Fluorescent Protein Discovery Project

Project Overview

In a semester-long project, we will utilize modern genetic engineering methods to evolve new proteins and explore the applications of fluorescent proteins in research and industry. Specifically, we will develop new color variants from the gene that encodes red fluorescent protein (RFP).

The protocols we will use include mutagenic PCR of the *mRFP1* gene, insertion of these randomly mutated gene copies into pET-15B plasmids, and transformation of competent *E. coli* BL21-DE3 cells. These protocols will generate bacteria that express the mutated *mRFP1* gene, which we will screen under UV light for the expression of new variants of RFP, some of which might no longer be red! Each student team will choose a few candidate gene variants to sequence. The genetic sequence will allow you to identify the changes in the genetic code that resulted in the change in fluorescence from the original red colour.

The original DsRFP protein, isolated from the coral species *Discosoma striata*, posed challenges to researchers because its tetrameric quaternary structure tends to aggregate in host cells, which results in toxicity. The variant of the RFP we will be working with, encoded by a gene called *mRFP1*, is monomeric and distantly related to the original DsRFP (Shaner *et al.* 2004). Notably, the monomeric RFP was originally created through mutagenic PCR (Shaner *et al.* 2004), the same process we will be using this term, and is now a widely used RFP in research.

The project this semester is inspired by Project Light-Bright, a kickstarter-like campaign conceived of and launched by Eric Gaucher and members of his lab group at Georgia Tech in 2014. Their goal was to develop an open source library of fluorescent proteins that could be made freely available to other researchers, specifically those studying cancer. You can view the Gaucher group's video about the project at https://youtu.be/zVQs-yqhCFU

Fluorescent Protein Background

The discovery and development of fluorescent proteins has had far ranging impacts in the fields of cell, molecular, and developmental biology. Most commonly, genes that encode fluorescent proteins are used to indicate whether a gene of interest is being transcribed and translated into protein. The fluorescent gene is inserted into the DNA sequence near the promoter of the gene of interest. When the gene of interest is transcribed, the fluorescent protein is also transcribed and

lights up to indicate where the protein of interest is, reporting out to the researcher that the gene of interest also has that behaviour. When we use the flourescent protein as a visable marker, we call it a "reporter gene." As a corallary, the amount the gene of interest is expressed in living cells can be measured using the intensity of fluorescence, usually without disrupting normal cellular processes.

Fluorescent proteins, also called fluorophores, can be attached to molecular probes that selectively bind and visually identify certain DNA or RNA sequences in protocols like fluorescence *in situ* hybridization, or FISH. In immunofluorescence, fluorophores can be attached to antibodies and used to target a wide range of biomolecules. For instance, proteins unique to a cancer cell can be targeted by a fluorescent probe to assist in real-time removal of cancerous tumors. Our project this semester aims to expand on the open source library of fluorescent proteins currently available to researchers.

Eric Gaucher and colleagues (Randall et al 2016) derived a set of *mRFP1* fluorescent variants using multiple rounds of mutagenic PCR, where each dot represents a fluorescent type (Figure 1). Ten of these mRFP1 variants are available to us this semester (boxes). These gene variants express a rainbow of colour options, so each lab team will have a gene of interest that encodes a different color protein.

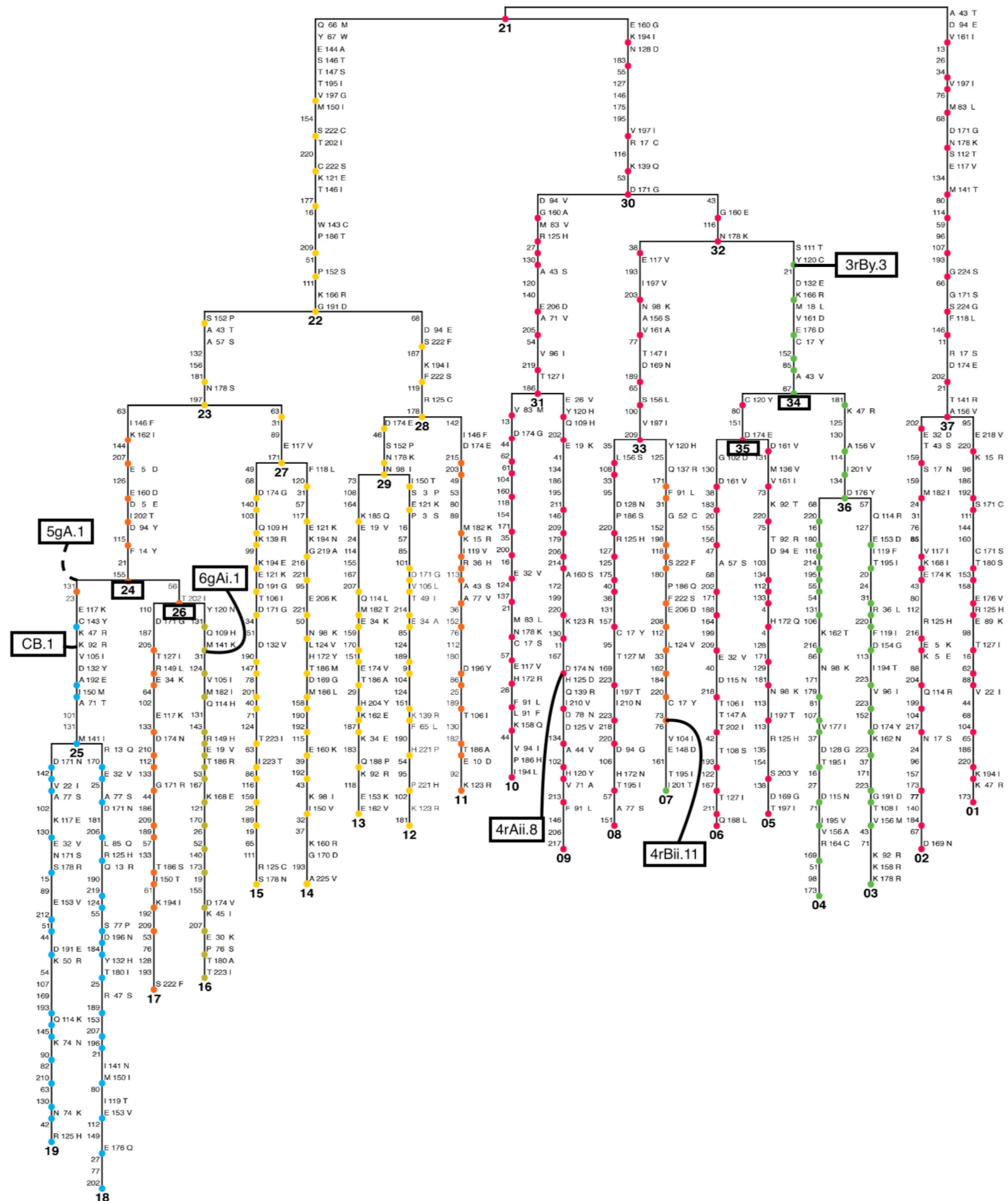


Figure 1. Branching diagram of multiple rounds of mRFP1 mutagenesis. Filled circles represent each round of mutagenic PCR, where the color of the circle indicates the flourescent protein color, and the adjacent text indicates additional genotype information about each variant (see Randall et al 2016). Boxes show the variants available to lab teams in BIOL 2345. The precise origin of genotype 5gA.1 is unknown, but genotype 24 is a recent ancestor. Sequences for these ancestral types are available for analysis and comparison with the sequence work we will do on these variants and their derivatives.

Plasmids

The gene of interest will come to you inserted into a plasmid vector, a small circular piece of DNA. Compared to the *E. coli* genome, which has between 4 and 5 million base pairs depending on the strain, plasmids are tiny but the genes they carry can have a powerful impact on phenotype. While plasmids are naturally occuring in bacteria, they have been co-opted by molecular biologists for molecular cloning, gene transfer, and recombinant DNA work. The plasmid vector we will be using this term is called **pET-15b** and was created by Novagen to meet a wide range of research needs. The entire plasmid is a circular piece of DNA that contains 5,708 base pairs. To understand how the pET-15b plasmid works in our host cells, we need a little background information first:

Generally, plasmids are circular, double stranded DNA, separate from a cell's chromosomal DNA. Plasmids naturally occur in many bacteria and can be transferred from one bacterium to another through the processes of conjugation, transduction, and transformation. If the transferred plasmid contains genes beneficial for the bacterial cell in its current environment (such as antibiotic resistant genes to a bacterium swimming in antibiotic), the bacterial cells with the plasmid will survive and reproduce, making more bacteria with the same plasmid (Figure 2). This also increases the chance that the plasmid will spread via conjugation, transduction, and transformation to other cells in the microbial community.

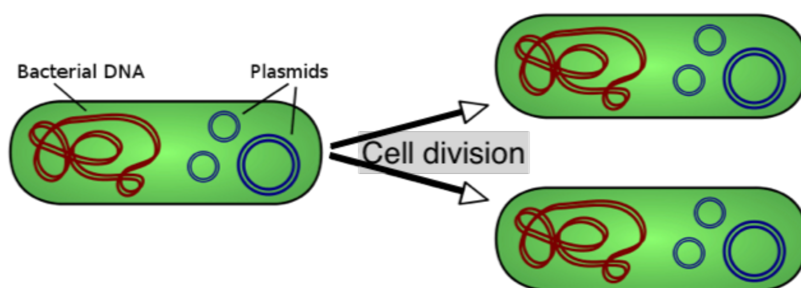


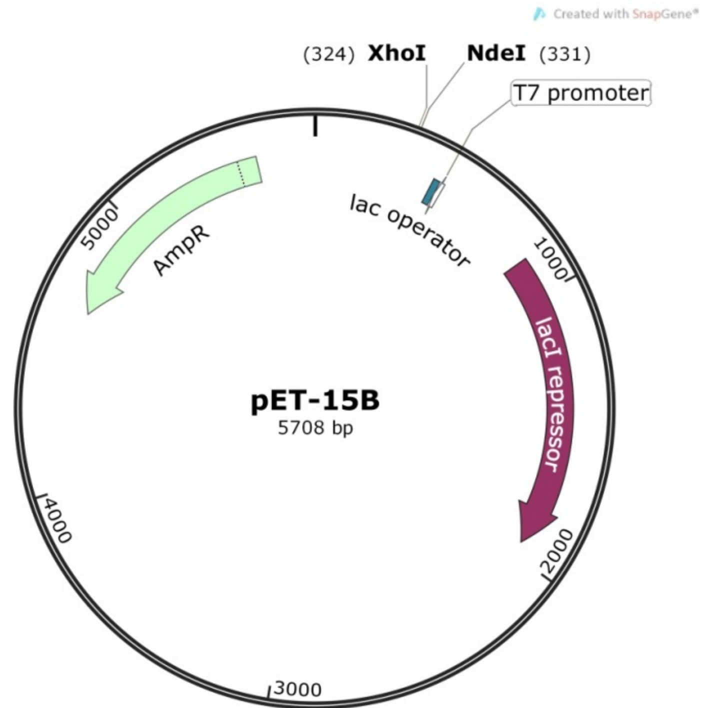
Figure 2. A bacterial cell (green) always contains genomic or bacterial DNA (red) and often contains circular extra-chromosomal DNA (blue). Before the bacterium divides, both types of DNA are replicated. Upon division, both are faithfully inherited by the two daughter cells. Image modified from Spaully 2007: Creative Commons BY-SA 2.5.

About pET-15b

The pET-15b artificial plasmid (map[1] shown at right) contains several genes and DNA sequences that are useful for molecular biology and therefore important to understand:

1. A selectable marker
2. An inducible gene expression system
3. Restriction sites to allow us to insert the gene of interest to a specific location on the plasmid.

Each of these is explained below:



Selectable marker: The gene *ampR* confers resistance to ampicillin, and related antibiotics like carbinicillin, by encoding the enzyme β-lactamase, which is expressed constituitvely (all the time). We can use it as a selectable marker by adding an antibiotic to our bacterial growth media to select for growth only of cells that contain this plasmid.

Inducible gene expression: The gene of interest itself is inserted near a *lac* operon-based inducible gene expression system that will allow us to turn on or off expression of the target gene (*mRFP1*). The lacI repressor protein (also consitutively expressed) binds to the *lac* operator and prevents transcription of any associated genes. When added, lactose, or its molecular mimic IPTG (Isopropyl β-D-1-thioglactopyranoside), will bind the lacI repressor protein, removing the barrier and inducing transcription of the gene of interest.

Restriction endonucleases are necessary to cut the plasmid and prepare the *mRFP1* gene for insertion. Restriction enzymes will cut the double-stranded DNA of the plasmid but only in very specific locations, called restriction sites. Each restriction enzyme has its own unique restriction site. We will be using the XhoI and NdeI enzymes, whose restriction sites are labeled on the plasmid map. The specific DNA sequences that they cut are shown at the arrows on the image below.

NdeI

XhoI

[1] www.snapgene.com/resources/plasmid_files/pet_and_duet_vectors_(novagen)/pET-15b/

To create recombinant DNA and clone our gene of interest, we cut both the gene of interest and the plasmid with the same restriction enzyme, then insert the gene of interest into the plasmid (Figure 3).

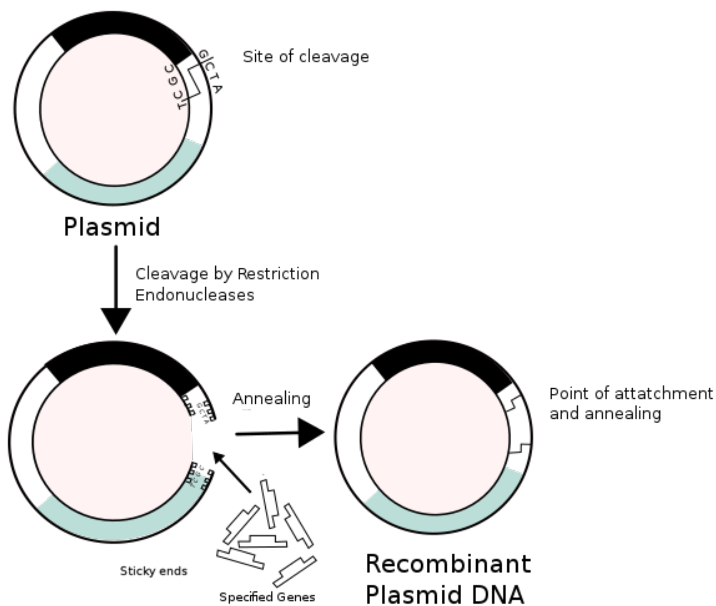


Figure 3. A plasmid is cleaved or cut at the restriction site, leaving sticky ends of DNA exposed. The gene of interest, if cut with the same restriction enzyme, hsas the matching sticky ends for the plasmid to adhere to. Image modified from Minestrone Soup 2007 Creative Commons BY-SA 3.0.

Fluorescent Protein Discovery Project – Week 2

This week we will begin the wet lab portion of the project. Please review the Lab Notebook instructions posted on the course website and be prepared to take careful notes regarding samples, procedures, and anything else that happens to your sample during during the lab period. In each of the following weeks, your team will continue to use the samples and results from today's work, so creating complete notes will be invaluable for an accurate lab notebook entry and when writing up the final Lab Report.

- Form project groups: 2 students (one group of 3 if necessary)
- Each lab team selects a variant of RFP
- Extract and purify the pET-15B + RFP plasmid – 1 reaction / group
- Quantify the plamid concentration using the Nanodrop
- Set up mutagenic PCR of the RFP gene – 2 reactions / group

Bacteria cultures

Several pure cultures of *E. coli* carrying pET-15B plasmids with differnet varients of the RFP gene will b available in lab. These are grown overnight in LB broth containing 100 µg/ml CARB in the shaking incubator at 37°C at 120 rpm.

Plasmid Extraction

The gene of interest is provided to you in a plasmid inside bacteria, so carefully freeing the plasmid DNA from the bacterium while keeping it intact and pure is the first step to ensure we have tigh quality template DNA for PCR. To extract DNA, we will use a Qiagen DNA extraction kit, specially designed for use with plasmids. Commercial kits are used frequently in research settings because they offer reliable and repeatable results. This kit selectively binds DNA to a silica resin spin column, where it is treated with chemicals to remove non-DNA, then released into solution. Here is a little more detail:

The general steps of DNA isolation begin with cell lysis, where salts and detergents damage cell membranes, exposing the contents of the cell while inactivating the bacterial defense nucleases that could damage the exposed genetic material. The salts, along with alcohol, prepare the DNA to bind to the silica resin membrane in the spin column, while the rest of the solubilized contents pass through. The protocol includes several wash steps to remove detergents, salts, proteins and other impurities from the bound DNA. A final drying step removes any residual ethanol, which can prevent the DNA from releasing into solution in the final elution step.

Plasmid extractions have the additional challenge of separating the smaller plasmid from the larger genomic DNA. This is accomplished with solution P2 that lyses cells while disrupting the hydrogen bonding between nucleotides, which causes the all the DNA in the sample—both plasmid and genomic—to become single stranded. Buffer N3 then reverses the conditions to allow the tiny plasmid DNA to re-form quickly into double-stranded DNA. The double-stranded plasmid DNA stays suspended in solution while the large and unwieldy genomic DNA will not have enough time to come back together. Instead, the genomic DNA binds with other cell contents to form a white precipitate that can be pelleted and discarded. In this step, excessive mixing can break the genomic DNA into small enough parts that they can contaminate the plasmid product.

Want more information? See the links in the footnote[2] below.

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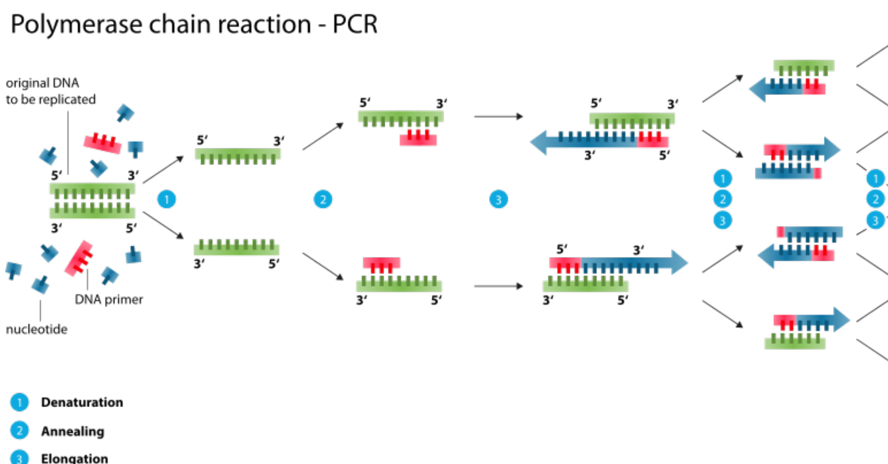
[2] See bitesizebio.com/180/the-basics-how-alkaline-lysis-works/ or the Qiagen product site https://www.qiagen.com/us/shop/sample-technologies/dna/plasmid-dna/qiaprep-spin-miniprep-kit/#orderinginformation

Mutagenic Polymerase Chain Reaction (Mutagenic PCR)

The polymerase chain reaction (PCR) uses polymerases and thermal cycling to create large quantities of gene copies necessary for many downstream applications such as DNA sequencing. Kary Mullis, a Georgia Tech alumnus (BS Chemistry), won the Nobel prize in Chemistry in 1993 for developing PCR.



(Image modified from Enzoklop CC BY-SA 3.0)

For PCR, polymerase enzymes are usually chosen for their high fidelity and ability to avoid mistakes in replication. Because DNA polymerases effectively double the number of copies each cycle, any mutation introduced in one cycle will be propagated in future cycles allowing mutations to accumulate. However, in this experiment, we *want* to generate a few mutations during PCR. We will be using the GeneMorph II Random Mutagenesis Kit by Agilent Technology. The kits combines several polymerases with poor proofreading ability with a proprietary reaction buffer called Mutazyme II to create a randomly mutagenized PCR product. By adjusting the amount of template DNA and number of cycles, we can expect between 4.5 – 9 mutations per thousand base pairs (kb).

The primers chosen for this reaction flank the *RFP* gene and also contain restriction sites for the restrction enzymes XhoI and NdeI, underlined in the primer sequences below. These restriction sites will be important next week when we insert the mutagenized *RFP* genes into plasmids.

RFP Forward (NdeI): 5'-CTGGTCGGCCATATGGCGTCTTCTGAAGACGTTATC-3'
RFP Reverse (XhoI): 5'-CGGATCCTCGAGCTATTACGCACCGGTAGAGTG-3'

Plasmid Extraction Protocol using Qiagen QIAprep® Spin Miniprep kit – 1 per group

1. Transfer 10 ml of overnight culture into a 15 ml centrifuge tube using a sterile serological pipet. Label the tube with group, section, and contents, to prevent potential mix-ups.

2. Centrifuge the tube at 4,000 g for 5 minutes using the large bench-top centrifuge.

3. Invert and shake the tube over a liquid waste container to remove all the supernatant.

4. Re-suspend the cell pellet in 500 µl of Buffer P1. Gently pipet the solution up and down until no cell clumps remain – be persistent. Transfer the solution into a sterile 2 ml centrifuge tube.

5. Add 500 µl of Buffer P2, and mix by inverting the tube 4 – 6 times. The mixture should appear a uniform light blue color. **Do not** vortex or allow the reaction to proceed for longer than 5 minutes before moving on to the next step.

6. Add 700 µl of Buffer N3, and invert the tube repeatedly until the solution is mixed thoroughly. A white precipitate will form, and the blue color will completely disappear when it is fully mixed. This may take up to a minute. Centrifuge the tube at 13,000 rpm (17,900 g) for 10 minutes. A gelatinous white pellet will form.

7. Avoid disturbing the pellet and carefully transfer 800 µl of the supernatant into a blue QIAprep 2.0 spin column nested inside an outer tube. Centrifuge the tube at 13,000 rpm (17,900 g) for 60 seconds.

8. Discard the flow through by carefully removing the spin column and pouring out the contents of the outer tube into the liquid waste; replace the column.

9. Collect all the remaining supernatant and repeat steps 7 and 8. The more supernatant recovered the higher the higher the concentration of purified plasmid.

10. Add 500 µl of Buffer PB to the spin column. Centrifuge the tube at 13,000 rpm (17,900 g) for 60 seconds.

11. Discard the flow through into the liquid waste and replace the column in the tube.

12. Add 750 µl of Buffer PE. Centrifuge the tube at 13,000 rpm (17,900 g) for 60 seconds.

13. Discard flow through, and then replace the column in the tube, and centrifuge the empty column for 60 seconds to remove any residual wash buffer.

14. Carefully remove the column from the outer tube and transfer it to a sterile 2 ml tube.

15. Carefully add 50 µl of PCR water to the center of the column. Allow to sit for 60 seconds, and then centrifuge at 13,000 rpm (17,900 g) for 60 seconds. The cap of the outer tube won't close over the column, so be sure to secure the inner lid of the centrifuge.

16. Discard the column. <u>The flow through now contains a purified plasmid solution</u>. Label the tube as directed. When not being used, store in your group freezer box.

NanoDrop Lite Protocol for measuring DNA concentration and purity

The Nanodrop Lite is a microvolume spectrophotometer specially designed to measure and calculate the concentration and purity of nucleotides (DNA, single or double stranded, and RNA) or proteins in solution.

1. From the start-up screen, select 'DNA' and then 'dsDNA' to indicate double stranded DNA.

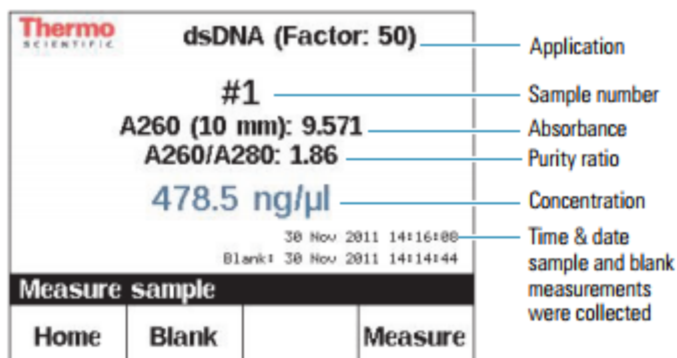
2. Blank the spec with an appropriate solution (usually PCR grade water) by carefully pipetting 2 µl onto the bottom pedestal. The liquid should form a dome in the center of the tiny metal stage.

3. Gently lower the arm. The drop will form a column between the top and bottom stages. Press 'Blank'.

4. Use a kimwipe to wipe the solution off the top and bottom stages. Press hard to remove all traces of the solution.

5. Repeat the process with 2 µl of sample and select 'Measure'. The DNA concentration will be presented in nanograms per microliter (ng/µl)

6. Pure nucleic acids have a purity ratio (A260nm/A280nm) of ~1.8. Any deviation suggests contamination.



7. Record the results, firmly wipe the top and bottom pedestal with a kimwipe to remove all traces of the solution.

Random Mutagenesis via PCR using:

Agilent Technology GeneMorph II Random Mutagenesis Kit – 2 reactions per group

In this experiment each group will use their previously extracted and purified plasmid DNA containing the RFP gene as a template to create many flawed copies of RFP through the process of mutagenic PCR. These reactions work best with a well-defined concentration of template DNA. In this case we will use between 425 - 625 ng of plasmid DNA.

1. Calculate how many microliters (µl) of your plasmid solution should be added to the reaction to obtain between 425 and 625 ng of total DNA.

   ___________ µl = ___________ ng

2. Considering all the reagents listed below, calculate how much PCR grade water is needed to create a final reaction volume of 50 µl. Have a TA check your work before starting. Keep all tubes on ice. Add reagents to the mastermix tube in the order listed: water, plasmid, and enzyme.

   6.5 µl Mastermix provided, will contain:
   - 0.25 µl Forward primer 250 ng/µl
   - 0.25 µl Reverse primer 250 ng/µl
   - 1 µl 40 mM dNTP
   - 5 µl 10X Mutazyme II reaction buffer

   _____ µl PCR grade water

   _____ µl Plasmid template DNA

   1 µl Mutazyme II DNA polymerase (TA will add last)

   50 µl = Total volume

3. Label your group's PCR tube, spin it for a few seconds in the PCR centrifuge to bring all reagents together in the bottom of the tube. Keep on ice until the class is ready to place tubes into the thermal cycler and initiate the polymerase chain reaction.

Step	Conditions	
Initial Denaturation:	95˚C for 2 minutes	
Denaturation	95˚C for 30 seconds	Repeat 29 times
Annealing	59˚C for 30 seconds	
Extension	72˚C for 60 seconds	
Final Extension:	72˚C for 10 minutes	