

MODULE E

USING BIOTECHNOLOGY TO CHANGE GENE EXPRESSION



EDUCATIONAL OBJECTIVES

- Analyze and compare genetic sequences
- Practice aseptic technique and spectrophotometry
- Observe a blue/white assay
- Recognize methods to modify organisms and appropriate reasons to do so
- Design a DNA construct to increase protein production
- Write a technical report

SCIENTIFIC QUESTIONS

BUILDING NEW BIOMOLECULES

Much of modern molecular biology research is done by combining and "recombining" DNA sequences to learn how they function and make useful products. This is known as **Recombinant DNA**, where pieces of DNA from different organisms are combined to make new products, or to make more of existing products.

One classic example of this technology is the use of *E. coli* to make insulin for diabetic patients. In this case, the human version of the gene that codes for insulin is combined with a strong promoter element on a circular piece of DNA. These are then moved into *E. coli* cells, which transcribe the DNA and translate the resulting RNA to make protein (Figure 1). These proteins are purified and checked for quality before being packaged and distributed to patients.

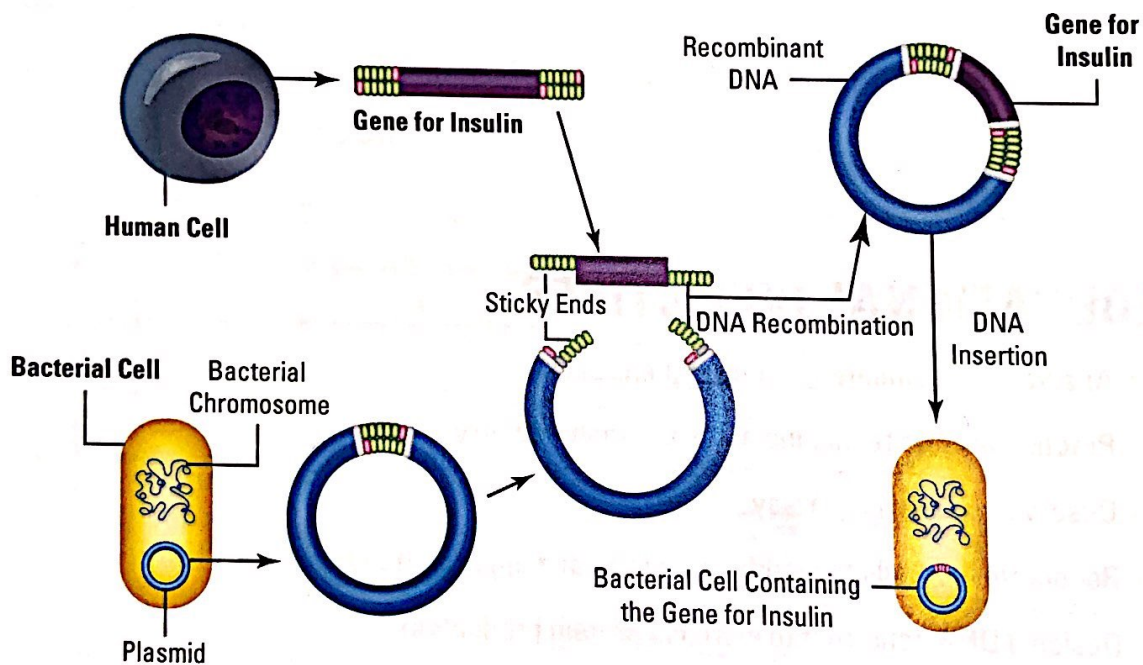


Figure 1. A schematic of recombinant DNA, in which human DNA is combined with a bacterial plasmid and moved into bacterial cells to make human proteins. Modified from <https://pmgbiology.com/2014/11/30/recombinant-dna-a-understanding-for-igcse-biology/>

Recombinant DNA can also be used to understand how specific DNA sequences function in cells and what happens if they are broken. For example, the sequences that regulate protein-coding genes in *cis* (like promoters, enhancers, silencers) make up about 10% of the human genome and are not well understood. If we combine these regulatory sequences with protein-coding sequences we do understand, we can learn about how the regulatory sequences work. For example, we can combine new regulatory sequences with a reporter

gene, like GFP, and measure how much of the green fluorescent protein is made in cells. When we put these reporter constructs in other organisms, we can answer questions like where, how much and under what circumstances is the gene expressed?

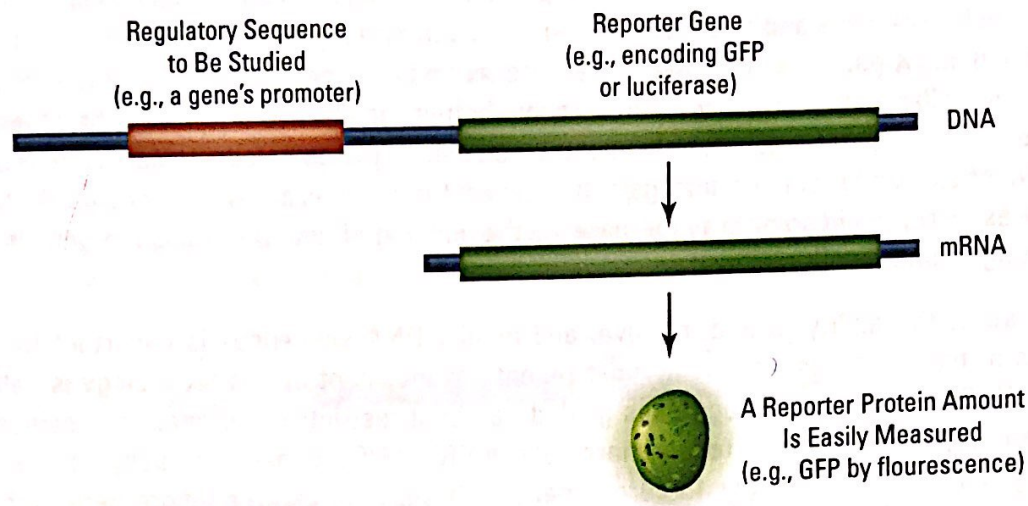


Figure 2. A schematic of a reporter construct. Modified from https://en.wikipedia.org/wiki/Reporter_gene

Originally, recombinant molecules were built as plasmids for bacterial cells. Bacterial plasmids are pieces of circular DNA with three defining features:

1. The bacterial **origin of replication** allows the vector (and its modified DNA) to be replicated by the bacteria. This results in two simultaneous types of amplification. First, each cell contains between 10–200 copies of the plasmid per cell. Second, each time the bacterium replicates and divides, it also replicates the plasmid. Thus, if cells are allowed to grow and divide overnight, millions of copies of the plasmid can be obtained. This is helpful if you need to make a lot of a specific protein, like insulin. It can also help if you need to amplify a signal for detection, such as when we build fluorescently labeled proteins.
2. The recombinant DNA molecule is added to the recipient cells by transformation. The efficiency of transformation is low; bacteria usually take up about 1 in 10,000 DNA molecules. Thus, it is necessary to have a method for **selecting** those bacteria that have been transformed. Bacteria that have successfully taken up the plasmid can be isolated by growing the cells on antibiotics. The *E. coli* we are working with are not naturally resistant to antibiotics. However, the plasmid contains a gene that renders cells resistant to ampicillin. Thus, only cells that contain the plasmid will be able to survive on plates containing the antibiotic ampicillin.

3. Plasmids also typically have a place where new DNA can be easily inserted. This region is known as the Multiple Cloning Site (MCS) and contains sites recognized by diverse restriction enzymes. In some plasmids, this site is in the middle of a copy of the *LacZ* gene. As you know, *LacZ* encodes the protein, beta-galactosidase, that can digest lactose into glucose and galactose. The enzyme can also digest other analogs of lactose, including X-gal. When digested, X-gal produces a blue product that the bacteria cannot metabolize further. Thus, we can screen whether the plasmid added to cells contains an active copy of *LacZ* by looking at the colonies. If the colonies are blue, *LacZ* is still functional and produces beta-galactosidase. If the colonies are white, *LacZ* production was disrupted in some way (perhaps by the addition of new DNA) and the gene is no longer functional.

Ultimately, the ability to add, remove, and modify DNA sequences is important for all types of biological research. The most recent advancement of this technology is called CRISPR/Cas9. This technology uses guided bacterial restriction enzymes to specifically cut and repair DNA. Some researchers believe CRISPR/Cas9 may eventually be used to edit genomes in human cells to cure genetic diseases. You can read more here: <https://www.neb.com/tools-and-resources/feature-articles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology>

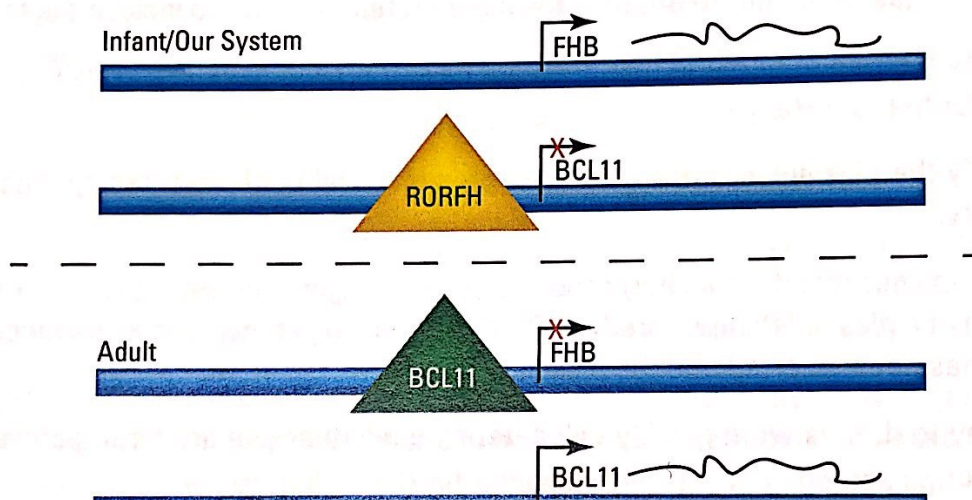
INTRODUCTION TO THIS EXPERIMENT

In this experiment, we will use the *Lac* operon to understand recombinant DNA technologies. We will use the amount of beta-galactosidase protein produced as a measure of how "strong" a promoter is. The regulatory elements of the *Lac* operon have been well-studied. By modifying these regulatory sequences, we should be able to increase or decrease the amount of RNA produced, which is correlated to the amount of protein produced. You will need to think critically about how changes to the DNA sequence could alter protein production. Importantly, changes to either the *cis*- or *trans*-elements, or the gene itself can alter gene expression. Also, not all alterations are bad. It is possible to alter the DNA sequence such that an activator binds **better** (or a repressor binds less well) and induces more expression. Once we understand our plasmid and have identified the strongest promoter, we will consider how we could insert a new gene to produce a more useful protein.

Ultimately, your goal is to design a plasmid that induces high concentrations of fetal hemoglobin in human cells, which can be used to treat sickle cell anemia.¹ Sickle cell anemia is a disease that arises from mutations in the adult hemoglobin gene (Hb). Mutations to Hb cause the hemoglobin molecules to bind together and create long protein chains. This changes the phenotype of the cells, which become sickle shaped; they can break easily, causing anemia, and can get stuck in blood vessels, causing pain.

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Fetal hemoglobin is a variant of the adult version of the protein. Fetal hemoglobin is typically only expressed during fetal development and the first 6 months of infancy. Fetal hemoglobin holds oxygen more tightly than the adult version and therefore can be used to transport oxygen in sickle cell patients. Furthermore, fetal hemoglobin does not form protein chains in adult cells and therefore will compete with mutated adult hemoglobin proteins and decrease symptoms of sickle cell anemia. Because hemoglobin is a tetramer with complicated quaternary structure, it's hard to produce outside of human cells. However, the genetic code for fetal hemoglobin is still present in adult cells; it is just repressed by a protein called BCL11A. If we can repress this repressor by increasing the amount of RORFH (the repressor of the repressor), presumably the gene encoding fetal hemoglobin will be induced in human cells and sickle cell patients can be treated.



You have obtained a job working at a biotechnology company. The company has recently discovered a gene that codes for a protein, *Repressor of Repressor of Fetal Hemoglobin* (RoRFH). You have been tasked with designing a plasmid that will produce this protein at levels that can be injected into patients with Sickle Cell Anemia.

There's just one problem. The lab has several bacterial strains that could be transformed, but your manager did not keep a useful laboratory notebook. You'll need to identify which *E. coli* strains are missing their *LacZ* gene. This is important; if the *E. coli* already have *LacZ* in their genomes, you can't use beta-galactosidase as a reporter gene. Additionally, the lab has several plasmids with different promoters. You'll need to identify which one has the strongest promoter to produce the most protein. Your tasks include the following:

1. Identify a strain of bacteria that does **not** have the *LacZ* gene in it. This strain can be used for the reporter assay.
2. Identify the plasmid with the strongest promoter and confirm it through spectrophotometry.
3. Write up your results in a short report to your manager. Your report should include the design of a plasmid that will produce RoRFH and an experimental plan to check that the gene has been inserted into the plasmid.

Your ability to do this work quickly will determine whether you are hired permanently to this position.

SCIENTIFIC BASIS OF OUR EXPERIMENT

- Some strains have been transformed with a plasmid. Some strains already have a functional *LacZ* gene in their genomes. You will be looking for the *E. coli* strain that is **NOT** transformed and does **NOT** have a functional *LacZ* gene.

You will analyze the sequence of your plasmid and predict whether you expect the strain to produce beta-galactosidase under various nutrient conditions.

- You will be given a set of DNA sequences.
- You will then compare your plasmid to the wild-type using the DNA alignment program ClustalOmega.
- You will draw a map of the plasmid. Then, identify the mutations in the plasmid and use your knowledge of the Lac operon to predict the outcome of the mutation on gene expression.

You will isolate total protein and perform an enzyme assay using spectrophotometry.

- You will induce protein production using several sugar supplements.
- You will permeabilize the cells with toluene. Toluene is used here because it permeabilizes the cell wall and membrane without adversely affecting the proteins. **Toluene is toxic if inhaled. DO NOT leave bottles open any longer than necessary.**
- You will incubate your protein mixtures with ONPG (o-nitrophenyl-beta-D-galactoside), a synthetic analog of galactose. ONPG is hydrolyzed by beta-galactosidase to produce galactose and O-nitrophenol. The O-nitrophenol is yellow and absorbs light at 420 nm. Thus, we can identify the increase in product over time using spectrophotometry. Remember from *Module B* that the amount of product produced can be used as a measure of enzyme function and, consequently, protein abundance.
- Review how spectrophotometry works in *Appendix 5*.
- The bacteria themselves also absorb light between 400 and 600 nm. Thus, we will also measure the absorbance at 550 nm and use this value to correct for the turbidity of the solution.

EXPERIMENTAL PROTOCOLS

EXPERIMENTAL OUTLINE

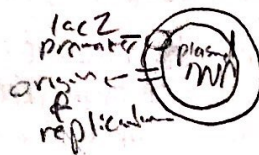
DAY 1: OBSERVING A BLUE/WHITE ASSAY AND SEQUENCE ANALYSIS**PRE-LAB**

- 5 1. We will be looking for an *E. coli* strain that is not transformed and does not yet contain *LacZ*. Given the data below, does this strain contain a plasmid? Does it contain a functional *LacZ* gene? How do you know?

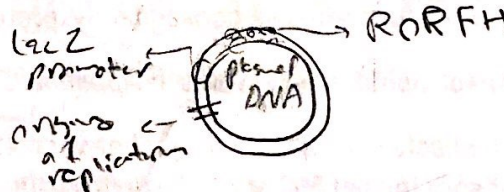
Reagent	-X-gal	+X-gal
No Ampicillin	White	White
+ Ampicillin	Dead	Dead

only cells that have been taken the plasmid will be capable of surviving and growing by adding new DNA it will be white so the strain is not Ampicillin resistant

2. Draw a picture of a plasmid that has a) selective marker, b) origin of replication, and c) a *LacZ* promoter upstream of the multiple cloning site within the *LacZ* gene.



- 3 Draw the same plasmid with the RoRFH gene incorporated into the multiple cloning site.



4. a. If you plated cells containing the plasmid from Question 2 on plates + amp, + X-gal, what color would they be?

blue color



- b If you plated the cells containing the plasmid from Question 3 on plates +amp, +X-gal, what color would they be?



1. Obtain plates of transformed and untransformed cells. The lab managers plated cells from several *E. coli* strains, which are labeled with numbers. The cells are plated on either LB+X-gal or LB+X-gal+ampicillin.
2. Count the number and color of colonies on each plate and record your data in the lab notebook page(s) you will turn in with your technical report. Use this data to determine which strains have plasmids and which already contain plasmids.

_____ Recommended strain for lab managers to transform with plasmids.

PART 2: ANALYZE DNA SEQUENCES

1. Open the website <http://www.bioservers.org/bioserver/>
 - a. Load your assigned plasmid sequence and the wild-type sequence into the sequence server and align them with ClustalOmega.
 - b. Note where the mutations are and compare them to the location of the *LacZ* gene and promoter on the map you were given in class.
 - c. Use the literature to predict the consequence of these mutations.
2. Record your class data.

plasmid X

DAY 2: INDUCE BETA-GALACTOSIDASE ACTIVITY AND MEASURE ENZYME ACTIVITY

Tube Number	Amount of Culture	Supplement	Volume of Sugar	Volume of Water	Final Volume
1	5 mL wild-type	Lactose	200 mL	220 mL	5.42 mL
2	5 mL wild-type	Glucose	200	220	5.42 mL
3	5 mL wild-type	Both	200/200	20	5.42 mL
4	5 mL wild-type	None	0	420	5.42 mL
5	5 mL mutant	Lactose	200	220	5.42 mL
6	5 mL mutant	Glucose	200	220	5.42 mL
7	5 mL mutant	Both	200/200	20	5.42 mL
8	5 mL mutant	None	0	20	5.42 mL

6. Add water to each tube as necessary so that the final volume in each tube is 5.42 mL.

7. Return all cultures to the shaking incubator for **45 minutes**. During this time, the cells will transcribe mRNAs and translate proteins.

During this time, we will discuss your plasmid maps, how to insert new DNA sequences, and how to assay for recombinant plasmids.

8. Shortly before the end of the 45-minute incubation, label 8 culture tubes (1–8 as above) and add two drops of toluene to each tube. **Cap the tubes immediately and place them on ice.**

9. At the end of the incubation, add 2 mL of the appropriate bacterial culture to each tube. Vortex and return the tubes to ice.

10. When you have collected all samples, place the tubes in a 28°C water bath for **30 minutes**, swirling occasionally. During this time, the cell walls will weaken, allowing them to take up the substrate.

During this time, we will discuss the writing expectation for this lab.

11. Transfer all of your tubes to the lab bench and add 0.4 mL of ONPG solution to each tube in intervals of 15 seconds.

12. Incubate your tubes on the bench for **20 minutes**. During this time, the ONPG will be hydrolyzed to produce a yellow color.

1	5 mL wild-type	Lactose	2.846	0.187	2.659	
2	5 mL wild-type	Glucose	0.425	0.222	0.203	
3	5 mL wild-type	Both	1.877	0.229	1.648	
4	5 mL wild-type	None	0.453	0.236	0.217	
5	5 mL mutant	Lactose	0.263	0.171	0.092	
6	5 mL mutant	Glucose	0.312	0.196	0.116	
7	5 mL mutant	Both	0.264	0.154	0.11	
8	5 mL mutant	None	0.315	0.218	0.097	

Make sure to obtain complete class data.

— ASSIGNMENT FOR NEXT WEEK