Fluorescent Protein Discovery Project – Part III

Over the next two weeks we will transform competent *E. coli* cells with our previously created recombinant DNA plasmids and compare fluorescent protein expression between the original and randomly mutated *RFP* genes.

First Week

- Measure concentration and purity of ligated plasmids
- Set up transformation reactions
- Plate transformed cells and incubate

Second Week

- Count colonies and calculate transformation efficiency
- Screen colonies under UV light
- Streak colonies that show changes in fluorescence

  OR
- Regroup and create a plan for troubleshooting

Transformation

Transformation is the final step in the process of molecular cloning where we will introduce our painstakingly created plasmids into competent *E. coli* cells, where "competent" simply means able to take up DNA from the environment. Ideally, each *E. coli* cell will take up one plasmid with insert, meaning the plasmid contains a copy of randomly mutagenized *mRFP1*. We will then grow up the *E. coli* to high population size so that each transformed cell will reproduce clonally, perhaps thousands of times. When *E. coli* reproduce on solid media, like an LB plate, they form colonies comprised of thousands of identical cells. Because the colonies are large enough to observe with the naked eye, we will be able to detect the colour that each colony flouresces, or the lack of fluoescence if our mutagenesis broke the gene's functionality. Using the colony fluorescence phenotype, we will isolate colonies with interesting fluorescence characteristics and use them to create a pure bacterial culture for further fluorescence characterization.

Bacterial transformation refers to the uptake of DNA from the environment. Known to occur naturally, this is one of only three ways asexually reproducing prokayrotes can exchange genetic material. Cells capable to transformation are considered competent. Natural competence evolves when alleles are selected for that promote attachment of DNA to the cell wall and subsequent DNA uptake into the cell. Genetically engineered competence takes advantage of these alleles and combines them into a single *E. coli* genome, such as in the strain we will use: BL21 (DE3) (New England BioLabs # C2527I).

In addition to a genetic propsensity to take up DNA, competence is also affected by abiotic factors. *E. coli* strain BL21 (DE3) is genetically engineered to increase the likelihood of uptake and expression of foreign proteins. This competence is further enhanced by chemical exposure and by heat shock, which exposes cells to high temperatures. Post heat shock, we'll incubate on ice and then allow the cells to recover in high nutrient (S.O.C.) medium, which will allow cells to repair their cell walls and begin to divide normally.

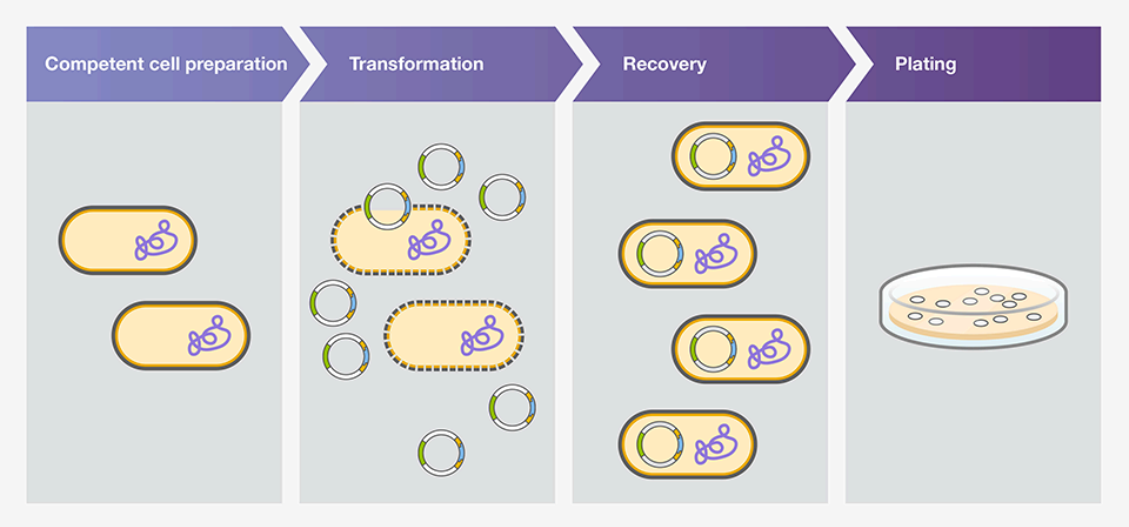


Figure 1. Diagram of competent cell uptake of foreign DNA via transformation, and the subsequent expression of those genes on a plate with bacterial colonies. Image credit: ThermoFisher Scientific, www.thermofisher.com.

Plasmids

The ligation reactions completed last week should have produced a plasmid with our gene of interest inserted, forming pET-15b + mutagenized *RFP*. As controls, we also ligated pET-15b alone to determine the rate of self-ligation (control) and added ligase to water (negative control). We also plated the original purified plasmid, extracted earlier, as a positive control.

- pET-15b + mutated *RFP*
- pET-15b + original *RFP*
- pET-15b alone
- Water

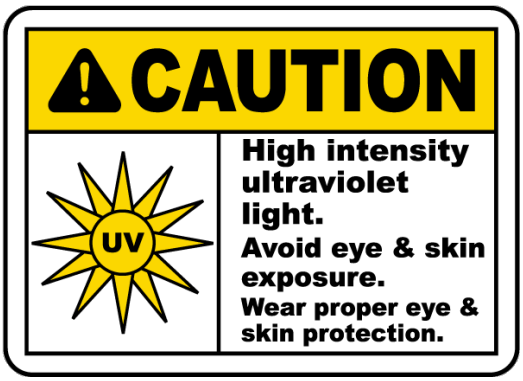
Plating

We will grow these different transformed bacteria on agar media poured into plates, where it will form a smooth, solid surface. The resources to allow growth and selective agents to prevent growth on those plates is important to consider at this point. Cells that have successfully taken up the pET-15b plasmid will show resistance to the antibiotic carbenicillin, meaning that cells with plasmids will grow on carbenicillin plates. Next, because *RFP* expression is inducible, it will only be visible in the presence of the chemical IPTG, so we'll use LB agar + CARB + IPTG plates as our selective media to isolate *E. coli* with plasmids that have fluorescing variants of our gene of interest.

To allow you create additional controls, we'll provide plates with just LB agar or with LB agar + CARB (no IPTG). These will be invaluable to troubleshoot our results should the transformation reactions spread on LB +CARB +IPTG not grow cells. We can use them to assess if the media additives working properly, if competent cells were not viable (able to live), etc. Good experimental design anticipates these questions and integrates controls on these media.

Therefore, with your team, you'll want to plan ahead and decide which samples will be plated onto what media. Each transformation reaction will produce enough product to spread onto two plates, which will allow for the use of one LB agar + CARB + IPTG plate and one of the control plate options. To maximize the number of potential new fluorescent proteins to select from next week, we recommend that each team spread the cells transformed with pET-15B + mutagenized *RFP* onto two LB agar + CARB + IPTG plates. Once you've plated, complete the provided worksheet to predict your expected results for each combination of media with bacterial culture.

Fluorescence Screening



In the week following plating, we'll be able to observe whether colonies grew on the various media types, as well as observe them under UV light from both long wave (365 nm) and short wave (302 nm) lamps. Some proteins may fluoresce differently when exposed to one or the other. Caution: Ultraviolet light is a mutagen that can damage skin. Always wear full PPE (gloves, lab coat, safety glasses, and face shield) when working with high intensity UV radiation.

Using the fluorescence observed from the original RFP plasmid as a benchmark, identify any colonies containing a randomly mutagenized *RFP* gene that show departures in fluorescence. Using aseptic technique, carefully touch a single target colony with a sterile toothpick or loop and streak it onto a new LB agar + CARB + IPTG plate. Repeat the process for five different candidate colonies to create the collection of mutant strains that you will characterize for their modified fluorescent traits. After the bacteria grow at room temperature for a week, they will be ready for further phenotypic characterization, as well as genotyping.

Transformation Efficiency

In addition to qualitative descriptions and images of the growth on plates from the previous week, we can calculate the transformation efficiency of the competent cells. This is the number of successfully transformed cells per µg of plasmid DNA. Measure the concentration of each plasmid on the nanodrop. (Hint: Blank the nanodrop with 1X T4 buffer instead of water for best results). The number of visible colonies is a good estimate of the number of successfully transformed cells, after you accounts for any dilutions you may have made when plating. This estimate assumes that each colony originates as a single successful cell and all transformed cells result in visible colonies. Because this is a relatively safe pair of assumptions, transformants are often referred to as colony forming units (CFUs).

You can (and should) calculate the transformation efficiency for each reaction by counting the colonies, then dividing by the amount of plasmid DNA, in µg, actually plated (recall only a portion of the 3 µl of ligated plasmid was transferred to each plate).