientific) to do this. Follow the steps below to perform your PCR clean up.

a. Add 1:1 volume of Binding Buffer (BB) to your left over template reaction, **not your NTC.** (You used 5 µl of PCR product in your gel so you should have 20 ul left). This means add 20 µl of (BB) to your 20 µl of PCR product in the PCR tube.
b. Mix thoroughly by vortex or flicking. Tube should be yellow for good DNA binding.
c. Transfer the 40 µl of solution from your PCR tube to the spin column.
d. Centrifuge at max speed for 1 min.
e. Discard flow-through and put binding column back in bottom collection tube.
f. Add 700 µl of Wash solution (WS) to the column.
g. Centrifuge at max speed for 1 min.
h. Discard flow-through.
i. Put column back in collection tube and spin at max speed for 1 min.
j. Discard flow-through.
k. Transfer binding column to new sterile 1.5 ml tube.
l. Add 50 µl Elution buffer (EB) to column and let sit for 1 min. at room temperature.
m. Spin at max speed for 1 min. Bottom solution in 1.5 ml tube is your clean DNA – **keep this! Dump column.**

We will store your clean DNA in the freezer (- 20 ºC) until next week.

Chemistry of this clean-up, essentially all we are doing is binding big (<100 bp) of DNA to the column and washing off all the other stuff that was in the PCR reaction. Then eluting the PCR product in the end.

At home or other F2F partner: Your job this week will be to keep track of steps for your lab partner. If streaming, check off steps for the person in lab.

Week 3: Restriction Digestion and Ligation

Now that we have confirmed that PCR has worked and we have amplified the GFP gene with restriction sites *Hin*dIII and *Eco*RI attached, we can start our cut and paste – cutting the GFP restriction sites and 'pasting' into pUC19, a plasmid vector. Plasmids are extra-chromosomal circular pieces of DNA found naturally in bacteria such as *E. coli*. We (mankind) have manipulated these plasmids for our use. Since plasmids are naturally accepted into bacteria cells, can replicate, and have genes on them expressed in bacterial cells, we will use a plasmid to integrate our GFP gene and express the green fluorescent protein from this gene in *E. coli*.

In Lab:

We will be setting up two digests, one for your clean PCR product (GFP with restriction sites) and one for pUC19. Both will be a double digest meaning two restriction enzymes are used in the same reaction. This will be done to give orientation to the insert when

being placed into the pUC 19 vector. (Most of this will make more sense once the at home or other partner has gone over the bioinformatics portion of this week's lab with you.

1. To start though, we need to set-up our digests.

Set-up the following in the order listed:

PCR digest:		pUC 19 digest:
10 X Fast Digest Buffer	2 µl	2 µl
*Eco*RI Fast Digest enzyme	1 µl	1 ul
*Hin*dIII Fast Digest enzyme	1 µl	1 ul
Template DNA	10 ul of clean GFP PCR	1 ul of pUC 19 (0.5 ug/ µl)
Sterile water	up to total volume of 20 µl	up to total volume of 20 µl

2. Place tubes at 37 ^{o}C (heat block in back of lab) for 15 minutes.
3. Place tubes at 80 ^{o}C for 10 minutes to heat denature enzymes.

Now we set up the ligation reaction.

4. Add in the order listed:

10 X Ligation buffer	2 µl
PCR digest	1 µl
pUC 19 digest	1 µl
DNA ligase	2 µl
Sterile water	up to 20 µl

5. Leave in rack up front until next week. The ligation reactions have to incubate at room temperature overnight. I'll place them in the freezer for you tomorrow.

Bioinformatics (at-home/ other partner)

Among the other files included with this module is a file titled pUC19.ape. You need to download and open this file in ApE. Also, keep your GFP file with the restriction sites open. Let's start with the GFP file. Below is the *Hin*dIII and *Eco*RI restriction sites.

*Hin*dIII	*Eco*RI
A'AGCTT	G'AATTC
TTCGA'A	CTTAA'G

1. On your GFP file – cut (delete) nucleotides to get the appropriate overhangs that would result from a *Hin*dIII and *Eco*RI digest. Save this file under a new name.
2. Do the same for the pUC 19 file leaving the opposing cut ends and cut out (delete) the region of pUC19 between the *Hin*dIII and *Eco*RI sites. Save this file under a new name. Easiest way to do this is to:
    a. Go to 'Enzyme' drop-down menu and click 'enzyme selector'.
    b. Select *Hin*dIII and *Eco*RI and then select to 'Highlight'. This will highlight the two restriction sites in the file making it easier for you to identify where to cut.
3. Now copy your GFP 'digested' sequence over and paste it into the area you deleted from pUC19. If done correctly, you should now have the same restriction sites back to their original form (because you sealed the fragments together), and the program should show GFP inserted into pUC 19.
4. Lastly, click the 'Graphic Map' icon and make sure to click 'circular' in the box at the far top left of the screen. This should show you a circular map of your new recombinant plasmid, the plasmid we are hoping to make in lab. Print-out the picture of your recombinant plasmid and paste it in your notebook.
5. Make sure to share this information with your partner(s) doing work in lab and explain to them what you did to get the recombinant molecule.

Week 4: Heat shock transformation

Background: The recombinant plasmid DNA molecules produced in the previous lab are inserted into *E. coli* cells, this process is called transformation. The *E. coli* cells are then cultured overnight so that each cell divides many times to produce many new cells (typically there are 2 billion cells per ml after overnight culturing). Each new cell receives a copy of the vector, with each vector containing a copy of the inserted PCR product. As long as our plasmid contains an 'origin of replication' it will be replicated using the host cell's machinery many times over.

We will be using a strain of *E. coli* call JM109 today for transformation. The heat shock technique for bacterial transformation was developed by two scientists named Mandel and Higa in 1970. This technique requires using early mid-log growth, cold $CaCl_2$ competent *E. coli* cells and a process of cold, hot, cold to incorporate foreign DNA into a bacteria cell. The $CaCl_2$ provides a shield around the *E. coli* membrane of positive charge to avoid repulsion of the negatively charged plasmid DNA from the normally negative charge of the interior of *E. coli* cell membrane. Cold stabilizes pores of adhesion to enable plasmid transfer. Heat opens the pores to allow plasmid in, cold then shuts the pores behind.

The cells used for transformation must be kept on ice constantly until the heat shock treatment.

In lab

Transformation protocol:

1. Add 20 ul of *E. coi* DH5 alpha cells to a 1.5 ml tubes on ice.
2. Add 2 ul of recombinant plasmid to the cell an keep on ice for 10 min.
4. **Carry your ice box to the hot water bath.** Heat shock the cells for 45 seconds by transferring the tube **directly** from ice to heating block set at 42 ^{o}C then return **directly** to ice for 2 min.
6. Add 250 ul of SOC. Place in shaking incubator set at 37 ^{o}C for 40 min.
8. Get a LB Amp plate and add 100 ul of your culture to the center of the plate. These plates also have X-gal and IPTG on them. Spread your culture sample on the plate as demonstrated by your instructor for the negative control plate. Incubate at 37 ^{o}C overnight. I will place the plates in the fridge the next day. The importance of X-gal and IPTG is in induction of β-galactosidase, an enzyme encoded in part but the *lacZ* gene that you have inserted your GFP inside of.

Bioinformatics part

Please watch the following video for more about heat shock transformation. https://www.youtube.com/watch?v=7UI9RVYG5CM so that you can better understand heat shock transformation.

Week 5: Analysis of Plates

Following the transformation protocol, the contents of each of these tubes were spread on a separate plate last week (all plates contained ampicillin as well as IPTG and x-gal). IPTG induces expression from the *Lac* operator by inhibiting the repressor. *LacO* is located in pUC 19 right in front of the *lacZ* gene/ where you inserted GFP. The addition of IPTG is to twoX-gal is an artificial substrate of galactose. β-galactosidase normally cleaves galactose. The 'X' here indicates a bromide ring which is clear when attached to galactose but will turn blue if freed from galactose due to enzymatic activity by β-galactosidase (Fig. 1). If you have successfully inserted a GFP into the β-galactosidase gene (alpha subunit of *lacZ* gene) located on the pUC19 plasmid, will β-galactosidase still be active? This is how we 'select' for positive transformants. If β-galactosidase is active, the bacteria should be blue, if not functional the bacteria will be white. This is called blue/ what selection.

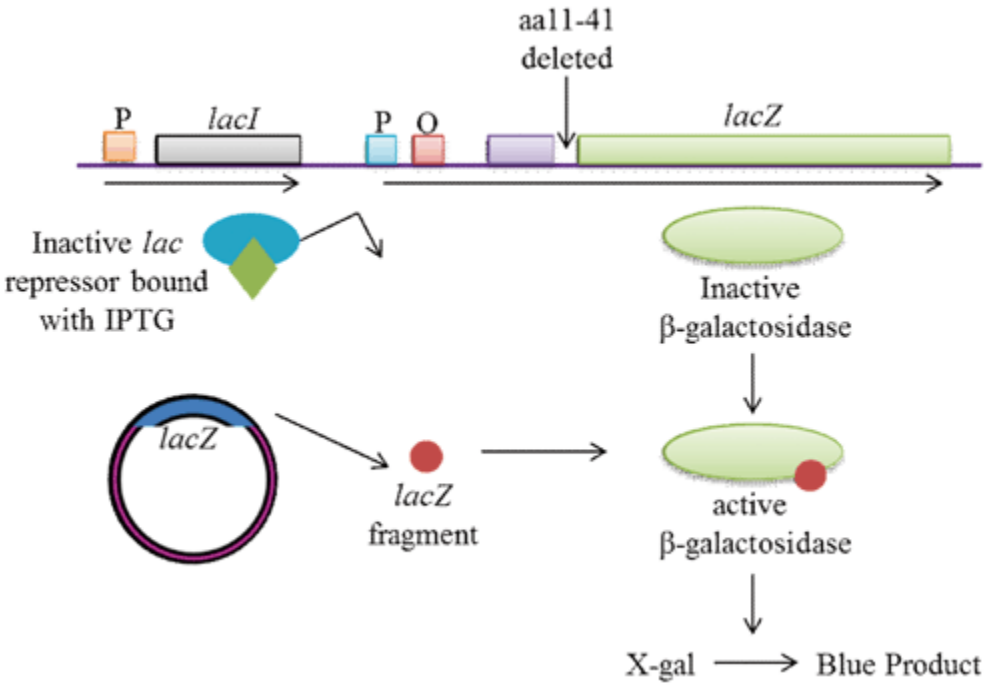
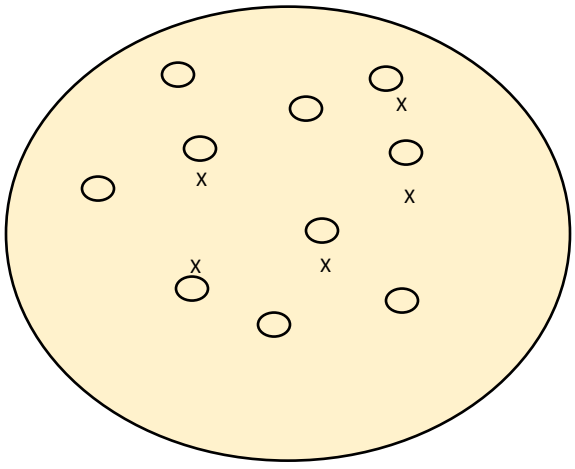


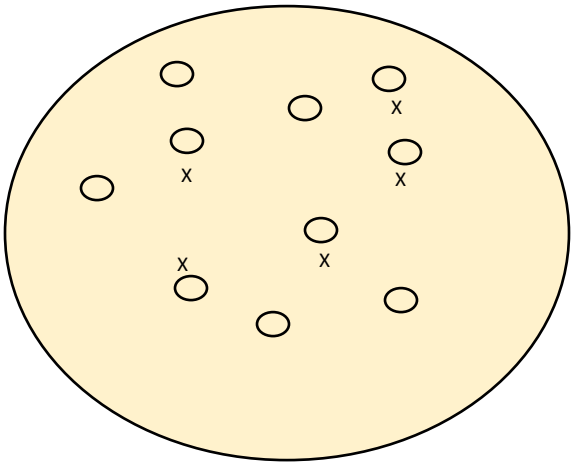
Figure 1. Image of X-gal/ IPTG induction from Das S., Dash H. (2015) Cloning and Transformation. In: Microbial Biotechnology- A Laboratory Manual for Bacterial Systems. Springer, New Delhi. https://doi.org/10.1007/978-81-322-2095-4_2.

1. Look up pictures of blue/ white selection to get a better idea on what this would look like.
2. Fill in the picture below with your prediction of which colonies would be blue vs. white. A recombinant clone is marked with an X next to the colony.
3. Fill in the picture below with your prediction on which colonies (same ones as in the blue/ white picture) will glow green due to expression of the GFP **and make sure to include these predictions in your lab notebook.**

Blue/ white selection



GFP fluorescence



4. Take a picture of your plate and analyze your results. Record findings in your notebook. End of Module IV! Congratulations! You have made a genetically-modified recombinant bacteria glow!