Fluorescent Protein Discovery Project – Part II

In lab this week, we will be using two restriction enzymes to make precise cuts in two different DNAs:

- the randomly mutagenized *mRFP1* gene copies created in last week's PCR protocol
- pET-15b plasmids, provided in lab today

in preparation for joining them next week in a ligation reaction. Both sets of protocols are provided below.

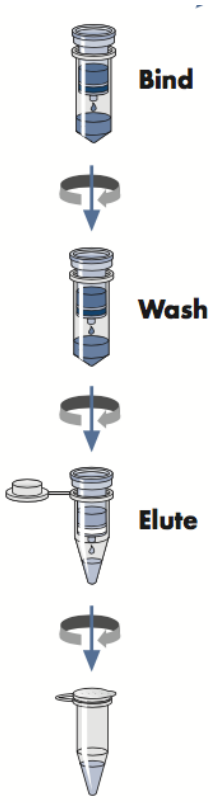
Week 3

- Purify PCR product
- Measure concentration of PCR product and provided plasmid
- Set up double restriction enzyme digest reactions

Week 4

- Purify restriction enzyme digest products
- Quantify DNA yields using the Nanodrop
- Visualize results using gel electrophoresis
- Set up ligation reactions

PCR purification kit



Mutagenic PCR tubes contain enzymes and buffering salts as well as the remnant primers, all mixed in with the target gene's mutated products. To make the downstream protocols more targeted to the gene of interest, we will remove the extraneous salts, DNA, and protein from the PCR product using a Qiagen PCR purification kit designed to remove enzymes and DNA fragments smaller than 100 bp, like primers. The kit works similarly to the plasmid isolation kit we used previously. In the kit protocol, DNA fragments of a desired length are bound to a silica membrane, washed to remove contaminants, and then brought back into solution. We will use this kit several times to provide clean DNA for upcoming the double restriction enzyme digestion and subsequent ligation. The three main ingredients of the kit are:

"Binding" Buffer PB contains a high concentration of salts that promote DNA binding to the silica membrane within the spin column. Binding is highly dependent on having a pH at or below 7.5 to insure recovery of 95% of desired DNA.

"Wash" Buffer PE contains ethanol that promotes adhesion of DNA to the silica membrane while allowing smaller fragments of DNA, unincorporated dNTPs, protein contamination, and buffer salts to wash through the membrane. Ethanol in necessary for this step but can interfere with the function of restriction enzymes and ligase in upcoming steps, so a dry spin step is incorporated to completely remove ethanol from the column.

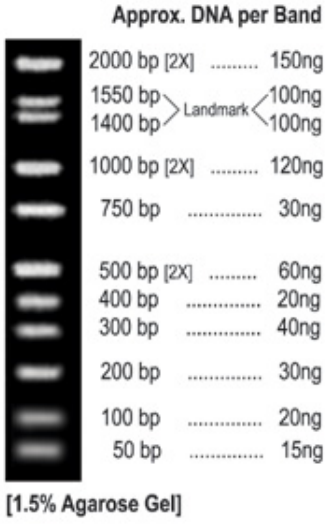
PCR grade water is free of nucleases and salts, both of which can interfere with downstream steps in our experiment. When stored in water at room temperature, DNA will degrade over time, so it is important to store your DNA samples frozen at -20˚C.

Gel Electrophoresis

Gel electrophoresis separates and allows analysis of charged macromolecules, like DNA from our mutagenic PCR, based on size. We'll use gel electrophoresis to determine if the expected sized fragments are present in our cleaned-up mutagenic PCR product. The gel will also confirm that the PCR clean up didn't accidentally remove the DNA containing the desired mutagenized *mRFP1* gene.

Gel electrophoresis separates molecules by passing an electrical current through a prepared gel containing the samples. Since DNA is negatively charged, it will move through the agarose gel away from the negatively charged cathode (black wire) and towards the anode (red wire). Smaller fragments will negotiate the web of gel molecules more easily than large molecules allowing them to move more quickly and grouping together into size classes that will be visible as bands in the gel.

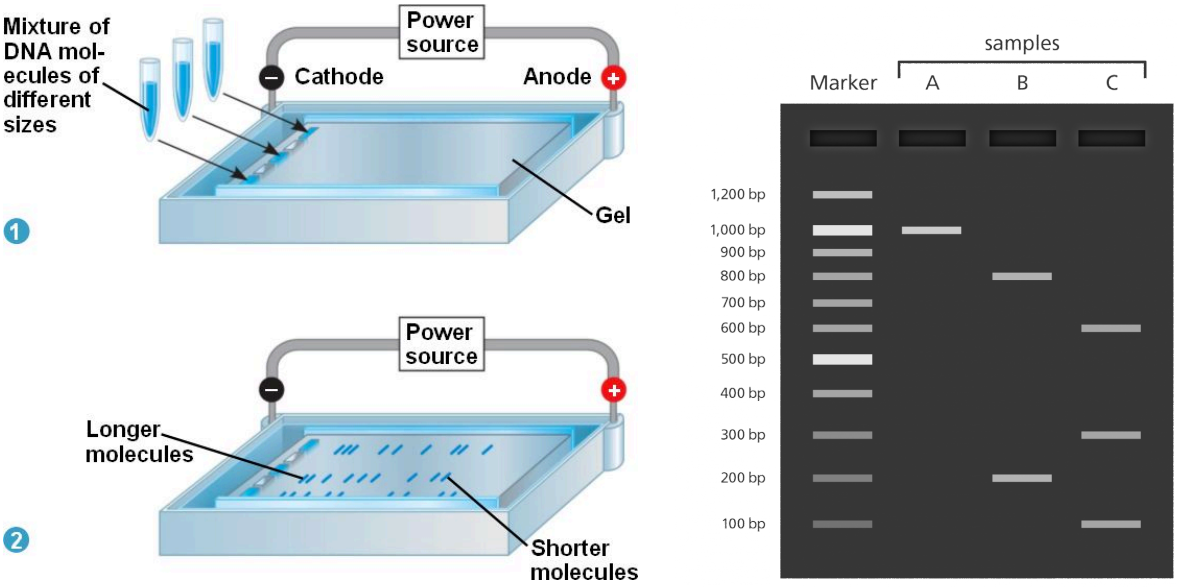
DNA doesn't naturally fluoresce under UV light. To visualize the DNA bands, GelRed has been added to the 1% agarose gels. This molecule binds to DNA and fluoresces under UV light. You'll run a DNA ladder, or marker, alongside the samples for a size reference. The DNA ladder contains fragments of known size and concentration (see image to right). The brightness or intensity of bands can be used as a rough estimate of the quantity of DNA in each band.



(Bionexus LO DNA marker)

Procedurally, cover your gels with a thin layer of conductive TAE buffer before adding the samples. To increase visibility and

viscosity of the samples, mix the PCR product with loading dye to help with loading the samples underwater. The loading dye pigment is also charged and migrate move down the gel ahead of any DNA fragments. This is useful in preventing the samples from running of the end of the gel and being lost in the buffer solution.



(Image : https://socratic.org/questions/why-is-gel-electrophoresis-used)

Restriction enzymes

Restriction enzymes, also called restriction endonucleases, are a class of enzymes that break double-stranded DNA by catalyzing the hydrolysis of phosphodiester bonds that form the backbone connector between individual nucleotides. Discovered in bacteria and archaea lineages, restriction enzymes selectively digest foreign DNA and offer prokaryotic cells protection from viral attack.

Unlike standard nucleases, restriction enzymes cut in precise locations in response to specific palindromic sequences of nucleotides, called restriction sites. Because the restriction sequences are palindromes, the enzyme actually recognizes and makes two cuts, one in each strand. If the cuts are offset from one another, the enzyme creates an overhang with a fragment of single stranded DNA at the ends of the cut region. The two enzymes we will use are shown below, with their cut sites:

Xho I

5′. . . C|TCGAG . . . 3′

3′. . . GAGCT|C . . . 5′

Nde I

5′. . . CA|TATG . . . 3′

3′. . . GTAT|AC . . . 5′

The strands break apart along the red lines, creating sticky ends of DNA that biochemically seek a stable matching sticky end to bind to. Only a matching sticky end will pair up and reconnect once cut, like two puzzle pieces that interlock. We will use two *different* restriction endonucleases to create a small DNA puzzle with two pieces that can only only interlock in a way that incorporates our gene of interest into the plasmid.

Of the hundreds of commercially available unique restriction enzymes, the pET-15B plasmid has restriction sites for dozens. The two we are using, XhoI and NdeI, are adjacent restriction sites on the plasmid. Adjacency ensures that the key genes on the plasmid are intact and functional for gene expression later in the experiment.

In preparation for this digestion, restriction sites were incorporated into the PCR primers we used in the mutagenesis protocol last week. The forward primer contains a site for NdeI, and the reverse primer a site for XhoI, which means all the PCR replicated *mRFP1* gene copies contain both restriction sites.

The process of using two different restriction enzymes on a single piece of DNA is called a "double digestion."

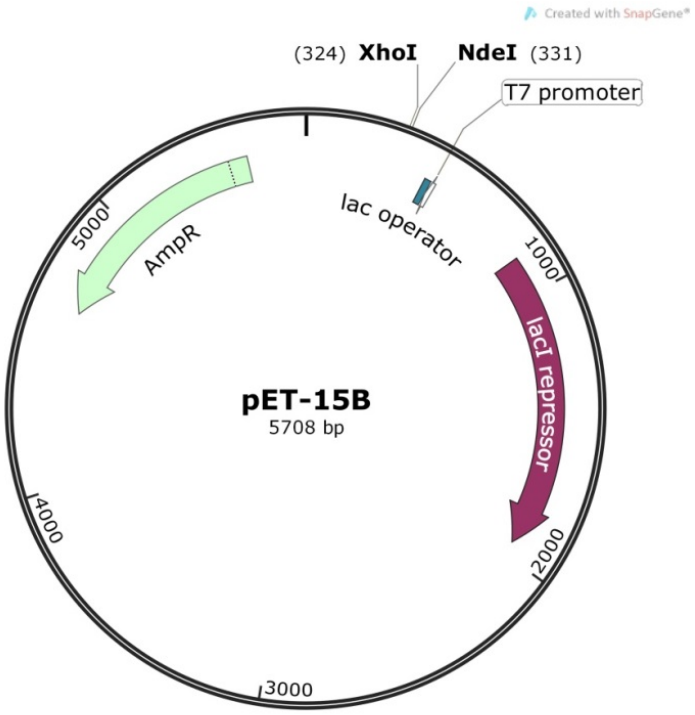


Figure 1. Plasmid pET-15b map.

Ligation

When the doubly digested plasmids and PCR product are combined, their complementary sticky ends can bind together by forming hydrogen bonds between nucleotides along each strand, inserting a mutagenized *mRFP1* gene into to the gap created in the pET-15b plasmid (Figure 2). To close the gap fully and create a continuous plasmid, we'll use the enzyme ligase to complete a ligation reaction to form covalent bonds along the sugar-phosphate backbone (Figure 2).

We are completing a double digestion to reduce the probability of unwanted byproducts, where the plasmid simply reattaches to itself without incorporating an insert. Even with this precaution,

these reactions contain such large numbers of molecules that nearly all-possible combinations of ligation products will be produced in some quantities. Many will subsequently be screened out during the transformation process.

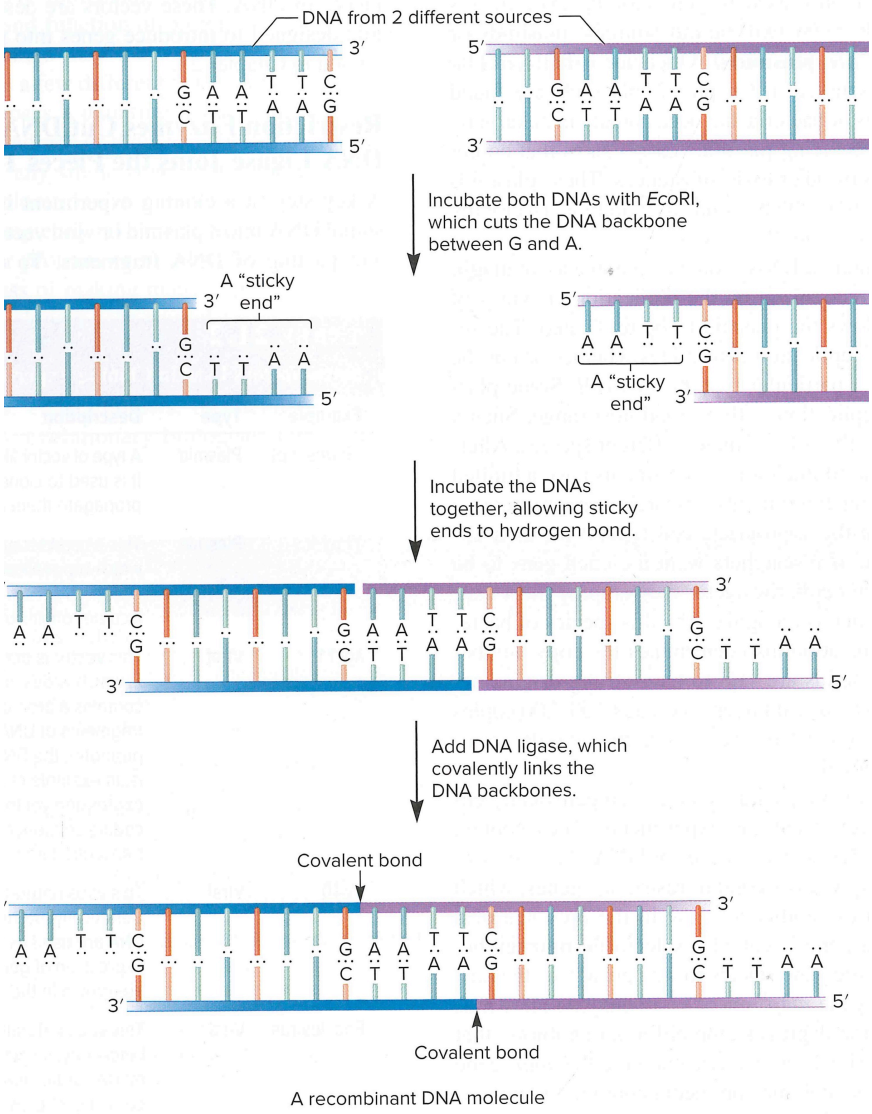


Figure 2. A time progression from top to bottom shows two different DNAs (blue and purple) that both have the same restriction site. Once cut, both have sticky ends that match the other. These sticky ends align and hydrogen bonds can form between the bases. The addition of DNA ligase creates recombinant DNA by forming a covalent bond along the phosphate backbone. Image credit: Brooker, Genetics 6th edition, Figure 21.1.

PCR Purification Protocol Qiagen QIAquick PCR Purification Kit – 1 per group

1. Briefly centrifuge the PCR tube containing the product from the mutagenic PCR, in the small PCR centrifuge to bring the solution to the bottom of the tube.
2. Transfer the entire contents, 50 µl, to a sterile 1.5 ml tube.
3. Add 250 µl of Buffer PB (5X the volume of product to purify) and mix by slowly pipetting the solution up and down a few times.
4. A yellow color indicates the pH of the solution is ≤ 7.5. If the mixture is orange or violet, add 10 µl of 3 M sodium acetate pH 5.0, mix and check for a yellow color.
5. Obtain a QIAquick spin column with 2 ml collection tube.
6. Add the entire 300 µl solution into the QIAquick column and centrifuge for 45 s. All microcentrifuge steps for this protocol will be carried out at 13,000 rpm (17,900 g).
7. Discard the flow-through in the collection tube. Place the QIAquick column back into the same tube.
8. Add 750 µl Buffer PE to the QIAquick column and centrifuge for 45 s.
9. Discard flow-through and place the QIAquick column into a new collection tube.
10. Centrifuge the column for an additional 60 seconds. This dry spin will remove any residual ethanol from the column, as long as the previous flow-through was discarded (Important).
11. Transfer the QIAquick column to a sterile labeled 1.5 ml tube.
12. Add 50 µl PCR grade water directly to the center of the QIAquick membrane, let the column stand for 60 seconds, and then centrifuge for 45 seconds.
13. For the mutagenic PCR product, check the concentration using the nanodrop. Low measurements could indicate that the PCR was not successful.
14. Keep the purified PCR product frozen at -20˚C when not in use, and for long term storage.

Restriction Enzyme Digest Protocol

New England Biolabs, Inc. XhoI (Catalog #R0146S), NdeI (Catalog #R0111S), BSA (bovine serum albumin) (Catalog #B9000S)

Only digest one of your two samples using this protocol. Save the other sample to run your gel later. Have your TA approve your final calculations before you begin. Keep enzymes on ice when in use and store in the freezer at -20˚C.

1. PCR product double digestion (1 reaction / group)
   Add the following components in the order they are listed to a sterile labeled PCR tube for a total volume of 50 µl.

   30 µl Purified PCR product
   8.5 µl PCR grade water
   5 µl Buffer 4
   0.5 µl BSA
   3 µl XhoI
   3 µL NdeI

2. Spin the tube briefly in the PCR centrifuge to bring all the components together.

3. Using the thermal cycler, incubate the tubes at 37˚C for 3 hours, followed by 65˚C for 20 minutes to deactivate the enzymes. Digests will be stored in the freezer at -20˚C until next week.

4. pET-15B plasmid double digestion (1 reaction / group)
   Add the following components in the order they are listed to a sterile labeled PCR tube:

   ____ µl of pET-15B plasmid to equal 1.5 µg
   ____ µl PCR Water to equal 50 µl total reaction volume
   5 µl Buffer 4
   0.5 µl BSA
   3 µl XhoI
   3 µl NdeI

5. Spin the tube briefly in the PCR centrifuge to bring all the components together.

6. Using the thermal cycler, incubate the tubes at 37˚C for 40 hours, followed by 65˚C for 20 minutes to deactivate the enzymes. Digests will be stored in the freezer at -20˚C until next week.

Ligation Reaction Protocol

New England Biolabs, T4 DNA Ligase (Catalog #M0202S)

Have your TA approve your final calculations before you begin.

1. Clean up both digestion reactions from last week using the PCR purification protocol.

2. This reaction requires the addition of specific amounts of digested plasmid and PCR product. Measure the concentration of each purified digest and calculate the volume needed to meet the conditions of the reaction.

3. Ligation reaction (1 reaction / group)
   Add the following components in the order they are listed to a sterile labeled PCR tube.

   2 µl of 10X T4 Ligation Buffer
   _____ µl PCR Water to equal 20 µl total reaction volume
   _____ µl of digested pET-15B plasmid to equal 50 ng
   _____ µl of digested PCR product to equal 42 ng
   1 µl of T4 DNA ligase

4. Self ligation reaction (1 reaction / group)
   Add the following components in the order they are listed to a sterile labeled PCR tube.

   2 µl of 10X T4 Ligation Buffer
   _____ µl PCR Water to equal 20 µl total reaction volume
   _____ µl of digested pET-15B plasmid to equal 50 ng
   1 µl of T4 DNA ligase

5. Negative control (1 reaction / group)
   Add the following components in the order they are listed to a sterile labeled PCR tube.

   2 µl of 10X T4 Ligation Buffer
   _____ µl PCR Water to equal 20 µl total reaction volume
   1 µl of T4 DNA ligase

6. Spin the tubes briefly in the PCR centrifuge to bring all the components together.

7. Incubate at 4˚C for 12 hrs in the refrigerator. Ligation reactions will be stored in the freezer at -20˚C until next week.

Gel Electrophoresis Protocol

1. Obtain a tray containing a 1% agarose gel stained with GelRed.

2. Place the tray into a gel box so the DNA will 'run to red' that is, travel towards the red lead, when the lid is in place.

3. Pour 1X TAE buffer into both ends of the box until the buffer is just covering the surface of the gel, and has filled the wells.

4. This process will allow us to visualize the size of DNA fragments and plasmids in each of the following samples:

    a. Purified RFP plasmid
    b. Purified PCR product
    c. Purified empty pET-15b plasmid
    d. Digested PCR product
    e. Digested pET-15b plasmid

5. Record where your group plans to put each sample and the DNA reference ladder.

6. Transfer 10 µl of each sample into a new PCR tube. As little as 5 µl will work, if your group is running low on any of the products/samples listed.

7. Add 1.7 µl of 6X loading dye to each tube and gently mix by pipetting up and down several times.

8. Slowly and carefully transfer each sample with loading dye into a designated well. The loading dye increases the density of samples so they sink to the bottom of each well.

9. Add 10 µl of Bionexus All purpose LO DNA ladder (BN2051) to a designated well. This already contains loading dye.

10. Slide the gel box lid into place and plug the leads into the power source.

11. Turn on the power source and set the voltage to 100V. If current is flowing through the gel, then tiny bubbles will be visible rising from one end of the box.

12. Allow the gel to run for 60 minutes or until the loading dye has traveled at least half way down the gel.

13. When complete, turn of the power before removing the gel. Obtain a picture of your gel under UV light using the Bio-Rad Gel Documentation system in the back of 123.

Troubleshooting Unexpected Results

The pursuit of new scientific knowledge requires persistence in the face of setbacks. Troubleshooting laboratory work is necessary at every level, whether developing new techniques, adapting others for new uses, or replicating work done in another lab. To successfully troubleshoot a problem, the researcher needs to know what each buffer and step of the protocol was expected to do and how.

In the event of unexpected results, describe the issue, possible causes, and potential solutions in your lab notebook and work with your TA to create a new plan.

1. Describe the unexpected results and your interpretation of them.

2. Describe in detail several potential scenarios that could have generated these results.

3. Is more information needed to distinguish between two potential causes? If yes, explain.

4. Manufacturers of kits and reagents have a vested interest in the success of their products and often provide advice and tips for troubleshooting. Did your group find this information helpful in identifying potential causes?

5. Propose a new action plan and schedule. Include a list of supplies and reagents that will be needed. Consult with your TA or lab instructor before proceeding.